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Studies of Ethanolic Extract from *Lentinus edodes On Different Cell Lines And Lymphocytes Separated From Cancer Breast Patients*

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Abstract: *Lentinus edodes* a Chinese edible and medicinal mushroom was grown using submerged culture on different standard liquid media and different food wastes by products. Shiitake broth medium was the medium that showed the maximum biomass for both tested strains. The biomass obtained from cultivation on all tested media was extracted with ethanol to obtain crude extract that tested for its biological activity. Also, SM broth medium showed the highest crude extract yield for the two strains. Some physiological factors were studied to optimize the conditions for cultivation on SM broth medium for maximum biomass and crude extract yield. Although recent studies have demonstrated that *L. edodes* separate compounds can inhibit the proliferation of cancer cells *In vitro* and *in vivo*, crude extract of *L. edodes* mycelia was tested in this study *In vitro* and *in vivo*. The Invitro cytotoxic activity of the crude extracts of mycelia of the two strains were examined on human breast cancer (MCF7) cell line using trypan blue exclusion assay and MTT cytotoxicity assay. The results showed that the crude extract of *Lentinus edodes LC2141* inhibited the proliferation of MCF7 at high concentration with IC_{50} 178.8µg/ml while *Lentinus edodes LC202* extract inhibited the proliferation of MCF7 at high concentration with IC_{50} 94.025µg/ml. Also, The treatment of Hep-G2 cells with different concentrations of ethanolic extract of both *Lentinus edodes LC2141* and *Lentinus edodes LC202* dramatically inhibited the cell growth in a dose-dependent manner, with high IC_{50} values of 953.1µg/ml and 1868.3µg/ml, respectively. Immunomodulating activity of the tested extracts was determined on normal and breast cancer patient lymphocyte using MTT and trypan blue exclusion assays. The treatment of lymphocyte with different concentrations of the crude extract showed an increase in lymphocyte proliferation by increasing concentration (dose dependant).
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Keywords: Lentinus edodes**,** Ethanolic crude extract**,** submerged culture, antitumor activity.

1. Introduction

Medicinal mushroom extracts have been considered as important remedies for the prevention and treatment of many diseases for thousands of years especially in the Orient (Israilides and Philippoussis, 2003; Kidd, 2000; Wasser and Weis, 1999). A plethora of medicinal effects has been demonstrated for mushrooms including antibacterial, antiviral, antifungal, antitumor and immuno-potentiating activities (Hobbs, 2003; Ooio and Liu, 1999). Among the various bioactive components which have been demonstrated to be most effective as antitumor and immunomodulatory agents are polysaccharides and polysaccharopeptides.

Nowadays macrofungi are distinguished as important natural resources of immunomodulating and anticancer agents and with regard to the increase in diseases involving immune dysfunction, cancer, autoimmune conditions in recent years, applying such immunomodulator agents especially with the natural original is vital. These compounds belong mainly to polysaccharides especially β-D-glucan derivates, glycopeptide/protein complexes (polysaccharide-peptide/protein complexes), proteoglycans, proteins and triterpenoids. Among polysaccharides, β (1 →3) - D-glucans and their peptide/protein derivates and among proteins, fungal immunomodulatory proteins (Fips) have more important role in immunomodulating and antitumor activities.

Lentinus edodes is the source of many therapeutic polysaccharide macromolecules among which the ones with proven pharmacological effects are lentinan, LEM and KS-2. Lentinan is a high molecular weight (about one million) homopolysaccharide in a triple helix structure, with linear chains consisting of (1 -3)- β - D-glucopyranosyl (Gl*cp*) residues with two β-(1 - 6)-linked Gl*cp* branchings for every five β -(1 - 3)-Gl*cp* residues (Aoki, 1984). LEM is a mycelial extract

preparation of *L. edodes* harvested before the cap and stem grow. It is a heteroglycan–protein conjugate containing 24.6% protein and 44% sugars, comprising mostly pentoses as well as glucose and smaller amounts of galactose, mannose and fructose (Lizuka, 1986; Sugano *et al.*, 1982). It also contains nucleic acid derivatives, B complex vitamins, ergosterol, eritadenine (an anticholesteremic amino acid), and water- soluble lignins (Sugano *et al.*, 1985). KS-2 is a peptide –polysaccharide complex. Besides its anti-tumour activity, it has been demonstrated to increase the host resistance to bacterial and viral infections (Jong and Birmingham, 1993). Lenthionine, a sulphur-containing compound, has antibacterial and antifungal activity (Yasumoto *et al.*, 1971; Morita and Kobayashi, 1967), and bis [(methylsulfonyl) methyl] disulphide, a derivative of lenthionine, has strong inhibitory effects against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* (Takazawa *et al.*,1982). The chloroform and ethyl acetate extracts of the dried mushroom have antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia* (Hirasawa *et al.*, 1999). Both fruiting body and the mycelium contain compounds with wide-ranging antimicrobial activity (Jong and Birmingham, 1993). Several fractions of LEM (an aqueous extract of the *L. edodes* mycelium and its solid culture medium) have immunoactive properties such as the induction of interferon in vitro (Hibino *et al.*, 1994) and in vivo (Suzuki *et al.*, 1979), inhibition of the infectivity and cytopathic effect of human immunodeficiency virus (Suzuki *et al.*, 1989; Tochikura, 1988) and blockade of the release of herpes simplex virus type 1 from tissue culture cells (Sarkar *et al.*, 1993).

Since many of the compounds, which are found in *L. edodes* have been shown to act synergistically (Yamasaki *et al.*, 1989), it is worth testing the biological activity of the whole mushroom and mycelium extract rather than its individual components. This principle (synergy) is compatible with similar natural biological products like the essential oils, which allow the achievement of strong effects when used as whole products, while quenching or nullifying potential unwanted side effects by the presence of individual components.

The aim of this work is study the antitumor activity (*In vitro*) and immumomodulating activity of ethanolic extract from mycelia of two different strains of *Lentinus edodes.*

2. Material and Methods

Fungal Strains:

The two edible fungal strains *Lentinus edodes LC2141* and *Lentinus edodes LC202* were kindly obtained from Fujian Agriculture Univ., China. The culture was maintained on Potato Dextrose Agar (PDA) medium and stored in refrigerator at 5 -7 °C after growth as recommended by Stamets, (1993) for routine culture and storage purposes.

Culture Media:

Medium for Submerged Culture of Shiitake(SM) (Mizuno, 1995):

Mycelia were grown in a submerged liquid culture in 250ml conical flasks. The medium composition for strain *Lentinus edodes LC2141* was: Thiamine -Hcl, 1.0 mg; KH_2PO_4 , 1.0g; $Cacl_2 .2H_2O$, 0.5g; $MgSO_4 . 7H_2O$, 0.5g; $FeSO_4. 7H_2O$, 10mg; $MnSO_4 .6H_2O$, 7.2mg; $ZnCl_2$, 4mg; $CuSO_4$, 1.0mg; Starch, 70g; corn steep liquor, 10g; Fructose, 15g; $NaNO_3$, 2g and distilled water, 1 L; Initial pH= 7). While the medium composition for strain *Lentinus edodes LC202* was: Thiamine -Hcl, 1.0 mg; KH_2PO_4 , 1.0g; $Cacl_2 .2H_2O$, 0.5g; $MgSO_4 . 7H_2O$, 0.5g; $FeSO_4. 7H_2O$, 10mg; $MnSO_4 .6H_2O$, 9mg; $ZnCl_2$, 4mg; $CuSO_4$, 0.8mg; Starch, 70g; corn steep liquor, 10g; Fructose, 10g; yeast extract,5g and distilled water, 1 L; Initial pH= 7). Each flask was inoculated with 25 agar plugs 0.7cm covered by the mycelium obtained from a 15 days old plate culture for 15 days incubation period in case of strain *Lentinus edodes LC2141* and 13 days in case of strain *Lentinus edodes LC202*.

Preparation of Crude Ethanol Extract (Turkoglu et al., 2007):

The mycelia of each strain were dried at 40°C before analysis. These dried mycelia were pulverized and 20.0g each of the powdered samples were soaked separately in 200 ml of 95% ethanol in an Erlenmeyer flask. The sample was extracted by stirring at 30°C at 150 rpm for 24 h. The mixture was filtered through Whatman's filter paper no 4. The residue was then extracted with two additional 200ml of ethanol as described above. The combined ethanolic extracts were concentrated in a rotary evaporator at 40°C. The ethanol was recovered and the extract was collected and dried and stored at 4°C for further use.

Tumor cell lines:

Ehrlich ascites carcinoma (EAC) cells (mouse tumor):

Ehrlich ascites carcinoma (EAC) cells were used in *In vitro* and *in vivo* experiments. The parent cell line was kindly supplied by National Cancer Institute, Cairo University, Egypt. The tumor cell line was maintained in female mice Swiss albino through serial intraperitoneal transplantation of 1x 10^6 viable tumor cells in 0.2 ml of saline. The tumor is characterized by

moderately rapid growth, which kill mice in 16 to 18 days due to accumulation of ascetic fluid and seldom shows distal metastasis or spontaneous regression. Ehrlich ascites carcinoma (EAC) cells were obtained by needle aspiration of ascetic fluid from the preinoculated mice under aseptic conditions using ultraviolet laminar air flow system. The cells in the ascetic fluid were tested for viability and contamination by staining 0.1 ml of this fluid by 0.1 ml of trypan blue dye which stains only the dead cells (Lazarus *et al*., 1966). Preliminary test for Invitro antitumor activity of the crude extracts was done by using Ehrlich ascites carcinoma (EAC) cells by trypan blue exclusion test (Sheldon and Preskorn, 1996). EAC cells were incubated with RPMI medium in a tissue culture plate, then the extract concentrations were added that content of each well was (0.8 ml medium + 0.1 ml cells + 0.1 ml extracts). The final concentrations of extract were as follows (25, 50, 100 and 200 mg/ml) dissolved in PBS. After 24 hrs incubation of cells with extract, the cells were stained with trypan blue dye and percent survival of cells was determined by counting dead and viable cells using haemocytometer. Control treatment in which EAC cells were cultured without extracts was evaluated and The dose response curve of viable cells was determined.

*Percent survival of the cells **= T/C x 100**
Where:
T …. Number of viable cells in unit volume of the test drug well.
C …. Number of viable cells in unit volume of the control well.

Human tumor cell lines:

Human carcinoma cell lines were used in this study, were MCF7 (Breast carcinoma cell line) and Hep-G2 (Liver carcinoma cell line). It was obtained frozen in liquid nitrogen (-180^{o}C) from the American Type Culture Collection. The tumor cell line was maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. For the assessment of the cytotoxic and cytostatic activities of *L. edodes* extracts cells were seeded in 96- well flat-bottomed microtiter plates at a density of approximately ($0.5X10^5$ cells/well), in complete RPMI-1640 Medium. After 24 h to ensure cell attachment, serial dilutions of the extracts in physiological saline were prepared. 100 µl of different concentrations of each tested extracts were added for 24 h at 37^{o}C, in a humidified 5 % CO_2 atmosphere. Cytotoxicity was determined using the MTT assay (Hansen *et al.*,1989). After incubation, 10 µl MTT reagent solution/well was added and incubated for an additional 4 h. MTT crystals were solubilized by added 100 µl of MTT detergent/well then the plate was shacked at room temperature. It was followed by photometric determination of the absorbance at 570 nm using microplate ELISA reader (Meter tech. Σ 960, USA) after development of violet color. Control cells were treated with vehicle alone. For each compound concentration, 3 wells were used (triplicate wells were prepared for each individual dose). The average was calculated. Data was expressed as the percentage of relative viability compared with the untreated cells. The cytotoxicity dose was calculated as a dose induced ≈100% relative non viability.

Calculation:

Percentage of relative viability was calculated using the following equation:
[Absorbance of treated cells /
Absorbance of control cells] X 100

Then the half maximal inhibitory concentration IC_{50} was calculated by the trend line equation.

Immunomodulating activity of the two tested ethanolic extracts:

Collection of blood:

Venous blood (5ml) was drawn from healthy volunteers and breast cancer patient volunteers. Human peripheral blood was collected in sterile heparin tube and lymphocyte separated according to the method described by (Boyum, 1976). Lymphocyte proliferation was determined by MTT assay and trypan blue exclusion assay for both control and breast cancer human lymphocytes.

Statistical analysis:

Statistical analysis of data was carried out by using one way analysis of variance (ANOVA) followed by homogenous subsets (Duncun[a]) at confidence level of 5% (0.05) using the Statistical Package for the Social Science (SPSS) version8. Duncan's multiple range tests were used to compare between means of treatments according to Walter and Duncan (1969) at probability 5%.

3. Results and Discussion

In vitro "cell culture" experiments

The effect of mycelia ethanolic extract on EAC viability by Trypan blue exclusion test:

As shown in data that have been summarized in the table **(1)** and figures (1 and 2), viability was measured and expressed as the survival fraction compared with untreated control cells. Ehrlich cells were treated with concentrations (400, 200, 100, 50 and 25µg/ml) of the ethanolic extracts of Lentinus edodes LC2141 and Lentinus edodes

LC202 for 24 h. percent of dead cells increased by increasing concentration. At concentration 200µg/ml, the viability percentage in comparison with control was the lowest.

Table (1): The effect of *Lentinus edodes LC2141* and *Lentinus edodes LC202* mycelia ethanolic extract on EAC viability at 24 hrs of exposure determined by Trypan blue exclusion test.

Concentration (µg/ml)	*Lentinus edodes LC2141*		*Lentinus edodes LC202*	
	Viability (control %) (24 hrs exposure)	% of dead cells (24 hrs exposure)*	Viability (control %)(24 hrs exposure)	% of dead cells (24 hrs exposure)*
25	94.60	5.40^{e}	96.80	3.20^{e}
50	72.00	28.00^{d}	64.50	35.50^{d}
100	36.60	63.40^{c}	38.70	61.30^{c}
200	15.20	84.80^{a}	20.50	79.50^{a}
400	22.60	77.40^{b}	26.90	73.10^{b}

*: Means in the same column with different letters have significant differences between each other.

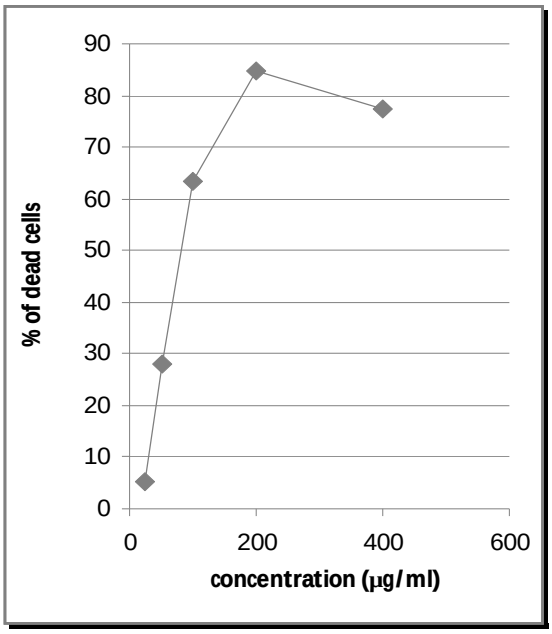

Fig. 1: Dose response curve on the effect of *Lentinus edodes LC2141* ethanolic extract on EAC viability at 24 hrs of exposure determined by trypan blue exclusion test.

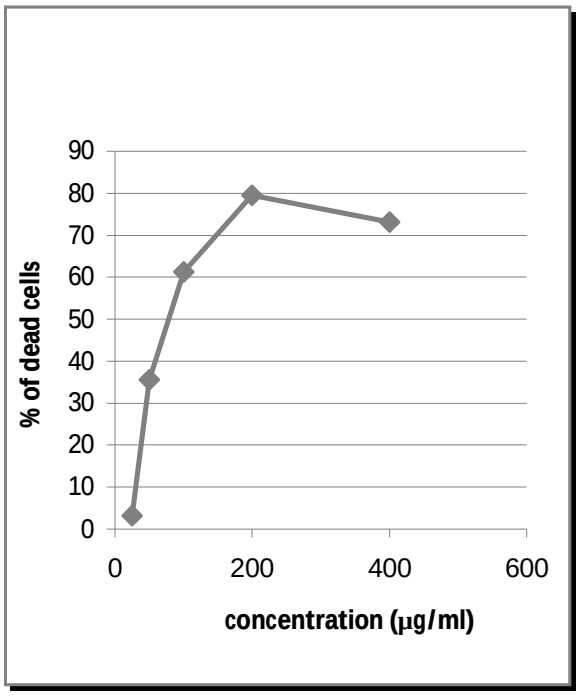

Fig. 2: Dose response curve on the effect of *Lentinus edodes LC202* ethanolic extract on EAC viability at 24 hrs of exposure determined by trypan blue exclusion test.

Anti-tumor activity against human breast cancer cell line (MCF7) and human hepatocellular carcinoma cell line (Hep-G2):

cytotoxicity was measured and expressed as the survival fraction compared with untreated control cells. The possible anti-proliferative effect of ethanolic extract of both Lentinus edodes LC2141 and Lentinus edodes LC202 was studied on the growth of MCF7 cell line after incubation for 24 h as shown in Fig. (3 and 4). The treatment of MCF7 cells with different concentrations of ethanolic extract of both Lentinus edodes LC2141 and Lentinus edodes LC202 dramatically inhibited the cell growth in a dose-dependent manner, with high IC_{50} values of 132.05µg /ml and 153.59 µg/ml, respectively. Also, the possible anti-proliferative effect of ethanolic extract of both Lentinus edodes LC2141 and Lentinus edodes LC202 was studied on the growth of Hep-G2 cell line after incubation for 24 h as summarized in Fig. (5 and 6). The treatment of Hep-G2 cells with different concentrations of ethanolic extract of both Lentinus edodes LC2141 and Lentinus edodes LC202 dramatically inhibited the cell growth in a dose-dependent manner, with high IC_{50} values of 953.1µg/ml and 1868.3µg/ml, respectively. Similar results were reported by Israilides et al., 2007 who found that Aqueous extracts of Lentinus edodes can significantly suppress the proliferation of cancer cell line MCF-7 in vitro. This is reflected by the comparative low IC_{50} values and the simultaneous higher IC_{50} values on normal cells. L. edodes mushroom water extracts are more cytotoxic than mycelial aqueous extracts. Methanolic extracts of either mushroom or mycelia of L. edodes do not exhibit any inhibitory (cytostatic) effect on MCF-7 cancer cell line supports the direct cytostatic/cytotoxic action of the L. edodes extracts on cancer cells, which is in parallel action with its host-mediated antitumor activity.

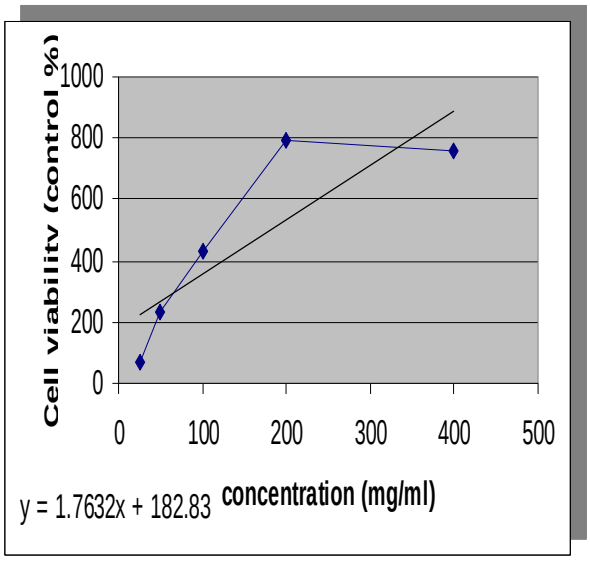

Fig. 3: Cell viability of breast carcinoma cell line MCF7 with ethanolic extract of both *Lentinus edodes LC2141* at concentration range from 400 to 25 µg/ml.

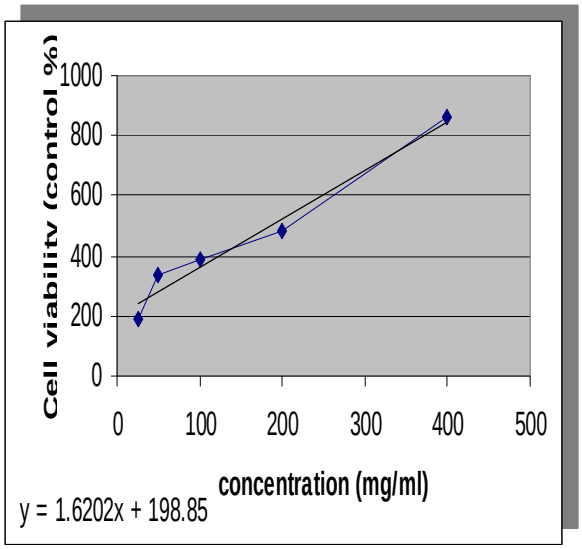

Fig. 4: Cell viability of breast carcinoma cell line MCF7 with ethanolic extract of both *Lentinus edodes LC202*at concentration range from 400 to 25 µg/ml.

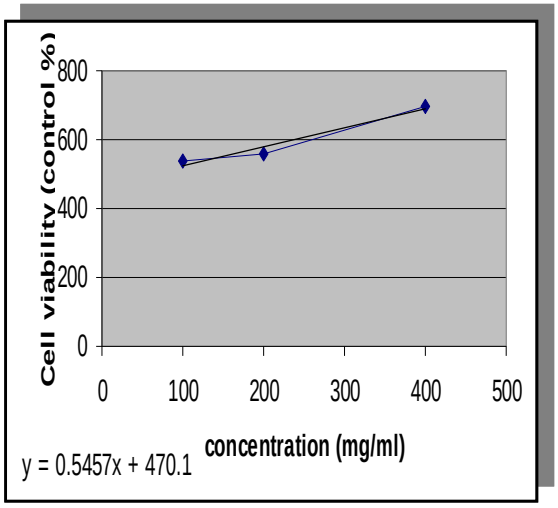

Fig. 5: Cell viability of liver carcinoma cell line Hep-G2 with ethanolic extract of both *Lentinus edodes LC2141*at concentration range from 400 to 100 µg/ml.

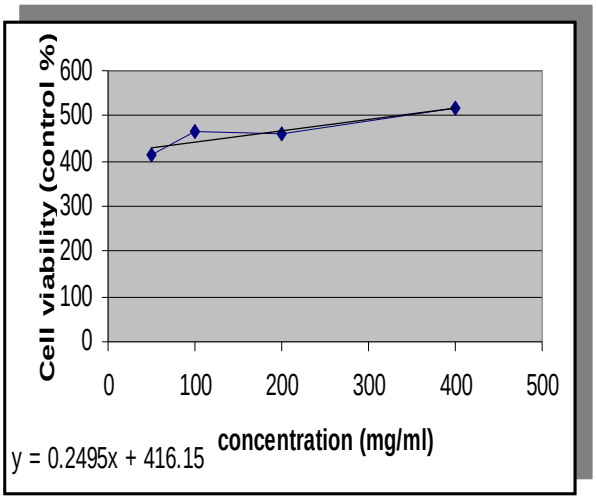

Fig. 6: Cell viability of liver carcinoma cell line Hep-G2 with ethanolic extract of both *Lentinus edodes LC202*at concentration range from 400 to 50 µg/ml.

The effect of the ethanolic extract from the two different strains of Lentinus edodes on lymphocyte proliferation:

Lymphocyte viability and proliferation were determined by trypan blue exclusion assay and MTT assay. Normal and breast cancer patient human lymphocyte was assayed. Proliferation was measured and expressed as count and absorbance compared with untreated control cells. The possible proliferative effect of ethanolic extract of both Lentinus edodes LC2141 and Lentinus edodes LC202 was studied on the growth of lymphocyte after incubation for 24 h. The results represented in Fig. (7 and 8) showed that the treatment of control human lymphocyte cells with different concentrations of ethanolic extract of Lentinus edodes LC2141 and Lentinus edodes LC202 resulted in dramatically increase in the lymphocyte proliferation in a dose-dependent manner. The highest count and absorbance was observed at concentration 400mg/ml. Also, treatment of lymphocyte cells of control human with the combination of the two ethanolic extract of strain Lentinus edodes LC2141 and strain Lentinus edodes LC202 showed significant increase in cell number and lymphocyte proliferation in a dose-dependent manner. The results represented in Fig. (48 and 49) showed that the treatment of lymphocyte of breast cancer patient with different concentration of ethanolic extracts of Lentinus edodes LC2141 and Lentinus edodes LC202 mycelia showed a significant increase in cell number and cell proliferation in a dose-dependent manner. Treatment with concentration 400µg/ml showed the highest increase in lymphocyte proliferation in comparison with control. Israilides et al., 2007 who found that L. edodes extracts supports the direct cytostatic/cytotoxic action of on cancer cells, which is in parallel action with its host-mediated antitumor activity. Furthermore, it was demonstrated that L. edodes can act as an immunomodulator to augment the proliferative response of rat thymocytes to T mitogens in vitro, indicating another mechanism for immunostimulatory activity. Overall there seems to be a therapeutic advantage in using L. edodes extracts orally administered instead of a single substance like Lentinan given intravenously. In addition, Nitha et al., 2007 reported that the ethanolic extract of M. esculenta mycelium is also found to possess significant antitumor activity against both ascites and solid tumour. The results indicate that the extract possessed both curative and preventive properties against solid tumour in a dose-dependent manner. The extract is also significantly effective against ascites tumour. These results suggest that M. esculenta mycelia contain compounds that may modulate tumourigenesis at different stages or may act at the same stage. Polysaccharide isolated from the fruiting bodies of M. esculenta has been reported to exhibit immunostimulatory activity (Duncan et al., 2001). Similarly, Lobanok et al., 2003 showed that the submerged mycelium and fruit bodies of L. edodes contain significant amounts of biologically active substances and exhibit immunomodulatory activity.

Table (2): The effect of *Lentinus edodes* ethanolic extracts on normal human lymphocyte proliferation "control group" at 24 hrs of exposure determined by Trypan blue exclusion test and MTT assay.

Concentration (µg/ml)	Ethanolic extract of *Lentinus edodes LC2141*mycelia		Ethanolic extract of *Lentinus edodes LC202* mycelia		Combination of the two ethanolic extract of strain *Lentinus edodes LC2141* and strain *Lentinus edodes LC202* mycelia	
	Count*	Absorbance *	Count *	Absorbance *	Count*	Absorbance *
Control	**5050^{c}**	**0.55^{c}**	**5050^{d}**	**0.55^{d}**	**5050^{d}**	**0.55^{d}**
100	**4166^{d}**	**0.53^{d}**	**6633^{c}**	**0.65^{c}**	**5425^{c}**	**0.83^{c}**
200	**6516^{b}**	**0.62^{b}**	**8410^{b}**	**0.89^{b}**	**6966^{b}**	**0.88^{b}**
400	**7523^{a}**	**0.74^{a}**	**16466^{a}**	**0.97^{a}**	**8600^{a}**	**0.95^{a}**

*****: Means in the same column with different letters have significant differences between each other.

Table (3): The effect of *Lentinus edodes* ethanolic extracts on breast cancer patient lymphocyte proliferation at 24 hrs of exposure determined by Trypan blue exclusion test and MTT assay.

Concentration (µg/ml)	Ethanolic extract of *Lentinus edodes LC2141*mycelia		Ethanolic extract of *Lentinus edodes LC202* mycelia	
	Count*	Absorbance *	Count*	Absorbance *
Control	8500^{e}	0.02^{e}	8500^{e}	0.02^{e}
50	9900^{d}	0.31^{d}	9250^{d}	0.16^{d}
100	10450^{c}	0.43^{c}	10120^{c}	0.21^{c}
200	14750^{b}	0.50^{b}	12650^{b}	0.23^{b}
400	22275^{a}	0.59^{a}	16850^{a}	0.26^{a}

*****: Means in the same column with different letters have significant differences between each other.

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Measurement of Scattered Radiation Dose to The Eyes, Breasts and Gonads of Patients During External Beam Radiation Therapy.

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Abstract: In Nigeria data on radiation doses to critical organs outside primary radiation beams during radiotherapy is sparse therefore the extent to which other parts of patient's body are protected during treatment could not be quantified. In clinical studies on measurement of radiation dose to critical structures, even though the doses are relatively low, have been associated with cardiac toxicity and increased risk of secondary cancer. This study is aimed at measuring scattered radiation to the eyes, breasts and gonads, of patients during Cobalt-60 external beam radiotherapy. Thirty patients with malignancy in the abdomen, breast, cervix and head and neck who consented to participate were studied. Scattered radiation was measured with thermoluminescence dosimetry (TLD) using calibrated Lithium Fluoride (LiF) phosphor and TLD Reader, Harshaw 4500. Scattered radiation dose to the eyes, breast and gonads from the treatment fields considered are: Abdomen (0.46 ± 0.10 Gy, 0.52 ± 0.10 Gy and 0.76 ± 0.50 Gy); Breast (0.58 ± 0.10 Gy, 1.10 ± 0.40 Gy and 0.50 ± 0.10 Gy); Head and neck (1.42 ± 1.10 Gy, 0.45 ± 0.10 Gy and 0.49 ± 0.10 Gy); Pelvis (0.50 ± 0.10 Gy, 0.48 ± 0.10 Gy and Nil). Gonads were not measured during irradiation of pelvic region. In this preliminary study, the scattered doses to the critical organs were found to be higher than the radiation level (0.1 Gy) at which cancer risk is considered unlikely. Further study is aimed at exploring treatment approach that would reduce scattered dose to the bearest minimum.
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Keywords: Scattered radiation, Critical organ dose, Cancer patient, External Beam Radiation Therapy

Introduction

Radiation therapy has been in use as cancer treatment for more than 100 years, with its earliest roots traced from the discovery of x-rays in 1895 by Wilhelm Roentgen[1]. Radiation therapy could be administered by teletherapy or brachytherapy. The main goal of radiation therapy is to deliver maximum percentage of prescribed dose to the tumour volume while the surrounding healthy structures (or critical organs) receive as low as reasonable achievable doses. This makes it crucial in radiation therapy to know the exact doses received by critical organs, which are outside the primary radiation beams[2] and make sure that their tolerance is not exceeded. While doses delivered outside the field are small relative to the primary field doses, they are still of clinical interest because they are given to a large parts of the body where there is potential for residual long term adverse effects[3]. It has been reported in literature[4] that patients who received external beam radiotherapy had significantly higher risk of developing cancer in organs adjacent and distant to the treatment volume than those treated with brachytherapy. Moon et al[5] reported male patients, who developed second cancer in the bladder, rectum, cecum, lung and brain; five years (latency period) after radiotherapy to the prostate. Despite critical organs' high risk attributed to external beam radiotherapy, its cost benefit to patients can not be overemphasized. External beam radiotherapy improves 5- year disease free survival and local tumour control of patient, who presented at the clinic early and for those patients who presented late, external beam radiotherapy is giving to relief pain, stress and for general improvement of quality of life. This is by far cheaper than when radiotherapy is not administered.

The critical organs selected for this study have high sensitivity to radiation damage and this was shown in the latest report (ICRP, 103) of the International Commission on Radiological Protection, ICRP[6], where the tissue weighting factors of the eyes, breast and gonads were slightly modified on grounds of high susceptibility to radiation damage.

It is generally accepted that low doses of ionizing radiation to healthy critical organs could induce cancer. In Nigeria, the burden of cancer is gradually increasing. The World Health Organization reported an estimation of 100,000 new cancer cases (in the year 1990) diagnosed in the country and gave a projection of 300,000 and 500,000 by the year 2010 and 2015 respectively[7]. Till date, there is no substantial data in Nigeria to support the development of secondary malignancies following radiation therapy.

There are many variables that could influence the occurrence of secondary cancers in

patients who have received radiation therapy. A clinically diagnosed cancer patient has a higher probability to develop second cancer at any site than a non cancer patient. Also, there are cancers, such as Retinoblastoma, which are known to be markers for genetic susceptibility to other cancers[8]. Administering radiation therapy at a younger age could increase the probability of developing a second cancer[9, 10]; other factors include gender, diet (smoked food), cigarette smoking and chemotherapy agents. The manifestation of secondary cancer is time dependent. For solid cancer, the latency is of the order of decades[11] while leukemia may be less than 5 years.

In a radiation therapy centre with Cobalt-60 machine, the radioactive source is expected to be replaced after five years of installation but in most centres with limited funding such as our centre, the radioactive source is usually over spent. This implies that patient spent longer treatment time and the possibility of exposure to scattered radiation becomes higher.

At our centre, where this study was conducted, the radioactive source is in its second half life and the extent to which patient are exposed to scattered radiation is not known. Hence, this present study is carried out to measure scattered radiation doses to critical organs that are outside the treatment field during radiation therapy.

Materials and Method

This study was carried out in the Department of Radiotherapy, University College Hospital (UCH), Ibadan between March 2008 and September 2009. The durability of Cobalt-60 machine at this centre makes it the most functional Radiotherapy centre in the whole of Nigeria. Also, this centre is always participating in the IAEA/WHO postal dose quality audit for Co-60 and megavoltage x-ray beams organized by the International Atomic Energy Agency (IAEA), Vienna. The TLDs results for our centre are usually within the acceptable limit of 5 %.

Following the UCH/UI ethical review committee's approval to conduct this study, the consent form to participate was explained and distributed to patients at the Radiotherapy clinic. Thirty cancer patients, who consented to participate, were included in the study. Their weights and heights were measured with Weylux scale, model 424. It has dual weighing scales for measuring both height and weight. In order to facilitate post treatment follow-up of patients, their contact addresses and mobile phone numbers were documented.

The treatment machine used for all the patients was Cobalt-60 machine, model Theratron 780 C. It was manufactured by the Atomic Energy of Canada and installed in the year 1987. This machine is a rotational unit with beam stopper and the treatment head is shielded with depleted Uranium. The piston within the treatment head moves the source to "on" and "off" positions, electronically. It has a collimator, which shapes the radiation beam to the desired treatment field. The minimum treatment square field size obtainable from the machine is 4 cm x 4 cm and the highest is 35 cm x 35 cm at source to skin distance (SSD) of 80 cm.

The radioactive Cobalt-60 source at the time of this study was installed in the year 2002 and its activity at the time of installation (March, 2002) was 303.7 TBq. The source, which is encapsulated in a stainless steel, is 2 cm x 2 cm in size. The monthly calibration of the source for determination of its dose-rate (cGy/min) is based on the IAEA protocol[12]. The treatment techniques adopted at the centre for patient treatment is fixed SSD of 80 cm and the daily workload on the machine is about 100 patients.

The scattered radiation dose to critical organs was measured with TLD system comprising of LiF (LiF-100) cards and Harshaw (Thermo Electron, USA) dual channel TLD reader (model 4500). Each TLD card consists of two 0.4 cm diameter LiF chips. The TLD reader has an in-built computer system to facilitate accurate dose assessment after appropriate calibration and it is programmed to anneal TLD chips automatically for fresh use after each measurement.

The calibration of the TLD system was carried out by irradiating a set of ten TLD cards; each consisting of two freshly annealed LiF chips A and B, to known doses (1 – 4 Gy) in the Cobalt-60 machine, acting as a standard source. The standard deviation, which represents the spread in the TLD response of each chip during the calibration process, was 1.3 % and 1.1 % respectively, showing a very good precision. The mean TLD reader response, R, for each TLD card was plotted against the standard absorbed dose, D (Gy). The calibration lines for chips A and B with correlation coefficients 0.9983 and 0.9834, respectively are shown in fig. 1. The lines were fitted with equations below:

$$R_A = 0.953\ D - 0.511 \quad (1)$$

$$R_B = 0.738\ D - 0.263 \quad (2)$$

Where, D, is the actual radiation dose that gives response R_A in chip A and R_B in chip B.

A set of three annealed TLD detector chips, labeled for each organ was used for each patient. Care was taken to ensure that the chips were placed

within the centre of each organ of interest as soon as treatment set-up was accomplished. The TLD chips were fixed at this position with the help of paper tape. In order to obtain accumulated dose received by critical organs throughout the period of treatment, the same TLD chips, duly marked for that particular organ was used. The treatment period for all patients was between 3 to 6 weeks. The exposed detectors were thereafter placed in a folder customized for each patient and kept in a box provided in a radiation free room. Some unexposed annealed TLD detectors chips were also kept in this room as control detectors.

At the completion of all treatment fractions, the exposed TLD chips were read with the calibrated TLD system. The scattered dose D received by each of the critical organ was calculated as the mean of the slightly varying dose values obtained from chips A and B using equations 1 and 2. In all dose calculations, the overall environmental effects of storage and handling of TLD chips were taken into consideration by subtracting the dose value obtained from the control detector from the mean absorbed dose in the exposed TLD detector.

The results of the scattered radiation to each of the critical organ with respect to the treatment fields were analysed and presented in tables and figure.

Results

A total of thirty patients who consented to participate were considered in this study. Out of these, 20 (67%) were females and 10 (33%) were males; their mean age, weight and height was 48 ± 20 years, 57 ± 17 kg; and 156 ± 23 cm respectively (Table 1). All of them completed their treatment within 6 weeks of enrollment.

The parts of the patients' body where treatment was administered were abdomen, breast, pelvis and head and neck. The head and neck region constitutes treatment to the brain, neck, parotid and tongue. Out of all these treatment sites, the most frequent was breast 11 (37%), followed by the head and neck 10 (33%), pelvis 7 (23%) and abdomen 2 (6 %) (Table 2). The mean field sizes selected for treatment was obtained from the approved treatment planning of the target volume. The largest field sizes obtained in this study was 23 ± 2 cm^2 and it was selected for abdomen, this was followed by 16 ± 1 cm^2 for pelvis, 14 ± 1 cm^2 for breast and 12 ± 3 cm^2 for head and neck (Table 2).

The mean distance between the critical organs (eye, breast, gonads) considered in this study and the centre of the treatment field varies with respect to the treatment site. The mean distance of the (eye, breast and gonads) from the centre of the respective fields are: Abdominal treatment field (49 ± 3 cm; 24 ± 2 cm and 20 ± 2 cm); Breast treatment field (24 ± 1 cm; 15 ± 3 cm and 48 ± 2 cm); Pelvis treatment field (61 ± 8, 43 ± 3 and Nil); Head and neck treatment field (10 ± 2 cm; 31 ± 3 cm and 73 ± 6 cm). Gonad was considered to be within the pelvic treatment field hence, it was not measured during pelvic treatment (Table 3).

The mean scattered radiation doses to the studied critical organs during radiation therapy vary with respect to the treatment site. The scattered radiation dose to the eye, breast and gonads from different treatment sites are: Abdomen (0.46 ± 0.10 Gy, 0.52 ± 0.10 Gy and 0.76 ± 0.50 Gy); Breast (0.58 ± 0.10 Gy, 1.10 ± 0.40 Gy and 0.50 ± 0.10); Pelvis (0.47 ± 0.10 Gy, 0.48 ± 0.10 Gy and Nil); head and neck (1.42 ± 1.10 Gy, 0.45 ± 0.10 Gy and 0.49 ± 0.10 Gy) (Table 4).

Table1: Patients' Demographic data

Parameter	No. of patient (%)
Sex: Female Male	20 (67 %) 10 (33 %)
Age, years Mean ± Std. dev	 48 ± 20
Weight, kg Mean ± Std. dev	 57 ± 17
Height, cm Mean ± Std. dev	 156 ± 23

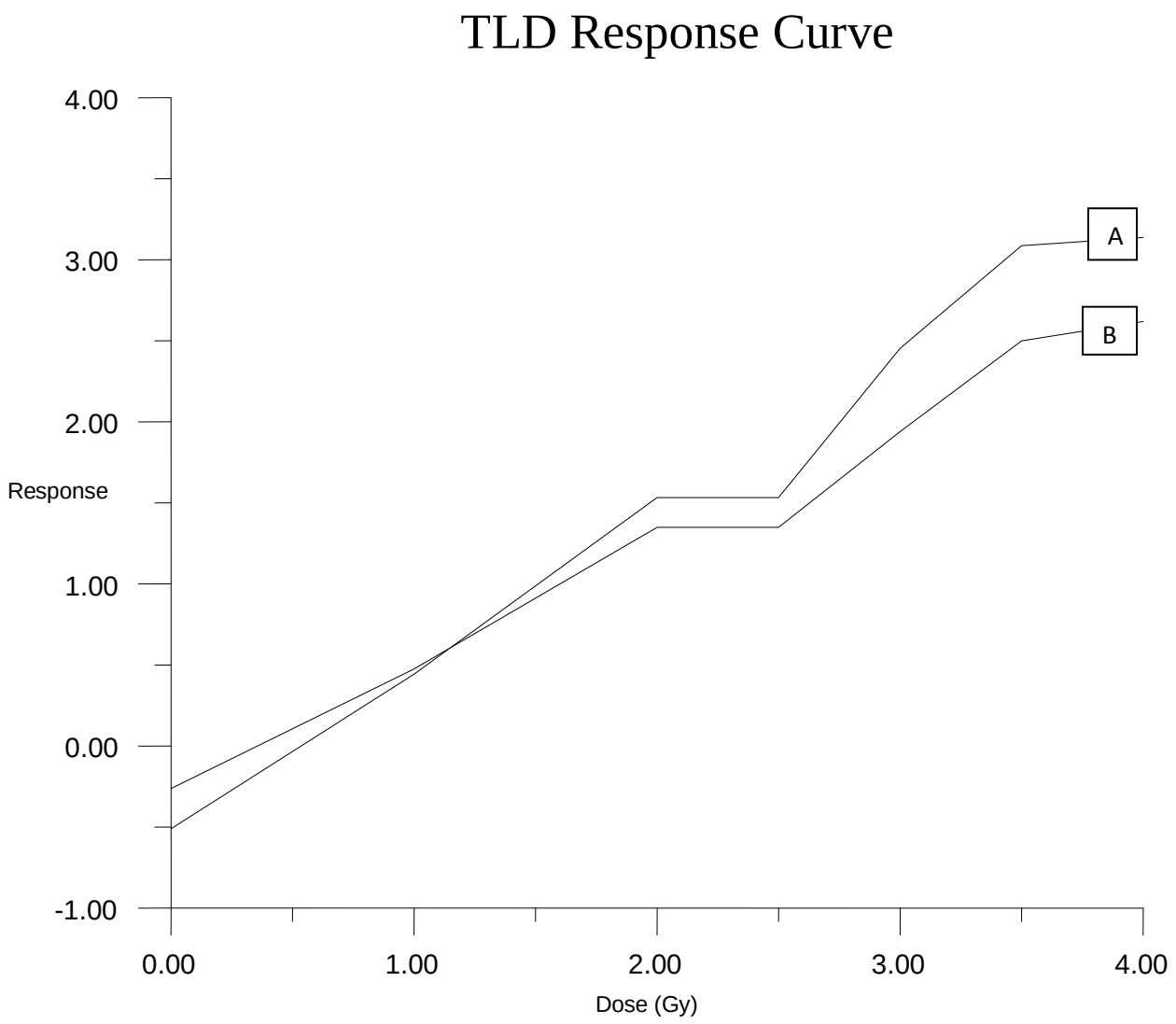

Fig. 1: Calibration Curve for chips A and B

Table 2: Number of Patients with respect to Treatment Site

Treatment Site	Mean field size, cm^2	Number of patient (%)
Head & Neck	12 ± 3	10 (33%)
Breast	14 ± 1	11 (37%)
Abdomen	23 ± 2	2 (6%)
Pelvis	16 ± 1	7 (23%)

Table 3: Mean distance of critical organ from the centre of treatment field

	Abdomen	Breast	Pelvis	Head & Neck
Eye	49 ± 3 cm	24 ± 1 cm	61 ± 8cm	10 ± 2 cm
Breast	24 ± 2 cm	15 ± 3 cm	43 ± 3 cm	31 ± 3 cm
Gonads	20 ± 2 cm	48 ± 2 cm	-	73 ± 6 cm

Table 4: Mean Scattered Dose to critical organ with respect to the treatment site

	Abdomen	Breast	Pelvis	Head & Neck
Eye	0.46 ± 0.10 Gy	0.58 ± 0.10 Gy	0.47 ± 0.10 Gy	1.42 ± 1.10 Gy
Breast	0.52 ± 0.10 Gy	1.10 ± 0.40 Gy	0.48 ± 0.10 Gy	0.45 ± 0.10 Gy
Gonads	0.76 ± 0.50 Gy	0.50 ± 0.10 Gy	-	0.49 ± 0.10 Gy

Discussion

The gender bias observed in this study reflected the distribution of patients at our radiotherapy clinics where, female specific malignancy namely, breast and cervical cancer predominates. In Dhaka, Bangladesh[12], where similar study was performed, the incidence of female malignancy, that is, breast cancer and cervical cancers, was 29 % and 37 % respectively, whereas in our centre and among the studied patients, the percentage of breast cancer and cervical cancer was 37 % and 23 % respectively. The mean age (years) of patients was 48 ± 20. All of them completed their treatment within 5 weeks of enrollment. This period is similar to what is practiced in Bangladesh, a developing country like Nigeria, where treatment of cancer of any organ took a period of 4 – 5 weeks duration. The part of the patients, where treatment was administered, was abdomen, breast, pelvis and head and neck. The head and neck region constitutes treatment to the brain, neck, parotid and tongue.

The amount of scattered radiation that is present during a particular treatment set-up is a function of treatment field sizes and this is derived from the approved planning target volume. There are various field sizes selected for patients during this study. The minimum equivalent square field size at the surface of the patient was 144 cm^2 and the maximum was 529 cm^2. It was reported by Miah et al[12] that different parts of cancer patients received scattered radiation dose in increasing order of field sizes during radiotherapy.

In this study, it was found that the maximum field size (529 cm^2) selected for abdominal treatment region did not result in higher dose to the studied critical organs. This implies that, apart from field sizes, there are other factors that could determine the amount of scattered radiation to different parts of patient during radiotherapy and one of these factors is distance. According to the inverse square law, the intensity of radiation at a particular point varies inversely as the square of its distance from the radiation source.

Among the studied critical organs, the eye is the closest (10 ± 2 cm) organ to the centre of the head and neck treatment field. This explains why the eye received the highest dose (1.42 ± 1.10 Gy) during head and neck treatment. The breast is the closest (15 ± 3 cm) organ to the centre of the contra-lateral breast treatment field and the highest radiation dose to the breast (1.10 ± 0.40 Gy) was obtained from this treatment field. The gonad is the closest (20 ± 2 cm) organ to the centre of the abdominal treatment field and the highest radiation dose (0.76 ± 0.50 Gy) to gonads was obtained from this treatment field. Miah et al also reported that scattered radiation dose to different organs varies with the height of the patient.

The induction of cancer and other stochastic health effects of ionizing radiation have not been observed consistently at low doses (≤ 0.1 Gy). This is because the existence of a risk at such level is so low that it could not be detected by current epidemiological data and method. However, the health Physics Society[13] recommended assessments of radiogenic health risks of radiation dose estimated above 0.1 Gy. In a study conducted by Wolfgang et al[14], it was found that significant number of secondary cancer was induced at the site, outside the treatment field, that received radiation dose of less than 6 Gy. This implies that the amount of scattered radiation dose measured in the critical organs considered in this study has potential to induce secondary cancer between 5 – 10 years after radiation therapy. The latency period for the manifestation of most secondary cancer is about 5 – 10 years.

In general, to minimize scatter radiation to critical organs, radiation therapy centre should choose the field sizes without compromising the tumour volume and should carefully make use of multi-leaf collimator if available.

Conclusion

The highest scattered dose (1.42 ±1.10 Gy) measured among the studied critical organs (eye) was found to be higher than the threshold (0.1 Gy) for cancer induction but far less than the maximum dose (9.096 ± 25 Sv) obtained in the similar study conducted in Dhaka Bangladesh. While following up the patients for possible occurrence of secondary cancer in the studied organs, patients would be

counseled to avoid as much as possible such factors that could dispose them to secondary cancer.

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Antitumor effects of osmium (II) and ruthenium (II) bipyridine complexes containing the acetylacetonato ligand against the growth of Eherlich Ascites Cell Carcinoma

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Abstract: The development of metal-based antitumor drugs has been stimulated by the clinical success of cisplatin and its analogs and by the clinical trials of other platinum and ruthenium complexes with activity against resistant tumors and with reduced toxicity of normal cells. In the present study, the newly synthesized [OsII(bpy)2(acac)](PF6) and [RuII(bpy)2(acac)](PF6) complexes were tested for their cytotoxicities against Eherlich Ascites Cells (EAC) carcinoma, for their superoxide dismutase (SOD)- like activities and for their cytoprotective effects of the normal human red blood cells (RBCs) against photo-irradiation induced by UV-lamb in the presence of m-chloroperbenzoic acid, in vitro. Also, their killing capabilities for the growth of EAC carcinoma in vivo and the measurements of the biochemical changes accompanying such killing were investigated. The in vitro study revealed that the average cytoprotective effects of RBCs, SOD- like activities and the cytotoxicity of EAC by similar concentrations of rutheniumII (RuII) and osmium (OsII) complexes were 91.5%, 89.5% and 90% and 98.3%, 89.8% and 92.8%, respectively. In the in vivo study, the mean SOD activities in both RBCs and liver of the tumorized mice were statistically significantly inhibited compared with those of the control group ($P<0.0001$). After treatment either with RuII and OsII complexes, the activities of the latter enzyme in RBCs and liver were elevated ($P<0.0007$, $P<0.04$ and $P<0.09$ and $P>0.05$, respectively). Also, the mean activity of catalase was inhibited in liver tissues in the tumorized animals and re-elevated after complexes treatment. In addition, treatment with these complexes elevate the glutathione (GSH) levels in liver tissues of the tumorized and normal mice with simultaneous reduction in the mean levels of the corresponding values of malondialdehyde. On the other side, the mean levels of triglycerides and cholesterol were reduced in serum but the mean levels of total lipids and total proteins were elevated in liver tissues after treatment. Moreover, the mean levels of DNA and RNA were significantly elevated in liver tissues of the tumorized animals and significantly reduced after treatment of the tumorized mice with the complexes. The previous results reflect tumor growth inhibition and prevention of EAC carcinoma metastasis into the liver. In conclusion, RuII and OsII bipyridine complexes are promising free radical scavengers in phototherapy and may be used as anti-tumor and anti-metastatic agents in the clinical trials in the future.
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1. Introduction

Transition-metal-based compounds constitute a discrete class of chemotherapeutics which were widely used in the clinic as antitumor and antiviral agents. However, drug resistance and side effects have limited their clinical utility (Chen et al., 2003). These limitations have prompted a search for more effective and less toxic metal-based antitumor agents. The wide range of coordination numbers and geometries, accessible redox states, thermodynamic and kinetic characteristics, and the intrinsic properties of the cationic metal ion and ligand itself offer the medicinal chemist a wide spectrum of reactivities that can be exploited. Although metals have long been used for medicinal purposes in a more or less empirical fashion (Thompson and Orvig, 2006), the potential of metal-based anticancer agents has only been fully realized and explored since the landmark discovery of the biological activity of cisplatin (Jung and Lippard, 2007). Recently, some of the efforts have been directed to ruthenium complexes. This is because these complexes demonstrate similar ligand exchange kinetics to those of platinum (II) while displaying only low toxicity. In addition, the redox potential between the different accessible oxidation states occupied by ruthenium complexes enables the body to catalyze oxidation and reduction reactions, depending on the physiological environment (Dougan et al., 2008). Moreover, the biochemical changes that accompany cancer alter physiological environment, enabling ruthenium complexes to be selectively activated in cancer tissues (Peacock and Sadler, 2008). Also, Brabec and Nováková in 2006 found that, ruthenium compounds bind to DNA affecting its conformation differently than cisplatin and its analogues. In addition, non-nuclear targets, such as

the mitochondrion and the cell surface, have also been implicated in the antineoplastic activity of some ruthenium complexes. Brabec and Nováková (2006) added that, some chemical properties make ruthenium compounds well suited for medicinal applications and as an alternative to platinum antitumor drugs in the treatment of cancer cells resistant to cisplatin.

Superoxide radical (O_2^{-}) is produced at any location where an electron transport chain is present, and hence O_2 activation may occur in different compartments of the cell. Therefore, SOD as a defense mechanism, is found in all subcellular locations (Alscher et al., 2002). It was showed that, a shift to a more oxidative state might result in uncontrolled lipid peroxidation, protein oxidation and ultimately cell death (Halliwell, 1999). For these reasons, the search for novel organometallic complex that defend against ROS and acting as anti-tumor agents must be the target of many studies including this study. In this study, the biological effects of osmium (II) complex with the structure [OsII(bpy)2(acac)](PF6) and ruthenium (II) complex with the structure [RuII(bpy)2(acac)](PF6) where (bpy = 2,2'-bipyridine, acac)= acetylacetone, and PF6= hexafluorophosphate) were evaluated both in vivo and in vitro. These complexes were tested in vitro for their cytotoxicities against Eherlich Ascites Cells (EAC) carcinoma, for their SOD-like activities and for their cytoprotective effects of the normal human red blood cells (RBCs) against photo-irradiation induced by UV-lamb in the presence of m-chloroperbenzoic acid. Also, their killing capabilities for the growth of EAC carcinoma and the measurements of the biochemical changes accompanying such killing were investigated in vivo.

2. Materials and methods

A-Materials:

1-Animals and tumor cell line: Adult female Swiss common bred albino mice purchased from Theodore Bilharz Institute, Giza, Egypt, with an average body weight of 25 to 30 gm were used. Ehrlich ascites carcinomas (EAC), a mammary origin, were used to give liquid tumor. These cells were kindly supplied by Doctor C. Benckhujsen, Netherland Cancer Institute, Amsterdam, Netherland. The tumor line was maintained in the Oncology Unit at the Egyptian National Cancer Institute, by serials of intraperitoneal (I.P.) transplantation in female Swiss Albino mice at 7 to 10 days intervals since 1982 up till then and was kindly supplied by such Institute. The mice were randomly divided into six groups (eight mice each) namely; normal mice (group 1), normal mice complex-treated (group 2), normal mice dimethy sulphoxide (DMSO)-treated (group 3), tumor bearing-complex treated mice (group 4), tumor-bearing-DMSO treated mice (group 5) and tumor-bearing mice only (group 6). The mice of the last 3 groups were i.p. inoculated with 1×10^6 EAC cells to produce the liquid tumor. 24 hours after tumor inoculation, the mice of group 2 and group 4 were i.p. treated with the OsII or RuII complex with a daily dose of 10 mg/kg/day (1/5 of LD50) day after day for 14 days starting from the first day after tumor inoculation. The normal- complex treated mice's group was treated with the same complex's dose as that of group 4.

2-Collection of samples: One day after the last treatment, the ascitic fluids containing EAC cells were collected and their volumes were measured. Livers were quickly dissected, rinsed with isotonic saline and dried. Then, 10 % liver tissues homogenized in cold phosphate-buffer (w/v) were prepared. After the removal of the cellular depris via centrifugation, the supernatants were used for biochemical analysis. Blood samples were also collected by tail vein cutting and their sera were used for subsequent analysis.

B-Methods:

1-Source and synthesis of the complexes:
The new complexes, [RuII bpy)2L](PF6) and [OsII (bpy)2L](PF6) (where, RuII= ruthenium(II), OsII= Osmium(II), bpy= 2,2- bipyridine, L= acetylacetone and PF6= hexafluorophosphate) have been prepared and characterized by spectroscopic measurements and also investigated by cyclic voltammetry by El-Hendawy et al. (1997) and El-Hendawy (2011), respectively. These complexes were kindly provided by Dr/ Ahmed El-Hendawy, Faculty of Science (Damietta), Mansoura University, Egypt and were used in both the in vitro and in the in vivo treatment of EAC carcinoma in the present study.

2- In vitro study:

2-1-Preparation of red blood cells (RBCs) samples: Heparinized fresh blood samples were withdrawn from five healthy volunteers and centrifuged at 3000 rpm/min. The pellets were separated and washed 3 times with phosphate buffered saline (PBS, pH 7.4, 0.01 M containing 0.135 NaCl) and centrifuged again. The cells were resuspended in PBS and 1x 106 cells were used in fluorohemolysis and the antihaemolytic effects of the RuII and OsII complexes in vitro.

2-2-Effects of m-chloro-perbenzoic acid (m-CPBA) on the fluorohemolysis and evaluation of the antihaemolytic effects of the complexes: To test

the antihaemolytic effect of the Ru(II) and Os(II) complexes, a photohaemolytic damage of normal human red blood cells (RBCs) was performed by exposing these cells to a UV-lamp in the above PBS containing 200 µM of m-CPBA (the acid concentration which gave the maximum haemolytic effect) for 30 minutes. After the completion of the incubation period, the tubes were centrifuged and the absorbance of the supernatants, as a measure of the photohaemolytic effect, was read at 546 nm in each case (Dacie and Lewis, 1984).

2-3- SOD and SOD-like activities of the complexes: SOD-like activities of the complexes and that in liver haemogenate were assayed by the method of Dechatelet et al. (1974). Simply each complex was added to a mixture of nitro blue tetrazolium salt and NADH in a pyrophosphate buffer (pH 8.3).The changes in the optical density was recorded/minute after the addition of phenazine methosulphate. The percent of inhibition of the colour development was calculated based on that of a control tube containing no complexes.

2-4-EACs cytotoxicity in vitro: The cytoxicity was determined using trypan blue exclusion by the method of MacLimans et al. (1957).

3- In vivo study:

3-1-Tumor volume: The tumor volumes were volumetrically measured in each case.

3-2-Antioxidants:

3-2-1-SOD and catalase activities in liver tissue haemogenate and SOD in RBCs: SOD activity in liver homogenate was assayed by Dechatelet et al. (1974) and that in RBCs was assayed by the procedure of Winterbourn et al. (1975). The catalase activity was determined according to Chance and Mackley (1955).

3-2-2-Glutathione (GSH) in liver tissue haemogenate and in RBCs and malondialdehyde (MDA) in liver tissues: GSH was determined in liver tissues and RBCs by the method of Beutler et al. (1963) but MDA was determined by the method of Stock and Donnandy (1971).

3-3-DNA and RNA contents of the liver tissues: The levels of DNA in liver tissues were evaluated according to Dische and Schwartezm (1937) and the RNA content was measured by the orcinol procedure of Mejbaum (1939).

3-4-Lipids profile and total proteins: The serum cholesterol was determined according to Richmond (1973) and the triglycerides were enzymatically hydrolyzed and determined according to the method of Fossati and Principe (1982). The total lipids and total proteins contents of liver tissues were determined by the methods of Knight et al. (1972) and Lowry et al. (1951), respectively.

3-5-Liver functions: Serum albumin was done according to the method of Doumas et al. (1971). Also, the activities of serum glutamic pyruvic transaminase and that of gamma-glutamyl transpeptidase (GGT) were determined by the method of Reitman and Frankel (1957) and by Szasz et al. (1969), respectively.

4-Statistical analysis: The biochemically collected data were characterized by their mean and standard deviations using instat software, version 2.03 (Graphed, USA). In addition, the student t-test was evaluated and the one-tailed P-values were also used for the statistical analysis of the results. The probability values at 0.05 up to more than 0.001 levels were considered statistically significant and 0.001 or less were considered highly significant (Snedecor and Cochran, 1969).

3. Results

1-Antihaemolytic, superoxide dismutase (SOD)-like activity and cytotoxicity effects of the complexes: The *in vitro* study revealed that the average cytoprotective effects of the complexes for the human RBCs from the photo-irradiative damage induced by UV-lamb in the presence of m-CPBA were 91.5% and 98.3 %. Also, the SOD-like activities and the cytotoxicity effects of the complexes for the viable EAC by similar concentrations of RuII and OsII complexes were 89.9% and 89.8% and 90 % and 92.8 %, respectively**.** In addition, the antihaemolytic and the cytotoxicity effects of the OsII complex were higher than those of the RuII complex **(Table 1).**

2-The activities of superoxide dismutase in red blood cells (SOD/RBCs) and the activities of both superoxide dismutase (SOD/Liver) and catalase in liver tissues: In the *in vivo* study, RuII and OsII complexes reduce the activities of SOD/RBCs, SOD/Liver and catalase in liver tissues and causes no change in the mean blood malondialdehyde (MDA) levels in the normal mice' group. On contrary, the mean activities of the former enzymes in the tumorized-non treated mice were statistically

significantly inhibited compared with those of the control group (P<0.0001). On the other hand, after treatment of the tumorized mice either with RuII and OsII complexes, the activities of these enzymes were re-elevated (P<0.0007, P<0.04 and P<0.0002 for RuII and P<0.09, P<0.13 and P<0.0005 for OsII, respectively (**tables 2 and 3**).

3-Glutathione reduced form (GSH) and malondialdehyde (MDA) in RBCs and in liver tissues: From **tables 2 and 3**, treatment with the divalent complexes elevates the mean GSH levels in liver tissues of the normal-complex treated compared to that of the normal nontreated group (P<0.0015 and P<0.0009, respectively) and in the tumorized-treated mice compared to that of the tumor nontreated group (P<0.0001 and P<0.014, respectively). In addition, simultaneous reductions in the mean MDA levels in liver tissues of the tumor-bearing mice compared to the tumorized-treated group were observed (P<0.009 and P<0.0001, respectively **and** tables 2 and 3).

4- Levels of DNA and RNA: The mean levels of DNA and RNA were significantly elevated in liver tissues of the tumorized mice compared with those of normal liver tissues (P<0.0001) and significantly reduced after complexes treatment compared with those of the tumorized non-treated mice (P<0.007 and P<0.01 for RuII and OsII complexes, respectively), a phenomenon which reflects inhibition of the tumor growth. In addition, OsII caused less damage of DNA of normal cells compared to RuII (P<0.05 and table 4 and 5).

5- Levels of lipids profile and the total proteins in liver tissues: Firstly, the treatments of normal mice with any of the complexes (OsII and RuII complexes, respectively) significantly reduce their mean serum levels of triglycerides (P<0.01 and P<0.33) and total lipids in their liver tissues (P<0.05 and P<0.03) compared with those of the normal control group. In addition, after killing of the tumor cells by the complexes, the mean serum levels of triglycerides (P<0.0001 in each case) and cholesterol in their sera were significantly reduced (P<0.04 in case of RuII only) but the mean levels of total lipids (P<0.08 and P<0.002, respectively) and total proteins (P<0.0001 in each case) were elevated in the same organ compared with those of the tumorized non-treated mice (**Table 4 and 5)**.

6- Liver function tests:

6-1- Serum albumin: The tumorized- non-treated mice showed significantly lowered albumin levels in their sera compared with that of normal mice (P<0.005), indicating a state of liver damage due to metastasis of EAC into such organ. In addition, complexes treatment did not affect serum albumin levels **(P<0.4** and **tables 6 and 7**).

6-2- Serum γ-GT and serum glutamic pyruvic transaminase (SGPT**):** As shown in tables 6 and 7, γ-GT mean activities are much elevated in sera of the tumorized non-treated mice than that of the normal controls (P<0.0001). In addition, the treatment with the complexes caused dramatic decrease in γ-GT activities compared with that of the tumorized non-treated mice (P<0.0001). On the other hand, DMSO did not affect the latter enzymes activities in the normal-DMSO treated animals. On the other side, the normal mice treated with the complexes showed reduction in the mean activities of catalase (P<0.01 in case of RuII complex) and slight elevations in the mean activities of SGPT compared with those of the normal control. Surprisingly, OsII complex is less toxic to normal liver cell compared with RuII one. This is because the mean activity of SGPT in normal OsII- treated mice is lowered than that of normal RuII- treated mice (P< 0.053 and **tables 6 and 7**).

6- Tumor volume: The mean volumes of the ascitic fluids after treatment of any of the complexes were highly significantly reduced (P<0.0001) compared with those of the tumorized non-treated mice **(Tables 6 and 7**).

4. Discussions

The field of medicinal inorganic chemistry is rapidly advancing. In particular organometallic complexes have much potential as therapeutic and diagnostic agents (Peacock and Sadler, 2008). The development of metal-based antitumor drugs has been stimulated by the clinical success of cisplatin in the treatment of resistant tumors and by the clinical trials of other platinum and ruthenium complexes showing reduced toxicities Brabec (2002). It is therefore of great interest, in this study, to understand the details of molecular and biochemical mechanisms underlying the biological efficacy of ruthenium and osmium complexes both *in vitro and in vivo*.

In the present study, two complexes of RuII and OsII with the same ligand were tested in vitro for their capabilities to prevent the photohaemolysis of human RBCs sensitized by m-CPBA via scavenging the produced free radicals, to mimic SOD activity and to kill EAC carcinoma. In this study, it was found that the complexes scavenge the free radicals produced from the photosensitization of m-CPBA

Table 1: Antihaemolytic effects, superoxide dismutase (SOD)-like activities and cytotoxicity of ruthenium and osmium bipyridine complexes.

Effects / Volume	Antihaemolytic effects©		SOD-like activities©		Cytotoxicity©	
	RuII	OsII	RuII	OsII	RuII	OsII
20 µL	90.3 %	97.7 %	78.2 %	76.9%	82 %	86 %
50 µL	91.3 %	98.1%	87.1 %	79.5 %	89 %	92 %
100µL	91.8 %	98.4 %	91.2%	96.2 %	91%	94 %
150 µL	91.9 %	98.6 %	93 %	96.6 %	94 %	96 %
200 µL	92.0 %	98.6 %	100%	100%	94%	96 %
Average effects %	91.5 ± 0.7	98.3 ± 0.4	89.9 ± 8.0	89.8 ± 10.8	90 ± 4.9	92.8 ± 4.1
Effects' range %	91.3 - 92	97.7 - 98.6	78.2 - 100	76.9 - 100	82 – 94	86 – 96

© = Values are the average of 5 different readings.

Table (2): Mean activities of superoxide dismutase in both red blood cells (SOD/RBCS) and liver tissues (SOD/Liver), catalase in liver tissues and the mean levels of both glutathione reduced form (GSH) and malondialdehyde in RBCs and in liver tissues of mice treated with Ruthenium bipyridine complex.

Parameters / *Group*	SOD/RBCS (U/0.01 gm haemoglobin)	SOD/Liver (% of inhibition/ 0.01 gm tissue)	Catalase (U/0.01 gm tissue)	GSH /RBCS (Mol/L packed cells)	GSH /Liver (mmol/ gm protein)	MDA/RBCs (Mol/ml packed cells $\times 10^{-5}$)
Normal	8.7 ± 1.8 (7.0 -10)	58 ±11 (43 - 70)	21 ± 5.0 (13 - 25)	0.8 ± 0.1 (0.6 - 0.8)	2.3 ± 0.4 (1.7- 2.7)	0.8 ± 0.2 (0.7 - 1.1)
Normal + DMSO	7.5 ± 2.6 (4.0 -10)	47 ± 8.0[i] (39 - 54)	20 ± 4.0 (15 - 24)	0.9 ± 0.2 (0.7 - 1.0)	2.2 ± 0.5 (1.6 - 2.7)	0.8 ± 0.3 (0.5 - 1.2)
Normal + Ru(II) treatment	6.3 ± 2.5[i] (4.0 - 10)	40 ± 8.0[i] (34 - 53)	14 ± 3.0[i] (10 - 17)	0.6 ± 0.1[i] (0.4 - 0.8)	3.9 ± 1.2[i] (2.7 -5.7)	1.0 ± 0.1[i] (0.9 - 1.1)
Tumor only	3.7 ± 0.8[ii] (2.9 - 5.0)	27 ± 7.0[ii] (21 - 37)	7.0 ± 4.0[ii] (5.0 - 13)	0.6 ± 0.1[i] (0.5 - 0.7)	1.5 ± 0.8[i] (0.5 - 2.4)	2.0 ± 0.2[ii] (1.7 - 2.2)
Tumor + DMSO	3.6 ± 1.8[ii] (2.0 - 7.0)	28 ± 7.0[ii] (21 - 37)	7.0 ± 3.0[ii] (5.0 - 11)	0.7 ± 0.2 (0.5 - 1.0)	1.2 ± 0.6[ii] (0.6 - 1.9)	2.0 ± 0.3[ii] (1.6 - 2.3)
Tumor + Ru(II) treatment	7.5 ± 2.6[i,**] (4.0 -10)	36 ± 11[ii,*] (19 - 46)	16 ±4.0[i,**] (12 - 23)	0. 5 ± 0.2[ii] (0.3 - 0.6)	3.5 ± 0.6[ii,**] (3.1- 4.5)	1.4 ± 0.6[i,*] (0.4 - 1.8)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (3): Mean activities of superoxide dismutase in both red blood cells (SOD/RBCS) and liver tissues (SOD/Liver), Catalase in liver tissues and the mean levels of both glutathione reduced form (GSH) and malondialdehyde in RBCs and in liver tissues of mice treated with osmium bipyridine complex.

Parameters / *Group*	SOD/RBCS (U/0.01 gm haemoglobin)	SOD/Liver (% of inhibition/ 0.01 gm tissue)	Catalase (U/0.01 gm tissue)	GSH /RBCS (Mol/L packed cells)	GSH /Liver (mmol/ gm protein)	MDA/RBCs (Mol/ml packed cells $\times 10^{-5}$)
Normal	8.7 ± 1.8 (7.0 -10)	58 ±11 (43 - 70)	21 ± 5.0 (13 - 25)	0.8 ± 0.1 (0.6 - 0.8)	2.3 ± 0.4 (1.7- 2.7)	0.8 ± 0.2 (0.7 - 1.1)
Normal + DMSO	7.5 ± 2.6 (4.0 -10)	47 ± 8.0[i] (39 - 54)	20 ± 4.0 (15 - 24)	0.9 ± 0.2 (0.7 - 1.0)	2.2 ± 0.5 (1.6 - 2.7)	0.8 ± 0.3 (0.5 - 1.2)
Normal + Os(II) treatment	5.6 ± 2.8[i] (3.3 - 10.0)	31 ± 9.4 (21 - 43)	± 3.0 16 (10 - 19)	0.8 ± 0.2 (0.6 -1.1)	3.5 ± 0.8[ii] (2.7 - 4.5)	1.0 ± 0.1[i] (0.9 - 1.1)
Tumor only	3.7 ± 0.8[ii] (2.9 - 5.0)	27 ± 7.0[ii] (21 - 37)	7.0 ± 4.0[ii] (5.0 - 13)	0.6 ± 0.1[i] (0.5 - 0.7)	1.5 ± 0.8[i] (0.5 - 2.4)	2.0 ± 0.2[ii] (1.7 - 2.2)
Tumor + DMSO	3.6 ± 1.8[ii] (2.0 - 7.0)	28 ± 7.0[ii] (21 - 37)	7.0 ± 3.0[ii] (5.0 - 11)	0.7 ± 0.2 (0.5 - 1.0)	1.2 ± 0.6[ii] (0.6 - 1.9)	2.0 ± 0.3[ii] (1.6 - 2.3)
Tumor + Os(II) treatment	4.7 ±1.8[ii] (3.3 – 6.7)	31 ± 7.0[ii] (22-38)	13 ± 4.0[i,*] (8.0 - 19)	0.6 ± 0.1[ii] (0.6 – 0.7)	2.2 ± 0.1[*] (2.0 - 2.4)	1.4 ± 0.1[ii,**] (1.2 - 1.6)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (4): Mean serum levels of triglycerides and cholesterol and the mean total Lipids, DNA and RNA in liver tissues of mice treated with ruthenium bipyridine complex.

Parameters Group	*Tri- glycerides* (mg %)	*Cholesterol* (mg %)	*Total Lipids* (mg / gm tissue)	*Total proteins* (mgm/gm tissue)	*DNA* (mgm/gm tissue)	*RNA* (mgm/gm tissue)
Normal	118 ± 17 (101-142)	130 ± 35 (96-185)	18 ± 5.0 (13 - 26)	388 ± 5.0 (382 -395)	9.0 ± 2.0 (7 - 11)	27 ± 5.0 (21 - 32)
Normal + DMSO	105 ± 16 (80-121)	158 ± 32 (111-190)	20 ± 4.0 (13 - 24)	380 ± 4.0[i] (375-386)	9.0 ± 2.0 (7 - 18)	28 ± 4.0 (24 - 32)
Normal + Ru(II) treatment	124 ± 34 (99-175)	123 ± 27[i] (95-166)	14 ± 2.0[i] (11 - 18)	382± 2.0[i] (380-384)	14 ± 4.0[i] (8 - 18)	29 ± 3.0 (28 - 34)
Tumor only	216 ± 40[ii] (180-260)	126 ± 29 (99-167)	11 ± 5.0[i] (6 -16)	368 ± 5.0[ii] (360-372)	19 ± 4.0[ii] (15 - 23)	36 ± 3.0[ii] (32 - 40)
Tumor + DMSO	218 ±35[ii] (182-260)	129 ±31 (99-168)	11 ± 3.0[i] (7.0 -16)	366 ± 6.0[ii] (356- 372)	14 ± 2.0[ii] (12 -18)	36 ± 6.0[i] (26 - 40)
Tumor + Ru(II) treatment	106±26[**] (73-140)	105 ±11[*] (93-120)	19 ± 5.0[*] (15 - 27)	388 ± 2.0[ii,**] (377-382)	13± 5.0[i,*] (8 - 18)	31 ± 5.0[*] (28 - 39)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (5): Mean serum levels of triglycerides and cholesterol and the mean total Lipids, DNA and RNA in liver tissues of mice treated with osmium bipyridine complex.

Parameters Group	*Tri- glycerides* (mg %)	*Cholesterol* (mg %)	*Total Lipids* (mg / gm tissue)	*Total proteins* (mgm/gm tissue)	*DNA* (mgm/gm tissue)	*RNA* (mgm/gm tissue)
Normal	118 ± 17 (101-142)	130 ± 35 (96-185)	18 ± 5.0 (13 - 26)	388 ± 5.0 (382 -395)	9.0 ± 2.0 (7 - 11)	27 ± 5.0 (21 - 32)
Normal + DMSO	105 ± 16 (80-121)	158 ± 32 (111-190)	20 ± 4.0 (13 - 24)	380 ± 4.0[i] (375-386)	9.0 ± 2.0 (7 - 18)	28 ± 4.0 (24 - 32)
Normal + Os(II) treatment	101 ±10[i] (88-116)	116 ± 33 (75-155)	15 ± 2.0[i] (13 - 17)	381 ± 3.0 (380 - 384)	10 ± 3.0[i] (5 - 13)	24 ± 3.0[i] (2.1-2.7)
Tumor only	216 ± 40[i] (180-260)	126 ± 29 (99 - 167)	11 ± 5.0[i] (6 - 16)	368 ± 5.0[ii] (360-372)	19 ± 4.0[ii] (15 - 23)	36 ± 3.0[ii] (32 - 40)
Tumor + DMSO	218 ±35 (182-260)	129 ±31 (99 - 168)	11 ± 3.0[i] (7.0 -16)	366 ± 6.0[ii] (356- 372)	14 ± 2.0[ii] (12-18)	36 ± 6.0[i] (26 - 40)
Tumor+ Os(II) treatment	97 ±12[i,**] (84-114)	114 ± 45 (60 - 162)	14 ± 3.0[i] (9.0 - 16)	382 ± 2.0[i,**] (377-382)	13 ± 5.0 [i,*] (9.0 -20)	29 ± 4.0[*] (26 - 35)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (6): Mean levels of albumin and the activities of γ- glutamyl transpeptidase (γ- GT) and glutamic pyruvic transaminase in sera of mice treated with Ruthenium bipyridine complex.

Parameters / Group	Albumin *(gm%)*	GGT *(IU/l)*	SGPT *(IU/ml)*	Tumor volume *(ml)*
Normal	3.9 ± 0.3 (3.6 - 4.2)	26 ± 12 (19 - 47)	26 ± 6.0 (20-32)	--
Normal + DMSO	3.3 ± 0.6[i] (2.6 - 4.2)	27 ± 8.0 (19 - 40)	25 ± 7.0 (19-34)	--
Normal + Ru(II) treatment	3.2 ± 0.7[i] (2.1 - 3.7)	14 ± 6.0[i] (8 - 23)	35 ± 8.0[i] (22-41)	--
Tumor only	3.1 ± 0.7[i] (2.4 - 3.7)	119 ± 18[ii] (90 - 141)	37 ± 9.0[i] (26-50)	3.8 ±1.3 (2.0- 5.0)
Tumor + DMSO	2.9 ± 0.5[ii] (2.2 - 3.5)	113 ± 17[ii] (94 - 132)	41 ± 9.4[ii] (27 - 53)	3.6 ±1.14 (2.0- 5.0)
Tumor + Ru(II) treatment	3.1 ± 0.6[i] (2.4 - 3.9)	20 ± 11[**] (10 - 34)	32 ± 7.0[i] (22-41)	0.4 ± 0.22 (0.0 - 0.5)[**]

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (7): Mean levels of albumin and the activities of γ- glutamyl transpeptidase (GGT) and glutamic pyruvic transaminase in sera of mice treated with osmium bipyridine complex.

Parameters / Group	Albumin (gm %)	GGT (IU/l)	SGPT (IU/ml)	Tumor volume (ml)
Normal	3.9 ± 0.3 (3.6 - 4.2)	26 ± 12 (19 - 47)	26 ± 6.0 (20-32)	--
Normal +DMSO	3.3 ± 0.6[i] (2.6 - 4.2)	27 ± 8.0 (19-40)	25 ± 7.0 (19-34)	--
Normal + Os(II) treatment	3.36 ± 0.6[i] (2.4-3.9)	21 ± 4.0 (15-24)	27 ±9.6 (19-42)	--
Tumor only	3.1 ± 0.7[i] (2.4-3.7)	119 ±18[ii] (90-141)	37.4±9[i] (26-50)	3.8 ±1.3 (2.0- 5.0)
Tumor + DMSO	2.9 ± 0.5[ii] (2.2-3.5)	113 ±17[ii] (94-132)	40.8±9.4[ii] (27-53)	3.6 ±1.14 (2.0- 5.0)
Tumor + Os(II) treatment	3.1±0.6[i] (2.5-3.6)	28 ±11[**] (18-41)	17±5.7[i,**] (12-24)	0.6 ± 0.32[**] (0.4- 0.9)

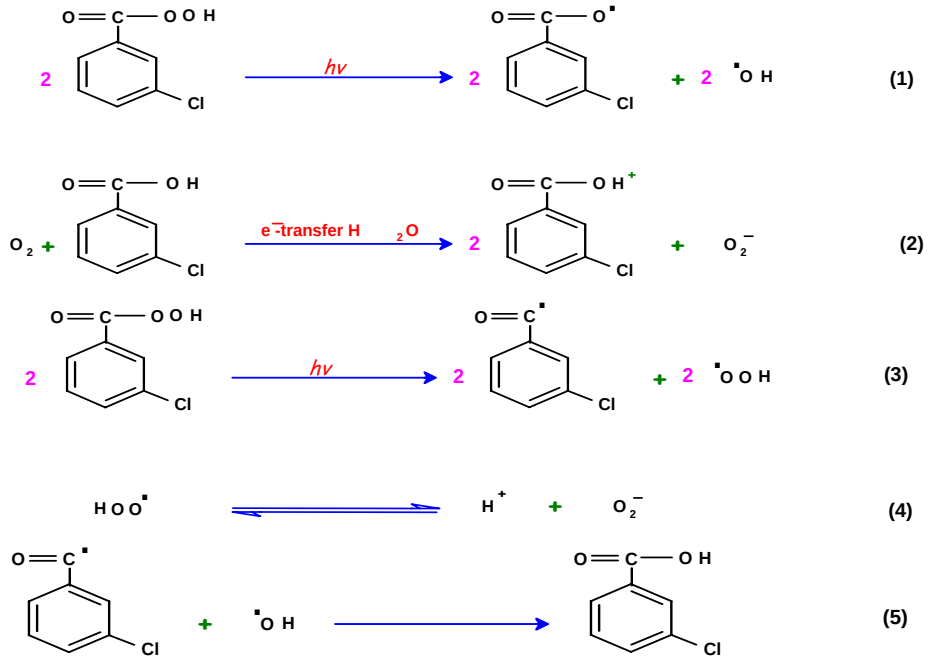
[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

and thus protect the RBCs from subsequent haemolysis.This is because the previous evidence indicated that ROS causes photohaemolysis of human RBCs photosensitized by m-CPBA (El-Naggar, 1997 and Abou-Seif and Elgendy, 1998). Such hemolytic state may be due to the formation of direct cellular membranes injury by the formed lipid peroxides. The latter peroxide formation may be attributed to the increase in the oxidative flux (Kurata et al., 1993) produced by the excessive free radicals e.g. $O_2^{-\cdot}$, $^{\cdot}OH$ and H_2O_2 synthesized during the photohaemolytic process. In addition, the involvement of the peroxidation process in the depletion of glutathione, NADH and adenosine triphosphate (ATP) causes more cellular destruction of RBCs (Halliwell, 1999). In the present study, the mean levels of GSH were significantly elevated in normal mice treated with RuII and OsII complexes compared with those of normal mice without treatment ($P<0.0001$) indicating that these complexes can be implicated in the redox cycle involved in GSH production (Dougan et al, 2008). Another proposed mechanism of RBCs lyses may be via the hydrophobic interactions between the formed hydroperoxides produced as a result of oxidative stress on the membrane double layers of RBCs. The latter interaction facilitates the penetration of water molecules into the inside of RBCs causing more haemolytic damage (Berround et al., 1996). Abou-Seif (2004) suggested that, the main initial radical generated from fluoroirradiation of m-CPBA is the $O_2^{-\cdot}$. He also, proposed two mechanisms to explain the production of the $O_2^{-\cdot}$ and/or $^{\cdot}OH$ radicals during the fluoroirradiation. The first involves fluorogeneration of hydroxyl radical ($^{\cdot}OH$) then $O_2^{-\cdot}$ radical. The $^{\cdot}OH$ could be produced by transforming e^- from the aqueous medium to the oxygen molecule (Packer, 1993) as presented in equations 1 and 2. In the second mechanism, $O_2^{-\cdot}$ radicals are generated firstly followed by $^{\cdot}OH$ radicals through hydrogen abstraction from the medium (equations 3 and 4).

In the present study, the RuII and OsII complexes showed similar percentages of SOD- like activities of about 90%. Such results confirm the tendency of such complexes to consume $O_2^{-\cdot}$ produced during photosensitization of RBCs. The abilities of such complexes to prevent RBCs destruction by 91.5% and 98.3%, respectively also confirm the abilities of these complexes to act as free radicals scavengers and to be used in photodynamic therapy. Also, the results favor the first proposed mechanism by Abou-Seif (2004) in which the main and the initial radical generated from fluoroirradiation of m-CPBA is the $O_2^{-\cdot}$. In addition, the ability of the OsII to protect the human RBCs and also its cytotoxicity for the normal EAC was higher than those of the RuII complex. This may be due to the fact that the liability of acetylacetone ligand in OsII complex is lower than that in ruthenium analogue which is similar to that of cis-platinum compared to cis-palladium complex (Hacker et al., 1984). The latter outhers stated that, the development of pd(II) anticancer drugs has not been promising, this is because pd(II) complex are about 105 times more reactive than their Pt(II) analogues leading to rapid hydrolysis of the leaving group(s).

Kostrhunova et al. (2008) showed that OsII arene complexes bind and distort polymeric DNA with a rate of binding comparable to that of cisplatin. They added that, the extent of the interaction of these complexes with DNA were correlated well with their cytotoxicities. In addition, the latter outhers reported that the osmiated-DNA inhibits RNA synthesis like that of cisplatin and the ruthenium analogue. Moreover, these outhers added that the unwinding angle induced in supercoiled plasmid DNA by osmium (II) arene complexes is larger (21-27°) in comparison to the ruthenium analogues (7-14°) or cisplatin. Such outhers attributed this to the intercalation of the arene interacts into the duplex. Finally, they reported that complexes with extended electron-rich π-systems can displace the intercalator ethidium bromide from DNA, *in vitro*, so supporting the previous intercalation hypothesis for the previous metal complexes. In fact, the osmium (II) and ruthenium (II) bipyridine complexes of the present study contain both the arene system bipyridyl and the electron-rich π-systems; namely, acetylacetone ligand suggesting the possible involvement of the previous mechanism in EAC carcinoma killing.

Vock et al. (2006) tested ten of ruthenium arene complexes with the general formula [Ru(eta6-arene)Cl_2(L)], (arene=benzene, p-cymene; L= imidazole, benzimidazole, N-methylimidazole, N-butylimidazole, N-vinylimidazole, N-benzoylimidazole; X = Cl, BF4, BPh4) for their selectivity toward cancer cells in vitro, those which showed higher cytotoxicity to the tumor cells but they were less (or not) cytotoxic toward nontumorigenic cells have been selected for a more detailed *in vivo* evaluation. In the present study, both of RuII and OsII showed higher EAC carcinoma toxicities *in vitro*, and therefore, were more evaluated *in vivo*. The *in vivo* results of the present study showed that, the mean levels of total lipids and total proteins in liver tissues of the tumorized mice were reduced and those of triglycerides in their sera were elevated confirming the existence of a catabolic state accompanying the growth of the tumor cells (Korekane et al., 2003).



These findings were confirmed by the re-elevation of the mean levels of the formers and the reduction of the latter after tumor killing by any of the two complexes and also by the reduction of the liver DNA and RNA contents after treatment of EAC with the complexes than those of the tumorized untreated animal. The latter results led one to confirm the abilities of the studied complexes not only to treat the tumor cells but also to prevent their metastasis to the liver (Tables 4 and 5). The reduction of the mean activities of both GGT (a tumor marker and a liver function enzyme) and SGPT (a liver function enzyme) after tumor treatment with both RuII and OsII complexes compared with those of the tumorized non-treated mice can confirm the latter two abilities. In addition, γ-GT is considered to be much more sensitive than either SGPT and/ or albumin in reflecting liver affection due to EAC implantation. This is because the former enzyme, activities were much elevated in sera of the tumorized non-treated mice than that of the normal controls ($P<0.0001$). In addition, the treatment with the complexes caused dramatic decrease in GGT activities compared with the tumorized non-treated mice ($P<0.0001$ for both RuII and OsII complexes (Tables 6 and 7).

In the present study, the decrease in the mean activities of SOD in RBCs and liver and that of catalase in liver tissues of the tumorized animals can cause a state of oxidative stress and thence formation of lipid peroxide causing cellular and organ damage. Such damage may include the EAC carcinoma and liver tissues. This is actually the case, because MDA was elevated in liver tissues of the tumorized animals and the activities of both SOD and catalase enzymes were re-elevated after treatment of the tumorized mice with the complexes. Such elevations can protect liver tissues from the oxidative stress, via scavenging the substrates of the latter two enzymes; namely, superoxide radicals and the formed H_2O_2, respectively. Also, such scavenging effect may participates in EAC killing throughout the prevention of their metastasis into the liver. Alessio et al. (2004) added that the use of a well known RuII complex that successfully completed a Phase I trial had the capacity to modify important parameters of metastasis such as tumor invasion, matrix metalloproteinases activity and cell cycle progression. One cannot neglect the involvement of such mechanisms during killing of EAC by the bipyridyl acetylacetenato RuII and OsII complexes.

Moreover, Kostrhunova et al. (2008) investigated the interactions between the potential biological target DNA and four OsII arene complexes, where arene = biphenyl or p-cymene and showed that these complexes bind to DNA. In their study, some of the OsII complexes exhibit promising cytotoxic effects in ovarian tumor cell lines. They also showed that such complexes produced DNA adducts and largely distort DNA conformation. The authors concluded that, the cytotoxicities of the complexes are consistent with their DNA binding and the binding involves combined coordination to guanine residues together with noncovalent interactions between the arene ligand and the DNA. In the present study, both RuII and OsII bipyridyl complexes containing the O,O-donor; acetylacetonato ligand showed higher percentages of EAC toxicities both in vitro and in vivo. Also, Pizarro and Sadler (2009) suggested that, DNA is believed to be the primary target for many metal-based drugs. These drugs can form specific lesions on DNA that induce its apoptosis. Therefore, it was concluded that the present complexes can follow a similar mechanism to that of Kostrhunova et al. (2008) and Pizarro and Sadler (2009) during killing of EAC. This conclusion is based on the reduction in the mean levels of nucleic acids after treatment of the tumorized mice with any of the complexes compared to the non-treated mice.

Pizarro and Sadler (2009) also added that, the newly emerging ruthenium (II) complexes not only bind to DNA coordinately, but also by both H-bonding and hydrophobic interactions triggered by the introduction of the extended arene rings into their versatile structures. In the present study, the bipyridyl moities of the studied complexes can participate, at least in part, in the hydrophobic interaction of both complexes with DNA causing its damage. Intriguingly, Pizarro and Sadler (2009) and Kostrhunova et al. (2008) added that osmium (the heavier congener of ruthenium) reacts differently with DNA but can also give rise to the high cytotoxic effects of the organometallic complexes. This is already the case in the present study, because OsII complex had significantly higher superoxide scavenging activity which protect the human RBCs from the photohaemolytic effects of m-CPBA. Süss-Fink (2010) also concluded that, the neutral or cationic arene ruthenium complexes provide both hydrophilic as well as hydrophobic properties due to the robustness of the ruthenium-arene unit that hold a high potential for the development of metal-based anticancer activity against a variety of cancer cells. *In conclusion,* RuII and OsII complexes can be used as promising free radical scavengers in phototherapy and may be used as anti-tumor, with slight normal cells toxicities, and anti-metastatic agents in the clinical trials in the future.

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Clinical characters of adrenocortical tumors in Chinese childhood

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Abstract: Kidney cancer includes both cancer of the renal parenchyma and cancer of the pelvis (ie transitional cell cancer). In adults, 85%-90% of kidney cancer cases are renal cell cancer. Renal cell cancer accounts for 2% - 3% of all malignancies in western countries and 1% -2% in Japan. The incidence of renal cell cancer is high in Western, and Northern Europe and North America while it is low in Asia. Children with adrenal tumors adrenal tumor is most common, children with precocious puberty may consider this disease, its clinical manifestations was different from adults, should be combined with clinical, laboratory and imaging features comprehensive diagnosis. The incidence of renal cell cancer has been increasing worldwide. Although the incidence of renal cell cancer in Japan is lower than the rates in the other industrialized countries, there is no doubt that it is increasing.
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Key words: tumor; adrenal tumor; adrenal cortex; Cushing's syndrome

1. Introduction

Great strides have been made in the understanding of molecular mechanisms of adrenocortical tumor. This has led this field to the enviable position of having a range of molecularly targeted therapies. Large sequencing efforts are now revealing more and more risk factors responsible for tumour development and progression, offering new targets for therapy. adrenocortical tumor is a rare disease, the world's children under the age of 15, the incidence is only 0.3/million-0.38 / million [1]. Adrenocortical tumors accounted for 0.2% of all childhood tumors, the peak age of onset is less than 5 years of age [2]. As the source of the heterogeneity of adrenal tissue and the children may have a tumor of embryonic tissue sources, various types of adrenal tumors of children, due to the clinical manifestations,are different. clinical manifestations of adrenal tumors and adrenal abnormalities were high cortisol symptoms,alone or mixed performance, high aldosterone hyperlipidemia is rare. Different from adult,children with adrenocortical tumors or abnormal adrenal androgen increased more common. In this paper, we would like to introduce the result of our studies, which evaluate the risk factors of renal cell cancer in a Chinese population. This study retrospectively analyzed the symptoms, laboratory tests and imaging inspection, compared the the clinical features between adrenal adenoma and adenocarcinoma, to improve the cognitive ability of clinical manifestations and diagnosis.

2. Methods and Materials

84 cases were reviewed for the pathologic diagnosis of adrenocortical tumor between 2000 and 2010. They were diagnosed as adrenal tumors (adenomas 43 cases, adenocarcinoma 41 cases, Table 1), containing 36 males and 48 females, male:female is 3:4. Mean diagnosis age at (5.63 ± 2.84) years. 57 cases (67.9%) are less than 5 years; 27 cases (32.1%) are more than 5 years. 13 cases adrenal tumors, 4 cases was found by prenatal B super, the other 9 cases was born for the clitoris hypertrophy, represented a penis enlargement and appeared acne after born 1 week, 7 cases is adenoma, 6 patients were adenocarcinomas, There was no evident difference in gender, onset doctor age, no tumor family history. the clinical characteristics, endocrine change, imaging findings，pathological diagnosis and the relationships were statistically analyzed by this research.

3. Statistical analysis

All the data were presented as mean±standard deviation or as percentage. Chi-squared test were used to analysis the difference of genotypic and allelic frequencies between patients and controls by SPSS 11.0 software. A P-value of less than 0.05 was viewed as statistically significant.

4. Results

43 cases (51.2%) performed precocious puberty, 31 cases (36.9%) precocious puberty were girls, and 12 cases were boys (14.3%). There is statistically significant difference between men and women (P <0.05).9 patients had hypertension,5 cases even caused paralysis led clinic. 9 cases had no abnormal symptoms, discoveryed due to abdominal pain, bloating, and (or) treatment abdominal mass, or accidental (Table 1).

Table 1. Main patient characteristics and outcomes

Clinical manifestations	cases	（%）
Precocious puberty	43	51.2
Earlier pubic hair	30	35.7
Penis enlargement	23	27.4
clitoris hypertrophy	11	13.1
Breast development	14	16.7
Growth acceleration	8	9.5
voice low	2	2.4
Other	9	10.7
Abdominal mass	7	8.3
Abdominal pain/abdominal distention	7	8.3
Fever, emaciated, tired	5	5.6
Physical examination/examination revealed	2	2.4
Cushing's syndrome	32	38.1
Central obesity	27	32.1
Hairy	20	23.8
Acne	14	16.7
High blood pressure	10	10.7
Hyperthyroidism appetite	7	8.3
Much blood quality	1	1.2

79 cases detected the blood levels of sex hormones, 75 cases (94.9%) testosterone increased, The value is higher than the corresponding age and 1.93 ~ 201.4 times of the upper limit.23 cases (27.7%) examined DHEA sulfate (DHEAS), 18 patients (21.4%) increased, adenoma and adenocarcinoma DHEAS level have no difference. 51 (60.7%) were detected estradiol (both have higher testosterone at the same time), 34 patients (40.5%) value increased,which is higher than the corresponding age and developmental level of 1.24 to 18.78 times. 21 cases with estradiol elevated DHEAS. 18 patients carryed dexamethasone suppression test, 3 cases of testosterone can be suppressed (Table 2).

Table 2 sex hormone levels in children with adrenocortical tumors

Sex hormones	N	negative	positive	positie（%）
testosterone	75	4	75	94.9
DHEAS	23	5	18	78.3
estradiol	51	17	34	66.7

76 cases (90.5%) blood lactate dehydrogenase (LDH) were more than the normal range (114 ~ 240 U / L), 29 adenomas (34.5%) increased,41 (48.8%) adenocarcinoma increased, both the positive rate is differences (P <0.05). Adenocarcinoma blood LDH average (724.1 ± 415.3) U / L, LDH adenoma average (412.7 ± 115.2) U / L, both were significantly different (P <O. 01).serum LDH of Adenoma and adenocarcinoma is the biochemical and endocrine markers,they were the only statistically significant difference. Table 3.

Table 3 adrenal cortical tumors in children with serum LDH enzyme

serum LDH	N	negative	positive	positie（%）
adenoma	43	14	29	67.4
adenocarcinoma	41	0	41	100

In this study,adenocarcinoma and adenoma underwent B-ultrasound and CT.Only 17.6% B ultrasonic diagnosis were consistent with pathology, so the B- ultrasound as a means of detection of cancer screening. 2 cases were misdiagnosed as adrenal neuroblastoma,the final pathological diagnosis was adrenal cortical carcinoma. CT diagnosis was only 31.7% in line with the pathology, including 3 cases adenoma,because the tumor has a large central necrosis,which was diagnosed by adenocarcinoma image, while the small cancer can not be qualitative.Therefore, clinicians must be combined with comprehensive clinical and laboratory tests，not to pander to the initial imaging diagnosis.

5. Discussions

Kidney cancer includes both cancer of the renal parenchyma and cancer of the pelvis (ie transitional cell cancer). In adults, 85% -90% of kidney cancer cases are renal cell cancer [3]. Renal cell cancer accounts for 2% - 3% of all malignancies in western countries [4-7] and 1% -2% in Japan[7-8].The incidence of renal cell cancer is high in Western, and Northern Europe and North America while it is low in Asia [3-5,7].children adrenal tumors can be divided into the adrenal cortex and medulla by adrenal tissue sources, and Children with adrenocortical tumors， which a simple expression of peripheral precocious puberty，was the most common, followed by both peripheral precocious puberty and Cushing syndrome,Cushing's syndrome again for the performance alone, finally, no endocrine abnormalities.Up to now,little attention has been paid to interethnic variability or individual differences, whereas，this is an important aspect in the current TKI era [9].Some studies have found that 125 children with cortical tumors purely peripheral precocious puberty accounted for 51.2%, simple Cushing's syndrome 0.8%, both 42%, non-functional 4.8% [10]. Children's non-iatrogenic Cushing's syndrome is more than 80% due to adrenal tumors, Cushing's syndrome in adults is more common. In this study, 43 precocious puberty (51.2%), 31 cases early-maturing girls (36.9%), and 12 boys (14.3%), precocious performance ratio between men and women was significant difference ($P<0.05$).Clinical manifestations of female masculinity and male peripheral precocious was puberty performance.27 Cushing's syndrome with central obesity in 32 cases, 9 patients had high blood pressure, stroke and 5 cases even caused paralysis led clinic. This is clearly different from adults with symptoms of Cushing's performance-based. So the peripheral precocious puberty in children should be on high alert.Adrenal androgen secretion is mainly DHEA, DHEAS and androstenedione [11], feminine sexuality cortical tumor cell aromatase activity was significantly higher in normal adrenal Cushing's syndrome and to the performance of cortical tumor cells. Studies confirmed the adrenal cortex tumor cells of ACTH and angiotensin lI (Ang Ⅱ) receptor expression, the expression of all kinds of P450 enzymes, exogenous ACTH can increase its activity [12], so the adrenal cortex hormones can be increased in the male various types of adrenal hormone secretion. No matter what age, gender，95%blood DHEAS are from the adrenal gland, the adrenal lesions prompted DHEAS. In this study, 79 cases examined the blood hormone levels, 75 patients (94.9%) testosterone increased， detection value is higher than the corresponding age.23 cases (27.7%) examined DHEA sulfate (DHEAS), 18 patients (21.4%) increased adenoma and adenocarcinoma.DHEAS level was no difference. 51 (60.7%) were detected estradiol (both have higher testosterone at the same time), the detection value increased in 34 patients (40.5%),Which is higher than the corresponding age.23 cases DHEAS were elevated. The incidence of renal cell cancer has been increasing around worldwide.The rapidly increasing incidence of renal cell carcinoma may be partly explained by the rising numbers of the new imaging techniques and diagnosis[13].the diagnosis of B ultrasound and CT is a common diagnosis of adrenal tumor, CT scanning is considered as the imaging diagnosis of adrenal cortical carcinoma and the primary means. Tumors were large, uneven density, with irregular low-density area or hemorrhage, and there is a strengthen uneven calcification as the typical CT, CT found the tumor, but three cases is negative in B ultrasound[1]. If CT is still negative and cortisol decreased, we should regularly review B ultrasound or CT. For the diagnosis of CAH patients, if poorly controlled disease, adrenal gland should be regularly reviewed by B ultrasound,to see whether hyperplasia of the adrenal adenoma. This study for the occurrence of abnormal adrenal and sex characteristics (or) symptoms of Cushing's patients underwent B ultrasound and CT. Only 17.6% B ultrasonic diagnosis was consistent to pathology, so the B ultrasonic is as a means of cancer screening. 2 cases were misdiagnosed as adrenal neuroblastoma, the final pathological diagnosis was adrenal cortical carcinoma.only 31.7% CT diagnosis wasin line with the pathology, including 3 cases adenoma,because the tumor has a large central necrosis,which was diagnosed with adenocarcinoma, while the small cancer can not be qualitative.Therefore, clinicians must be combined with comprehensive clinical and laboratory tests,not pander to the initial imaging diagnosis. In summary, the adrenal cortex tumor showed the signs of clinical abnormalities and (or) Cushing's syndrome, or no endocrine abnormalities.laboratory tests and common hormone increased the cortisol circadian rhythm disappeared and the clinical features of cancer,Unlike adults, children with adrenogenital disorders increased performance of the more common androgen. A few may be due to adrenal cortical tumors,early B ultrasonic exceptions have been misdiagnosed as CAH. In short,the distribution of children's tumor type and clinical manifestations are different from adults, we should summarize the clinical characteristics according to the early identification of adrenal tumors, clinical presentation, laboratory examination, imaging studies， We can make the final diagnosis timely，accurate treatment decisions and prognosis. Further studies may be necessary to provide a better understand of this important clinical issues,because the number of renal

cancer cases was small in our studies.

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Refereces

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Antitumor effects of osmium (II) and ruthenium (II) bipyridine complexes containing the acetylacetonato ligand against the growth of Eherlich Ascites Cell Carcinoma

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Abstract: The development of metal-based antitumor drugs has been stimulated by the clinical success of cisplatin and its analogs and by the clinical trials of other platinum and ruthenium complexes with activity against resistant tumors and with reduced toxicity of normal cells. In the present study, the newly synthesized [OsII$(bpy)_2$(acac)](PF6) and [RuII$(bpy)_2$(acac)](PF6) complexes were tested for their cytotoxicities against Eherlich Ascites Cells (EAC) carcinoma, for their superoxide dismutase (SOD)- like activities and for their cytoprotective effects of the normal human red blood cells (RBCs) against photo-irradiation induced by UV-lamb in the presence of m-chloroperbenzoic acid, *in vitro*. Also, their killing capabilities for the growth of EAC carcinoma *in vivo* and the measurements of the biochemical changes accompanying such killing were investigated. The *in vitro* study revealed that the average cytoprotective effects of RBCs, SOD- like activities and the cytotoxicity of EAC by similar concentrations of rutheniumII (RuII) and osmium (OsII) complexes were 91.5% and 98.3%, 89.9% and 89.8% and 90% and 92.8%, respectively**.** In the *in vivo* study, the mean SOD activities in both RBCs and liver of the tumorized mice were statistically significantly inhibited compared with those of the control group ($P<0.0001$). After treatment either with RuII and OsII complexes, the activities of the latter enzyme in RBCs and liver were elevated ($P<0.0007$, $P<0.04$ and $P<0.09$ and $P>0.05$, respectively). Also, the mean activity of catalase was inhibited in liver tissues in the tumorized animals and re-elevated after complexes treatment. In addition, treatment with these complexes elevate the glutathione (GSH) levels in liver tissues of the tumorized and normal mice with simultaneous reduction in the mean levels of the corresponding values of malondialdehyde. On the other side, the mean levels of triglycerides and cholesterol were reduced in liver tissues but the mean levels of total lipids and total proteins were elevated after treatment. Moreover, the mean levels of DNA and RNA were significantly elevated in liver tissues of the tumorized animals and significantly reduced after treatment of the tumorized mice with the complexes. The previous results reflect tumor growth inhibition and prevention of EAC carcinoma metastasis into the liver. ***In conclusion,*** RuII and OsII bipyridine complexes are promising free radical scavengers in phototherapy and may be used as anti-tumor and anti-metastatic agents in the clinical trials in the future.

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Key words: Photo-irradiation, complexes, cytotoxicity, tumors, malondialdehyde and metastasis

Introduction

Transition-metal-based compounds constitute a discrete class of chemotherapeutics which were widely used in the clinic as antitumor and antiviral agents. However, drug resistance and side effects have limited their clinical utility **(Chen et al., 2003)**. These limitations have prompted a search for more effective and less toxic metal-based antitumor agents. The wide range of coordination numbers and geometries, accessible redox states, thermodynamic and kinetic characteristics, and the intrinsic properties of the cationic metal ion and ligand itself offer the medicinal chemist a wide spectrum of reactivities that can be exploited. Although metals have long been used for medicinal purposes in a more or less empirical fashion **(Thompson and Orvig, 2006)**, the potential of metal-based anticancer agents has only been fully realized and explored since the landmark discovery of the biological activity of cisplatin (**Jung and Lippard, 2007)**. Recently, some of the efforts have been directed to ruthenium complexes. This is because these complexes demonstrate similar ligand exchange kinetics to those of platinum (II) while displaying only low toxicity. In addition, the redox potential between the different accessible oxidation states occupied by ruthenium complexes enables the body to catalyze oxidation and reduction reactions, depending on the physiological environment **Dougan et al. (2008).** Moreover, the biochemical changes that accompany cancer alter physiological environment, enabling ruthenium complexes to be selectively activated in cancer tissues **(Peacock and Sadler, 2008).** Also, **Brabec and Nováková in 2006** found that, ruthenium compounds bind to DNA affecting its conformation differently than cisplatin and its analogues. In addition, non-nuclear targets, such as the mitochondrion and the cell surface, have also been implicated in the antineoplastic activity of some ruthenium complexes. **Brabec and Nováková (2006)** added that, some chemical properties make ruthenium compounds well

suited for medicinal applications and as an alternative to platinum antitumor drugs in the treatment of cancer cells resistant to cisplatin.

Superoxide radical ($O_2^{\cdot-}$) is produced at any location where an electron transport chain is present, and hence O_2 activation may occur in different compartments of the cell. Therefore, SOD as a defense mechanism, is found in all subcellular locations **(Alscher et al., 2002).** It was showed that, a shift to a more oxidative state might result in uncontrolled lipid peroxidation, protein oxidation and ultimately cell death **(Halliwell, 1999).** For these reasons, the search for novel organometallic complex that defend against ROS and acting as anti-tumor agents must be the target of many studies including this study. In this study, the biological effects of osmium (II) complex with the structure [OsII(bpy)2(acac)](PF6) and ruthenium (II) complex with the structure [RuII(bpy)2(acac)](PF6) where (bpy = 2,2'-bipyridine, acac)= acetylacetone, and PF6= hexafluorophosphate) were evaluated both *in vivo* and *in vitro*. These complexes were tested *in vitro* for their cytotoxicities against Eherlich Ascites Cells (EAC) carcinoma, for their SOD-like activities and for their cytoprotective effects of the normal human red blood cells (RBCs) against photo-irradiation induced by UV-lamb in the presence of m-chloroperbenzoic acid. Also, their killing capabilities for the growth of EAC carcinoma and the measurements of the biochemical changes accompanying such killing were investigated *in vivo*.

Materials and Methods

A-Materials:

1-Animals and tumor cell line: Adult female Swiss common bred albino mice purchased from Theodore Bilharz Institute, Giza, Egypt, with an average body weight of 25 to 30 g were used. Ehrlich ascites carcinomas (EAC), a mammary origin, were used to give liquid tumor. These cells were kindly supplied by Doctor C. Benckhujsen, Netherland Cancer Institute, Amsterdam, Netherland. The tumor line was maintained in the Oncology Unit at the Egyptian National Cancer Institute, by serials of intraperitoneal (I.P.) transplantation in female Swiss Albino mice at 7 to 10 days intervals since 1982 up till then and was kindly supplied by such Institute. The mice were randomly divided into six groups (eight mice each) namely; normal mice (group 1), normal mice complex-treated (group 2), normal mice dimethy sulphoxide (DMSO)-treated (group 3), tumor bearing-complex treated mice (group 4), tumor-bearing-DMSO treated mice (group 5) and tumor-bearing mice only (group 6). The mice of the last 3 groups were i.p. inoculated with 10^6 EAC cells to produce the liquid tumor. 24 hours after tumor inoculation, the mice of group 2 and group 4 were i.p. treated with the OsII or RuII complex with a daily dose of 10 mg/kg/day (1/5 of LD_{50}) day after day for 14 days starting from the first day after tumor inoculation. The normal- complex treated mice's group was treated with the same complex's dose as that of group 4.

2-Collection of samples: One day after the last treatment, the ascitic fluids containing EAC cells were collected and their volumes were measured. Livers were quickly dissected, rinsed with isotonic saline and dried. Then, 10 % liver tissues homogenized in cold phosphate-buffer (w/v) were prepared. After the removal of the cellular depris via centrifugation, the supernatants were used for biochemical analysis. Blood samples were also collected by tail vein cutting and their sera were used for subsequent analysis.

B-Methods:

1-Source and synthesis of the complexes:

The new complexes, [RuII bpy)$_2$L](PF$_6$) and [OsII (bpy)$_2$L](PF$_6$) (where, RuII= ruthenium(II), OsII= Osmium(II), bpy= 2,2- bipyridine, L= acetylacetone and PF6= hexafluorophosphate) have been prepared and characterized by spectroscopic measurements and also investigated by cyclic voltammetry by **El-Hendawy *et al.* (1997)** and **El-Hendawy (2011),** respectively. These complexes were kindly provided by Dr/ Ahmed **El-**Hendawy, Faculty of Science (Damietta), Mansoura University, Egypt and were used in both the *in vitro* and in the *in vivo* treatment of EAC carcinoma in the present study.

2- In vitro study:

2-1-Preparation of red blood cells (RBCs) samples:

Heparinized fresh blood samples were withdrawn from five healthy volunteers and centrifuged at 3000 rpm/min. The pellets were separated and washed 3 times with phosphate buffered saline (PBS, pH 7.4, 0.01 M containing 0.135 NaCl) and centrifuged again. The cells were resuspended in PBS and 1x 10^6 cells were used in fluorohemolysis and the antihaemolytic effects of the RuII and OsII complexes *in vitro.*

2-2-Effects of m-chloro-perbenzoic acid (m-CPBA) on the fluorohemolysis and evaluation of the antihaemolytic effects of the complexes:

To test the antihaemolytic effect of the Ru(II) and Os(II) complexes, a photohaemolytic damage of normal human red blood cells (RBCs) was performed by exposing these cells to a UV-lamp in the above PBS containing 200 µM of m-CPBA (the acid concentration which gave the maximum haemolytic effect) for 30 minutes. After the completion of the incubation period, the tubes were centrifuged and the

absorbance of the supernatants, as a measure of the photohaemolytic effect, was read at 546 nm in each case **(Dacie and Lewis, 1984)**.

2-3- SOD-like activities of the complexes:

SOD-like activities of the complexes and that in liver haemogenate were assayed by **Dechatelet *et al.* (1974)**. Simply each complex was added to a mixture of nitro blue tetrazolium salt and NADH in a pyrophosphate buffer (pH 8.3).The changes in the optical density was recorded/minute after the addition of phenazine methosulphate. The percent of inhibition of the colour development was calculated based on that of a control tube containing no complexes.

2-4-EACs cytotoxicity *in vitro*:

The cytoxicity was determined using trypan blue exclusion by the method of **MacLimans et al**. (1957).

3- In vivo study:

3-1-Tumor volume: The tumor volumes were volumetrically measured in each case.

3-2-Antioxidants:

3-2-1-SOD and catalase activities in liver tissue haemogenate and SOD in RBCs:

SOD activity in liver homogenate was assayed **by Dechatelet *et al.* (1974)** and that in RBCs was assayed by the procedure of **Winterbourn et al. (1975).** The catalase activity was determined according to **Chance and Mackley (1955).**

3-2-2-Glutathione (GSH) in liver tissue haemogenate and in RBCs and malondialdehyde (MDA) in liver tissues:

GSH was determined in liver tissues and RBCs by the method of **Beutler et al. (1963)** but MDA was determined by the method of **Stock and Donnandy (1971).**

3-3-DNA and RNA contents of the liver tissues:

The levels of DNA in liver tissues were evaluated according to **Dische and Schwartezm (1937)** and the RNA content was measured by the orcinol procedure of **Mejbaum (1939).**

3-4-Lipids profile and total proteins:

The serum cholesterol was determined according to **Richmond (1973)** and the triglycerides were enzymatically hydrolyzed and determined according to the method of **Fossati and Principe (1982).** The total lipids and total proteins contents of liver tissues were determined by the methods of **Knight et al. (1972) and Lowry et al. (1951),** respectively.

3-5-Liver functions: Serum albumin was done according to the method of **Doumas et al. (1971).** Also, the activities of serum glutamic pyruvic transaminase and that of gamma-glutamyl transpeptidase (GGT) were determined by the method of **Reitman and Frankel (1957)** and by Szasz et al. (1969), respectively**.**

4-Statistical analysis:

The biochemically collected data were characterized by their mean and standard deviations using instat software, version 2.03 (Graphed, USA). In addition, the student t-test was evaluated and the one-tailed P-values were also used for the statistical analysis of the results. The probability values at 0.05 up to more than 0.001 levels were considered statistically significant and 0.001 or less were considered highly significant (**Snedecor and Cochran, 1969**).

Results

1-Antihaemolytic, superoxide dismutase (SOD)-like activity and cytotoxicity effects of the complexes:

The *in vitro* study revealed that the average cytoprotective effects of the complexes for the human RBCs from the photo-irradiative damage induced by UV-lamb in the presence of m-CPBA were 91.5% and 98.3 %. Also, the SOD-like activities and the cytotoxicity effects of the complexes for the viable EAC by similar concentrations of RuII and OsII complexes were 89.9 % and 89.8 % and 90 % and 92.8 %, respectively**.** In addition, the antihaemolytic and the cytotoxicity effects of the OsII complex were higher than those of the RuII complex **(Table 1).**

2-The activities of superoxide dismutase in red blood cells (SOD/RBCs) and the activities of both superoxide dismutase (SOD/Liver) and catalase in liver tissues:

In the *in vivo* study, RuII and OsII complexes reduce the activities of SOD/RBCs, SOD/Liver and catalase in liver tissues and causes no change in the mean blood malondialdehyde (MDA) levels in the normal mice' group. On contrary, the mean activities of the former enzymes in the tumorized-non treated mice were statistically significantly inhibited compared with those of the control group ($P<0.0001$). On the other hand, after treatment of the tumorized mice either with RuII and OsII complexes, the activities of these enzymes were re-elevated ($P<0.0007$, $P<0.04$ and $P<0.0002$ for RuII and $P<0.09$, $P<0.13$ and $P<0.0005$ for OsII, respectively and **tables 2 and 3**).

3-Glutathione reduced form (GSH) and malondialdehyde (MDA) in RBCs and in liver tissues:

From **tables 2 and 3**, treatment with the divalent complexes elevates the mean GSH levels in liver tissues of the normal-complex treated compared to that of the normal nontreated group (P<0.0015 and P<0.0009, respectively) and in the tumorized-treated mice compared to that of the tumor nontreated group (P<0.0001 and P<0.014, respectively). In addition, simultaneous reductions in the mean MDA levels in liver tissues of the tumor-bearing mice compared to the tumorized-treated group were observed (P<0.009 and P<0.0001, respectively **and** tables 2 and 3).

Table 1: Antihaemolytic effects, SOD-like activities and cytotoxicity of ruthenium and osmium bipyridine complexes.

Effects / Volume	Antihaemolytic effects©		SOD-like activities©		Cytotoxicity©	
	RuII	OsII	RuII	OsII	RuII	OsII
20 µL	90.3 %	97.7 %	78.2 %	76.9%	82 %	86 %
50 µL	91.3 %	98.1%	87.1 %	79.5 %	89 %	92 %
100µL	91.8 %	98.4 %	91.2%	96.2 %	91%	94 %
150 µL	91.9 %	98.6 %	93 %	96.6 %	94 %	96 %
200 µL	92.0 %	98.6 %	100%	100%	94%	96 %
Average effects %	91.5 ± 0.7	98.3 ± 0.4	89.9 ± 8.0	89.8 ± 10.8	90 ± 4.9	92.8 ± 4.1
Effects' range %	91.3 - 92	97.7 - 98.6	78.2 - 100	76.9 - 100	82 – 94	86 – 96

© = Values are the average of 5 different readings.

4- Levels of DNA and RNA:

The mean levels of DNA and RNA were significantly elevated in liver tissues of the tumorized mice compared with those of normal liver tissues (P<0.0001) and significantly reduced after complexes treatment compared with those of the tumorized non-treated mice (P<0.007 and P<0.01 for RuII and OsII complexes, respectively), a phenomenon which reflects inhibition of the tumor growth. In addition, OsII caused less damage of DNA of normal cells compared to RuII (P<0.05 and table 4 and 5).

5- Levels of lipids profile and the total proteins in liver tissues: Firstly, the treatments of normal mice with any of the complexes (OsII and RuII complexes, respectively) significantly reduce their mean serum levels of triglycerides (P<0.01 and P<0.33) and total lipids in their liver tissues (P<0.05 and P<0.03) compared with those of the normal control group. In addition, after killing of the tumor cells by the complexes, the mean serum levels of triglycerides (P<0.0001 in each case) and cholesterol in their sera were significantly reduced (P<0.04 in case of RuII only) but the mean levels of total lipids were elevated (P<0.08 and P<0.002, respectively) and total proteins (P<0.0001 in each case) in the same organ compared with those of the tumorized non-treated mice (**Table 4 and 5)**.

6- Liver function tests:

6-1- Serum albumin:

The tumorized- non-treated mice showed significantly lowered albumin levels in their sera compared with that of normal mice (P<0.005), indicating a state of liver damage due to metastasis of EAC into such organ. In addition, complexes treatment did not affect serum albumin levels **(P<0.4** and **tables 6 and 7**).

6-2- Serum γ-GT and serum glutamic pyruvic transaminase (SGPT**):**

As shown in tables 6 and 7, γ-GT mean activities are much elevated in sera of the tumorized non-treated mice than that of the normal controls (P<0.0001). In addition, the treatment with the complexes caused dramatic decrease in γ-GT activities compared with that of the tumorized non-treated mice (P<0.0001). On the other hand, DMSO did not affect the latter enzymes activities in the normal-DMSO treated animals. On the other side, the normal mice treated with the complexes showed reduction in the mean activities of catalase (P<0.01 in case of RuII

complex) and slight elevations in the mean activities of SGPT compared with those of the normal control. Surprisingly, OsII complex is less toxic to normal liver cell compared with RuII one. This is because the mean activity of SGPT in normal OsII- treated mice is lowered than that of normal RuII- treated mice (P< 0.053 and **tables 6 and 7**).

6- Tumor volume:

The mean volumes of the ascitic fluids after treatment of any of the complexes were highly significantly reduced (P<0.0001) compared with those of the tumorized non-treated mice (Tables **6 and 7**).

Table 2): Mean activities of superoxide dismutase in both red blood cells (SOD/RBCS) and liver tissues (SOD/Liver), catalase in liver tissues and the mean levels of both glutathione reduced form (GSH) and malondialdehyde in RBCs and in liver tissues of mice treated with Ruthenium bipyridine complex.
[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Parameters / ***Group***	**SOD/RBCS (U/0.01 gm haemoglobin)**	**SOD/Liver (% of inhibition/ 0.01 gm tissue)**	**Catalase (U/0.01 gm tissue)**	**GSH /RBCS (Mol/ ml cells)**	**GSH /Liver (mMol/ gm protein)**	**MDA/RBCs (Mol/ml packed cells)**
Normal	8.7 ± 1.8 (7.0 -10)	58 ±11 (43 - 70)	21 ± 5.0 (13 - 25)	0.8 ± 0.1 (0.6 - 0.8)	2.3 ± 0.4 (1.7- 2.7)	0.8 ± 0.2 (0.7 - 1.1)
Normal + DMSO	7.5 ± 2.6 (4.0 -10)	47 ± 8.0[i] (39 - 54)	20 ± 4.0 (15 - 24)	0.9 ± 0.2 (0.7 - 1.0)	2.2 ± 0.5 (1.6 - 2.7)	0.8 ± 0.3 (0.5 - 1.2)
Normal + Ru(II) treatment	6.3 ± 2.5[i] (4.0 - 10)	40 ± 8.0[i] (34 - 53)	14 ± 3.0[i] (10 - 17)	0.6 ± 0.1[i] (0.4 - 0.8)	3.9 ± 1.2[i] (2.7 -5.7)	1.0 ± 0.1[i] (0.9 - 1.1)
Tumor only	3.7 ± 0.8[ii] (2.9 - 5.0)	27 ± 7.0[ii] (21 - 37)	7.0 ± 4.0[ii] (5.0 - 13)	0.6 ± 0.1[i] (0.5 - 0.7)	1.5 ± 0.8[i] (0.5 - 2.4)	2.0 ± 0.2[ii] (1.7 - 2.2)
Tumor + DMSO	3.6 ± 1.8[ii] (2.0 - 7.0)	28 ± 7.0[ii] (21 - 37)	7.0 ± 3.0[ii] (5.0 - 11)	0.7 ± 0.2 (0.5 - 1.0)	1.2 ± 0.6[ii] (0.6 - 1.9)	2.0 ± 0.3[ii] (1.6 - 2.3)
Tumor + Ru(II) treatment	7.5 ± 2.6[i,**] (4.0 -10)	36 ± 11[ii,*] (19 - 46)	16 ±4.0[i,**] (12 - 23)	0. 5 ± 0.2[ii] (0.3 - 0.6)	3.5 ± 0.6[ii,**] (3.1- 4.5)	1.4 ± 0.6[i,*] (0.4 - 1.8)

Table 3): Mean activities of superoxide dismutase in both red blood cells (SOD/RBCS) and liver tissues (SOD/Liver), Catalase in liver tissues and the mean levels of both glutathione reduced form (GSH) and malondialdehyde in RBCs and in liver tissues of mice treated with osmium bipyridine complex.

Parameters ***Group***	**SOD/RBCS (U/0.01 gm haemoglobin)**	**SOD/Liver (% of inhibition/ 0.01 gm tissue)**	**Catalase (U/0.01 gm tissue)**	**GSH /RBCS (Mol/ml packed cells)**	**GSH /Liver (mMol/ gm protein)**	**MDA/RBCs (Mol/ml packed cells)**
Normal	8.7 ± 1.8 (7.0 -10)	58 ±11 (43 - 70)	21 ± 5.0 (13 - 25)	0.8 ± 0.1 (0.6 - 0.8)	2.3 ± 0.4 (1.7- 2.7)	0.8 ± 0.2 (0.7 - 1.1)
Normal + DMSO	7.5 ± 2.6 (4.0 -10)	47 ± 8.0[i] (39 - 54)	20 ± 4.0 (15 - 24)	0.9 ± 0.2 (0.7 - 1.0)	2.2 ± 0.5 (1.6 - 2.7)	0.8 ± 0.3 (0.5 - 1.2)
Normal + Os(II) treatment	5.6 ± 2.8[i] (3.3 - 10.0)	31 ± 9.4 (21 - 43)	16 ± 3.0 (10 - 19)	0.8 ± 0.2 (0.6 -1.1)	3.5 ± 0.8[ii] (2.7 - 4.5)	1.0 ± 0.1[i] (0.9 - 1.1)
Tumor only	3.7 ± 0.8[ii] (2.9 - 5.0)	27 ± 7.0[ii] (21 - 37)	7.0 ± 4.0[ii] (5.0 - 13)	0.6 ± 0.1[i] (0.5 - 0.7)	1.5 ± 0.8[i] (0.5 - 2.4)	2.0 ± 0.2[ii] (1.7 - 2.2)
Tumor + DMSO	3.6 ± 1.8[ii] (2.0 - 7.0)	28 ± 7.0[ii] (21 - 37)	7.0 ± 3.0[ii] (5.0 - 11)	0.7 ± 0.2 (0.5 - 1.0)	1.2 ± 0.6[ii] (0.6 - 1.9)	2.0 ± 0.3[ii] (1.6 - 2.3)
Tumor + Os(II) treatment	4.7 ±1.8[ii] (3.3 – 6.7)	31 ± 7.0[ii] (22-38)	13 ± 4.0[i,*] (8.0 - 19)	0.6 ± 0.1[ii] (0.6 – 0.7)	2.2 ± 0.1[*] (2.0 - 2.4)	1.4 ± 0.1[ii,**] (1.2 - 1.6)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table 4): Mean serum levels of triglycerides and cholesterol and the mean total Lipids, DNA and RNA in liver tissues of mice treated with ruthenium bipyridine complex.

Parameters **Group**	***Tri- glycerides*** *(mg %)*	***Cholesterol*** *(mg %)*	***Total Lipids*** *(mg / gm tissue)*	***Total proteins*** ***(mgm****/gm tissue)*	***DNA*** ***(mgm****/gm tissue)*	***RNA*** ***(mgm****/gm tissue)*
Normal	118 ± 17 (101-142)	130 ± 35 (96-185)	18 ± 5.0 (13 - 26)	388 ± 5.0 (382 -395)	9.0 ± 2.0 (7 - 11)	27 ± 5.0 (21 - 32)
Normal + DMSO	105 ± 16 (80-121)	158 ± 32 (111-190)	20 ± 4.0 (13 - 24)	380 ± 4.0[i] (375-386)	9.0 ± 2.0 (7 - 18)	28 ± 4.0 (24 - 32)
Normal + Ru(II) treatment	124 ± 34 (99-175)	123 ± 27[i] (95-166)	14 ± 2.0[i] (11 - 18)	382± 2.0[i] (380-384)	14 ± 4.0[i] (8 - 18)	29 ± 3.0 (28 - 34)
Tumor only	216 ± 40[ii] (180-260)	126 ± 29 (99-167)	11 ± 5.0[i] (6 -16)	368 ± 5.0[ii] (360-372)	19 ± 4.0[ii] (15 - 23)	36 ± 3.0[ii] (32 - 40)
Tumor + DMSO	218 ±35[ii] (182-260)	129 ±31 (99-168)	11 ± 3.0[i] (7.0 -16)	366 ± 6.0[ii] (356- 372)	14 ± 2.0[ii] (12 -18)	36 ± 6.0[i] (26 - 40)
Tumor + Ru(II) treatment	106±26[**] (73-140)	105 ±11[*] (93-120)	19 ± 5.0[*] (15 - 27)	388 ± 2.0[ii,**] (377-382)	13± 5.0[i,*] (8 - 18)	31 ± 5.0[*] (28 - 39)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table 5): Mean serum levels of triglycerides and cholesterol and the mean total Lipids, DNA and RNA in liver tissues of mice treated with osmium bipyridine complex.

Parameters **Group**	***Tri- glycerides*** *(mg %)*	***Cholesterol*** *(mg %)*	***Total Lipids*** *(mg / gm tissue)*	***Total proteins*** ***(mgm****/gm tissue)*	***DNA*** ***(mgm****/gm tissue)*	***RNA*** ***(mgm****/gm tissue)*
Normal	118 ± 17 (101-142)	130 ± 35 (96-185)	18 ± 5.0 (13 - 26)	388 ± 5.0 (382 -395)	9.0 ± 2.0 (7 - 11)	27 ± 5.0 (21 - 32)
Normal + DMSO	105 ± 16 (80-121)	158 ± 32 (111-190)	20 ± 4.0 (13 - 24)	380 ± 4.0[i] (375-386)	9.0 ± 2.0 (7 - 18)	28 ± 4.0 (24 - 32)
Normal + Os(II) treatment	101 ±10[i] (88-116)	116 ± 33 (75-155)	15 ± 2.0[i] (13 - 17)	381 ± 3.0 (380 - 384)	10 ± 3.0[i] (5 - 13)	24 ± 3.0[i] (2.1-2.7)
Tumor only	216 ± 40[i] (180-260)	126 ± 29 (99 - 167)	11 ± 5.0[i] (6 - 16)	368 ± 5.0[ii] (360-372)	19 ± 4.0[ii] (15 - 23)	36 ± 3.0[ii] (32 - 40)
Tumor + DMSO	218 ±35 (182-260)	129 ±31 (99 - 168)	11 ± 3.0[i] (7.0 -16)	366 ± 6.0[ii] (356- 372)	14 ± 2.0[ii] (12-18)	36 ± 6.0[i] (26 - 40)
Tumor+ Os(II) treatment	97 ±12[i,**] (84-114)	114 ± 45 (60 - 162)	14 ± 3.0[i] (9.0 - 16)	382 ± 2.0[i,**] (377-382)	13 ± 5.0 [i,*] (9.0 -20)	29 ± 4.0[*] (26 - 35)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table 6): Mean levels of albumin and the activities of γ- glutamyl transpeptidase (γ- GT) and glutamic pyruvic transaminase in sera of mice treated with Ruthenium bipyridine complex.

Parameters **Group**	**Albumin** ***(gm%)***	**GGT** ***(IU/l)***	**SGPT** ***(IU/ml)***	**Tumor volume (ml)**
Normal	3.9 ± 0.3 (3.6 - 4.2)	26 ± 12 (19 - 47)	26 ± 6.0 (20-32)	--
Normal + DMSO	3.3 ± 0.6[i] (2.6 - 4.2)	27 ± 8.0 (19 - 40)	25 ± 7.0 (19-34)	--
Normal + Ru(II) treatment	3.2 ± 0.7[i] (2.1 - 3.7)	14 ± 6.0[i] (8 - 23)	35 ± 8.0[i] (22-41)	--
Tumor only	3.1 ± 0.7[i] (2.4 - 3.7)	119 ± 18[ii] (90 - 141)	37 ± 9.0[i] (26-50)	3.8 ±1.3 (2.0- 5.0)
Tumor + DMSO	2.9 ± 0.5[ii] (2.2 - 3.5)	113 ± 17[ii] (94 - 132)	41 ± 9.4[ii] (27 - 53)	3.6 ±1.14 (2.0- 5.0)
Tumor + Ru(II) treatment	3.1 ± 0.6[i] (2.4 - 3.9)	20 ± 11[**] (10 - 34)	32 ± 7.0[i] (22-41)	0.4 ± 0.22 (0.0 - 0.5)[**]

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table 7): Mean levels of albumin and the activities of γ- glutamyl transpeptidase (GGT) and glutamic pyruvic transaminase in sera of mice treated with osmium bipyridine complex.

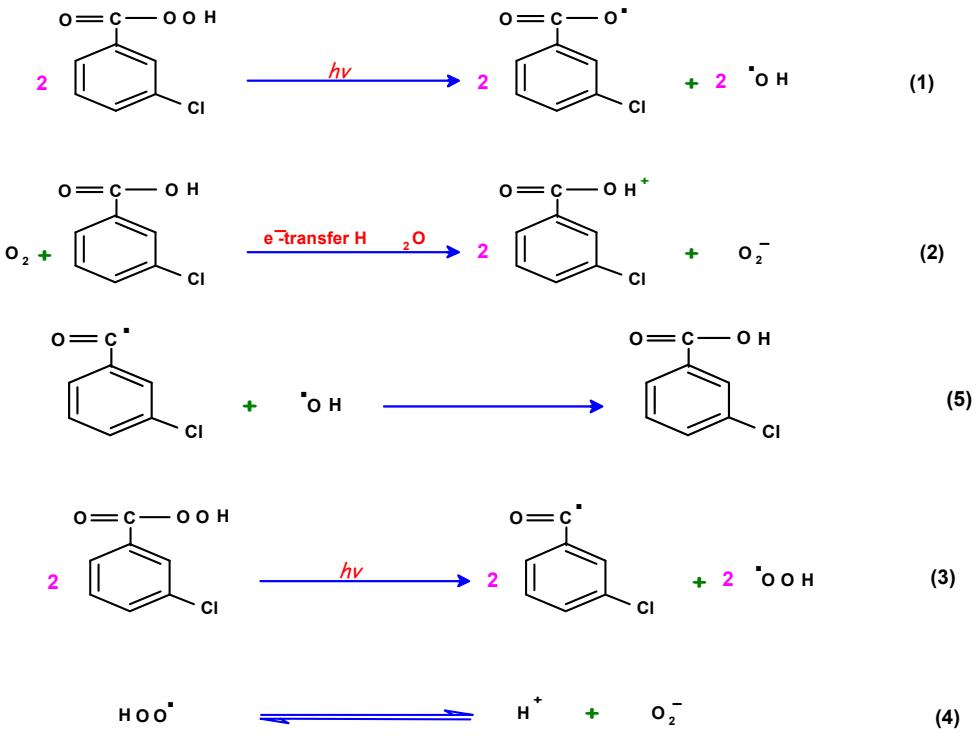
Parameters Group	Albumin (gm %)	GGT (IU/l)	SGPT (IU/ml)	Tumor volume (ml)
Normal	3.9 ± 0.3 (3.6 - 4.2)	26 ± 12 (19 - 47)	26 ± 6.0 (20-32)	--
Normal +DMSO	3.3 ± 0.6[i] (2.6 - 4.2)	27 ± 8.0 (19-40)	25 ± 7.0 (19-34)	--
Normal + Os(II) treatment	3.36 ± 0.6[i] (2.4-3.9)	21 ± 4.0 (15-24)	27 ±9.6 (19-42)	--
Tumor only	3.1 ± 0.7[i] (2.4-3.7)	119 ±18[ii] (90-141)	37.4±9[i] (26-50)	3.8 ±1.3 (2.0- 5.0)
Tumor + DMSO	2.9 ± 0.5[ii] (2.2-3.5)	113 ±17[ii] (94-132)	40.8±9.4[ii] (27-53)	3.6 ±1.14 (2.0- 5.0)
Tumor + Os(II) treatment	3.1±0.6[i] (2.5-3.6)	28 ±11[**] (18-41)	17±5.7[i,**] (12-24)	0.6 ± 0.32[**] (0.4- 0.9)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Discussion

The field of medicinal inorganic chemistry is rapidly advancing. In particular organometallic complexes have much potential as therapeutic and diagnostic agents **(Peacock and Sadler, 2008).** The development of metal-based antitumor drugs has been stimulated by the clinical success of cisplatin in the treatment of resistant tumors and by the clinical trials of other platinum and ruthenium complexes showing reduced toxicities **Brabec (2002).** It is therefore of great interest, in this study, to understand the details of molecular and biochemical mechanisms underlying the biological efficacy of ruthenium and osmium complexes both *in vitro and in vivo*.

In the present study, two complexes of RuII and OsII with the same ligand were tested in vitro for their capabilities to prevent the photohaemolysis of human RBCs sensitized by m-CPBA via scavenging the produced free radicals, to mimic SOD activity and to kill EAC carcinoma. In this study, it was proposed that the complexes scavenge the free radicals produced from the photosensitization of m-CPBA and thus protect the RBCs from subsequent haemolysis. This is because the previous evidence indicated that ROS causes photohaemolysis of human RBCs photosensitized by m-CPBA **(El-Naggar, 1997 and Abou-Seif and Elgendy, 1998).** Such hemolytic state may be due to the formation of direct cellular membranes injury by the formed lipid peroxides. The latter peroxide formation may be attributed to the increase in the oxidative flux **Kurata et al. (1993**) produced by the excessive free radicals e.g. $O_2^{\cdot-}$, $^{\cdot}OH$ and H_2O_2 synthesized during the photohaemolytic process**.** In addition, the involvement of the peroxidation process in the depletion of glutathione, NADH and adenosine triphosphate (ATP) causes more cellular destruction of RBCs **(Halliwell, 1999).** In the present study, the mean levels of GSH were significantly elevated in normal mice treated with RuII and OsII complexes compared with those of normal mice without treatment ((P<0.0001)) indicating that these complexes can be implicated in the redox cycle involved in GSH production (**Dougan et al, 2008).** Another proposed mechanism of RBCs lyses may be via the hydrophobic interactions between the formed hydroperoxides produced as a result of oxidative stress on the membrane double layers of RBCs. The latter interaction facilitates the penetration of water molecules into the inside of RBCs causing more haemolytic damage **(Berround et al., 1996). Abou-Seif (2004)** suggested that, the main initial radical generated from fluoroirradiation of m-CPBA is the $O_2^{\cdot-}$. He also, proposed two mechanisms to explain the production of the $O_2^{\cdot-}$ and/or $^{\cdot}OH$ radicals during the fluoroirradiation. The first involves fluorogeneration of hydroxyl radical ($^{\cdot}OH$) then $O_2^{\cdot-}$ radical. The $^{\cdot}OH$ could be produced by transforming e^- from the aqueous medium to the oxygen molecule **(Packer, 1993) as** presented in equations 1 and 2. In the second mechanism, $O_2^{\cdot-}$ radicals are generated firstly followed by $^{\cdot}OH$ radicals through hydrogen abstraction from the medium (equations 3 and 4).



In the present study, the RuII and OsII complexes show that of cisplatin and the ruthenium analogue. 90%. Such result radicals scavengers and to be used in photodynamic therapy. Also, the results favor the first proposed mechanism by **Abou-Seif (2004)** in which the main and the initial radical generated from fluoroirradiation of m-CPBA is the O_2^{-}. In addition, the ability of the OsII to protect the human RBCs and also its cytotoxicity for the normal EAC was higher than those of the RuII complex. This may be due to the fact that the liability of acetylacetone ligand in OsII complex is lower than that in ruthenium analogue which is similar to that of cis-platinum compared to cis-palladium complex (**Hacker et al., 1984).** The latter outhers stated that, the development of pd(II) anticancer drugs has not been promising, this is because pd(II) complex are about 105 times more reactive than their Pt(II) analogues leading to rapid hydrolysis of the leaving group(s).

Kostrhunova et al. (2008) showed that OsII arene complexes bind and distort polymeric DNA with a rate of binding comparable to that of cisplatin. They added that, the extent of the interaction of these complexes with DNA were correlated well with their cytotoxicity. In addition, the latter outhers reported that the osmiated-DNA inhibits RNA synthesis like Moreover, these outhers added that the unwinding angle induced in supercoiled plasmid DNA by osmium (II) arene complexes is larger (21-27°) in comparison to the ruthenium analogues (7-14°) or cisplatin. Such outhers attributed this to the intercalation of the arene interacts into the duplex. Finally, they reported that complexes with extended electron-rich π-systems can displace the intercalator ethidium bromide from DNA, *in vitro*, so supporting the previous intercalation hypothesis for the previous metal complexes. In fact, the osmium (II) and ruthenium (II) bipyridine complexes of the present study contain both the arene system bipyridyl and the electron-rich π-systems; namely, acetylacetone ligand suggesting the possible involvement of the previous mechanism in EAC carcinoma killing.

Vock *et al. (2006)* tested ten of ruthenium arene complexes with the general formula [Ru(eta6-arene)Cl_2(L)], (arene=benzene, p-cymene; L= imidazole, benzimidazole, N-methylimidazole, N-butylimidazole, N-vinylimidazole, N-benzoylimidazole; X = Cl, BF4, BPh4) for their selectivity toward cancer cells in vitro, those which showed higher cytotoxicity to the tumor cells but they

were less (or not) cytotoxic toward nontumorigenic cells have been selected for a more detailed *in vivo* evaluation. In the present study, both of RuII and OsII showed higher EAC carcinoma toxicities *in vitro*, and therefore, were more evaluated *in vivo*. The *in vivo* results of the present study showed that, the mean levels of total lipids and total proteins in liver tissues of the tumorized mice were reduced and those of triglycerides in their sera were elevated confirming the existence of a catabolic state accompanying the growth of the tumor cells **(Korekane et al., 2003).** These findings were confirmed by the re-elevation of the mean levels of the formers and the reduction of the latter after tumor killing by any of the two complexes and also by the reduction of the liver DNA and RNA contents after treatment of EAC with the complexes than those of the tumorized untreated animal. The latter results led one to confirm the abilities of the studied complexes not only to treat the tumor cells but also to prevent their metastasis to the liver (Tables 4 and 5). The reduction of the mean activities of both GGT (a tumor marker and a liver function enzyme) and SGPT (a liver function enzyme) after tumor treatment with both RuII and OsII complexes compared with those of the tumorized non-treated mice can confirm the latter two abilities. In addition, is considered to be much more sensitive than either SGPT and/ or albumin in reflecting liver affection due to EAC implantation. This is because the former enzyme, activities were much elevated in sera of the tumorized non-treated mice than that of the normal controls ($P<0.0001$). In addition, the treatment with the complexes caused dramatic decrease in GGT activities compared with the tumorized non-treated mice ($P<0.0001$ for both RuII and OsII complexes (Tables 6 and 7).

In the present study, the decrease in the mean activities of SOD in RBCs and iver and that of catalase in liver tissues of the tumorized animals can cause a state of oxidative stress and thence formation of lipid peroxide causing cellular and organ damage. Such damage may include the EAC carcinoma and liver tissues. This is actually the case, because MDA was elevated in liver tissues of the tumorized animals and the activities of both SOD and catalase enzymes were re-elevated after treatment of the tumorized mice with the complexes. Such elevations can protect liver tissues from the oxidative stress, via scavenging the substrates of the latter two enzymes; namely, superoxide radicals and the formed H_2O_2, respectively. Also, such scavenging effect may participates in EAC killing throughout the prevention of their metastasis into the liver. **Alessio et al. (2004)** added that the use of a well known RuII complex that successfully completed a Phase I trial had the capacity to modify important parameters of metastasis such as tumor invasion, matrix metalloproteinases activity and cell cycle progression. One cannot neglect the involvement of such mechanisms during killing of EAC by the bipyridyl acetylacetenato RuII and OsII complexes.

Moreover, **Kostrhunova et al. (2008)** investigated the interactions between the potential biological target DNA and four OsII arene complexes, where arene = biphenyl or p-cymene and showed that these complexes bind to DNA. In their study, some of the OsII complexes exhibit promising cytotoxic effects in ovarian tumor cell lines. They also showed that such complexes produced DNA adducts and largely distort DNA conformation. The authors concluded that, the cytotoxicities of the complexes are consistent with their DNA binding and the binding involves combined coordination to guanine residues together with noncovalent interactions between the arene ligand and the DNA. In the present study, both RuII and OsII bipyridyl complexes containing the O,O-donor; acetylacetonato ligand showed higher percentages of EAC toxicities both in vitro and in vivo. Also, **Pizarro and Sadler (2009)** suggested that, DNA is believed to be the primary target for many metal-based drugs. These drugs can form specific lesions on DNA that induce its apoptosis. Therefore, it was concluded that the present complexes can follow a similar mechanism in killing of EAC to that of **Kostrhunova et al. (2008) and Pizarro and Sadler (2009).** This conclusion is based on the reduction in the mean levels of nucleic acids after treatment of the tumorized mice with any of the complexes compared to the non-treated mice.

Pizarro and Sadler (2009) also added that, the newly emerging ruthenium (II) complexes not only bind to DNA coordinately, but also by both H-bonding and hydrophobic interactions triggered by the introduction of the extended arene rings into their versatile structures. In the present study, the bipyridyl moities of the studied complexes can participate, at least in part, in the hydrophobic interaction of both complexes with DNA causing its damage. Intriguingly, **Pizarro and Sadler (2009) and Kostrhunova et al. (2008)** added that osmium (the heavier congener of ruthenium) reacts differently with DNA but can also give rise to the high cytotoxic effects of the organometallic complexes. This is already the case in the present study, because OsII complex had significantly higher superoxide scavenging activity which protect the human RBCs from the photohaemolytic effects of m-CPBA. **Süss-Fink (2010)** also concluded that, the neutral or cationic arene ruthenium complexes provide both hydrophilic as well as hydrophobic properties due to the robustness of the ruthenium-arene unit that hold a high potential for the development of metal-based

anticancer activity against a variety of cancer cells. ***In conclusion,*** RuII and OsII complexes can be used as promising free radical scavengers in phototherapy and may be used as anti-tumor, with slight normal cells toxicities, and anti-metastatic agents in the clinical trials in the future. **Acknowledgements:** The author would like to express greatful thanks to Dr./Ahmed Hendawy, Faculty of Science (Damietta), Mansoura University, Egypt, for provision of metal complexes and for helpful discussion.

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STUDIES ON TICKS OF CATTLE AND THEIR BACTERIAL ISOLATES

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ABSTRACT: Entomological and bacteriological Studies on tick species infesting cattle was conducted in this investigation.A total of 504 ticks were found to infest the 50 cattle examined indicating a burden of 144 [28%.6%], 121 [24.0%], 117 [23.2%] and 122 [24.2%] for *Boophilus*, *Rhipicephalus*, *Hyalomma* and *Amblyomma* species respectively. Bacteriological examination revealed that 302 [59.9%] ticks were positive for bacterial growth viz; *Boophilus* 130 [90.3%], *Rhipicephalus* 101 [84.2%], *Hyalomma* 46 [39.3%] and *Amblyomma* 25 [20.5%]. Colony count [CC] from *Boophilus* was 58 [50.0%] for *Staph. aureus* and 29 [25.0%] each for *Proteus* and *Corynebacterium*.*Rhipicephalus* harboured 24 [33.3%] *Staph. aureus* and 48 [66.7%] *Corynebacterium*, *Amblyomma* harboured only 48 [100%] *Coryne*bacterium, while *Hyalomma* had 47 [66.2%] *Staph. aureus* and 24 [33.8%] *Corynebacterium* species. The disk diffusion sensitivity method revealed that both gram positive isolates were susceptible to CIP, GN, CO and OF and the gram negative *Proteus* to CIP only.
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Keyword: TICKS, CATTLE, BACTERIAL ,ISOLATES

INTRODUCTION

Ticks as obligatory blood sucking arthropods found world over, are the most important vectors of human and animal diseases (Turner and Stephens, 2008; Youssefi *et al.*, 2008) particularly bacteria associated with tick pyemia, acute and per acute mastitis, septicemia, abscessation and lameness (Shanson, 1983., Zaria *et al.*, 2009).

This study was conducted to indentify the bacterial isolates associated with tick species infesting cattle and to assess the antibiotic sensitivity of these isolates.

MATERIALS AND METHODS

Tick Collection and identification:

Cattle brought for sale at the Maiduguri Cattle market were examined and 504 ticks were manually collected using hand forceps and put into 70% alcohol to sterilize. Ticks were then identified to the genus level as described by Soulsby (1982) at the Veterinary Parasitology Laboratory of University of Maiduguri, Nigeria.

Bacteriological examination:

Different scalpel blades were used to dissect each tick, and their intestinal contents inoculated on blood and Mac Conkey agar, and incubated at 37^{o}C for 48hours to observe for bacterial growth as described by Murray *et. al.,* (1995). The growth on each culture media was transferred to a drop of distilled water on grease free glass slides and made into a thin smear which was allowed to air-dry and then fixed with heat form a Bunsen burner, gram stained, air dried and observed at x100 of the light microscope. Standard biochemical tests were performed on the isolates as a means to further characterize them.

Sensitivity testing:

This was performed by the disk diffusion method according to CLSI standards (Clinical and Laboratory Standards Institute) and the zone of growth /inhibition read as described by Habrun *et. al.,* (2010).

RESULTS

The results of this study as shown in Table I indicated that a total of 504 ticks examined for bacterial isolates, 302 (59.9%) were infected with various species comprising of *Boophilus* 130 (90.3%), *Rhipicephalus* 101 (84.2%), *Hyalomma* 46 (39.3%) and *Amblyomma* 25 (20.5%).

A total of 116 (37.8%) Isolates were obtained from *Boophilus* comprising of *Staph. aureus* 58 (50.0%), *Proteus* 29 (25.0%) and *Coryne*. 29 (25.0%).

Rhipicephalus had 72 (23.5%) isolates comprising of *Staph. aureus* 24 (33.3%) and *Coryne.* 48 (66.7%).

Hyalomma had 71 (23.1%) comprising of *Staph aureus* 47 (66.2%) and *Coryne*. 24 (33.8%).

Amblyomma had 48 (15.6%) of only *Coryne.* species (100%).

Table 2 shows the disk diffusion sensitivity test results with both gram positive isolates susceptible to CIP, CN, CO and OF and the gram negative *Proteus* to CIP only.

Table 1: Isolation rate of bacteria from various tick species examined

Tick	No. bacterial	No (%) infected isolates	No (%) isolates	No (%) of isolates genera examined *Staph.*	*Coryne*	with bacterial *Proteus*
Boophilus	144	130(90.3)	116(37.8)	58(50.0)	29(25.0)	29925.0)
Rhipicephalus	121	101(84.2)	72(23.5)	24(33.3)	48(66.7)	-
Hyalomma	117	46(39.3)	71(23.1)	47(66.2)	24(33.8)	-
Amblyomma	122	25(20.5)	48(15.6)	-	48(100)	-
Total	504	302(59.9)	307	129(42.0)	149(48.5)	29(9.5)

Table 2: Antibiogram of isolates

Bacteria	Width (mm) of zone of clearance/resistance (R)									
Gram +ve:	CIP	GN	CX	CO	FX	AP	CD	AU	OF	E
Staph.	27	14	10	22	R	R	R	R	23	R
Coryne.	29	18	R	19	R	R	R	R	25	R
Gram –ve:	CIP	TE	NB	AX	OF	C	CF	AP	GN	N
Proteus	25	R	R	R	17	R	R	R	R	R

DISCUSSION

This study has revealed the close association of cattle ticks *Boophilus*, *Hyalomma*, *Rhipicephalus* and *Amblyomma* with bacterial species of *Staphylococcus aureus*, *Corynebacterium Spp*. and *Proteus Spp.*

Boophilus was the most frequently infected with bacteria (90.3%) from which *Staph. aureus*, *Coryne. Spp.* and *Proteus Spp*. were isolated while *Rhipicephalus* and *Hyalomma* harboured *Staph. aureus* and *Coryne Spp.* and *Amblyomma* had only *Coryne. Spp.* These findings conform with those by Tomasz *et. al.,* (2009) and Zaria *et. al.,* (2009) that ticks are reservoirs of *Staph. aureus* known to predispose livestock to abscessation, septicemia, tick borne fever, tick borne typhus and complicated dermatophilosis.

Corynebacterium species are known to cause nasal, nasopharyngeal and tonsillar diphtheria often with marked oedema of the neck, and some species produce strong exotoxins and if absorbed unto broken mucous membrane causes toxaemia leading to cardiac and neural complications (Jesus *et al.,* 2008).

Proteus species are known to cause urinary tract infection (UTI), abdominal and wound infections and serve as a secondary invader in ulcers, pressure sores, and burns and damaged tissues. It also causes septicemia, meningitis and chest infections (Parola and Rault, 2001).

The organisms isolated were found to have high antibiotic resistance indicating a need for tick control so as to reduce economic losses accrued from tick and tick borne diseases as the saying goes "prevention is better than cure''.

In conclusion, ticks carry pathogenic bacteria that show multiple antibiotic resistances and could play an important role in the epidemiology of bacterial diseases of man and his livestock.

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Radiotherapy Alone Versus Combined-Modality Therapy for Initial Treatment of Early Stage Hodgkin's Lymphoma

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Abstract: Purpose: Retrospective analysis and comparison of the efficacy, feasibility and long term side effects, of combined-modality therapy (CMT) versus radiotherapy alone as front-line therapy in early stage Hodgkin's lymphoma (HL). **Patients and Methods:** Between January 1998 and January 2008, 115 patients with early stage IA and IIA Hodgkin's lymphoma were allocated to receive either radiotherapy alone (n= 43), with a mean dose, of 40 Gy, or radiochemotherapy (n = 72) with low dose involved field radiotherapy (LDIFRT), with a mean dose, of LDIFRT of 30 Gy. The primary endpoint of this study was overall and disease -free survival time at 5 and 10 years in both treatment arms. Secondary endpoints included treatment response, tolerability and late treatment related events of each schedule. Kaplan-Meier method estimated overall survival (OS) and disease -free survival (DFS). Log rank test compared survival curves with p value ≤ 0.05 considered significant. **Results:** A total of 115 eligible patients were analyzed. Adverse prognostic factors were almost higher in the CMT group. Both treatment protocols could be delivered in an optimal dose and without significant delay. After 10 years of follow-up CMT produced significantly less nausea and vomiting ($p = 0.023$), as well as less incidence of second malignancy ($p = 0.001$), also less pulmonary toxicity ($p = 0.11$), Hyporthyroidism ($p = 0.07$), cardiac complications ($p = 0.38$), and Hyperthyroidism ($p = 0.19$) but without statistical significance. For CMT arm, the 10-years DFS and OS were 87% and 83%, respectively, compared with 75% and 71%, respectively, for the radiotherapy alone arm. Elevated ESR > 50 ($p = <0.001$), stage IIA disease ($p = 0.01$), and involvement of > 3 lymph node sites ($p = 0.003$) had statistically significant adverse effect on the OAS. However age, sex, pathological subtype, and bulky mediastinum had no statistically significant effect on the OAS (all p = NS). Univariate analysis of factors that might affect DFS showed that patients with involvement of < 3 lymph node sites ($p = 0.007$), ESR < 50 ($p = 0.001$) and stage IA disease ($p = 0.01$), had statistically significant longer DFS. Conclusion: In patients with early stage HL, a CMT results in less incidence of late treatment related events, and has a trend toward better DFS and OS, when compared with radiotherapy alone, however a larger number of patients and longer follow-up is required for a definitive statement on survival.

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Key words: early stage IA and IIA Hodgkin's lymphoma, combined-modality therapy, radiotherapy alone, prognostic factors, toxicity.

1. Introduction

Hodgkin's lymphoma (HL) is one of the most common malignancies in young adults[1,2]. It has become a highly curable cancer[3,4]. Patients have a relatively long survival, although a few studies have concluded that the death rate of patients with HL still is greater than that of the general population[5,6]. This may relate to various complications, such as second malignancies[7-11], cardiac toxicity[12-14], and infections[6]. Twenty years after their treatment, more patients have died from other causes than from HL[6,15]. Given an overall 5-year relative survival rate of 85% for patients with HL[16], and the fact that the highest incidence rates occur between ages 20 and 34 years, large numbers of patients remain at lifelong risk for the late effects of treatment. Because treatment efficiency has improved dramatically, one way to improve long-term survival is to reduce mortality from causes other than HL.

In limited-stage disease (stages I and II), HL can be cured in the majority of patients by a variety of treatments used on their own or in combination. Combined chemotherapy and radiation is the most effective treatment approach for early stage Hodgkin lymphoma. Chemotherapy with involved field radiation therapy (IFRT) is shown to be superior to radiation therapy alone in a large EORTC clinical trial[17]. Most trials have shown that two to six cycles of chemotherapy with IFRT is adequate treatment for early stage HL[17-22], as regard, cure and in minimizing toxicity, especially late toxicity that has an impact on future quality of life and survival[23].

We now present our experience with the combined-modality therapy versus radiotherapy alone schedule at our Clinical Oncology Department, Tanta University Hospital in 115 patients with limited-stage HL and report on efficacy and late treatment related events of each schedule.

2. Patients and Methods

Patients

Between January 1998 and January 2008, 115 eligible patients had histologically verified HL based on the World Health Organization (WHO) histologic classification, stages IA and IIA according to the American Joint Committee on Cancer (AJCC), Seventh Edition, 2010 for HL. The medical records of all patients at our Clinical Oncology Department, Tanta University Hospital were properly revised, organized, and analyzed to achieve the goal of our work.

Exclusion criteria included the following: KPS scale < 70, age greater than 75 years or less than 18 years, previous treatment with chemo- immuno-, or radiotherapy for HL, inadequate bone marrow function (WBC count < 3.0 x 10^9/L or platelet count < 100 x 10^9/L), inadequate renal function (serum creatinine of no more than 1.25 x upper normal limit or creatinine clearance < 60 mL/min/1.73 m^2), and inadequate liver function (serum bilirubin of more than 1.25 x upper normal limit). Also patients were excluded if they had a history of ventricular arrhythmia, congestive heart failure, or documented myocardial infarction. Pregnant and lactating patients were also excluded, as were patients with inadequacy of follow-up.

Investigations

The following parameters were assessed at baseline: KPS, weight, nodal examination, computed tomography (CT) scan of the neck, chest, abdomen and pelvis, ECG, echocardiography, ESR, LDH, blood counts (hemoglobin, granulocytes, and platelets), and blood chemistry (renal and liver function tests). All baseline parameters, except the CT scan of the neck, chest, abdomen and pelvis, and ECG, were performed before each cycle.

Blood counts were performed weekly during treatment. Assessment of nodal areas, 1 month after the patient stopped therapy and then every 3 months together with assessments of ESR, LDH, blood counts, chemistry, weight, performance status, toxicity, and general examination. Scans were performed at the time when progressive disease was suspected on clinical examination or every follow-up visit. Follow-up visits were scheduled every 3 months in the first 2 years after cessation of treatment and every 6 months thereafter, for a median follow-up time of 132 months (mean; 113.4 months, range; 1 - 137 months).

Treatment

Patients were allocated to receive either radiotherapy alone (n= 43), or radiochemotherapy (n= 72) with low dose involved field radiotherapy (LDIFRT). The mean dose of radiotherapy in the radiotherapy alone arm was 40 Gy (range, 35 to 45 Gy). Standard radiotherapy treatment was 30 Gy to the primary wide field, with a smaller field boosted to a total dose of 40 Gy. In the radiochemotherapy arm (n= 72) the mean dose, of LDIFRT was 30 Gy (range, 20 to 36 Gy). The chemotherapy regimen in patients who were to receive the combined-modality therapy (CMT), was administered in the form of ABVD (Adriamycin "doxorubicin", bleomycin, vinblastine, dacarbazine). The schedule was repeated for 4- 6 cycles followed by LDIFRT. Before every cycle of chemotherapy, standard premedication was administered with dexamethasone 20 mg intravenously (IV), diphenhydramine 50 mg IV, and cimetidine 300 mg IV (or ranitidine 50 mg IV) were administered 30 minutes before chemotherapy. Antiemetics were administered at the oncologist's discretion.

In the CMT arm dose reductions were performed according to nadir and nadir duration. Most of the patients received at least 4 cycles of protocol treatment unless they developed progressive disease or unacceptable toxicity. In patients with assessable disease and no change in disease status after six cycles, treatment was continued by 2nd line therapy, but subsequent treatment protocol in these patients was left to the discretion of the oncologist and not reported in our study.

Toxicity and Response Criteria

Late complications were scored according to the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer late radiation morbidity scoring schema[24]. No special investigations were required. Tumor response was evaluated according to modified WHO criteria. Complete response was considered to be the disappearance of all known disease, together with a return to within-normal values of relevant blood chemistries, including ESR and LDH, for at least 4 weeks. Partial response was considered to be a ≥50% decrease in tumor area (calculated by multiplying the longest diameter by the greatest perpendicular diameter) or, in the case of multiple lesions, a ≥ 50% decrease in the sum of the products of the perpendicular diameters of the multiple lesions. Progressive disease was defined as a greater than 25% increase in the size of the target lesion or, in the case of several target lesions, a greater than 25% increase in the sum of the products of the perpendicular diameters of these lesions or the appearance of any new lesion. An increase in ESR and/or LDH levels not associated with radiologic or clinical evidence of tumor progression was not used as the sole indicator of progressive disease. Stable disease was defined as a bidimensionally measurable decrease of less than 50% or increase of less than 25% in the sum of the products of the largest perpendicular diameters of all measurable lesions for at least 6 months.

Outcome Measures

The primary endpoint of this study was overall and disease -free survival time at 5 and 10 years in both treatment arms. Secondary endpoints included treatment response, tolerability and late treatment related events of each schedule.

Statistical Methods

The date of final analysis was November 2011. Patients' first relapse (as measured by physical examination and ultrasound or CT scan) served as the end point for DFS. If clinical detection of disease was preceded by an elevation in ESR and/or LDH levels, the date that the ESR and/or LDH levels were first above normal was recorded.

Disease free survival was measured from the day of starting treatment until the date of documented disease relapse or to last follow-up. Overall survival (OS) time was calculated from the time of diagnosis until death from any cause or to the date of last follow-up. SPSS version 17.0 was used for data management. Kaplan Meier method[25] estimated OS and DFS. Log rank test compared survival curves with p value ≤ 0.05 considered significant.

3. Results

Between January 1998 and January 2008, the total number of patients eligible for this study was 115. Patient and tumor characteristics are listed in table 1. Adverse prognostic factors were almost significantly higher in the CMT group. The mean age in both treatment arms was identical: 30 years.

Treatment Compliance

Most patients (97.6% in the radiotherapy alone arm and 90.3% in the CMT arm) received the full dose of the planned treatment protocols. Treatment delays of 7 days or more occurred more frequently in the CMT arm than in the radiotherapy alone arm (9.3% versus 11.1%, respectively) but without statistically significant difference ($p = 0.94$), (Table 2).

Dose reductions were performed infrequently. Overall, only 8 patients (6.9%, 8/115) received at least one dose reduction (Table 2). There was no statistically significant difference between the treatment arms in the percentage of patients with dose reductions (2.4% in the radiotherapy alone arm versus 9.7% in the CMT arm; $p = 0.98$). The mean radiotherapy doses for all patients in the radiotherapy alone and CMT arms were 40 Gy and 30 Gy, respectively.

Response to Treatment and Survival

Although not statistically significant the CMT protocol was associated with more clinically complete response (Table 2) than the radiotherapy alone protocol (98.6% versus 95.3%, respectively, $p= 0.11$). The higher response rates following treatment with the CMT protocol did not result in a significant superior DFS ($p=0.12$), or a significant better OS ($p=0.17$) (Figs. 1, 2).

Patients were followed for a median of 132 months, range; 1 – 137 months (mean±SD = 130 ± 36.6 month). With respect to the primary endpoint, the difference in the DFS at 5 and 10 years was not statistically significant different between the 2 treatment arms (85% and 75%, respectively for the radiotherapy alone arm versus 90% and 87%, respectively for the CMT arm).

Univariate analysis of factors that might affect DFS showed that patients with a number of lymph node sites of less than 3 before start of treatment ($p = 0.01$), ESR < 50 ($p = 0.001$), and stage I disease ($p = 0.01$), had statistically significant longer DFS while age ($p = 0.12$), sex ($p = 0.11$), and pathological subtype ($p = 0.59$), had no significant impact on DFS (Table 3).

Elevated level of ESR > 50 ($p = < 0.0001$), stage II ($p = 0.01$) and number of lymph node sites > 3 ($p = 0.003$) had statistically significant adverse effect on the OS. However, age ($p = 0.19$), sex ($p = 0.15$), pathological subtype ($p = 0.66$), and presence of bulky mediastinum ($p = 0.26$) had no statistically significant impact on the OS (Table 4).

Late events after therapy

Late events after therapy were evaluated and summarized in table 5. After about 10 years of follow-up CMT produced significantly less nausea and vomiting ($p = 0.02$), as well as less incidence of second malignancy ($p = 0.001$). Other late events including pulmonary toxicity ($p = 0.11$), Hypothyroidism ($p = 0.07$), cardiac complications ($p = 0.38$), and Hyperthyroidism ($p = 0.19$) were more frequent in the radiotherapy alone arm than in the CMT arm but this difference was not statistically significant (all p = NS) (Table 5).

Table 1. Patient and tumor characteristics in patients with early stage HL by treatment arm

	Radiotherapy alone arm		Combined-modality therapy arm		*P* value
	No.	%	No.	%	
No. of patients	43		72		
Age					
≥50 years	19	44.2	28	38.9	0.57
< 50 years	24	55.8	44	61.1	
Stage					
I	18	41.9	7	9.7	<0.001
II	25	58.1	65	90.3	
Pathological type					
Nodular sclerosis (NS)	27	62.8	53	73.6	0.51
Mixed cellularity (MC)	10	23.3	9	12.5	
Lymphocyte depletion (LD)	1	2.3	2	2.8	
Lymphocyte predominance (LP)	5	11.6	8	11.1	
Lymph node sites					
≥3	6	13.9	21	29.2	0.06
<3	37	86.1	51	70.8	
Karnofsky performance status					
≥90%	31	72.1	44	61.1	0.52
<90%	12	27.9	28	38.9	
Bulky mediastinum					
Yes	1	2.3	20	27.8	0.001
No	42	97.7	52	72.2	
Sex					
Male	21	48.8	39	54.2	0.58
Female	22	51.2	33	45.8	
ESR					
High	4	9.3	17	23.6	0.05
Normal	39	90.7	55	76.4	

Table 2. Therapy and efficacy parameters in patients with early stage Hodgkin's disease by treatment arm

Parameters	Radiotherapy alone arm		Combined-modality therapy arm		*P* value
	No.	%	No.	%	
Dose reduction for any reason					
No	42	97.6	65	90.3	0.98
Yes	1	2.4	7	9.7	
Treatment delay, days					
0	33	76.7	53	73.6	0.94
1 – 6	6	14	11	15.3	
≥ 7	4	9.3	8	11.1	
Clinical response					
Complete	41	95.3	71	98.6	
Stable disease	1	2.4	0	0.0	0.12
Progressive disease	1	2.4	1	1.4	
Relapse of disease at 10 years					
No	33	76.7	64	88.9	0.11
Yes	10	23.3	8	11.1	

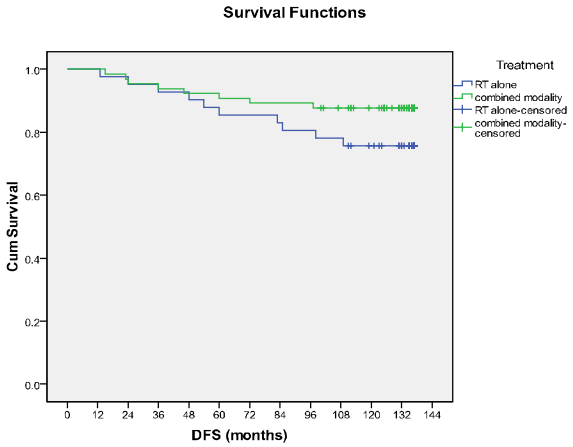

Figure 1. Kaplan–Meier Curves for Disease-Free Survival Time in Patients with Early Stage Hodgkin's Disease by Treatment Arm. Patients were assigned to receive either combined-modality therapy or radiotherapy alone. There was no statistically significant difference between the two treatment arms (p= 0.12).

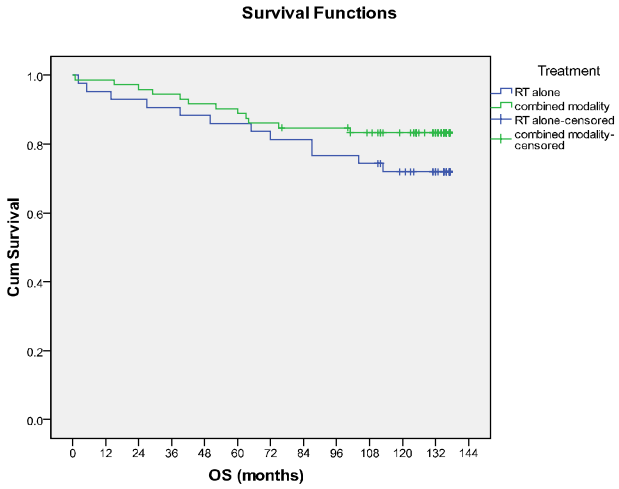

Figure 2. Kaplan–Meier Curves for Overall Survival Time in Patients with Early Stage Hodgkin's Disease by Treatment Arm. Patients were assigned to receive either combined-modality therapy or radiotherapy alone. There was no statistically significant difference between the two treatment arms (p= 0.17).

Table 3. Factors affecting disease-free survival in patients with early stage HL by treatment

Patient characteristics	Radiotherapy alone arm		Combined-modality therapy arm		*P* value
	5y DFS	10y DFS	5y DFS	10y DFS	
Age					
≥50	82	76	91	87	0.12
<50	83	75	90	88	
Sex					
Male	80	65	87	81	0.11
Female	90	85	93	93	
Pathological type					
NS	100	92	96	96	
MC	40	20	33	0.0	0.59
LD	0	0	0	0	
LP	100	100	100	100	
ESR					
>50	0.00	0.00	54	45	0.001
<50	89	79	98	96	
Stage					
I	100	94	100	100	0.01
II	73	60	89	86	
LN sites					
>3	4	0	60	46	0.01
<3	91	86	100	100	

Table 4. Factors affecting overall survival in patients with early stage HL by treatment arm

Patient characteristics	Radiotherapy alone arm		Combined-modality therapy arm		*P* value
	5y OS	10y OS	5y OS	10y OS	
Age					
>50	91	74	90	88	0.19
<50	78	68	85	75	
Sex					
Male	85	61	84	74	0.15
Female	86	81	93	93	
Pathological type					
NS	96	88	94	94	
MC	60	20	55	16	0.66
LD	0	0	50	0	
LP	100	100	100	100	
ESR					
>50	0	0	58	32	<0.001
<50	94	79	98	98	
Stage					
I	100	94	100	100	0.01
II	76	56	87	81	
LN sites					
>3	50	0	66	47	0.003
<3	91	83	98	98	
Bulky mediastinum					
YES	0	0	90	90	0.26
NO	88	73	88	80	

Table 5. Late events after therapy

Event	Number of Events After Therapy				*P*- value
	Radiotherapy alone arm		Combined-modality therapy arm		
	No.	%	No.	%	
Number of all Events	**38**		**25**		**0.18**
Second malignancy	6	14	0	0	0.001
Cardiac	5	11.6	5	6.9	0.38
Pulmonary Grade < 3 Grade > 3	 0 3	 0 7	 1 0	 1.4 0	0.11
Hypothyroidism	17	39.5	17	23.6	0.07
Hyperthyroidism	1	2.3	0	0	0.19
GIT	3	7	0	0	0.02
Other	3	7	2	2.8	0.28

4. Discussion

This study was designed to test the hypothesis that the tolerability advantage that CMT has over radiotherapy alone is maintained without affecting efficacy. To our knowledge, this is the first study at our Clinical Oncology Department, Tanta University Hospital focusing on patients with early stage Hodgkin's lymphoma that compares radiotherapy alone with CMT consisting of chemotherapy plus additional LDIFRT.

The primary endpoint of this study was to compare the efficacy of CMT with radiotherapy alone arms. The results of the CMT regimen were not statistically significantly different from those of the radiotherapy alone schedule in terms of DFS (p =0.113), or OS (p =0.173). The EORTC H7F trial compared radiotherapy alone schedule to CMT consisting of 6 cycles chemotherapy and involved-field radiotherapy [(26)]. Similarly, ten-year OS was not different in the 2 groups[(26)]. This notion was supported by a clinical trial comparing CMT with radiotherapy alone in which no significant survival disadvantage was observed in patients receiving CMT[(27)]. In this trial, 5-year DFS and OS was better but without statistical significant difference in the group receiving CMT[(27)].

In our study, the 5-year DFS rate was 85% and 90% for patients in the radiotherapy alone arm and CMT arm respectively, while the 5-year OS rate was 86% and 88% for patients in the radiotherapy alone arm and CMT arm respectively. This is comparable to that observed in other three published trials[(28-30)].

In this study elevated level of ESR > 50, stage II and number of lymph node sites > 3 had statistically significant adverse impact on the OS. Univariate analysis of our data revealed the well-known prognostic factors of number of lymph node sites of less than 3, ESR < 50, and stage I disease, to be statistically significant predictors for longer DFS. Similarly, early on the EORTC identified features at presentation that allow patients to be stratified into more favorable or less favorable prognostic groups. The unfavorable group comprised patients aged >50 years with clinical stage II and 2 to 5 involved nodal areas, if no B symptoms ESR >50 or with B symptoms ESR >30[(31)].

Secondary endpoints of our study included treatment response, tolerability and late treatment related events of each schedule. The CMT regimen was associated with a higher complete response rate than the radiotherapy alone schedule but without statistical significance. However, the results for the response analysis should not be over-interpreted because the minority of patients (9.7%) in the CMT arm had stage I compared to 41.9% of the patients in the radiotherapy alone arm (p = < 0.001). Moreover, the study population was not stratified with respect to presence or absence of bulky mediastinum (27.8% of cases in the CMT arm versus 2.3% in the radiotherapy alone arm { p = 0.001} had bulky mediastinum) or number of lymph node sites (29.2% of cases in the CMT arm versus 13.9% in the radiotherapy alone arm { p = 0.06} had > 3 lymph node sites), thus adverse prognostic factors were almost significantly higher in the CMT group which may have resulted in imbalances within this subset of patients. In their HD7 trial[(18)] the German Hodgkin Study Group (GHSG) showed in 650 patients that 2 cycles of ABVD followed by extended-field radiotherapy (EFRT) was superior to EFRT alone in clinical stages I-IIB. However, in the HD8 trial[(20)] 1204 patients with stages I and II, apart from more acute hematological, gastrointestinal and mucosal toxicity after extended-field radiotherapy, there was no difference between the trial arms in terms of complete response rates, freedom-from-treatment failure and overall survival, leading to the conclusion that when combined with effective chemotherapy a reduction in field size from extended to involved is entirely appropriate[(20)].

In this study, the radiotherapy in the CMT arm was able to be delivered in an adequate dose. The dose

of radiotherapy used in our study is accepted by many as the optimal dose for radiotherapy in a combination regimen, and proof is lacking that a higher dose is more efficacious[32,33]. Results in these trials suggested no relevant radiotherapy dose effect exists in the range of 20 to 40 Gy following 4 cycles of modern chemotherapy, indicating that doses of more than 30 Gy were no longer appropriate[32,33]. Thus devotees of the "more is better" school of oncology must face the uncomfortable truth that many trials have failed to show survival benefit associated with an increase in the total dose or dose-intensity of radiotherapy[32,33].

In 2005, the Institute of Medicine and the National Research Council of the National Academies issued the report *From Cancer Patient to Cancer Survivor: Lost in Transition*[34]. Recommendations in this report included the conduct of additional studies to measure the prevalence and risk of late effects. Thus, it becomes increasingly important for health-care providers to evaluate the risk of late sequelae, and to be able to critically evaluate research results. The need is especially important for clinicians who take care of patients with HL, in view of the escalating number of reports which document late effects[35].

Given in our study, that 5-year DFS rate was 85% and 90% for patients in the radiotherapy alone arm and CMT arm respectively, while the 5-year OS rate was 86% and 88% for patients in the radiotherapy alone arm and CMT arm respectively, and the fact that the highest incidence rates occur between ages 20 and 35 years, thus, large numbers of patients remain at lifelong risk for the late effects of treatment.

With respect to late events after therapy we found that after a median follow-up of 132 months, CMT produced significantly less nausea and vomiting, as well as less incidence of second malignancy. Other late events including pulmonary toxicity, Hypothyroidism, cardiac complications, and Hyperthyroidism were more frequent in the radiotherapy alone arm than in the CMT arm but this difference was not statistically significant (all p = NS). The results of our study were supported by the findings of other clinical trials, Hoppe[36] and Aleman et al[6] found that second primary cancers are the leading cause of death in patients with HD[6,36]. Another study demonstrated that survivors are also at elevated risks for cardiac disease, pulmonary disorders, endocrine dysfunction, and other sequelae[35]. For patients with early stage disease, the 20-year cumulative secondary malignancy rate is estimated between 4% and 20%[37,38]. Risk factors for secondary malignancies and cardiac disease are the choice and dose of chemotherapy and radiotherapy[5,6,37-42]. Koontz *et al.*[27] demonstrated that CMT resulted in statistically significant less incidence of Hypothyroidism (p = 0.0027), as well as less incidence of second malignancy (p = 0.023) than in the radiotherapy alone arm[27]. Our data confirmed this finding and this reduced late treatment related toxicities in the CMT arm was accompanied by an improvement in quality-of-life.

In summary, the results of our study showed that the CMT regimen used is safe and easy to administer in our patients. The regimen is overall less toxic than the wide field high dose radiotherapy alone schedule. Because the treatment related late events of the CMT regimen was lower than that of the radiotherapy alone regimen with comparable OS and DFS, the substitution of CMT for wide field high dose radiotherapy alone schedule is not only feasible, but may be in the patients' best interest. The regimen was so well associated with less treatment related late events that its widespread use at this point in time is recommended by several authorities in the field[17-23,27].

We recommend evidence-based treatment for early stage HL which will require large prospective randomized trials comparing efficacy, toxicity, and quality of life. Because of the complex relationship between treatment efficacy and toxicity and the diverse assumptions and expectations for treatment held by patients with cancer, the comprehensive measurement of health status should become an important and appropriate component of many clinical trials.

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The role of CDK8、STAT1 and TMEFF2 in colorectal cancer

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Abstract: In the development and progression of cancer, it is estimated that over a million new cases of colorectal cancer (CRC) are diagnosed yearly, accounting for more than 9% of all new cancer cases [Quinn M, et al.2001]. It is also the second most common cause of cancer-related deaths. Despite the recent advances in treating cancer, the 5-year survival rate from CRC remains at 50% and 10% for TNM stages III and IV, respectively[Jonathan A D Simpson,et al.2010]. In this study we investigated the molecular and clinical features of CDK8、STAT1 and TMEFF2-expressing colorectal cancers, the immunohistochemistry was used to detect the expressions of the CDK8、STAT1 and TMEFF2 in these colorectal tissues respectively. The expression of CDK8 locates in nucleus，the positive rates （100 cases colorectal cancer、15 cases adenomas and 15 cases normal mucous） were 37%（37/100)、0(0/15）and 0（0/15）respectively.The CDK8 expression in colorectal cancer was significantly higher than that in normal mucosa and colorectal adenomas (P <0.05); The CDK8 expression had no significant correlation with the clinicopathological factors in patients (P> 0.05)；there was a clear CDK8 protein loss for the older specimens(P＜0.05). The STAT1 expression locates in cytoplasm and nucleus,，and the positive rates were 26%（26/100)、73%(11/15）and 67%（10/15）respectively.The STAT1 expression in colorectal cancer was significantly lower than that in normal mucosa and colorectal adenomas（P＜0.05)；.The STAT1 expression in colon cancer was higher than that in rectal cancer.The TMEFF2 expression were all negative. The CDK8 abnormally high expressed in colorectal cancer,This study suggested that the CDK8 plays a important role in the development colorectal cancer.The decreased expression of STAT1 in colorectal cancer associated with the development of the colorectal cancer; the STAT1 expression was related with tumor location.The loss expression of the TMEFF2 may not be involved in the development of the colorectal cancer.

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[Key words] Colorectal cancer；CDK8；STAT1；TMEFF2

Colorectal cancer is a common digestive cancer, its occurrence is a multi-stage, multiple genes process, involving both the activation of multiple oncogenes and tumor suppressor gene inactivation. Currently, there are four treatment options for CRC: surgery, chemo-therapy, radiotherapy and monoclonal antibody therapy. These different modalities can be used alone or in combination, depending on the stage of disease and fitness of the patient. Despite the historical success of these modalities, none of them takes into account the individual tumour biology or the immune response to the cancer.Cyclin dependent kinase 8 (CDK8)，which encodes a member of the mediator complex, is located at13q12.13, a region of recurrent copy number gain in a substantial fraction of colon cancers.It has recently proven to be an effective cancer gene, including colorectal cancer protein and the development of a variety of malignancies,[Firestein R et al.2009];Signal transducers and activators of transcription 1(STAT1) inhibits tumor formation through a negative regulator of cell proliferation and angiogenesis [Klampfer L,et al.2006], the inactivation of the pathological process for colorectal cancer is important; transmembrane protein with EGF-like and two follistatin-like domains 2 (TMEFF2) is very limited in human normal tissues,Recent studies have shown that it did not exhibit the characteristics of tumor suppressor genes [Daniel E.H,et al.2004]. In this study, immunohistochemistry detected CDK8, STAT1 and TMEFF2 proteins in colorectal cancer, colorectal adenoma and colorectal normal mucosa, we analyzed clinical data and pathological features, explored the role of three proteins in colorectal cancer occurrence and development .Understanding the molecular pathology of colorectal cancer may improve therapeutic and diagnostic strategies.our data define CDK8 and STAT1expression in colorectal cancer as a biomarker with potentially important therapeutic implications.

Materials and Methodes

1. Study population

Three hundred and five cases were collectedfrom patients undergoing elective surgical

resection of a histologically proven colorectal cancer, colorectal adenoma and colorectal normal mucosa, The samples were collected between January 2002 and December 2012 from three hospitals,100 colorectal cancer in CDK8 group, 15 colorectal adenoma, 15 normal colorectal tissue;50 men and 50 women in colorectal cancer, aged 21-84 years, mean 64 years, 61 colon cancer, 39 rectal cancer, High and moderately differentiated was 57 cases, Low and undifferentiated was 43 cases,According to TNM staging, Ⅰ, Ⅱ stage was 46 patients, Ⅲ, Ⅳ was 54 cases. 15 casescolorectal adenoma, 15 cases normal colorectal tissue; 52 males and 48 females were colorectal cancer, aged 21 -86 years, mean age 64 years, 62 cases of colon cancer, rectal cancer, 38 cases well-differentiated, 59 cases moderately differentiated, poorly differentiated, undifferentiated 41 cases; Ⅰ, Ⅱ patients with 44 cases, Ⅲ, Ⅳ period of 56 cases,15 colorectal cancer, 15 colorectal adenoma and 15 normal colon mucosa in TMEFF2 group. All specimens received in the histopathology laboratory were incised, fixed immediately in formaldehyde and processed through to embedding in paraffin wax, ensuring optimal tissue fixation and preservation for histological examination. all patients were not receiving chemotherapy， radiotherapy or other treatment before biopsy, pathology diagnosed was definitude after biopsy.Informed consent was obtained from the patients for the use of the resected samples for research. The study complied with the appropriate institutional guidelines.

2. Immunohistochemistry

Tissue sections were placed in a hot oven at 60℃ for 30 min,dewaxed in xylene and then rehydrated in three baths of 100%,90% and 70% ethanol. Endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxide in Tris-buffered saline (TBS). Antigen retrieval was achieved using a rotary microwave oven; the slides were immersed in citrate buffer (pH6.5) and placed in the centre of the oven for 10 min at 800 W then for another 10 min at 300 W. The slides were cooled down immediately for 10 min with tap water,serum in TBS was added to block non-specific adsorption of the antibodies to the tissue.human monoclonal antibodies anti-CDK8 antibody (sigma, USA), anti-STAT1 antibody (sigma, USA) and anti-TMEFF2 antibody (sigma, USA) were incubated with the tissue sections for 1h at room temperature. Universal streptavidin-biotin-peroxidase and DAB kits were used to detect specific antibody binding according to the manufacturer's instructions.The slides were finally counterstained with haematoxylin (sigma, USA), then dehydrated and mounted.Negative controls were done by omitting the primary antibody.

3.Scoring

Positive: CDK8 appears clear tan or brown staining in the background of nucleus; STAT1 appears clear tan or brown stain in the background of the cytoplasm or nucleus; TMEFF2 appears clear background tan or brown stain in skin cells or fibroblasts cytoplasm or the nucleus[3].Accordance with the degree of positive staining cells (antigen content), can be divided into weakly positive (+); middle positive (++); strongly positive (+++). According to the number of positive cells, can be divided into:weak positive (+; the total number of positive cells in 25% or less); middle positive (++; the total number of positive cells in 25% -49%); strongly positive (+++; more than 50% positive cells of the total number). In this study, We used an integrated measurement points. The formula is: (+)% × 1+ (++)% × 2 +(+++)% × 3; total value of <1.0 for the (+) ,1.0-1 .5 were (++),> 1.5 were (+++).least 5-10 HPF were observed.

4 Statistical analysis

Data are expressed as mean ± SD. Differences between groups were compared using Student's t test. Pearson's $\chi 2$ test was used to analyze the correlation between variables. $P<0.05$ was considered to be statistically significant. Data were analyzed using SPSS 12.0.

Results

1. CDK8, STAT1 and TMEFF2 expression

CDK8 expression in colorectal cancer, colorectal adenoma and colorectal normal mucosa were 37% (37/100), 0% (0/15) and 0% (0/15) respectly, CDK8 expression in colorectal cancer tissue was significantly higher than the normal colorectal mucosa and adenomas ($\chi 2 = 6.575$ $p = 0.010$), As shown in Figure 1 A, B, C and Table 1; STAT1 in colorectal cancer, colorectal adenoma and colorectal normal mucosa were 26% (26/100), 67% (10/15) and 73% (11/15) respectly,STAT1 expression in colorectal cancer was significantly lower than normal colorectal tissue ($\chi 2 = 11.310$ $p = 0.001$) and adenoma ($\chi 2 = 8.229$ $p = 0.004$), but there was no significant difference in the expression between the normal mucosa and colorectal adenomas, such as Figure 1 D, E, F and Table1. As shown in Figure G, H,TMEFF2 was negativein in colorectal cancer, colorectal adenoma and colorectal normal mucosa.

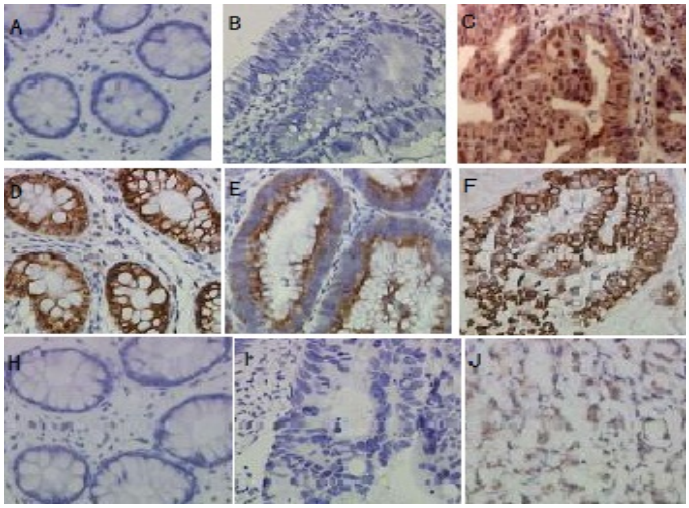

Figure 1. CDK8, STAT1, and TMEFF2 in colorectal carcinoma.
No CDK8 expression in normal colorectal tissue (A) and adenoma (B); visible expression in the cell nucleus of colorectal cancer (C); STAT1 expression in most normal colon tissue (D) and the cytoplasm or nucleus of colorectal adenomas (E), occasionally expressed in colorectal cancer cells (F); No TMEFF2 expression in normal colorectal tissue (H), colorectal cancer (J),visible expression in prostate cancer (J).Evision two-step method, DAB staining × 400.

Table 1. CDK8 and STAT1 expression in each group

pathological features	N	STAT1				N	CDK8			
		positive	negative	x^2	P		positive	negative	x^2	P
Normal mucosa	15	11	4	11. 310	0. 001	15	0	15	6. 575	0. 010
Adenoma	15	10	5	8. 789	0. 003	15	0	15	6. 575	0. 010
Colorectal cancer	100	26	74			100	37	63		

2. Clinical pathological and CDK8, STAT1 expression

STAT1 expression in colorectal cancer was significantly lower than the colon ($\chi 2 = 4.759$ $p = 0.0029$), there was no significant correlation in STAT1 expression (including age, sex, differentiation, stage, whether the lymph nodes transfer) ($p > 0.05$), As shown in Table 2; there was no significant correlation in CDK8 expression (including patient age, sex, tumor location, differentiation, stage, lymph node metastasis).

Table 2. The relationship of CDK8 and STAT1 between the expression and clinicopathological factors

pathological features		STAT1 N	positive	negative	x^2	P	CDK8 N	positive	negative	x^2	P
Age	＜65	44	14	30			45	17	28		
	≥65	56	12	44	1 .382	0 .240	55	20	35	0. 021	0. 884
Sex	M	52	15	37			50	17	33		
	W	48	11	37	0 .456	0. 499	50	20	30	0. 386	0. 534
Location	colon	62	21	41			61	20	41		
	rectum	38	5	33	5. 254	0 .022	39	17	22	1. 191	0. 275
Differ	H/ M	59	18	41			57	20	37		
	L/U	41	8	33	1. 520	0. 218	43	17	26	0. 208	0. 648
Tumor-stage	Ⅰ、Ⅱ	44	13	31			46	17	29		
	Ⅲ、Ⅳ	56	13	43	0. 513	0. 474	54	20	34	0. 000	0. 993
Lymph-metastasis	N	44	13	31			46	17	29		
	Y	56	13	43	0. 513	0. 474	54	20	34	0. 000	0. 993

Discussion

Colorectal cancer is the colorectal epithelium and glandular malignancies,ranking the second cause of cancer deaths in recent years, the incidence was significantly increased.The pathological process of colorectal cancer involved ina variety of molecular biological mechanisms,A variety of oncogenes and tumor suppressor genes were involved in the process of development of cancer from the normal mucosa.CDK8 resides on a region of Chromosome 13 that is known to undergo chromosomal gain in 60% of colorectal cancers[Firestein R,et al.2008;Tsafrir D,et al.2006; Sheffer M,et al.2009;Martin ES, et al.2007].Previous studies have shown that CDK8 is overexpressed in a subset o f colorectal cancers[Firestein R,et al.2008;Tsafrir D,et al.2006;Martin ES, et al.2007],4,28,30.Recently, CDK8 proved to be an effective cancer proteins, including a variety of malignancies, including colorectal cancer incidence and development. there is a typical WNT / β pathway of unusual activity in almost all colorectal cancer, the activity of this pathway was related to colorectal cancer growth, invasion, survival[Conaway,R.C,et al.2005], but the full formation of malignant tumors need other genes participation, such as CDK8. CDK8 regulated the activities of β-catenin gene in colorectal cancer, and play an important role in cancer cell proliferation [Firestein R,et al.2009].In this study, CDK8 expression in colorectal cancer was significantly higher than the normal colon mucosa and colorectal adenomas,so,CDK8 play an important role in the pathological process of colorectal cancer, indicated that CDK8 was the colorectal cancer gene. the high expression of CDK8 in a considerable part of colorectal cancer cases; the expression of 37% was higher than 26% of thef irst results of Firestein [Firestein R,et al.2009] but less than 70%-positive rate as shown in the second [Firestein R,et al.2010]; the results can be powerful description of CDK8 in the high expression rates of colorectal cancer.between CDK8 expression and clinicopathological factors,there were not relationship in age, sex, tumor location, tumor differentiation, tumor stage, lymph node metastasis,CDK8 expression is not correlated with the findings of Firestein, Men CDK8 expression results are slightly different from female colorectal cancer .

STAT1 gene is located on 2q32.2, encoding 750 amino acid residues with a molecular weight of 91kDa, which can be activated by a variety of ligands, including IFNα, IFNγ, EGF, PDGF and IL6. In contrast to other STATs, STAT1 played a negative regulatory role in tumor cell growth[Yu H,et al.2004]. STAT1 negatively regulated the cell proliferation and angiogenesis, thereby inhibiting tumor formation. STAT1 is an important molecule in IFN-γ signaling pathway,endogenous INF-γ and STAT1 form a basic

monitoring system to control the formation of chemically induced and spontaneous tumor development.but it played an important role in the regulation of tumor progression.STAT1 low expression is mainly due to DNA methylation of Colorectal cancer [Dhruva Kumar Mishra,et al.2010] and STAT1 miRNA interference [Gregersen LH,et al.2010].Our study found that STAT1 expression in Colorectal cancer was significantly lower than intestine colorectal adenomas and large intestine normal mucosa,As a tumor suppressor gene，STAT1 which downregulated in colorectal lose its inhibitory role in tumor development, In addition, the absence of STAT1 expression was lower in rectal cancer than colon cancer,In other malignant consistent with the results. indicating the location of the tumor in the rectum or colon, Klampfer [Lidija Klampfer,t al.2008] thought that the STAT1 expression levels significantly decreased in transformed intestinal epithelial cells,In this study,Only the STAT1 expression decreased in colorectal cancer,compared with the STAT1 expression of normal colorectal mucosa,There was not statistically significant in colorectal adenomas; In addition, Simpson et al [Simpson JA,et al.2010] found that nucleus STAT1 was the independent prognostic indicators for colon cancer, compared to with low levels the nucleus STAT1 of rectum Cancer,Survival time of colon cancer patients with high levels of nucleus STAT1 was extension,All of these results also explain that STAT1 played an important role in the development and occurrence of colorectal cancer.

TMEFF2 located in 2q32.3, 246,666 base pairs,17 exons,encoding the transmembrane protein containing 374 amino acid, it was tissue-specific expression,its expression is very limited in normal tissue, mainly expressed in the prostate and central nervous system, in the colon tissue,only expressed TMEFF2 mRNA and without protein express [Young J,et al.2001]. TMEFF2 in prostate cancer was over-expression [Daniel E.H,et al.2004], people thought that TMEFF2 acted as a tumor suppressor gene, TMEFF2 may inhibit tumor cell growth, prostate cancer showed a higher degree of malignancy without TMEFF2 expression;But Afar [Daniel E.H,et al.2004] proved that TMEFF2 did not show the characteristics of tumor suppressor genes, overexpressing TMEFF2 did not inhibited cell proliferation in prostate cancer PC3 cell line. In addition, there is a wide range of TMEFF2 methylation [Qiong He,et al.2010] and the phenomenon of frequently loss of heterozygosity in colorectal cancer [Fabian Model,et al.2007], so that the TMEFF2 mRNA had no expression in the majority of colon tumors and other tumors, TMEFF2 can not act as a tumor suppressor gene. In this study, TMEFF2 protein expression can be detected in prostate cancer,However, TMEFF2 protein expression can not be detected in 15 cases of colorectal cancer, 15 cases of colorectal adenomas and 15 normal colorectal mucosa tissues, our results further confirmed that there was no TMEFF2 protein in the large intestine, TMEFF2 was tissue-specific expression; the same time, we provided further evidence for the questioned whether TMEFF2 was the tumor suppressor gene, at least, TMEFF2 did not expresse tumor suppressor protein in colorectal cancer.we must recognized that there was TMEFF2 mRNA expression, but no protein product in the normal colon tissue, and did not shows no methylation in most of the normal colon and inflammatory bowel disease tissue, the imechanism needs further study.

In summary, we have conducted a large sample of CDK8, STAT1, TMEFF2 express.Our findings show that CDK8 is expressed in a high fraction of colorectal cancers,over-expression CDK8 played an important role in the development of colorectal cancer; STAT1 were down-regulated in colorectal cancer, the absence of STAT1 expression was related to the occurrence and development of colorectal cancer;compared to rectum cancer,the absence of STAT1 expression is more obvious than the colon; there was no TMEFF2 protein expression in colorectal tissue, TMEFF2 is not the tumor suppressor gene of colorectal cancer. Thus, CDK8 and STAT1 may be the new indication and new therapeutic targets for the detection of colorectal cancer.these findings may be of great use in defining patients that may be distinctly susceptible to small molecule-based therapies.

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Determinants of Child Mortality in Rural Nigeria

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Abstract: This study examined the determinants of child mortality in rural Nigeria employing the 2008 Nigeria Demographic and Health Survey (NDHS) data. Data were analyzed using Descriptive Statistics and the Logit regression model. The result of analysis showed that the average age of the respondents at first birth is 19 years; while more than half of them had no formal education, and about three-fifths had less than 24 months birth interval. Secondary and higher education of mother, age of mother at first birth, place of delivery, type of birth, child ever breastfed, sex of child, were among the significant factors influencing child mortality in rural Nigeria. Maternal education, access to adequate health care (especially for pregnant women and children under five years) and increased awareness of benefits of breastfeeding were identified as the key factors to reducing child mortality in rural Nigeria.

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Keywords: Determinants**,** Child mortality, Rural Nigeria, Logit model.

1. Introduction

Child mortality defined as the likelihood for a child born alive to die between its first and fifth birthday, is one of the most sensitive and commonly used indicators of the social and economic development of a population. Thus, it is frequently on the program of public health and international development agencies and has received renewed attention as a part of the United Nation's Millennium Development Goals (MDG; Espo, 2002). The MDG target is to reduce child mortality by two thirds in the year 2015. This is pertinent as the progress and future of any country depends on how healthy the children are. This is reflected in their access to basic health care, nutritious food and a protective environment, and if these are not available, the country's mortality rates would increase and economic potentials diminish (WHO, 2008).Globally, according to the UN Inter-agency Group on Child Mortality Estimation (2011) a significant amount of progress has been made towards achieving the target of reducing mortality rate by two thirds among children under five. For instance the number of under-five deaths worldwide has declined from more than 12 million in 1990 to 7.6 million in 2010. However, the highest rates of child mortality are still in Sub-Saharan Africa-where 1 in 8 children dies before the age of 5 years, more than 20 times the average for industrialized countries (1 in 167) and South Asia (1in 15) despite action plans, interventions and broad approaches toward improving child's health in the region (WHO, 2005). Further, West African countries in particular experienced mortality up to three times higher than neighbouring countries in Northern and Southern Africa (Balk *et al.,* 2004) and of all the under-five deaths which occur, five countries namely; India, Nigeria, Democratic Republic of the Congo, Pakistan and China account for about 50% with India (22%) and Nigeria (11%) together accounting for a third of all under-five deaths.

Nigeria, despite its wealth of human and natural resources, the Federal Ministry of Health's Integrated Maternal, Newborn and Child Health Strategy and the fact that it is one of the first African countries with an integrated plan to look after mothers, newborns and children right through from conception to the child's fifth birthday, is one of the least successful of African countries in achieving improvements in child survival in the past four decades (Nigeria Health Journal, 2011).

Childhood deaths in Nigeria are usually caused by avoidable environmental threats to health which stem most often than not from traditional problems that have long been resolved in the wealthier countries, such as a lack of clean water, sanitation, adequate housing, and protection from mosquitoes, other insects and animal disease vectors and in people's beliefs and attitudes concerning childcare and behavioural practices into health strategies (Feyisetan & Adedokun, 1992; Ogunjuyigbe,_2004). Children from poor households are more vulnerable to these attendant risks compared with children born to better off families. They are usually more exposed to risks such as inadequate water and sanitation, indoor air pollution, crowding and exposure to disease vectors and are more likely than not to be undernourished. They are ,therefore, at greater risk of severe disease, and are more likely to suffer from more than one disease when ill. They are less likely to have access and use preventive and curative

interventions, and those who do receive treatment are less likely to receive appropriate quality services (Wagstaff *et al.,* 2004). Thus, at the dawn of the 21st century, childhood mortality which is an indicator of health status of a country is very crucial and remains a daunting issue for these developing countries and rural Nigeria in particular where poverty rates are disproportionately high. Based on this foregoing, apart from contributing to literature on child mortality in Nigeria, this study attempts to provide empirical information on the factors that contribute to high childhood mortality, its slow decline in the country and help suggest appropriate health interventions or programs.

2. Review of Empirical Studies on Determinants of Child Mortality

Determinants of childhood mortality have been viewed from a number of analytical frameworks. This dates back to Mosley and Chen (1984) and Schultz (1984) who made the distinction between variables considered to be exogenous or socio-economic (i.e. cultural, social, economic, community, and regional factors) and endogenous or biomedical factors (i.e. breastfeeding patterns, hygiene, sanitary measures, and nutrition). The effects of the exogenous variables were considered indirect because they operate through the endogenous biomedical factors while the bio-medical factors were called intermediate variables or proximate determinants because they constitute the middle step between the exogenous variables and child mortality.

Empirically, many studies have shown that child mortality is influenced by a number of socio economic and demographic factors such as sex of the child, mother's age at birth, birth order, preceding birth interval among others. For instance, Mondal *et al.* (2009) using the logistic regression model, investigated factors influencing infant and child mortality in Rajshahi District of Bangladesh. Findings revealed that the most significant predictors of neonatal, post-neonatal and child mortality levels are immunization, ever breastfeeding, mother's age at birth and birth interval. In a similar vein, Chowdhury *et al.* (2010) examined the effects of demographic characteristics on neonatal, post neonatal, infant and child mortality also using the logistic regression model. They identified the important predictors of neonatal mortality as breast feeding practice, of post-neonatal period as duration of marriage, order of birth and birth interval and of infant and child mortality as age at marriage, duration of marriage, birth interval, birth order and breast feeding practice.

Uddin et al. (2009) in their study, investigated child mortality in Bangladesh also using the logistic regression. Results of analysis showed that father's education, occupation of father, occupation of mother, standard of living index, breastfeeding status and birth order were significant determinants of child mortality in Bangladesh.

Hong (2006) showed that levels of infant and child mortality in many developing countries remain unacceptably high, and they are disproportionably higher among high-risk groups such as newborn and infant of multiple births. A mother's poor health and poor nutritional status may also have postnatal consequences such as impaired lactation and render her unable to give adequate care to her children (Retherford *et al.,* 1989). Some studies show that child mortality is lower for boys than for girls (Huq and Cleland, 1990; Kabir and Chowdhury, 1992) while, child mortality has been noted to peak in places where living conditions are lowest (Millard, Ferguson and Khali, 1990).

Kumar and File (2005) used data from the Ethiopia Demographic and Health Survey [EDHS] conducted in 2005 to investigate the predictors of child [0-5 years] mortality in Ethiopia. The cross tabulation technique was used to estimate the predictors of child mortality. Results revealed that birth interval with previous child and mother standard of living index were the vital factors associated with child mortality. Furthermore, Mother's education and birth order were found to have substantial impact on child mortality in Ethiopia. The study concluded that an increase in Mothers' education and improved health care services are significant in reducing child mortality in Ethiopia.

Mesike and Mojekwu (2012) in their study examined the environmental determinants of child mortality in Nigeria using principal component analysis and simultaneous multiple regression for child mortality modelling in Nigeria. Estimation from the stepwise regression model showed that household environmental characteristics do have significant impact on mortality as lower mortality rates were experienced in households that had access to immunization, sanitation facilities, good and proper refuse and solid waste disposal facilities, good healthy roofing and flooring materials as well as those using low polluting fuels as their main source of cooking.

3. Materials and Methods

Secondary data used for this study was the 2008 Nigeria Demographic and Health Survey (NDHS) data. The sampling frame used for the 2008 NDHS was the 2006 National Population and Housing Census of the Federal Republic of Nigeria. The survey covered all the 36 states and the Federal Capital Territory, Abuja. The primary sampling unit (PSU), referred to as a cluster, was defined on the

basis of Enumeration Areas (EAs) from the 2006 census frame. The sampling procedure used by 2008 NDHS was a stratified two-stage cluster design. In the first stage, 286 clusters were selected at the urban area while 602 clusters were selected in the rural areas. In the second stage of the selection, 41 households were selected in each cluster, by equal probability systematic sampling. Hence, a representative sample of 36,410 households were selected for the 2008 NDHS survey with 24,684 households from the rural areas and 11,726 households from urban areas. However, for this study, only 21034 households were used out of the 24,684 households canvassed for the study in the rural areas, due to incomplete information by some of the respondents. These 21,034 households constituted the sample size.

The analytical tools employed in this study include: Descriptive statistics and the Logistic Regression model. Descriptive statistics include the use of frequencies, mean, percentages and tables which was used to analyse the socio-economic characteristics of the respondents while the logistic regression method was used to identify the variables that have significant influence on under-five mortality among rural households.

The logistic regression model expresses a qualitative dependent variable as a function of several independent variables. It is used when the dependent variable is dichotomous and the independents are of any type. In this analysis, child mortality (Z) is the dependent variable which takes the value of 1, if mortality occurs among under- five year old children in the household and 0 if otherwise, i.e.

Z = 1, If mortality occurs for any child between ages 1 to 5 in the household
0, otherwise

The logit model postulates the probability (P_1) that child mortality is a function of an index (Z_i)

Where:

(Z_i) is an inverse of the standard logistic cumulative function of P_i i.e. $P_i(y) = f(Z_i)$

(Z_i) is also an inverse of the standard logistic cumulative function of P_i i.e $P_i(y=1) = f(Z_i)$

The probability of child mortality is given by $P_i\,(y=1) = \frac{1}{1+e}$-zi

The probability of no child mortality is given by $Q_1(y=0) = 1 - P_i\,(y=1)$

Since,

$$\begin{cases} 1\text{-Pi}(y=1) = \mathbf{1} - \frac{1}{1+e^{-zi}} \\ 1\text{- Pi}\,(y=1) = \frac{1+e^{-zi}-1}{1+e^{-zi}} \\ 1\text{-Pi}\,(y=1) = \frac{e^{-zi}}{1+e^{-zi}} \end{cases}$$

But

$$\frac{1}{P_i\,(y=1)} = \mathbf{1} + e^{-zi}$$

Thus, $\frac{P_i\,(y=1)}{1-P_i\,(y=1)} = \frac{1}{e^{-zi}}$

and $\frac{P_i\,(y=1)}{1-P_i\,(y=1)} = e^{zi}$

The probability that child mortality occurs is calculated from Z_i value

$Z_i = b_0 + b_1X_1 + b_2X_2$----------$+ b_nX_n$

Where:

X_1-X_n are the independent variables

Z =Under-five mortality in household (1 if yes, 0 Otherwise)

B_0 = constant

B_1 is the coefficient of the X's variables

4. Results and Discussion

4.1 Socio-economic and Demographic Characteristics of Respondents

The distribution by educational status of the respondents in table I revealed that more than half of the mothers (58%) do not have formal education as expected. This could be as a result of the fact that in the rural areas, formal education is not a requirement for fitting into the way of life. However, the implication of this is a low level of welfare of the child as a mother's education is directly related with the health of a child. This is because education makes a mother highly developed, free from traditional values, which leads to changes in behavioural patterns, attitude and improved welfare of the child (Mondal *et al.,* 2009).

With respect to the age of the respondents at first birth which ranged between 15 to 45 years, almost three-quarters (72.0%) were aged below 20 years at first birth with an average age at first birth of 19 years. This indicates a higher chance of child mortality due to complications in pregnancy and delivery, premature birth and other related causes

The incidence of infant and child mortality is expected to be lower among working women than those unemployed since a mother's occupation is usually associated with the nutritional status of their children. Women/mothers that have a source of income are able to provide food in the right quantity and quality and other essential needs for their children which otherwise would have been

impossible if they were unemployed. According to Table I, 32.0% of the respondents are unemployed, 24.4% are farmers while 29.6% are involved in trading as their primary occupation. Also, more than half of the children born were males while 49% were females.

Poverty influences health because it largely determines environmental risks, as well as access to resources to deal with those risks. Wealth index was used to evaluate the influence of social class on fertility behaviour and health of mother and child. The index was estimated using the respondent's assets and their standard of living (NDHS, 2008). Results from table I show that 64.6% of the respondents are poor, 19.7% are in the middle class while 15.7% are rich. This reveals the extent of poverty and inequality in rural Nigeria. It also corroborates the findings of IFAD (2010) in which about 80 % of the rural households were found to be poor.

Access to electricity and television were used as proxies for access to infrastructure and information respectively. Table I revealed that while more than three-quarters of the respondents did not have access to electricity, more than four- fifths did not have access to television. This is an indication of inadequate infrastructure as well as decreased awareness about child health since households learn about childcare, proper hygiene and sanitation through the various programmes on public health. Usually, poor households rely on biomass fuels for cooking and heating because they are unable to afford clean fuels such as kerosene and gas. In line with this, results show that the major cooking fuel in the study area is wood. Also, according to table I, more than half of the respondents (53.1%) do not have toilet facilities i.e. they make use of bush as their toilet while approximately 43.5% use pit latrine and only 3.4% use flush toilet. This is an indication of poor sanitary conditions in the rural areas of Nigeria.

Access to good sanitation facilities is believed to reduce morbidity and diarrhea which is one of the major causes of under-five mortality in Nigeria. Water could act as a medium for many diseases that is waterborne such as diarrhea, which poses the greatest threat to child survival in Sub-Saharan Africa countries. As shown in table I a greater proportion of the respondents (76.6%) do not have access to safe drinking water and as such are exposed to diseases and infection.

The household head (which is the father in most cases in rural households of Nigeria) is the main income earner and decision maker of a family. Highlights of the occupational distribution shows that most household heads (55.4%) are farmers while the remaining are engaged in other activities which include trading, teaching e.t.c. This confirms previous findings in other studies (Fayehun & Omololu, 2009; Uddin *et al.,* 2009; Mondal *et al.*, 2009) that farming is the predominant occupation in rural areas.

4.2 Determinants of Child Mortality in Rural Nigeria

Results of the logistic regression as shown in table II identifies factors such as secondary education, higher education, age at first birth squared, place of delivery, type of birth, ever breastfed, sex of child, father's education, father's occupation, type of toilet facility, rich class, North East, North West and South East as the major factors influencing child mortality in rural Nigeria. While the square of age at first birth, sex of child, North East, North West and South East increased child mortality in rural Nigeria, secondary education, higher education, place of delivery, type of Birth, ever breastfed, father's education, father's occupation and rich class were the factors associated with a reduction in child mortality in rural Nigeria. The chi-square value of 814.88 which is significant at 1% implies that all the independent variables jointly explain the likelihood of child mortality.

Secondary and tertiary education of the respondents had a negative impact on child mortality in rural Nigeria implying that the higher the level of educational attainment of the respondents the lower the level of child mortality in the household. This could be attributed to the fact that women or mothers with no or low educational attainment are unable to inculcate modern health knowledge and practices which are basic requirements for enhancing child health. The coefficient of primary education although not significant, was however positive indicating that respondents with primary education had a higher level of child mortality. This is consistent with the findings of (Chowdhury *et al.,* 2010) and (Iyun, 2000) that child mortality is higher among women with primary education and lower among women with higher education.

Proper medical attention and hygienic condition during delivery can reduce the risk of infections and facilitate management of complications that can be the cause death or various illnesses for the mother or the newborn child (Uddin *et al.,* 2009). The negative and significant coefficient of place of delivery implies that children born in hospital and maternity clinic have a lower risk of mortality as expected, owing to proper healthcare and attention from professionals compared with those born at home.

With respect to type of birth, the result indicates that the risk of childhood mortality is significantly lower among single births than multiple births. This could be as a result of the fact that babies of multiple births usually become physically weak when they are born (Chowdhury *et al.,* 2010). These children may face competition for resources such as food and medical care leading to increase in mortality.

The coefficient of ever breastfed was negative and significant at 1% indicating that children who are breastfed have a lower risk of mortality than those not breastfed. This could be attributed to the fact that breastfeeding combats various infectious disease and strengthens essential antibody system of the children (Chowdhury *et al.,* 2010). This finding also corroborates the findings of (Mondal *et al.*, 2009) and (Uddin *et al.*, 2009) that breastfeeding has a beneficial effect on the nutritional status, morbidity and mortality of infants.

The negative correlation between father's education and child mortality indicates that the higher the level of education of the father the lower the level of mortality. The fact that the father's education plays an important role in earning income, which in turn ensures adequate nutrition, clothing and housing for the household, is a reflection of the direct relationship between father's education and access to child health facilities (Mondal *et al.,* 2009). Moreover, it is likely that higher educated people belong to higher economic class. This finding is consistent with (Uddin *et al.*, 2009) and(Iyun, 2000) that mortality is higher among children whose father's have no formal education and lower among children whose father's have higher education.

The positive co-efficient of father's occupation implies higher level of child mortality with fathers who are engaged in farming as the primary source of income. For many households in Nigeria, especially in the rural areas, agriculture is the main occupation. However, previous and current analyses of poverty have shown that poverty is disproportionately concentrated among households whose primary livelihood depends on agriculture. This can be attributed to the fact that farming is highly prone to natural hazards like drought, flood, pest and disease infestation and so on. These factors and many more (low prices during peak of harvesting, poor infrastructural facilities) contribute to a reduction in the returns that can be reaped from farming which invariably leads to a sizeable reduction in incomes of the individuals belonging to these households and consequently lead to a low level of welfare of the households.

The negative effect of type of toilet facilities on child mortality implies that access to sanitary means of excreta disposal reduces the risk of mortality and is a strong predictor of infant mortality. In other words, access to modern sanitation facilities (flush toilets) reduces diarrhea incidence which is a major cause of child mortality. This result is consistent with the findings of (Mondal *et al.,* 2009) and (Jacoby *et al.*, 2003). Similarly, there is a negative association between the wealth index for the rich class and child mortality indicating that children born in wealthier households experience lower mortality (Uddin *et al.*, 2009). This is because a child born to a financially privileged and well educated family is less likely at risk of dying prenatally or within the first month of life, since the mother was probably well nourished during pregnancy, and is likely to have delivered at a health facility.

The square of age of mother at first birth had a positive effect on child mortality indicating that as age of mother at first birth increases, child mortality increases. This is because older mothers are usually at a higher risk of pregnancy related complications. On the other hand, the significant and negative coefficient of 0.165 of sex of child indicates that mortality is higher among female children than male children in the study area. This is expected particularly in the context of Nigeria, where gender discrimination in favour of the male child is the norm. This is because the male child is expected to continue the family lineage and provide old age security.

The positive and significant coefficient of the North East, North West and South East regional dummies implies that the rate of child mortality is higher in these zones. This differential in childhood mortality among various zones in Nigeria is a pointer to where resources should be effectively targeted for the country to achieve the MDG on child mortality.

Table I: Socioeconomic and Demographic Characteristics of Respondents

Variable	Frequency	Percent
Level of Education		
No education	12,204	58.0
Primary	4,820	22.9
Secondary	3,577	17.0
Higher	433	2.1
Age of Mother at first birth		
Under 20	15,287	71.0
21-30	5503	26.2
>30	244	2.8
Sex of Child		
Male	10,744	51.1
Female	10,290	48.9
Mother's Occupation		
Unemployed	6,752	32.0
Farming	5,125	24.4
Trading	6,216	29.6
Others	2,941	14.0
Wealth Index		
Poor	13,587	64.6
Middle	4,149	19.7
Rich	3,298	15.7
Access to Electricity		
Yes	5,148	24.5
No	15,886	75.5
Access to Television		
Yes	3,967	18.8
No	17,067	81.2
Types of Cooking Fuel		
Wood	12729	60.8
Charcoal	6225	30.2
Kerosene	1655	7.8
Gas	425	1.2
Type of Toilet Facility		
No facility	11,158	53.1
Pit latrine	9,143	43.5
Flush Toilet	733	3.4
Source of Drinking Water		
Tap water/Borehole	4,931	23.5
Well Water	6,125	29.1
Stream/River/Lake	9,978	47.4
Household Head's Occupation		
Farming	11,648	55.4
Trading	3,039	14.4
Others	6,347	30.2

Source: NDHS, 2008

Table II: The Determinants of Child Mortality in Rural Nigeria

Variables	Coefficients	Standard Error	Z-statics
Constant	-0.021	0.405	-0.05
Primary education	0.083	0.066	1.26
Secondary education	-0.278	0.086	-3.24***
Higher education	-0.920	0.228	-4.03***
Age at 1st birth	0.041	0.036	1.14
Age at 1st birth squared	0.034	0.020	1.65*
Mother's occupation	-0.053	0.048	-1.11
Place of delivery	-0.221	0.064	-3.47***
Type of birth	-1.106	0.089	-12.38***
Ever breastfed	-0.975	0.044	-22.04***
Sex child	-0.165	0.043	-3.81***
Father's Education	-0.323	0.112	-2.88***
Father's occupation	0.082	0.049	1.68*
Access to electricity	-0.046	0.068	-0.67
Access to television	0.049	0.089	0.55
Type of floor materials	0.086	0.066	1.31
Type of cooking materials	0.084	0.124	0.68
Source of water	-0.033	0.047	-0.70
Type of toilet facility	-0.158	0.051	-3.09***
Middle class	-0.079	0.092	-0.86
Rich class	-0.422	0.216	-1.96**
North East	0.203	0.077	2.65***
North West	0.226	0.077	2.94***
South East	0.172	0.103	1.66*
South West	-0.058	0.092	-0.63
South South	-0.003	0.056	-0.06

Source: Regression Results, 2010

Log likelihood = -7372.116; Chi Squared (X^2) = 814.88; Pseudo R^2 = 0.0524;

[xxx]Significant at 1%, [xx] Significant at 5%, [x] Significant at 10%

5. Conclusion

Nigeria has an accelerated population growth with the resources not growing at the same rate. Thus poverty level has increased in the country drastically and has led to illiteracy, increased risks of illnesses, such as malaria and diarrhea, due to poor living conditions, limited access to safe water and other basic infrastructure, inadequate sanitation, malnutrition from household food insecurity, child labour, early marriage and other causes of child mortality.

Since child mortality is a powerful indicator for measuring the overall health situation of a country and also a powerful social indicator, special attention should be given to it as children are not only assets but they are the future of a country or a nation. Based on the findings of this study, this study suggests that:

- maternal education should be advocated as a strategy to reduce child mortality. This could be achieved through female literacy programmes in the rural areas. Also, women should be enlightened on the attendant risk of early marriage and child birth, small birth interval and its implication on child mortality. This could be incorporated into the female literacy programmes.
- government should see to the provision of more primary healthcare facilities in the rural areas to ensure adequate coverage of and accessibility to the health facilities.
- there is a need to intensify awareness on the benefits of breastfeeding since breastfeeding has been found to combat various infectious diseases and strengthens essential antibody system of the children. This could be achieved through awareness campaigns and enlightenment programs by effective participation of government, NGOs, religion leaders and mass media. .
- government should intensify efforts at providing rural infrastructure for instance potable water, modern waste disposal

facilities to reduce the risk of exposure to diseases such as malaria and diarrhea which are the leading causes of infant and child mortality as well as make public and private investments in health infrastructure.

Appropriate health interventions be targeted to regions with high infant and child mortality in rural Nigeria.

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HEPATOTOXIC EFFECT OF SUB-ACUTE EXPOSURE OF TREATED CARBANACEOUS EFFLUENT ON MICE

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ABSTRACT: The present study examined the hepatotoxic effects of carbonaceous wastewater in mice, the mice were exposed to five different concentrations of the waste water. Cyclophosphomide was used as the positive control and distilled water was used as a negative control, for a period of 35das. At post exposure, the activities of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and Alkaline phosphatase (ALP) in serum were measured as indicators of liver function. The weights of the animals were recorded weekly after which their liver were harvested. Organ weight was measured at post exposure and preserved afterwards for histology. The physical, chemical and heavy metal composition of the wastewater was also analysed. The liver weight of the exposed mice was however significantly different from that of the negative control in the 25% and 75% concentration of the wastewater administered at $p<0.05$. The activities of ALP, ALT, and AST in the serum of exposed mice were significantly increased compared to the negative control mice and this increase was concentration dependent at $P<0.05$. The histological lesions observed in the liver at various concentrations examined included Kupfer cell hyperplasia, severe portal congestion, portal and central venous congestion and mild hydrople degeneration of hepatocytes. The results of the study showed that the observed hepatotoxic effect in the exposed mice may be caused by the presence of heavy metal and other physical and chemical substances present in the wastewater. This suggests a higher risk to liver damage in humans and other organisms exposed to this wastewater and may also be deleterious to the surrounding environment.

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Keywords: Alanine aminotransferase, Aspartate aminotransferase, Alkaline phosphatise, carbonaceous effluent, hepatotoxic effect, Mice, wastewater

1.0 INTRODUCTION

The liver serves many vital functions in the body. It has a large capacity to hold blood and thus serves as a blood storage site (LeBlanc and Dauterman, 2001). The liver synthesizes and secretes many substances that are necessary for normal bodily function. It cleanses the blood of various endogenous and foreign molecules (Rankin, 1998). It biotransforms both endogenous and exogenous materials, reducing their bioreactivitiy and preparing them for elimination. It eliminates wastes and foreign materials through biliary excretion (Klaassen, 2001). Three of these functions occur in a manner that makes the liver a major organ of chemical elimination: chemical uptake from blood, chemical biotransformation, and biliary elimination of chemicals. This process is called entero-hepatic circulation. A chemical may undergo several cycles of entero-hepatic circulation resulting in significant increase in the retention time for the chemical in the body and increased toxicity. The liver functions to collect chemicals and other wastes from the body. Accordingly, chemicals may be retained in the liver at high concentrations resulting in toxicity. The biotransformation of chemicals that occur in the liver sometimes results in the generation of reactive compounds that are more toxic than the parent compound thus causing damage to the liver (Le Blanc and Dauterman, 2001)

Alanine Aminotransferase (ALT) is the enzyme produced within the cells of the liver. The level of ALT abnormality is increased in conditions where cells of the liver have been inflamed or undergone cell death (Cheesbrough, 2006). As the cells are damaged, the ALT leaks into the bloodstream, leading to a rise in serum levels (Cheesbrough, 2006). Any form of hepatic cell

damage can result in an elevation in the ALT. The ALT level may or may not correlate with the degree of cell death or inflammation. ALT is the most sensitive marker for liver or cell damage. This enzyme also reflects damage to the hepatic cell. It is less specific for liver disease. It may be elevated and lead to other conditions such as myocardial infarction (heart attack). The ratio between ALT and AST are useful to physicians in accessing the etiology of liver enzyme abnormalities.

Alkaline phosphates are an enzyme, which is associated with the biliary tract though it is not specific to it. It is also found in the bone or placenta. Renal or intestinal damage can also cause the alkaline phosphatise to rise. If the alkaline phosphatise is elevated, biliary tract damage and inflammation should be considered. However considering the above, other aetiologies must also be entertained. One way of assessing the aetiology of the alkaline phosphates is to perform a serological evaluation called isoenzyme. Another more common method to assess the aetiology of the elevated alkaline phoshatase is to determine whether the GGT is elevated or whether other function tests are abnormal (such as bilirubin). Alkaline phosphatise may be elevated in primary biliary cirrhosis, alcoholic hepatitis, PSC, gallstones in choledocholithiasis (Cheesbrough, 2006).

The aim of this study is to determine the hepatotoxic effect of wastewater discharged from a carbonaceous industry on mice (*Mus musculus*). The aim would be achieved through examination of liver function test. These will include the monitoring of the following parameters; AST, ALP, and ALT, histopathology of the liver, body and organ weight index. The waste water will also be analysed for the presence of some physical, chemical and heavy metals. The findings from this study may be useful in the assessment of the toxic effects of waste water samples on public health and environment.

2.0 MATERIALS AND METHODS

2.1. Sources of Experimental Materials and Treatment

2.1.1 Laboratory Mice

Forty two male albino mice were obtained from the animal breeding unit at the Institute for Advanced Medical Research and Training (IMRAT), College of Medicine, University of Ibadan, UCH, Ibadan, Nigeria. Mice were acquired and quarantined in a pathogen-free, well ventilated room in order to enable the animals acclimatize to their environment. During the period of acclimatization, the animals were supplied with food (pelleted foods) and drinking water on a daily basis. Their beddings were also changed daily (disinfected and discarded). The mice were maintained in the departmental animal and breeding unit at IMRAT where each cage contains six animals.

2.1.2. Waste water

The waste water sample was sourced from the drainage pipes of the Effluent Treatment Plant of a beverage producing company in Lagos State, Nigeria. This company is well known for the production of drink beverages which are consumed nationwide.

2.1.3. Storage of Effluent

The collected effluents were stored in plastic bottles and refrigerated at 4°C until when needed. They were then brought out and diluted to various concentrations at room temperatures. The various concentrations were in-turn stored in plastic bottles and refrigerated all through the experiment.

2.2. Preparation of Controls

2.2.1. Negative Control

Distilled water was used as a vehicular solvent for the dilution of the waste water used.

2.2.2. Positive Control

The drug cyclophosphamide was used. Administered dosage depends on the average body mass of the animal per kg, which is 40mg/kg. The value/information in mg provided by the manufacturer was taken into consideration when the calculation was made. Average body weight of animal for positive control per kg is 24.5g. 40mg ---→ 1000g, X ---→ 24.5g, X = 40 x 24.5/1000 = 0.98mg of cyclophosphamide dissolved in 1000ml distilled water and administered orally for 35 days.

2.3. Exposure of Animals to the Samples

The animals were randomly divided into seven groups. Each group was made up of six individuals. Five groups were each injected with a different dilution of the effluent. One group was administered a positive control and another group the negative control sample. The dosage that was administered depends on the average body weight. 0.3ml of the sample was administered orally to each animal for a period of 35 consecutive days.

2.4. Determination of Serum Aspartate Aminotransferase (AST), Alanine

Aminotransferase (ALT) and Alkaline Phosphatase (ALP) Activities

These were determined following the principle described and standardized by Reitman and Frankel (1957).

2.5. Tests and Methodology

pH determination, alkalinity, total sulphate, total chloride, total hardness, total dissolved solids, total suspended solids, biochemical oxygen demand (BOD), chemical oxygen demand (COD) and determination of metals.

2.6. Statistical Analysis

Results were analysed by the mean standard deviation and statistical analysis of variance (ANOVA).

3. RESULTS ANALYSIS

Table 1 shows the effects of industrial carbonaceous waste water on the body weights of the experimental animals.

Table 1: Effects of Industrial Carbonaceous Waste Water on the Body Weights of the Experimental Animals

Periods	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5
Positive Control	23.00 ± 2.68	25.83 ± 3.10	26.17 ± 2.71	26.83 ± 2.04	29.33 ± 1.03
Negative Control	25.50 ± 1.38	27.67 ± 1.37	28.00 ± 0.90	30.00 ± 1.10	29.00 ± 1.26
10%	24.33 ± 2.80	27.17 ± 2.71	28.20 ± 2.50	29.80 ± 2.50	30.80 ± 1.80
25%	24.33 ± 1.75	26.67 ± 2.00	27.67 ± 2.33	29.50 ± 2.66	30.50 ± 2.35
50%	24.17 ± 0.75	26.50 ± 2.26	28.00 ± 0.63	29.50 ± 1.05	31.00 ± 2.00
75%	25.17 ± 4.02	27.50 ± 4.08	29.17 ± 2.32	29.67 ± 3.08	30.00 ± 3.35
100%	24.00 ± 2.76	26.83 ± 3.76	27.33 ± 5.54	28.17 ± 4.88	30.67 ± 4.50

Keys: Results are expressed in mean ± S.D., positive control = administered cyclophosphamide, negative control = administered distilled water, n = 5, 10% = 10% waste water + 90% distilled water, 25% = 25% waste water + 75% distilled water, 50% = 50% waste water + 50% distilled water, 75% = 75% waste water + 25% distilled water, 100% = 100% waste water

Table 2 shows the effects of industrial carbonaceous waste water on the organ weights of the experimental animals.

Table 2: Effects of Industrial Carbonaceous Wastewater on the Liver Weights of the Experimental Animals.

	LIVER (g)
Positive Control	1.20 ± 0.17
Negative Control	1.20 ± 0.17
10%	1.63 ± 0.31
25%	1.80 ± 0.50^{a*b**}
50%	2.00 ± 0.05 ^{a*b**}
75%	1.57 ± 0.31
100%	1.43 ± 0.23

Keys: a = $p<0.05$ compared with positive control (administered cyclophosphamide), b = $p<0.05$ compared with negative control (administered distilled water). Results are expressed in mean ± S.D., n = 5, 10% = 10% waste water + 90% distilled water, 25% = 25% waste water + 75 distilled water, 50% = 50% waste water + distilled water, 75% = 75% waste water + 25% distilled water, 100% = 100% waste water, * = significant difference compared with positive control, ** = significant difference compared with negative control.

Table 3 shows the effects of industrial carbonaceous waste water on some liver enzymes in hepatoxicity.

Table 3: Effects of Industrial Carbonaceous Wastewater on Some Liver Enzymes in Hepatoxicity.

Liver Enzymes	Serum ALP Activity (IU/L)	Serum ALT Activity (IU/L)	Serum AST Activity (IU/L)
Positive Control	54.76 ± 2.62	107.10 ± 5.27 ^{b**}	230.46 ± 2.00
Negative Control	27.27 ± 0.92 ^{a*}	82 ± 2.73 ^{a*}	127.21 ± 2.66 ^{a*}
10%	34.92 ± 3.18 ^{a*b**}	93.48 ± 3.14 ^{a*b**}	147.79 ± 5.61 ^{a*b**}
25%	40.15 ± 1.51 ^{a*b**}	109.16 ± 2.24 ^{b**}	189.21 ± 3.00 ^{a*b**}
50%	52.38 ± 1.90 ^{a*b**}	117.67 ± 2.40 ^{a*b**}	233.59 ± 10.90 ^{b**}
75%	69.09 ± 5.80 ^{a*b**}	134.43 ± 6.40 ^{a*b**}	328.45 ± 24.39 ^{a*b**}
100%	82.05 ± 9.22 ^{a*b**}	198.33 ± 2.73 ^{a*b**}	511.46 ± 21.60 ^{a*b**}

Keys: a = $p<0.05$ compared with positive control (administered cyclophosphamide), b = $p<0.05$ compared with negative control (administered distilled water). Results are expressed in mean ± S.D., n = 3, 10% = 10% waste water + 90% distilled water, 25% = 25% waste water + 75% distilled water, 50% = 50% waste water + 50% distilled water, 75% = 75% waste water + 25% distilled water, 100% = 100% waste water, * = significant difference compared with positive control, ** = significant difference compared with the negative control.

Table 4 shows the physico-chemical parameters of the industrial carbonaceous waste water used in this study.

Table 4: The Physico-Chemical Parameters of the Industrial Carbonaceous Waste Water

Parameters	Value (%)
Lead	0.010
Nickel	0.062
Cadmium	0.003
Magnesium	24.061
Manganese	11.257
Nitrate	7.295
Sulphate	2.700
Chloride	3.000
pH	7.80
BOD	2.50
Suspended Solid	0.50
Dissolved Solid	0.01
Hardness/ $CaCO_3$	100.00
Alkalinity	3.20

4. DISCUSSION

Hepatotoxicity is a predominant effect of high concentrations of inhaled toxic substances in animals (ATSDR/USEPA, 1989). Hepatotoxicity of toxic substance has been described and investigated in numerous oral studies of acute, intermediate and chronic duration in several animal species (ATSDR/USEPA, 1989). Hepatotoxicity is the most prominent and characteristic systemic effect of toxic substances and heavy metals, resulting in centrilobular necrosis and hemorrhage often leading to hemorrhagic ascites (ATSDR/USEPA, 1989).

The liver is important organs for metabolism, detoxification, storage and excretion of these chemicals and their metabolites and thus is vulnerable to damage. The similarity in body weight of mice exposed to waste water and control may be due to the ability of the mice to feed even while being exposed. The increase in liver may be due to inflammation of these chemicals present in waste water. Such observations were also observed in rats exposed to cadmium and alcohol (Brzoska *et al.*, 2003).

The observed dose-dependent increase in the activity of ALP, AST and ALT can be traced to possible necrosis of the liver where these enzymes are naive. The histological examination of the organ showed it clearly. Omotuyi *et al.* (2008 cited by Daramola, 2010) also observed this in rats exposed to an overdose of artesunate drug.

The histological lesions observed in the liver at various concentrations examined included Kupfer cell hyperplasia, severe portal congestion, portal and central venous congestion and mild hydrople degeneration of hepatocytes. The lesions, like severe portal congestion, hydrople degeneration of hepatocyte, occlusion of the tubular lumen and tubular necrosis observed in mice may be due to the chemicals present in the waste water. These chemicals are higher than standard limit from Nigeria and World Health Organization.

In other related studies, four cases of liver disease in humans resulting from inhalation exposure to Nitrosodimethylamine (NDMA) have been described in the literature (ATSDR/USEPA, 1989). Of the subjects who did not die, one was a chemist who was exposed to unknown concentrations of fumes and experienced exhaustion, headache, cramps in the abdomen, soreness on the left side, nausea and vomiting for at least two years (Freund, 1937; ATSDR/USEPA, 1989). The second case was an automobile factory worker who was exposed to unknown levels of NDMA and became violently ill with jaundice and ascites (Hamilton and Hardy 1974; ATSDR/USEPA, 1989).

In another related study, pathologic examination of dogs following exposure to Nitrosodimethylamine (NDMA) for 4 hours showed marked necrosis and varying degrees of hemorrhage in the liver (Jacobson et al. 1955). Doolittle et al. (1984) reported that the only toxic signs observed in rats exposed to NDMA for 4 hours were reddened eyes and piloerection. The only additional information reported in this study pertained to genotoxic effects (ATSDR/USEPA, 1989).

In this study, no mortality was reported among the mice exposed to the wastewater discharged from a carbonaceous industry. The lack of mortality in mice may be attributable to the fact that the animals were killed immediately following exposure and consequently not observed for subsequent death. This is similar to what was reported in other related studies. The lack of mortality in rats at the higher concentrations of NDMA in the Doolittle et al. (1984) study may also be attributable to the fact that the animals were also killed immediately following exposure and consequently not observed for subsequent death. However, Petechial and larger hemorrhages were observed in the lungs of two people following lethal poisoning with NDMA (Kimbrough

1982; ATSDR/USEPA, 1989). Myocardial and endocardial bleeding was observed in a person following lethal poisoning with NDMA (Kimbrough 1982; ATSDR/USEPA, 1989). Gastrointestinal hemorrhage occurred in humans following lethal poisoning with NDMA (Kimbrough 1982, Pedal et al. 1982; ATSDR/USEPA, 1989).

Also in other previously related studies, five members of a family who consumed unknown quantities of NDMA in lemonade became ill with nausea and vomiting associated with acute liver disease, generalized bleeding and low platelet counts (Kimbrough 1982, Cooper and Kimbrough 1980). Two of these people died; the other three were released from a hospital 4-21 days after admission. Another fatality due to ingestion of NDMA was attributed to liver failure (Fussgaenger and Ditschuneit 1980, Pedal et al. 1982; ATSDR/USEPA, 1989). Autopsies of the subjects described above showed that the primary effects were hemorrhagic and cirrhotic changes in the liver and necrosis and hemorrhage in other internal organs (ATSDR/USEPA, 1989).

In this study, organ weight was measured at post exposure and preserved afterwards for histology. The physical, chemical and heavy metal composition of the wastewater was also analysed. The liver weight of the exposed mice was however significantly different from that of the negative control in the 25% and 75% concentration of the wastewater administered at $p<0.05$. The activities of ALP, ALT, and AST in the serum of exposed mice were significantly increased compared to the negative control mice and this increase was concentration dependent at $P<0.05$. In mammals, several sub-chronic exposures of rats, mice and monkeys to substances such as perfluorooctanesulfonate (PFOS) have resulted in effects on body weight gain in females and in males (Seacat *et al*., 2002, 2003; Thibodeaux *et al*., 2003; Luebker *et al*., 2005a, b; Du *et al*., 2008) as reported in this present study with carbonaceous effluent. Based on existing data, substances such as perfluorooctanesulfonate (PFOS) has been shown to influence membrane function and structure of hepatocytes, as assessed by increase in serum alanine aminotransferase (ALT) activity in carp (*Cyprinus carpio*) (Hoff *et al*., 2003) and hepatic PFOS concentration was significantly and positively related to serum ALT activity in both feral carp (*C. carpio*) and eel (*Anguilla anguilla*) (Hoff *et al*., 2005; Du *et al*., 2008).

In reproductive and developmental toxicity, it appeared that plasma androgens and estrogens can be affected after fathead minnow (*Pimephales promelas*) exposed to heavy metals and toxic substances (Oakes *et al*., 2004, 2005; Ankley *et al*.,

2005; Du *et al*., 2008). Substances such as PFOS exposure to zebrafish embryos resulted in developmental toxicity and altered certain gene expression (Shi *et al*., 2008; Du *et al*., 2008).

Recently, several studies showed that estrogenic properties of PFOA (induction of vitellogenin, VTG) in rare minnow (*Gobiocypris rarus*) (Wei *et al*., 2007; Du *et al*., 2008), induction of VTG in cultured male tilapia hepatocytes (Liu *et al*., 2007; Du *et al*., 2008) and in male medaka (*Oryzias latipes*) treated with fluorotelomer alcohol (FTOHs) (Ishibashi *et al*., 2008; Du *et al*., 2008). Ankely *et al*. (2005) reported that no significant adverse effects on growth were observed in developing fathead minnows exposed PFOS for 24 h. In a study by Du *et al*. (2008), histological examination revealed that the most pronounced morphological alternation is accumulation of lipid in the liver of male fish, suggesting the hepatic toxicity is gender specific.

Studies have also shown that administration of substances such as carbon tetrachloride (CCl_4) to rats inhibits endoplasmic reticulum calcium pump activity and reduces the amount of calcium associated with subsequently isolated microsomal subcellular fractions (Ray and Moore, 1986). Calcium released from an intracellular pool(s) may initiate hepatotoxic changes in liver (Ray and Moore, 1986).

Our results showed that the observed hepatotoxic effect in the exposed mice may be caused by the presence of heavy metal and other physical and chemical substances present in the wastewater. This suggests a higher risk to liver damage in humans and other organisms exposed to this wastewater and may also be deleterious to the surrounding environment.

5. CONCLUSION

The carbonaceous wastewater caused liver dysfunction in mice at various concentrations. This suggests that exposure to these waste may pose risk to human health and will pollute the aquatic environment, contaminating the source of water supply for both domestic and commercial uses. Therefore it becomes imperative that environmental policy makers in Nigeria to take stringent decision in order to avert pollution of waste water and prevent or reduce risk to human other organisms.

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Tumour Necrosis Factor-Alpha affects Estrogen Metabolic Pathways in Breast Cancer Cells

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Abstract: The purpose of this study is to investigate the effect of the proinflammatory cytokine Tumor necrosis factor-alpha (TNF-α) on estrogen metabolic pathways in MCF-7, a breast cancer cell line. This aims to add to our understanding of the relationship between inflammation and breast carcinogenesis. MCF-7 cells where thus treated with different concentrations of TNF-α and different techniques were employed to assess its effect on the estrogen metabolic pathways: Capillary liquid chromatography/mass spectrometry (LC/MS) analysis was used for quantitative measurement of estrogens and estrogen metabolites (EM). High performance liquid chromatography (HPLC) was used for the analysis of estrogen-DNA adduct levels. Reporter gene assay, Real time reverse transcription polymerase chain reaction (real time RT-PCR) and Western blot analysis, were used to assess the expression of estrogen-metabolizing genes and enzymes. Our results indicated that compared to controls, TNF-α significantly increased the total EM (P<0.05) and decreased the estrone (E1) / 17-β estradiol (E2) ratio (P<0.05). Moreover, it significantly altered the expression of genes and enzymes involved in E2 activation and deactivation pathways e.g. Cytochrome P-450 1A1 (CYP1A1), Cytochrome P-450 1B1 (CYP1B1), Catechol-O-methyl transferase (COMT) and Nicotinamide adenine dinucleotide phosphate-quinone oxidoreductase 1 (NQO1). In addition, there were increased levels (P<0.05) of some catechol estrogens e.g. 4-hydroxy-estrone (4-OHE1) and 2-hydroxyestradiol (2-OHE2) with decreased levels of methylated catechols e.g. 2-methoxy estradiol (2-MeOE2) (P<0.05). DNA adducts especially 4-OHE1-[2]-1-N3 Adenine was significantly increased (P<0.05). It can thus be concluded that TNF-α directs the estrogen metabolism into more hormonally active and carcinogenic products in MCF-7. This may implicate a new possible explanation for inflammation associated breast cancer.
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Keywords: Breast cancer; Tumor necrosis factor-alpha; Estrogen metabolites; Estrogen metabolizing genes and enzymes; DNA adducts.

1. Introduction

Breast cancer is one of the most common malignancies accounting for nearly 1 in 3 cancers diagnosed among women in the United States, and it is the second leading cause of cancer death among women (DeSantis et al., 2011).

It is well established that prolonged exposure to estrogens especially estradiol, is an important risk factor for genesis and promotion of breast cancer (Handerson and Canellos, 1990; Handerson et al., 1988). The most acknowledged mechanism of estrogen carcinogenicity is its binding to its specific estrogen receptor alpha (ER-alpha) for exerting a potent mitogenic effect on cell proliferation (Russo and Russo, 1999). However, there is conceivable evidence that estrogen induced breast cancer can also occur by a non ER-alpha mediated mechanism involving the estrogen metabolic pathway (Bocchinfuso and korach, 1997).

Metabolism of estrogens is characterized by two major pathways: one is hydroxylation at the 16-position and the second is hydroxylation to form the 2- and 4-catechol estrogens. In the catechol pathway (Diagram 1), the metabolism involves further oxidation of 17- β estradiol (E2) to semiquinones and quinones which react with DNA to form depurinating adducts. The apurinic sites obtained by this reaction generate mutations that may lead to the initiation of

cancer. Oxidation of catechol estrogens to their quinones normally occurs in homeostasis which minimizes their reaction with DNA. When the homeostasis is disrupted, excessive amounts of catechol estrogen quinones are formed and the resulting increase in depurinating DNA adducts can lead to initiation of cancer (Cavalieri et al., 1997; Bradlow et al., 1986; Moobery, 2003; Cavalieri and Rogan, 2011). Moreover, 4-hydroxylated estrogens may exert proinflammatory roles by inducing reactive oxygen species (ROS) and DNA damage which is possibly decisive in chronic inflammation (Straub, 2007).

It has become increasingly clear that inflammation plays a major role in breast cancer pathogenesis. An inflammatory tumor microenvironment consists of infiltrating immune cells and activated fibroblasts, both of which can secrete cytokines, chemokines, and growth factors, as well as DNA-damaging agents (Coussens and Werb, 2002). Some studies show evidence that chronic inflammation is linked to breast cancer recurrence and that elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients (Cole, 2009; Pierce et al., 2009). In addition, experimental studies clearly indicate that inflammatory mediators promote tumor development in cancer prone animal strains (Rao et al., 2006). Moreover, inhibition of TNF-α and NF-kappaB (NF-κB) transcription factor is proved to be protective with respect to chemical induced mammary gland carcinogenesis (Connell et al., 2011). Further, the in vitro activation of the TNF-α/NF-κB axis has induced an invasive and malignant behaviour in breast cancer cells (Balkwill, 2009).

TNF-α is a major inflammatory cytokine shown to be highly expressed in breast carcinomas (Leek et al., 1988). Indeed, investigations strongly suggest that the chronic expression of TNF-α in breast tumors actually supports tumor growth. The number of cells expressing TNF-α in inflammatory breast carcinoma was found to be correlated with increasing tumor grade and node involvement, and TNF-α expression was suggested to play a role in the metastatic behavior of breast carcinomas (Miles et al., 1994). Furthermore, patients with more progressed tumor phenotypes were shown to have significantly higher TNF-α serum concentration (Sheen-Chen et al., 1997). The tumor-promoting functions of TNF-α may be mediated by its ability to induce proangiogenic functions, to promote the expression of matrix metalloproteinases (MMP) and endothelial adhesion molecules, and to cause DNA damage via reactive oxygen, the overall effect of which is promotion of tumor related processes (Balkwill, 2002).

In the current study we explored a new mechanism by which inflammation may influence breast carcinogenesis through the estrogen metabolic pathway. We thus investigated the effect of the TNF-α, a hallmark of inflammation, on the estrogen metabolic pathway in MCF-7 estrogen dependent breast cancer cells. Our choice of MCF-7 as our study model was built on several bases: First, studies have shown that regular use of nonsteroidal antiinflammatory drugs (NSAIDS), such as aspirin, significantly reduce the risk of ER-positive but not ER-negative breast cancers (Terry et al., 2004). Second, it was recently suggested that inflammation may promote more aggressive ER-positive tumors and that this may be one of the mechanisms by which a portion of ER-positive breast tumors fail to respond to endocrine therapy (Baumgarten et al., 2012). In general, we sense that further research is required to fully elucidate the mechanisms of action of TNF-α on breast carcinogenesis because TNF-α activity may vary under different physiological conditions and in a cell-type-dependent manner which contributes to a sense of ambiguity regarding its tumor effects. Most of the reports examining the effects of TNF-α on MCF-7 breast cancer cells demonstrated its ability to induce apoptosis, inhibit proliferation and promote migration, invasion as well as resistance to chemotherapeutic drugs. These effects may however vary with other estrogen dependent (T47D) or independent (MDA-MB-231) cell lines (Goldberg and Schwerfeger, 2010).

What encouraged us more to explore this area of research is that not many studies have examined the role of inflammation on the estrogen metabolic pathways in general inspite of this pathway being an important cause of carcinogenesis. In some of these studies, TNF-α has been found to have an important role in regulating estrogen synthesis in peripheral tissues, including normal and malignant breast tissues (Purohit and Newman, 2002; Hermann et al., 2002; Hong et al., 2004; Mikhaylova et al., 2007). Other studies have pointed that the effect of TNF-α on estrogen metabolism and homeostasis is mediated by its effect on the coordinated expression of the enzymes that are involved in estrogen biosynthesis and metabolism. For example, it has been suggested that TNF-α increases the local estrogen biosynthesis in human endometrial glandular epithelial cells and directs estrogen metabolic enzymes to produce more hormonally active and carcinogenic metabolites (Salama et al., 2008).

Collectively, the above mentioned information stimulated our interest to have a closer look on the estrogen metabolic pathway in MCF-7 breast cancer cells as a whole and examine the role

played by TNF-α in this pathway aiming to enrich our knowledge about how inflammation may influence breast carcinogenesis. Understanding the mechanisms by which inflammatory mediators promote breast cancer may shed the light on these cytokines being explored as potential therapeutic targets and hopefully will lead to novel therapeutic regimens to treat this devastating disease.

Table 1. The rate and profile of EM in MCF-7 cells treated with TNF-α.

Estrogen and Estrogen metabolites	Measurement (pmole/mg protein/48hours) (% from total EM)	
	Control	TNF-α (5ng/ml)
Parent estrogen		
Estrone (E1)	4889.02±162.19 (25.3±0.83)	3939.29±207.68* (14.8±0.78)
Estradiol (E2)	12385.67±511.8 (64.1±2.65)	17133.73±325.83* (64.4±1.22)
2-Hydroxylation pathway catechols		
2-Hydroxy estradiol (2-OHE2)	0	1394.1±358.2 (5.24±1.35)
2-Hydroxylation pathway methylated catechols		
2-Methoxyestrone (2-MeOE1)	31.25±2.8 (0.16±0.01)	47.2±5.8 (0.18±0.02)
2-Methoxyestradiol (2-MeOE2)	25.5±4.28 (0.13±0.02	6.34±0.85* (0.02 ±0.0027)
2-Hydroxyestrone-3 methyl ether (3-MeOE1)	20.122±0.964 (0.11±0.005)	49.88±4.24* (0.19±0.016)
4-Hydroxylation pathway catechols		
4-Hydroxy estrone (4-OHE1)	0	63.3±10.623 (0.24±0.04)
4-Hydroxylation pathway methylated catechols		
4-Methoxy estrone (4-MeOE1)	13.987±3.96 (0.07±0.02)	4.49±0.162 (0.02±0.0007)
4-Methoxy estradiol (4-MeOE2)	49.17±2.73 (0.254±0.014)	79.11±60.4 (0.3±0.23)
16α-Hydroxylation pathway		
Estriol (E3)	632.35±545.9 (3.27±2.82)	0
17-Epiestriol (17-epi E3)	1777.481±574.4 (9.2±2.97)	3669±1130.8 (13.8±4.25)
16-Epiestriol (16-epi E3)	449.29±418.12 (2.3±2.17)	0
Total EM	19321.13±1252	26600.56±1196.6*

Values represent the mean±SE of three experiments. *indicates significant difference of TNF-α treated cells compared to control at $P < 0.05$.

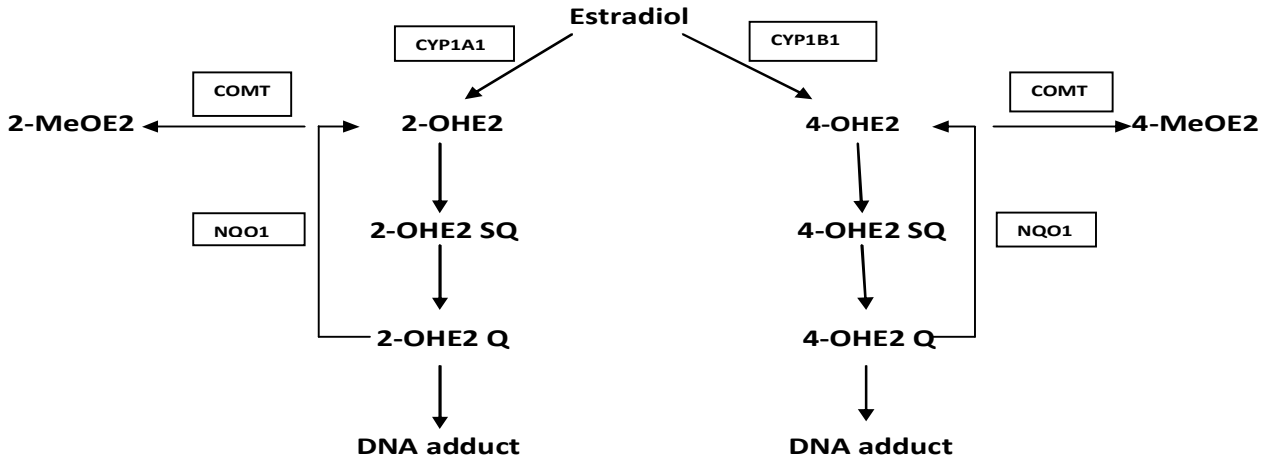

Diagram 1. Part of the metabolic pathway of estradiol and the role of various enzymes involved: Estradiol is metabolized into 2-hydroxyestradiol (2-OHE2) and 4-hdroxyestradiol (4-OHE2) by CYP1A1 and CYP1B1 respectively. These catechols undergo further oxidation into semiquinones and quinones that react with DNA to form depurinating adducts leading to mutations associated with breast cancer. NQO1 reduces these quinones back to catechols which are detoxified into methoxy derivatives by the action of COMT. This protects the cells against DNA adducts formation and lowers the potential for mutagenic damage.

Figure 1

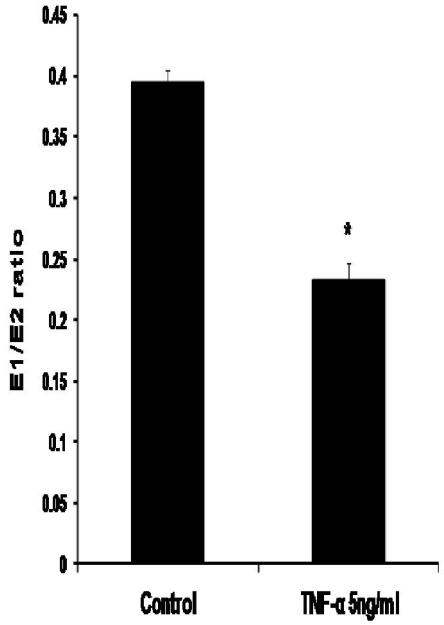

Figure 1. Effect of TNF-α on the ratio of E1/E2 in MCF-7 cells. Cells were grown in serum-free media and treated with E2 (10 nM) alone or with TNF-α (5 ng/ml) for 48 hours. Media were collected to quantify the mean rate of EM formation in (pmole/mg protein/48 hours) by LC/MS. Data were normalized against protein concentration. E1/E2 ratio was calculated. Values represent the mean±SE of three experiments.* indicates significant difference of TNF-α treated cells compared to control at $P < 0.05$.

Figure 2

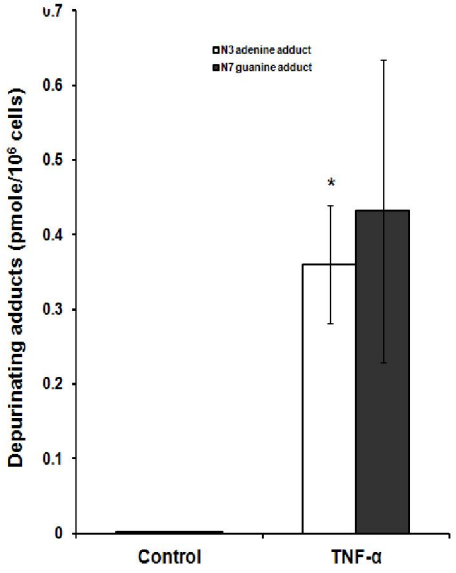

Figure 2. Effect of TNF-α on depurinating DNA adducts formation**.** Cells were grown in serum-free media and treated with E2 (10 nM) alone or with TNF-α (5 ng/ml) for 48 hours. The media were collected for DNA adducts analysis by HPLC. Values represent the mean±SE of three experiments. * indicates significant difference of TNF-α treated cells compared to control at $P < 0.05$.

Figure 3

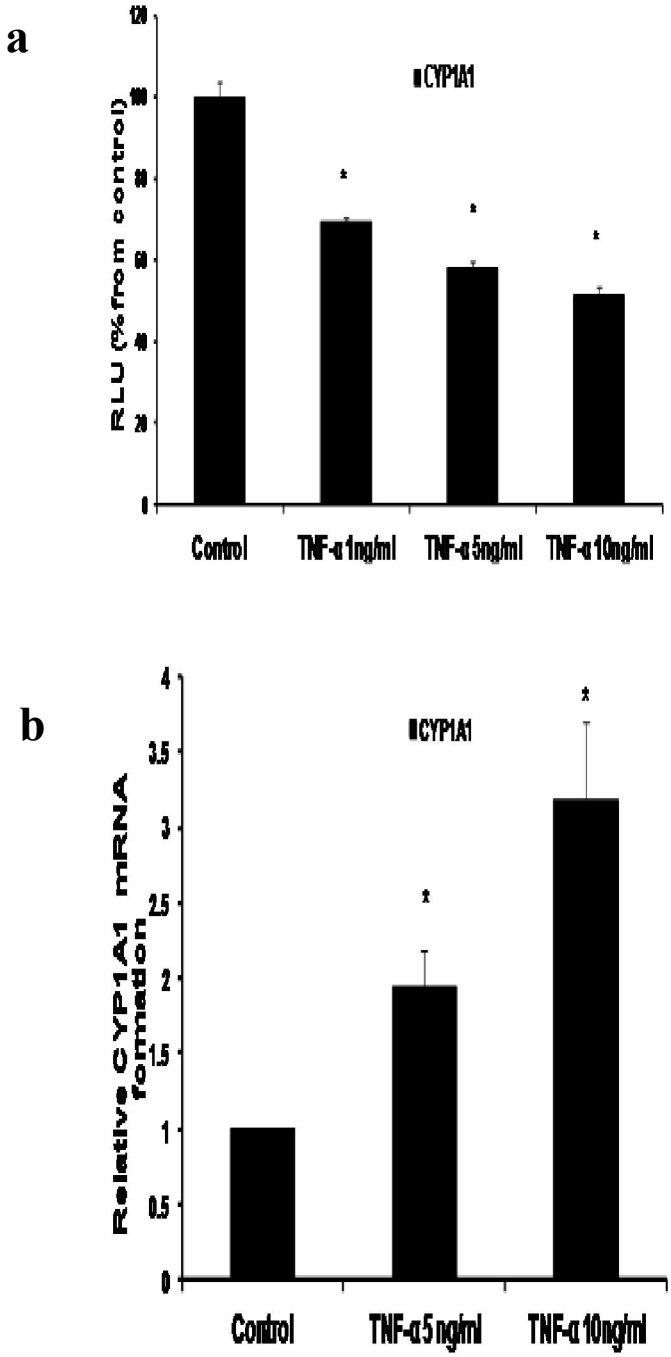

Figure 3. Effect of TNF-α on CYP1A1 in MCF-7 cells. (**a**) Luciferase reporter gene assay. The cells were stably transfected with reporter plasmids containing CYP1A1 promoters. After treatment with different doses of TNF-α, luciferase production was measured 48 hours later. Results represent the mean±SE of two experiments. (**b**) Real time RT-PCR analysis. The cells were treated with TNF-α 5 ng/ml and 10 ng/ml for 24 hours followed by total RNA isolation from the cells and the CYP1A1 mRNA expression was determined by real time RT-PCR. The threshold cycle value of CYP1A1 was normalized based on that of GAPDH. Results represent the mean±SE of two experiments. * indicates significant difference of TNF-α treated cells compared to control at $P < 0.05$.

Figure 4

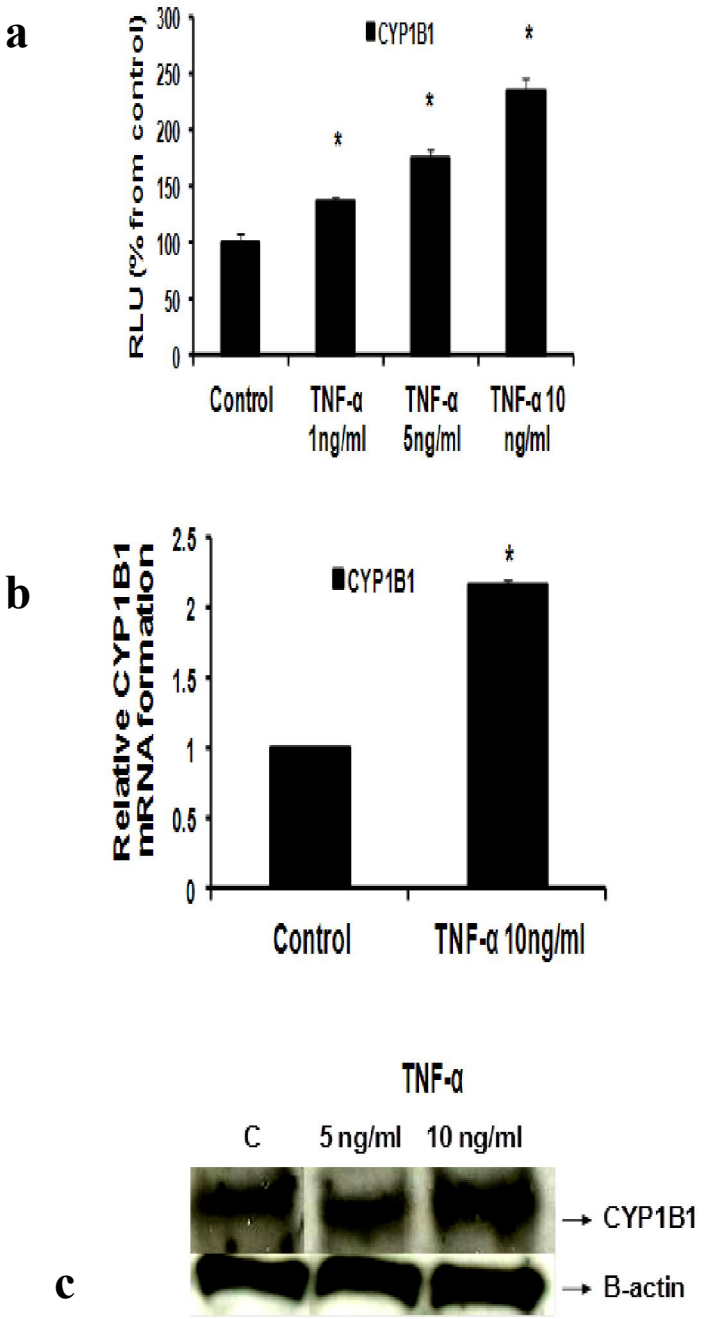

Figure 4. Effect of TNF-α on CYP1B1 in MCF-7 cells: (**a**) Luciferase reporter gene assay. The cells were stably transfected with reporter plasmids containing CYP1B1 promoters. After treatment with different doses of TNF-α, luciferase production was analyzed 48 hours later. Results represent the mean±SE of two experiments. (**b**) Real time RT-PCR analysis. The cells were treated with TNF-α 10 ng/ml for 24 hours followed by total RNA isolation from the cells, and the CYP1B1 mRNA expression was determined by real time RT-PCR. The threshold cycle value of CYP1B1 was normalized based on that of GAPDH. Results represent the mean±SE of two experiments. (**c**) Western blot analysis. CYP1B1 protein levels were determined 72 hours after treatment with different doses of TNF-α. * indicates significant difference of TNF-α treated cells compared to control at $P < 0.05$.

Figure 5

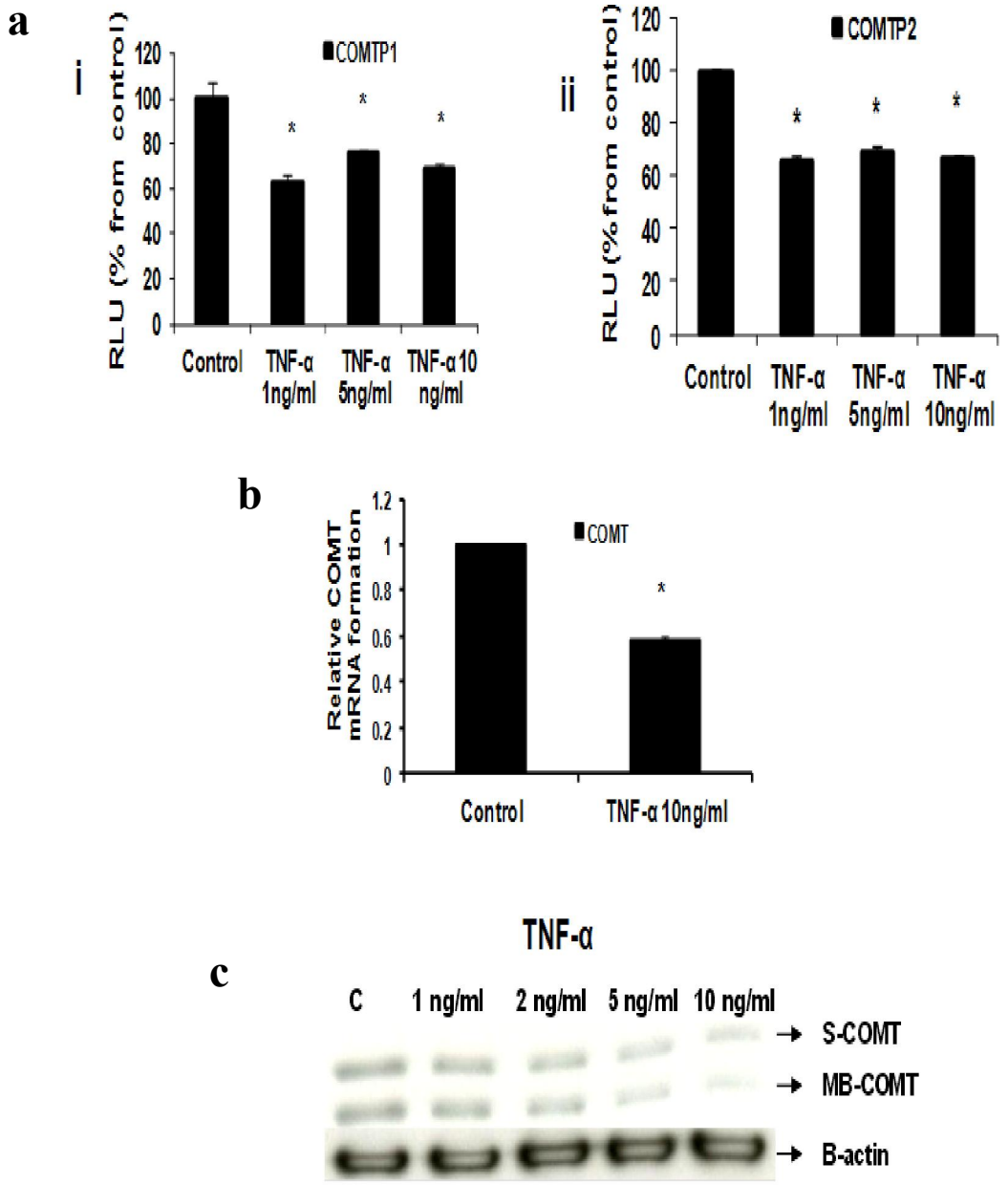

Figure 5. Effect of TNF-α on COMT in MCF-7 cells: (**a**) Luciferase reporter gene assay. The cells were stably transfected with reporter plasmids containing COMTP1 (**i**) and COMTP2 promoters (**ii**). After treatment with different doses of TNF-α, luciferase production was analyzed 48 hours later. Results represent the mean±SE of two experiments. (**b**) Real time RT-PCR analysis. The cells were treated with TNF-α 10 ng/ml for 24 hours followed by total RNA isolation from the cells and COMT mRNA expression was determined by real time RT-PCR. The threshold cycle value of COMT was normalized based on that of GAPDH. Results represent the mean±SE of two experiments. (**c**) Western blot analysis. Both S-COMT and MB-COMT protein levels were determined 72 hours after treatment with different doses of TNF-α. * indicates significant difference of TNF-α treated cells compared to control at $P < 0.05$.

Figure 6

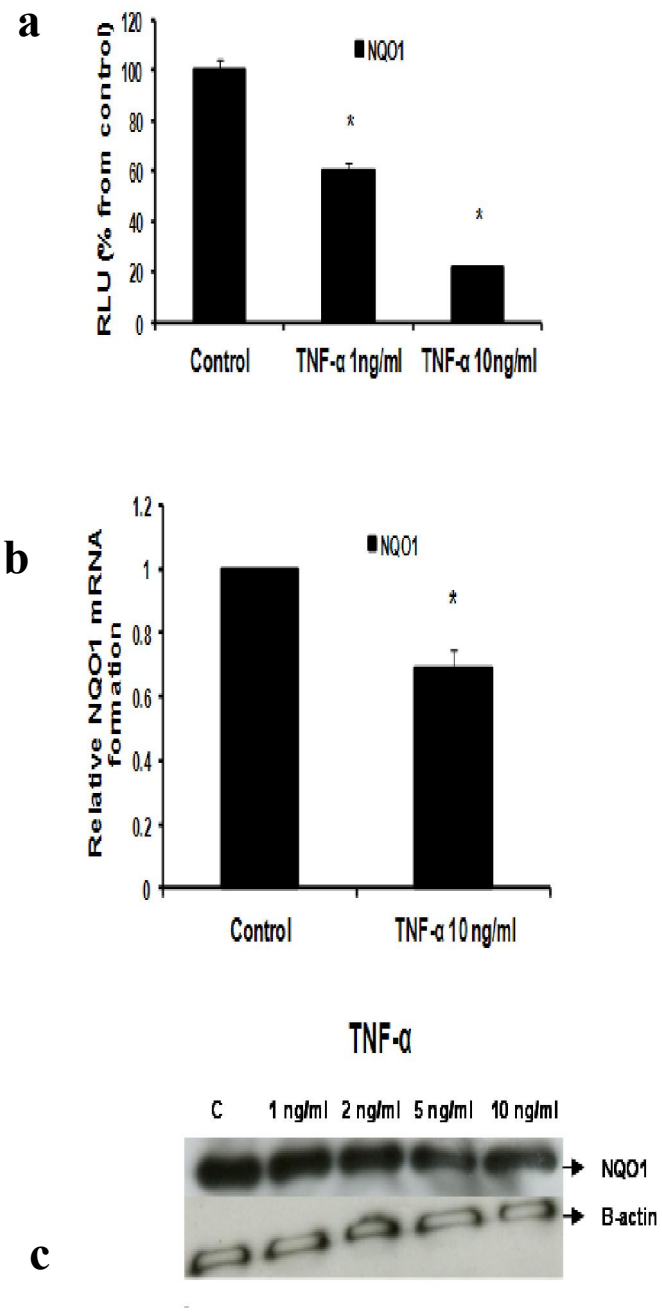

Figure 6. Effect of TNF-α on NQO1 in MCF-7 cells: (**a**) Luciferase reporter gene assay. The cells were stably transfected with reporter plasmids containing NQO1 promoters. After treatment with different doses of TNF-α, luciferase production was measured 48 hours later. Results represent the mean±SE of duplicate experiments. (**b**) Real time RT-PCR analysis. The cells were treated with TNF-α 10 ng/ml for 24 hours followed by total RNA isolation from the cells, and the NQO1 mRNA expression was determined by real time RT-PCR. The threshold cycle value of NQO1 was normalized based on that of GAPDH. Results represent the mean±SE of two experiments. (**c**) Western blot analysis. NQO1 protein levels were determined 72 hours after treatment with different doses of TNF-α. * indicates significant difference of TNF-α treated cells compared to control at $P < 0.05$.

2. Materials and Methods

Cells and Cell Culture

The human breast cancer cell line MCF-7 was purchased from (Rockville culture collection, Md, USA). The cells were maintained at 37ºC in a 95% humidified, 5% CO_2 atmosphere in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin and streptomycin (Gibco BRL, Life Technologies, Long Island, NY, USA). To study the effect of TNF-α on the profile of estrogen metabolism and DNA adduct analysis, the cells were grown in serum free, phenol red-free media for 24 hours. The cells were then treated with 10 nM of E2 with or without 5ng/ml TNF-α for 48 hours. The media were collected for estrogen metabolite measurements and DNA adducts analysis.

Determination of estrogen/estrogen metabolites (EM) by capillary LC/MS

The levels of estrogen and estrogen metabolites (EM) were determined by testing the conditioned media from cells treated with E2 alone or with different doses of TNF-α according to the method described by Xu et al. (2007). Briefly, 5 µL of the stable isotope labelled estrogen and estrogen metabolite (SI-EM) working internal standard solution (40 pg SI-EM) was added to 400 µl cell medium aliquot followed by 500 µl 0.15 M sodium acetate buffer (pH 4.6) containing 0.25% (w/v) L-ascorbic acid. Dichloromethane (6 ml) was added to the sample, which then underwent inverse extraction at 8 rpm (RKVSD™, ATR, Inc., Laurel, MD, USA) for 30 minutes. After extraction, the aqueous layer was discarded and the organic solvent portion was transferred into a clean glass tube and evaporated to dryness under a stream of nitrogen gas at 60°C (Reacti-Vap III™, Pierce, Rockford, IL, USA). The dried sample residue was then redissolved in 40 µl of 0.1 M sodium bicarbonate buffer (pH 9.0) and 40 µl of dansyl chloride solution (1 mg/ml in acetone). After sonication, the sample was heated at 60°C (Reacti-Therm III™ Heating Module, Pierce, Rockford, IL, USA) for 5 min to form the EM and SI-EM dansyl derivatives (EM-Dansyl and SI-EM-Dansyl), respectively. After derivatization, all samples were analyzed using capillary LC/MS using an Agilent 1200 series nanoflow LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a TSQ™ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA, USA). Quantitation of cell medium estrogen metabolites was carried out using Xcalibur™ Quan Browser (Thermo Electron). Absolute concentrations of individual EM were normalized against protein concentrations.

HPLC analysis of estrogen-induced depurinating DNA adduct

Sample preparation

MCF-7 cells were treated as described above. The growth media was used to measure the level of depurinating estrogen−DNA adducts according to the method described by Zahid et al. (2008). Briefly, cell culture media was extracted by using Varian C8 Certify II solid phase extraction cartridges (Varian, Harbor City, CA, USA) which were preequilibrated by sequentially passing 1 ml methanol, distilled water, and potassium phosphate buffer (100 mM, pH 8.0) through them. Culture media was adjusted with 1 ml of 1 M potassium phosphate to pH 8 and passed through the cartridge. After washing with 2 ml of 100 mM potassium phosphate, and 5 ml of distilled water, the analytes were eluted with 1 ml of elution buffer consisting of methanol/acetonitrile/water/TFA (8:1:1:0.1). The eluant was evaporated to about 100 ul by using Jouan RC10 Vacuum Concentrator and reconstituted with 100 ul of methanol/water (1:1). The solution was then passed through 5000 MW cut off filters and analyzed on HPLC connected with multiple electrochemical detectors.

HPLC analysis of adducts

Analysis of all samples was conducted on an HPLC system equipped with dual ESA Model 580 autosampler, and a 12-channel CoulArray electrochemical detector (ESA, Chelmsford, MA, USA). The two mobile phases used were as follows: A: acetonitrile/methanol/buffer/water (15:5:10:70) and B: acetonitrile/methanol/buffer/water (50:20:10:20). The buffer was a mixture of 0.25 M citric acid and 0.5 M ammonium acetate in triple-distilled water, and the pH was adjusted to 3.6 with acetic acid. The 95 ul injections were carried out on a Phenomenex Luna-2 C-18 column (250 x 4.6 mm ID, 5 mm; Phenomenex, Torrance, CA, USA), initially eluted isocartically at 90%A/10%B for 15 minutes, followed by a linear gradient to 90%B/10%A in the next 40 min, and held there for 5 minutes (total 50 minutes gradient) at a flow rate of 1 ml/minute and a temperature of 30 ^{o}C. The serial array of 12 coulometric electrodes was set at potentials of -35, 10, 70, 140, 210, 280, 350, 420, 490, 550, 620, and 690 mV. The system was controlled and the data were acquired and processed using the CoulArray software package (ESA). Peaks were identified by both retention time and peak height ratios between the dominant peaks and the peaks in the two adjacent channels. The metabolites, conjugates and depurinating adducts were quantified by comparison of peak response ratios with known amounts of standards. The level of adducts were normalized against cell numbers and the DNA contents.

Reporter assay

Stable transfection of MCF-7 with the reporter plasmids

Luciferase reporter plasmids containing the CYP1A1, CYP1B1, COMTP1 (proximal COMT-promoters) and COMTP2 (distal COMT-promoters) and NQO1 promoters were used in this study. These plasmids were stably transfected into MCF-7 according to the following: MCF-7 cells were cotransfected with individual reporter plasmid (10ug) and Neomycin-expressing vector (1ug) using Fugene 6 (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. Forty-eight hours post-transfection, the media was replaced with Genecitin-containing media (500 µg/ml media). Individual colonies were picked and propagated following 2 weeks of selection and were screened for luciferase activity. Colonies which showed positive luciferase activity indicated that they were successfully stably transfected and thus were selected and maintained in liquid nitrogen for further experiments.

Luciferase assay

Stably transfected cells containing the reporter gene vectors with the promoters of interest were grown in RPMI media, distributed in 6 well plates and treated with different concentrations of TNF-α. After 48 hours, the cells were harvested and luciferase activities were determined using luciferase enzyme assay systems according to the supplier's protocol (Promega, Madison, WI, USA). The luciferase activity was normalized against protein concentration using Bradford protein assay procedure.

Real time RT-PCR

MCF-7 cells were cultured, distributed in 6 well plates and treated with different concentrations of TNF-α. After 48 hours, Total RNA was isolated using RNA STAT-60 kit (Ambsio, lakeforest CA, USA) or RNA aqueous-Micro (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. cDNA synthesis was conducted using High-Capacity cDNA Reverse Transcription kit following manufacturer's protocol. PCR amplification was performed using Tagman Fast Universal PCR Master Mix. Quantitative real-time RT-PCR was carried out using Pre-developed Tagman Assay reagents Control Kits. Kits used were obtained from (Applied Biosystem, Foster city, CA, USA). Each assay was performed in duplicate.

Western blot analysis

MCF-7 cells were cultured, distributed in 6 well plates and treated with different concentrations of TNF-α. After 72 hours, the cells were washed with phosphate buffered saline (PBS) and whole cell lysates were prepared with Radioimmunopreciptation assay (RIPA) lysis buffer. The cell lysates were solubilized in sample buffer [60 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.7 mol/l β-mercaptoethanol; 0.01% bromophenol blue] and subjected to SDS polyacrylamide gel electrophoresis (PAGE). Proteins were electroblotted onto nitrocellulose membranes. Membranes were immunoblotted with the primary antibodies against CYP1B1 and COMT (Chemicon International Inc., Temecula, CA, USA), NQO1 (Novus Biologicals, Inc, Littleton, CO, USA) and β–actin (Sigma-Aldrich Co., St. Louis, MO, USA). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) and X-ray films where then developed.

Statistical Analysis

Data are presented as mean±Standard error (SE) of at least two or three individual experiments as indicated under the results section. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey test as a post-ANOVA multiple comparison test. Unpaired *t*-test was used for comparison between two groups. $P<0.05$ was considered statistically significant.

3. Results

TNF-α alters the profile of E2 and/or EM in MCF-7 cells (Table 1)

To evaluate the effects of TNF-α on estrogen metabolism, the EM levels were measured in conditioned media from MCF-7 cells treated with E2 (10 nM) alone or in combination with TNF-α (5 ng/ml) using LC/MS. It was found that TNF-α significantly increased the total EM compared with control. The mean rate of formation of total EM for control was 19321.13±1252 pmole/mg protein/48 hours, while in TNF-α treated cells the mean rate was 26600.56±1196.6 pmole/mg protein /48 hours. To further assess the effects of TNF-α on estrogen metabolism, we derived the ratios for E1/E2, which is a commonly used predictor for cancer risk (Zhang et al., 2012). TNF-α shifted the E1/E2 equilibrium towards the formation of more E2 (Figure 1). Thus, in MCF-7 cells treated with TNF-α, the E1/E2 ratio was 0.232±0.01423 which is significantly lower compared with control where the ratio was 0.395±0.0092. TNF-α also increased the rate of formation of 2-OHE2 accompanied by a decline in the rate of 2-MeOE2 by 75%. Concerning the other methylated catechols, there was non significant increase in the levels of 2-MeoE1 and 4-MeOE2 and non significant decrease in 4-MeOE1. There was

however a significant increase in 3-MeOE1 by 2.5 folds. The 4-OHE1 levels were not detected in control, but increased to 63.3±10.623 upon adding TNF-α. On the contrary E3 levels and 16-epiestrol levels were detected in control but not in TNF-α treated cells. There was also a non significant increase in the rate of formation of 17-epiestriol compared to control.

TNF-α increases the levels of DNA adducts in MCF-7 cells

To investigate the implications of the possible effects of TNF-α on E2 metabolism and the formation of depurinating DNA adducts, the levels of DNA adducts (4-OHE1 [2]-1-N3 Adenine and 4-OHE1 [2]-1-N7 Guanine) were explored in culture media from MCF-7 cells treated with either E2 alone or in combination with TNF-α by HPLC. Our results demonstrated that TNF-α significantly increased Adenine adduct levels while there was no significant increase of the Guanine adducts. Thus, in control MCF-7 cells and TNF-α treated cells, the levels of N3 Adenine adducts were 0.0025±0.0005 and 0.3604±0.078 (pmole/10^6cells) respectively. Also the levels of N7 Guanine adducts were 0.00293±0.00054 and 0.4319±0.2022 in control cells and TNF-α treated cells respectively (Figure 2).

Effect of TNF-α on estrogen-metabolizing genes and expression in MCF-7 cells

To elucidate the underlying mechanisms of the differential pattern of estrogen metabolites and the rate of estrogen-induced adducts formation between TNF-α treated and non treated cells, we assessed the effect of TNF-α on the expression of key genes and enzymes involved in estrogen metabolism. Thus, we treated MCF-7 cells with TNF-α and measured its effect on enzyme promoter activity using Reporter gene assays, enzyme mRNA levels using real time RT-PCR and enzyme protein levels using Western blot analysis.

a- TNF-α downregulates CYP1A1 promoter and upregulates CYP1A1 mRNA

In our study we demonstrated that treatment of MCF-7 cells with TNF-α (1, 5 and 10 ng/ml) significantly reduced CYP1A1 to 69.39 ±1.215, 58±1.74 and 51.38±2.091 RLU respectively as indicated by Reporter assay (Figure 3a) . However CYP1A1 mRNA was significantly upregulated to 1.938 ±0.23 and 3.1745±0.529 upon treatment with TNF-α 5 ng/ml and 10 ng/ ml respectively as indicated by real time RT-PCR (Figure 3b). This discrepancy between CYP1A1 luciferase activity and mRNA levels will be addressed in the discussion section. It's noteworthy that Western blotting for detecting CYP1A1 enzyme was attempted in this study but unfortunately, commercially available CYP1A1 antibodies didn't give satisfactory results.

b- TNF-α upregulates CYP1B1

Our study proved that treatment of MCF-7 cells with TNF-α (1, 5 and 10 ng/ml) significantly increased CYP1B1 levels in a dose dependent pattern as indicated by Reporter assay by 35.7 %, 75%, 134 % respectively (Figure 4a) while TNF-α (10 ng/ml) significantly increased CYP1B1 mRNA by 116% as determined by real time RT-PCR (Figure 4b). Western blot data also revealed that CYP1B1 protein expression was upregulated (Figure 4c).

c- TNF-α downregulates COMT

Our Reporter gene assay showed that TNF-α (1, 5 and 10 ng/ml) significantly reduced COMTP1 expression by 36.75%, 23.72% and 31.85% respectively (Figure 5ai). Similarly COMTP2 was significantly reduced by 34%, 30.4% or 33.6% respectively (Figure 5aii). Real time RT-PCR data revealed that COMT mRNA was reduced by 41.7% in cells treated with TNF-α 10 ng/ml compared with untreated control (Figure 5b). Consistent with the above two assays Western blotting confirmed that TNF-α downregulated COMT expression in its two forms: soluble (S-COMT) and membrane bound (MB-COMT) compared with control (Figure 5c).

d- TNF-α downregulates NQO1

Our data reflected that the effect of TNF-α on NQO1 occurred on the transcriptional and translational levels as indicated by Reporter, real time RT-PCR and Western blotting. In Reporter assay, treatment with TNF-α 1 ng/ml and 10 ng/ml resulted in significant downregulation of NQO1 promoter activity to 60.3±2.609 and 21.45±0.9192 RLU respectively (Figure 6a). In real time RT-PCR assay NQO1 mRNA levels were reduced by 30.5% using TNF-α 10 ng/ml (Figure 6b). Also, TNF-α decreased NQO1 protein levels as indicated by Western blotting (Figure 6c).

4. Discussion

Chronic inflammation represents a major risk factor for many cancer types, including liver, breast, prostate, pancreas, ovary, skin, gastric, colorectal and pulmonary carcinomas (Aggarwal et al., 2006).

TNF-α was identified as a keyplayer in the cytokine network and a major mediator of cancer related inflammation. It exerts its action via activation of NF-κB which is the pivotal regulator of cellular inflammatory responses (Moore et al., 1999;

Pikarsky et al., 2004; Balkwill, 2006; Sethi et al., 2008).

Numerous studies have linked TNF-α to breast cancer progression. As a result, the mechanisms by which NF-α promotes breast cancer have been recently explored using both in vitro and in vivo models (Goldberg and Schwertfeger, 2010). In this study we are trying to focus on the influence of TNF-α on the estrogen metabolic pathway in MCF-7 cells. This may help us better understand the mechanisms by which inflammation affects breast cancer.

One of the interesting findings in our study is that the total EM was significantly higher in MCF-7 treated cells compared to control. One reason for this may be due to that TNF-α increased the local estrogen biosynthesis in MCF-7 cells through stimulating aromatase expression as suggested by previous reports (Macdiamid et al., 2011; Morris et al., 2011). However, based on our results we are adding a new mechanism where by TNF-α may be directly interfering with the estrogen metabolic pathway. Besides increasing total EM, we also found that TNF-α significantly decreased the E1/E2 ratio due to a decrease in the level of estrogen as E1 with a concomitant increase in E2 concentration. The interconversion of weakly active estrone (E1) into highly potent estradiol (E2) and their relative abundance dictate the estrogenic environment and may be contributing to the development of breast cancer. This alteration in E1/E2 ratio could be explained in light of the experimental evidence that TNF-α modulates the expression of hydroxysteroid (17β) -dehydrogenase 1 (HSD17β1) in breast cancer, an enzyme that efficiently catalyzes the conversion of E1 into E2 (Zhang et al, 2012). Also, 4-OHE1, which in our study was detected only in TNF-α treated cells, was previously shown to be tumorigenic in animal models (Liehr and Ricci, 1996). It's noteworthy that 4-hydroxy catechols have greater carcinogenic potential than 2-hydroxy catechols due to different mechanisms of adduction leading to higher DNA adducts (Cavalieri and Rogan, 2011).

To understand why TNF-α altered the rate and pattern of estrogen metabolites, we further explored the effect of TNF-α on the expression of key genes involved in estrogen metabolism. Our data revealed that TNF-α increases the expression levels of CYP1A1 and CYP1B1. This was associated with a concomitant effect on the expression of detoxification enzymes COMT and NQO1 where TNF-α reduced the expression levels of these two enzymes. In agreement with our results several studies reported similar effects on these enzymes (Salama et al., 2008; Gharavi and El-Kadi, 2007; Tchivileva et al., 2009). Surprisingly, TNF-α was found to upregulate mRNA of CYP1A1 and to downregulate it's promoter activity as indicated by luciferase assay. This effect may be similar to that explained by Morel et al (Morel et al., 1999) where increasing the CYP1A1 activity in hepatoma cells elicited an oxidative stress and led to the repression of a reporter gene driven by the CYP1A1 gene promoter in a negative feedback loop. Our results also indicated that TNF-α induced the rate of formation of the catechol estrogen 2-hydroxy estradiol (2-OHE2). This catechol estrogen was found to redox cycle and to generate hydrogen peroxide (H_2O_2) and hydroxyl radicals in MCF-7. Depending on the localized concentrations of catechol estrogens and enzymes that mediate redox cycling, this may be an important mechanism contributing to the development of breast cancer (Fussel et al., 2011). Given that our results also indicated a significant downregulation of the detoxifying enzyme COMT accompanied by a reduction in the anticarcinogenic, antiangiogenic metabolite 2-MeOE2 (Pribluda et al., 2000), this may create a deleterious influence on estrogen carcinogenesis. Interestingly, we also found that E3 was detected in cells treated with E2 alone while it was not detected in cells treated with TNF-α. The effect of this hormone on breast cancer has been controversial. Some studies indicate that there's increased risk of development of breast cancer with E3 therapy (Rosenberg et al., 2006). Other studies however, support the hypothesis that E3 exert protective roles by antagonizing the carcinogenic effects of E2 (Cole and MacMahon, 1969). Recent studies indicate that E3 acts as a G-protein-coupled receptor 30 (GPR30) antagonist in estrogen receptor-negative breast cancer cells. GPR30 has been recently involved in rapid signalling triggered by estrogens (Lappano et al., 2010).

Studies on estrogen metabolism, formation of DNA adducts, carcinogenicity, cell transformation, and mutagenicity have led to the hypothesis that reaction of certain estrogen metabolites, predominantly catechol estrogen-3,4-quinones, with DNA forms depurinating adducts [4-OHE1(E2)-1-N3Adenine and 4-OHE1(E2)-1-N7Guanine]. Formation of these adducts and the concomitant apurinic sites in DNA have been shown to induce mutations that are associated with initiation of breast cancer (Mailander et al., 2006; Naushad et al., 2011). Indeed, our study demonstrated that treatment with TNF-α is associated with a significant increase in estrogen-induced depurinating adducts especially the Adenine adducts. It is likely that this resulted from the increased level of some catechol estrogens accompanied by downregulation of the detoxification

enzymes COMT and NQO1 as demonstrated in this report.

Taken altogether, it's clear from this study that TNF-α alters the balanced homeostatic set of activating and deactivating pathways of estrogen metabolism in MCF-7 breast cancer cells. It influences key genes and enzymes involved in estrogen metabolism leading to increased DNA adduct products. This may implicate a new potential mechanism for inflammation associated breast cancer and provide a plausible explanation for the effect of TNF-α on the advancement of breast cancer. Also it can be suggested that compounds which are known to have positive regulatory effects on estrogen metabolic pathways, e.g. indole-3-carbinol, can be useful in inflammation associated breast cancer (Aggarwal and Ichikawa, 2005).

For future research we would like to see whether the effect of TNF-α on estrogen metabolic pathways plays a role in ER-positive breast cancer patients who are resistant to endocrine therapy. Also whether it's involved in higher resistance to endocrine therapy among obese breast cancer patients who are also resistant to endocrine therapy. No doubts that further evaluation of human breast cancer samples and experimental models as well as development of novel in vivo models designed to specifically study inflammation in mammary tumorigenesis will help to elucidate many of these questions.

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cLEAD BIOACCUMULATION BY *PSEUDOMONAS* SPECIES ISOLATED FROM PIG WASTE.

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ABSTRACT: Living organisms are exposed in nature to lead commonly in their ionized forms, which at different concentrations affect microbial population. This can have significant impact given that many microorganisms are essential parts of the decomposing food chain. Their presence in the atmosphere, soil and water, even in traces, can cause serious problems to all organisms. Microorganisms are known to interact with heavy metals through a number of mechanisms including intracellular accumulation. *Pseudomonas* species isolated from pig waste was exposed to different concentrations of lead solution within 24 hours. The percentage log survival / growth rate in the different concentrations of lead was determined periodically. Bioaccumulation of lead by the test isolate was determined in the graded lead concentrations (0, 1.10, 100. 500 µg/ml). The result showed that the growth of the isolate was progressively inhibited by lead in a dose dependent fashion. The isolate showed a potential to survive lead intoxication and accumulated the toxicant. Therefore, *Pseudomonas* species isolated from pig waste shows a promise for its use in bioremediation of lead polluted environments and can be used remedy the toxic effect of heavy metals on plants. This can be applied as organic manure together with the microorganism in heavy metal-polluted site to prevent heavy metal toxicity and to enhance the growth of plants.
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Keywords: Bioaccumulation, Lead, *Pseudomonas* species, Pig, Waste

1. INTRODUCTION

There is a continuous influx of heavy metals into the biosphere from both natural and anthropogenic sources (Perelomov and Prinsky, 2003(1). This could be from industrial activities lead to substantial release of toxic metals into the environment. These heavy metals constitute a major hazard for the human health and ecosystem (Boopathy, 2000(2). Heavy metals, such as lead, copper, cadmium, chromium and mercury, are important environmental pollutants, particularly in areas with high anthropogenic pressure. Their presence in the atmosphere, soil and water, even in traces, can cause serious problems to all organisms. Heavy metal accumulation in soils is of concern in agricultural production due to the adverse effects on food quality (safety and marketability), crop growth (due to phytotoxicity) and environmental health (Augusto Costa and Pereira Duta, 2001(3).

Living organisms are exposed in nature to lead commonly in their ionized forms, which at different concentrations affect microbial population. This can have significant impact given that many microorganisms are essential parts of the decomposing food chain. The affected microbial population are likely to be replaced by same/other species that may be less efficient in organic matter decomposition, Nutrient recycling, soil formation etc. thereby putting a bridge to Agricultural sustenance / continuity (Yu 2005(4). Lead pollution affects a broad spectrum of species and its persistence in the environment is considered to be hazardous. It affects the human body organs and systems negatively especially the nervous system, (White et. al., 2007(5). It slows down photosynthetic processes, reduces essential nutrient and water absorption, retards plant growth and eventually plant death. Also, Grazing animals are directly affected by the consumption of forage and feed contaminated by air borne lead and somewhat indirectly by the uptake of lead through plant root which subsequently lead to reproductive failure and death (Casarett *et al*., 2007(6).

The metal ion toxicity is determined by many factors such as physio chemicals characters of metals ion including electro- negativity, reduction-oxidation potential, and etc. (Workentine et al., 2008(7). Chemical methods such as precipitation, oxidation or reduction have been widely used to remove metal ions from industrial waste water. Those methods are ineffective or expensive (Volesky, 1990(8). The activity of microorganisms is extended to environmental management, and microbes have superseded the conventional

techniques of remediation (Vidali, 2001(9). Biological methods such as biosorption and bioaccumulation provide promising alternative to chemical methods (Kapoor and Viraragharan, 1995(10). The mechanism by which microorganisms remove heavy metals can be divided into three categories; the first mechanism is the biosorption of metals ions on the cell surface, second intracellular uptake of metals ion and third chemical transformation of metal ions by microorganism (Pardo *et al*., 2003(11). Among the different technique employed for metals removal from multi elemental system, biosorption has been found to be highly selective (Knauer *et al.,* 1997(12).

Furthermore metal accumulating bacteria can be used to remove, concentrate and recover metals from industrial effluents (Malekzadeh *et al*., 2002(13) and Chowdhury *et al*., 2008(14)). The capacity of any biosorbent is mainly influenced by biomass characteristic, physiochemical properties of the target metals, and the micro environment of contact solution including pH, temperature and interaction with other ions (Chen and Wang 2007(15). Moreover once the toxic metals are adsorbed or transferred within organic materials they can be removed from waste water (Smith and Collins, 2007(16).

The present study has been able to show that microorganisms isolated from pig waste have the inherent capability of removing heavy metals from heavy metal-polluted soil. It implies that adverse effects of heavy metal on plants in heavy metal-polluted soil can be remedied using pig waste. This serves the double purpose of supplying nutrients to the plants while also removing the heavy metals from the soil.

2. MATERIALS AND METHODS

2.1. Sample preparation and isolation of lead-resistant *Bacillus*

Pig waste was collected using a clean polyethylene bag from the Department of Animal production in the School of Agriculture and Agricultural Technology (SAAT) of Federal University of Technology Owerri (F.U.T.O), lmo state, Nigeria. Two grams of the pig waste were homogenized in sterile water and serially diluted. Lead [$(PbNO_3)^2$] incorporated nutrient agar plates containing different concentrations (1, 10,100,500 µg/ml) of the lead salt were prepared and inoculated with 0.1 ml of the diluted samples. Incubation was done at 37^{o}C for 24 hours. Isolated colonies were purified by two subsequent single colony transfers. Pure colonies were specifically transferred into nutrient agar slants. The slants were incubated at 37°C for 18 - 24 h. These served as the stock cultures and were stored at 4°C in the refrigerator. The isolates were identified according to the method stipulated in Holt *et al*. (1994(17).

2.2. Preparation of stock solution of heavy metal salt

A weight of lead salt that gave 1 g of the heavy metal (metal without the salt) dissolved in 1000 ml of deionized water. It was left to stand for 30 min to obtain complete dissolution. This was followed by sterilization by membrane filtration.

2.3. Preparation of standard inoculum

A loopful of cells from the stock culture was inoculated into 100 ml sterile nutrient broth in triplicates and incubated at 37°C for 24 h with intermittent shaking. At the end of the incubation period, cells were harvested by centrifugation at 4000 rpm for 30 min and re-suspended in 100 ml sterile physiological saline. The total viable counts were carried out to estimate the number of viable organisms. During this process, the cultures were subjected to serial dilutions up to 10^6 dilutions. An aliquot (0.1 ml) from each dilution was inoculated by spread plate technique into freshly prepared nutrient agar plates, which were incubated at 37°C for 24 h. The dilutions producing between 30 - 300 colonies were chosen and served as inoculum for Percentage log survival test.

2.4. Percentage log survival test

Different concentrations of lead solution were prepared in deionized water to obtain 1.0, 10.0, 100.0 and 500.0 µg/ml. Ninety milliliters of each the different concentrations was put in 100 ml conical flask and inoculated with 10 ml of the standard culture with constant shaking. A control was set up with 90 ml of normal saline without toxicant and was inoculated with 10 ml of the standard culture. At exposure times of 0, 2, 4, 12, 24 h, 1 ml was aseptically withdrawn from each of the flasks for viable count using the spread plate technique. The percentage log survival of the isolate was calculated using the formula:

$$\text{Percentage log survival} = \frac{\log A}{\log B} \times 100$$

Where A = Count in toxicant concentration

B =Count in the control

2.5. Metal up take assay

The isolate was developed by growing in 100 ml of freshly prepared nutrient broth (pH 7.0) at 37^0C for 18-24hrs with constant shaking. Cells were harvested by centrifugation at 4000rpm for 30 min. they were washed thrice with sterile phosphate buffered saline and re-suspending in 100ml of deionized water. The viability of the cells was assessed by plating 0.1ml onto a nutrient agar plate. Stock solution of different concentrations (1.0, 10.0, 100, 500 µg/ml) of lead was prepared and adjusted to pH of 7.0 using 0.1 M sodium hydroxide and 0.1 M trioxonitrate (V) acid. From the various concentrations of the heavy metal salt, 40 ml were withdrawn using sterile pipette into duplicate set of 100 ml flask and inoculated with 10 ml of each of the standard inoculum. For the control, 40 ml of sterile normal saline was inoculated with 10 ml of the inoculum. All flasks were incubated at 25°C ± 2 for 24 h. At the end of the incubation period, cells were harvested by centrifugation at 4000 rpm for 30 min, washed thrice in sterile phosphate buffered saline, dried, weighed, digested and analyzed for heavy metal content using AAS.

2.6. STATISTICAL ANALYSES

Data obtained from this study were analyzed using a one-way analysis of variance (ANOVA) and values for P≤0.05 were considered statistically significant.

3. RESULT

The growth curve of the test organism relative to the control was calculated. This was done by measuring and plotting the absorbance as a function of time of incubation. The absorbance of the control after 24 hours of incubation was taken as the maximum growth of the test organism. The result is presented in figure 1. From the result, it was observed that the growth curve was concentration dependent. There was no significant effect of the lead on the growth curve of the organism when exposed to the toxicant up to the concentration of 100 µg/ml at P≤ 0.05. Conversely, there was a very high significant effect of the lead toxicant on the growth curve of the organism when exposed to 500 µg/ml concentrations after the incubation period. At this concentration, the organism entered the stationary phase after 16 hours of incubation.

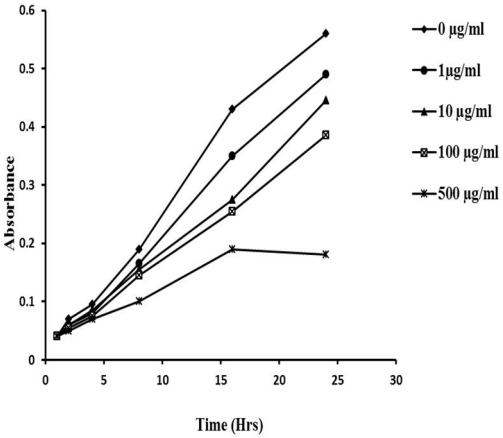

Figure 1. Growth curve of the test organism in different concentations of lead.

The result of the percentage log survival of the test organism in different concentrations of the lead toxicant and at different incubation times are presented in figure 2. The effect of lead concentrations on the percentage log survival of the test organism showed that at increasing concentrations of lead, the percentage log survival decreased with increase in time of exposure. At the initial hour of incubation, the test organism had 100% survival at all the lead concentrations. At subsequent hours of incubation, the survival rate of the test organism decreased significantly with increase in lead concentration and time of incubation (P≤0.05).

Dose response curve obtained from the plot of lead concentration (µg/ml) against the bioaccumulation of lead (mg/kg) by the test organism is presented in figure 3. The lead concentration correlated well with lead bioaccumulation with a very high R^2 value (R^2 = 0.9971). The bioaccumulation model gave a good linearization of the dose-response data. The equation of the curve is given as lead concentration (µg/ml) = 0.0725 lead bioaccumulation (mg/kg) + 2.3815. The result shows that the bioaccumulation increased significantly with increase in concentration of the lead toxicant with the highest

bioaccumulation observed in the test organism when exposed to 500 µg/ml concentrations.

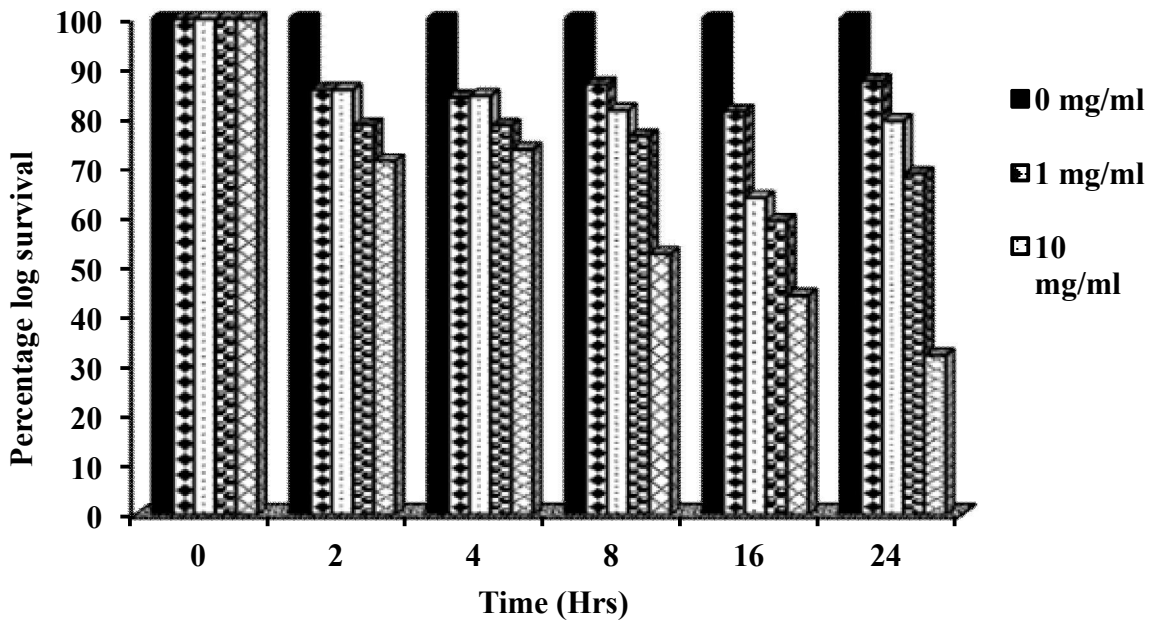

Figure 2. percentge log survival of the isolates in different concentrations of lead at different time intervals.

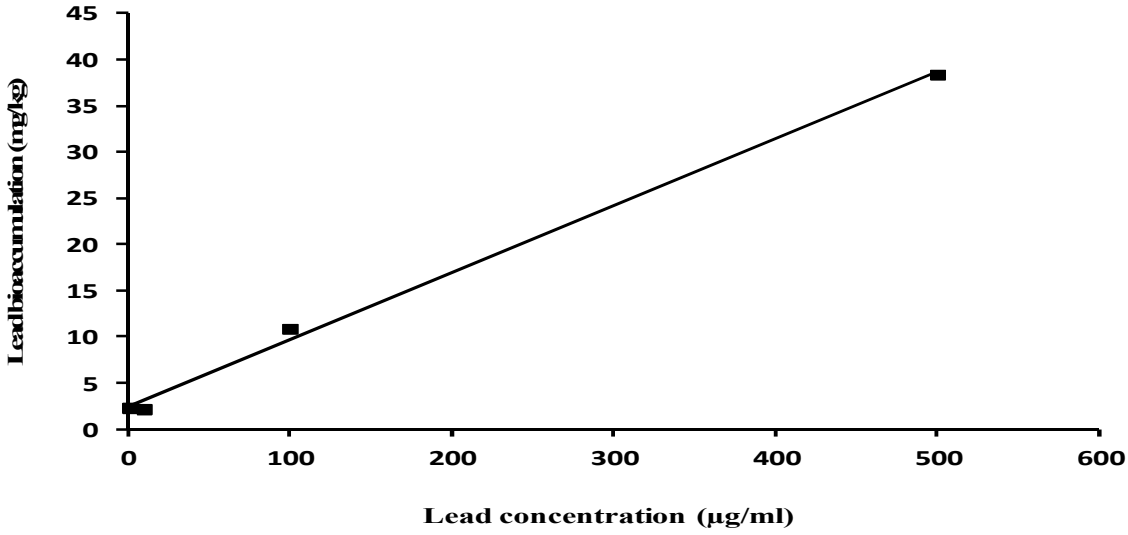

Figure 3. Lead bioaccumulation by *Pseudomonas* species in different concentrations of lead

4. DISCUSSION

Industrial activities led to substantial release of toxic metals into the environment. Heavy metals constitute a major hazard for the human health and ecosystem (Boopathy, 2000(2). Some metals including iron, zinc, copper and manganese are micronutrients used in the redox processes, regulation of osmotic pressure, enzymes cofactors and are also important in the maintenance of the protein structure (Vallee and Auld 1990(18). On the other hand metals including lead and cadmium do not play any known physiological role and\ are in fact toxic to cells. Lead reacts with the sulphydryl groups of protein and inhibits their function (Ron *et al.,* 1992(19). The metal ion toxicity is determined by many factors such as physio-chemicals characters of metals ion including electro- negativity, reduction-oxidation potential, etc. (Workentine *et al.,* 2008(7).

The results of the study showed that the *Pseudomonas* species is capable of surviving when exposed to various concentrations of lead salt within 24 hours exposure duration. This is in accordance with the works of Odokuma1 and Akponah (2010(20), Odokuma and Ijeomah (2003(21), Odokuma and Emedolu (2005(21). In their reports *Bacillus* sp. and *Aeromonas* sp. were shown to be resistant to the toxicity of heavy metals. The persistence of these isolates in the presence of the respective heavy metals may be as a result of the possession of heavy metal resistant plasmids (Odokuma and Oliwe, 2003). The spore forming ability of *Bacillus* sp. might also, have contributed to its ability to survive when exposed to the various concentrations of the heavy metal salt.

The result of the percentage log survival of the test organism in different concentrations of the lead toxicant and at different incubation times as presented in figure 2 revealed that at the initial hour of incubation, the test organism had 100% survival at all the lead concentrations. At subsequent hours of incubation, the survival rate of the test organism decreased significantly with increase in lead concentration and time of incubation ($P \leq 0.05$). This is in line with the works of Odokuma and Akponah, (2010) that showed that the percentage survival of their isolates decreased with increase in contact time as well as concentration when exposed to different concentrations of heavy metals. This shows that contact time is a very crucial factor in establishing the resistance of organisms to the toxic pressure of the metals.

At the initial hour of incubation, the test organism had 100 % survival in all the lead concentrations. At subsequent hours of incubation, the test organism had irregular rate of survival in the 1 µg/ml and 10 µg/ml concentrations respectively. When exposed to 100 µg/ml and 500 µg/ml, the rate of survival decreased with increase in the time of incubation. The effect of lead concentrations on the percentage log survival of the test organism showed that at high concentration lead, the percentage log survival decreased with increase in time of exposure. This is in line with the works of Odokuma1 and Akponah, (2010) that showed that the percentage survival of their isolates decreased with increase in contact time as well as concentration when exposed to different concentrations of heavy metals. This shows that contact time is a very crucial factor in establishing the resistance of organisms to the toxic pressure of the metals.

Dose response curve obtained from the plot of lead concentration (µg/ml) against the bioaccumulation of lead (mg/kg) by the test organism presented in figure 3 depicted that lead concentration correlated well with lead bioaccumulation with a very high R^2 value (R^2 = 0.9971). The bioaccumulation model gave a good linearization of the dose-response data. The equation of the curve is given as lead concentration (µg/ml) = 0.0725 lead bioaccumulation (mg/kg) + 2.3815. The result shows that the bioaccumulation increased significantly with increase in concentration of the lead toxicant with the highest bioaccumulation observed in the test organism when exposed to 500 µg/ml concentrations.

Bioaccumulation test carried out revealed that *Pseudomonas* species had an inherent capability to withstand the toxicity of lead and bioaccumulate the metal (Odokuma and Emedolu, 2005). Richard *et al.,* 2002 reported that Cu^{+2} and Pb^{+2} appear to bind to materials on the cell surface. Lead is precipitated in an insoluble form that is localized to the cell membrane or cell surface. Similar results were obtained by El-Hendawy (2009) which shows the localization of one or more metal to cell wall of *V. alginolyticus*. This could be generally explained by the fact that the negatively charged groups (carboxyl, hydroxyl and phosophryl) of bacterial cell wall absorb metal cations through various

mechanisms such as electrostatic interaction, van der Waals forces, covalent bonding or combination of such processes (Chojnacka *et al.,* 2005). Both dead and living cells adsorb metal ions (Ansari and Malik 2007).

Several principal sites of metal-complex formation in biological systems have been proposed (Vieira and Volesky, 2000). These processes involve a typical ion-exchange process where the metal ion is exchanged for a counter-ion attached to biomass. Bioleaching is a similar process where microbes dissolve the metals present in solid matrix into soluble form. Others include accumulation in the cell wall, carbohydrate or protein polyphosphate complexes, and complexion with carboxyl groups of the peptidoglycan in the cell wall. However, there are five basic mechanisms that convey an increased level of cellular resistance to metals: (1) efflux of the toxic metal out of the cell; (2) enzymatic conversion; (3) intra- or extracellular sequestration; (4) exclusion by a permeability barrier; and (5) reduction in sensitivity of cellular targets. In the present study, it was observed that there was an increase in bioaccumulation with increase in the lead concentration. These observations suggest that metal uptake may involve diffusion phenomenon whereby, metal ions move from regions of high concentrations to low concentrations and the fact that the steeper the concentration gradient, the more raped is the movement of molecules or ions (Taylor et al., 1997) or any of the above-mentioned mechanisms. The high R^2 values obtained in the regression plot indicated that lead concentration was a strong determinant of the bacterial accumulation. The *Bacillus* species can be used, in the future, for heavy metals removal, immobilized on waste biomaterials. Input of heavy metals imposes a selective pressure that may favor the growth and activity of resistant/tolerant microbes. The development of a metal-resistant population in a contaminated soil can result from: (i) vertical gene transfer (reproduction), (ii) horizontal gene transfer (including transposons and broad host range plasmids), and (iii) selection pressures on spontaneous mutants (due to the presence of metals). Transposable elements carrying mercury resistance genes have been linked to the distribution of this trait in nature (Khosro *et al*, 2011).

The present study has been able to show that microorganisms isolated from pig waste have the inherent capability of removing heavy metals from heavy metal-polluted soil. It implies that adverse effects of heavy metal on plants in heavy metal-polluted soil can be remedied using pig waste. This serves the double purpose of supplying nutrients to the plants while also removing the heavy metals from the soil.

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5/20/2012

POTENTIAL PRODUCTION OF LIPASES BY *PSEUDOMONAS* AND *STAPHYLOCOCCUS* SPECIES ISOLATED FROM PALM OIL CONTAMINATED TROPICAL SOIL

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ABSTRACT: This study reports on the potential production of lipases by *Pseudomonas* and *Staphylococcus* species isolated from palm oil contaminated tropical soil. The lipase activities of *Pseudomonas* species and *Staphylococcus* species at 37^{o}C and pH 7 were evaluated. Both organisms grew well and produced lipases at the prevailing assay conditions. The lipase activity by *Pseudomonas* species was significantly higher than that of *Staphylococcus* species at P< 0.05. Effect of pH on the enzyme activities of the isolates showed that lipase production varied according to the pH of the assay medium. This shows that pH is a determinant factor in lipase production. There were increases in lipase activities with increase in pH up to optimum pH of 7 in both organisms. The lipase activities decrease progressively after this optimum pH till the last pH of exposure (pH 10). At pH 7 and 8, the two organisms had equal lipase activities (0.64 and 0.63 U/ml/min, respectively), after which, the lipase activity of *Pseudomonas* species reduced gradually with increase in pH unlike *Staphylococcus* species whose lipase activity abruptly decreased with increase in pH. Lipase activities in both organisms were affected differently by varying temperature changes. In *Pseudomonas* species, there was a steady increase in lipase activity with increase in temperature up to the optimum at temperature of 30 ^{o}C (0.67 U/ml/min). The activity decreased slightly at temperature of 35 ^{o}C but effect of temperature against the activity was more pronounced at the temperatures of 40 and 45 ^{o}C. The lipase activity of *Staphylococcus species* was also affected by temperature changes. There was a steady increase in the activity up to the optimum at temperature of 35 ^{o}C. The activity started decreasing steady with further increase in temperature. In conclusion, the study showed that *Pseudomonas* and *Staphylococcus* species isolated from palm oil contaminated tropical soil has a great potential for the production of lipases.
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Keywords: potential, production, lipases, *Pseudomonas* species, *Staphylococcus* species, palm oil, tropical soil.

1. INTRODUCTION

Lipases are water soluble enzymes which have the ability to hydrolyze triacylglycerols to release free fatty acids and glycerol. Lipases constitute a major group of biocatalysts that have immense biotechnology applications. Lipases have been isolated and purified from fungi, yeast, bacteria, plant and animal sources (Joseph *et al* 2008). Of all these, bacterial lipases are more economical and stable (Immanuel *et al*., 2008). Lipases are currently attracting an enormous attention because of their biotechnological applications. Hence, they have become important biocatalysts in various industrial sectors, such as the agrochemical, pharmaceutical, detergent and food industries (Bouke *et al*., 2007). Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. Most of the bacterial lipases reported so far are constitutive and are non-specific in their substrate specificity and a few bacterial lipases are thermostable. Due to such attributes, lipases are used in detergents, manufacture of food ingredients pitch control in pulp and paper industry (Fariha *et al.*, 2006), production of aromas, production of insecticides and synthesis of drugs such as naxopren and ibuprofen and as a biocatalyst stereo selective transformations. The exponential increase in the application of lipases in various fields, in the past few years, necessitated both qualitative and quantitative improvement in enzyme production. The quantitative enhancement requires strain improvement and medium optimization for overproduction (Immanuel *et al*., 2008).

Microbial lipases constitute an important group of biotechnological valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production (liu *et al*., 2005). Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial application (Ruchi *et al.,* 2008). Lipases are used in

two distinct fashions. They are used as biological catalysts to manufacture other products (such as food ingredients) and by their application such as (in marking fine chemicals). Lipases have received increased attention recently, evidenced by the increasing amounts of information about lipase in the current literature. The renewed interest in this enzymes class is due primarily to investigations of their role in pathogenesis and their increasing use in biotechnological applications (Haki *et al*., 2003). The commercial use of lipases is a billion-dollar business that comprises a wide variety of different applications (Snellman *et al*., 2006).

The limited resources of fossil fuels, increasing prices of crude oil, and environmental concerns have been the diverse reasons for exploring the use of vegetable oil as alternative fuels (Shah *et al*., 2006). The biodiesel fuels from vegetable oil do not produce sulphur oxide and minimize the soot particulate one third times in comparison with the existing one from petroleum. Because of these environmental advantages biodiesel fuel can be exported as a substitute for conventional diesel fuel (Iso *et al*., 2007).

Immobilized *P. cepacia* Lipase was used for the transesterification of soya bean oil with methanol and ethanol (Noureddini *et al*., 2005). Fatty and esters have also been prepared from castor oil using n-hexane as solvent and two commercial lipases, Novozym 435 and lipozyme IM, as catalyst (de Oliveria *et al*., 2004). Fatty acids esters were produced from two Nigerian Lauric oils, palm kernel oil and coconut oil, by transesterification of the oil with different alcohols using PS30 lipase as a catalyst in the conversion of palm kernel oil to alkyl esters (biodiesel), ethanol gave the highest conversion of palm kernel oil to alkyl esters (biodiesel), ethanol gave the highest conversion of 72%. Some of the fuel properties compared favourably with international biodiesel specifications (Abigor *et al*., 2009). This study reports on the potential production of lipases by *Pseudomonas* and *Staphylococcus* species isolated from palm oil contaminated tropical soil.

2. MATERIALS AND METHOD

2.1. Sample Collection and Isolation of Test Organisms

The test organisms were isolated from soil samples contaminated with palm oil. About 1 g of soil sample was collected in a sterile container and transported to the laboratory on ice. The soil sample was serially diluted in sterile water and inoculated onto nutrient agar plates. After incubation at 37 ^{o}C for 18-24 hours, the colonies were isolated, purified and identified using their cultural and biochemical characteristics. Chromogenic substrate plates were prepared were prepared according to the method of Singh *et al* (2006) as reported by Amara *et al* (2009). Briefly, plates were prepared by using 0.01% of phenol red together with 1% olive oil which served as substrate, 2 % Arabic gum, 10 mM CaCl and 2% agar. The pH was adjusted to 7.3–7.4 using 0.1 N NaOH, where 2% olive oil, 4% Arabic gum and 20 mM CaCl were added to water (pH 7.3) and mixed using suitable mixture till complete homogenization was occurred then the mixture was added to the same volume of 4% melted agar (50°C). The phenol red was added in final concentration 0.01% to the mixture to give orange-reddish color. They were distributed to agar plates wells were punched in the agar plates using sterile cork borer (8 mm in diameter). The base of each hole was sealed with a drop of melted sterile water agar. The organisms were grown in nutrient broth and centrifuged after 24 hours of incubation. The supernatants were used to detect lipase activity. Aliquots (50 µl) of the supernatant were introduced into each well while sterile media served as control. Plates were incubated for 30 minutes at 37 ^{o}C. The changes in the color around the wells were taken as indicative of the presence of lipase activity.

2.2. Lipase Assays

Lipase activity was measured by titrimetric method using olive oil as a substrate. Olive oil (10% v/v) was emulsified with gum Arabic (5% w/v) in 100 mM potassium phosphate buffer (pH 7.0). Up to 100µl of supernatant was added to the emulsion and incubated for 15 minutes at 37^{0}C. The reaction was stopped and fatty acids were extracted by addition of 1.0 ml of acetone: ethanol solution and titrating with 0.05M NaOH until pH 10.5 was reached using phenophathelin as the indicator (Jensen, 1983). The unit of enzyme activity was defined as the amount of enzyme required to hydrolyze one µmol of fatty acids from triglycerides per minute.

2.3. Effect of Temperature on Lipase Activity:

For selection of optimum temperature for the production of lipases, the temperatures varying from 20 to 45°C were selected by keeping the remaining parameters same. The lipase assay was performed as reported above.

2.4. Effect of pH on Lipase Activity:

The optimum pH for enzyme production was selected by varying the pH of the assay medium from 4 to 10 whereas the other parameters were unaltered. The lipase assay was performed as stated above.

3. Result Analysis

Microbial lipases have been a kind of favorable enzymes with their actual and potential applications in household detergents, synthesis of pharmaceuticals or agrochemicals, processing of fats and food ingredients, dairy and textile industries and production of surfactants (Gupta et al., 2004). Lipases have been isolated and purified from fungi, yeast, bacteria, plant and animal sources. Of all these, bacterial lipases are more economical and stable (Joseph et al, 2008). The lipase activities of *Pseudomonas* species and *Staphylococcus* species at 37^{o}C and pH 7 were evaluated. Both organisms grew well and produced lipases at the prevailing assay conditions. The lipase activity by *Pseudomonas* species was significantly higher than that of *Staphylococcus* species at $P< 0.05$ (Figure 1). Some bacterial species have been shown to produce lipases. Among these are *Bacillus*, *Pseudomonas* and *Burkholderia* (Svendsen 2000). Patil *et al*, (2011) reported that lipase can be produced by a variety of organisms including bacteria, fungi, plants and animals. The present study evaluated the lipase production potentials of *Pseudomonas* and *Staphylococcus* species isolated from palm oil polluted soil.

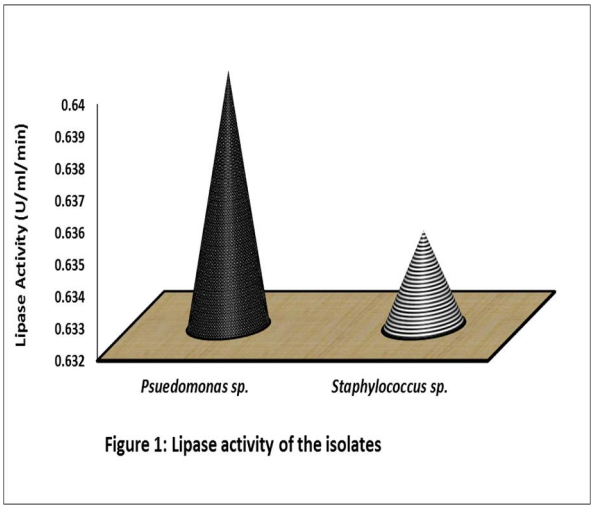

Figure 1: Lipase activity of the isolates

Effect of pH on the enzyme activities of the isolates showed that lipase production varied according to the pH of the assay medium. This shows that pH is a determinant factor in lipase production. There were increases in lipase activities with increase in pH up to optimum pH of 7 in both organisms (Figure 2). The lipase activities decrease progressively after this optimum pH till the last pH of exposure (pH 10). At pH 7 and 8, the two organisms had equal lipase activities (0.64 and 0.63 U/ml/min, respectively), after which, the lipase activity of *Pseudomonas* species reduced gradually with increase in pH unlike *Staphylococcus* species whose lipase activity abruptly decreased with increase in pH. This is at variance with the work of some researchers who reported the optimum lipase activity at alkaline pH (Yuan et al, 2010; Prazeres et al, 2006). But the present study is in consonance with the work of some other researchers whose bacterial species samples isolated from oil contaminated soil had optimum lipase activity at pH 7 (Sirisha et al, 2010; Vijayaraghavan et al, 2011).

Lipase activities in both organisms were affected differently by varying temperature changes. In *Pseudomonas* species, there was a steady increase in lipase activity with increase in temperature up to the optimum at temperature of 30^{o}C (0.67 U/ml/min). The activity decreased slightly at temperature of 35^{o}C but effect of temperature against the activity was more pronounced at the temperatures of 40 and 45 ^{o}C (Figure 3).

The lipase activity of *Stahpylococcus species* was also affected by temperature changes. There was a steady increase in the activity up to the optimum at temperature of 35 ^{o}C. The activity started decreasing steady with further increase in temperature. It has been demonstrated by many authors that temperature is a very strong determinant of enzyme activity (Sirisha et al, 2010; Vijayaraghavan et al, 2011; Prazeres et al, 2006; Yuan et al, 2010). This study agrees with the work of Guzman et al, (2008) who reported an optimum temperature for lipase activity between 35-45 ^{o}C. In conclusion, the study showed that *Pseudomonas* and *Staphylococcus* species isolated from palm oil contaminated tropical soil has a great potential for the production of lipases.

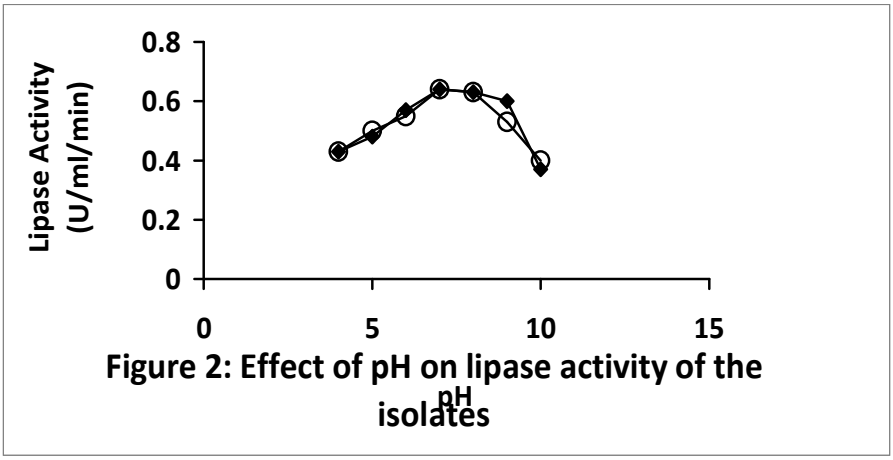

Figure 2: Effect of pH on lipase activity of the isolates

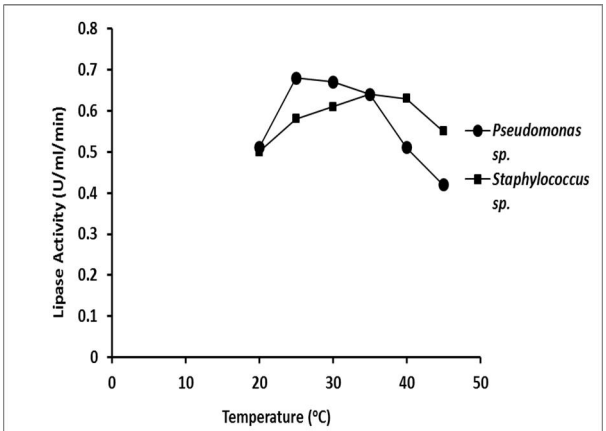

Figure 3: Effect of temperature on lipase activity of the isolates

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Isolation and Identification of *Candida albicans* and *Staphylococcus aureus* from Oral Swabs among Primary School Pupils in Uzuakoli, Abia State, Nigeria

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ABSTRACT: *Candida albicans* and *Staphylococcus aureus* were isolated from oral swabs of one hundred pupils, (aged 8 – 11 years) of Ngwu/ Amankwo Community Primary School, Uzuakoli, Abia – State. Sterile swab sticks were used for the collection of the specimens. Out of the 100 specimens collected, *Staphylococcus aureus* was isolated from (61.0%) samples, (65.0%) samples contained yeast – like organisms and (30.0%) samples had both *Staphylococcus aureus* and *Candida albicans,* forty of the yeast – like isolates were identified as *Candida albicans*. The highest carriage of *Staphylococcus aureus* and *Candida albicans* was found in the mouth of pupils using Charcoal and Chewing stick as dental agent. These pupils, who used chewing stick only, as their method of oral hygiene maintenance, had a carriage of 67.0%. Pupils that used the toothbrush infrequent had a carriage of 75.0%, those that alternated the use of tooth brush with chewing stick had a carriage of 42.0%, while those that used toothbrush regularly, as their method of oral hygiene, had the least carriage of 4.0%. This study showed that the use of tooth brush regularly, is the best method of oral hygiene.
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Keywords: *Candida albicans*, *Staphylococcus aureus*, Charcoal, Chewing stick, oral swabs, toothbrush, Nigeria

1. INTRODUCTION

Bacteria are the most obvious inhabitants of the oral cavity but other microorganisms are often seen. These include several species of fungi, viruses and protozoa (George et al., 1988). *Candida albicans* and *Staphylococcus aureus* are normal flora of the oral cavity and they are opportunistic pathogens which means that they are generally harmless in its normal environment but become pathogenic in a compromised host. A compromised host is seriously debilitated and has a lowered resistance to infection (Prescott *et al*., 2002).

The oral cavity is inhabited by more than 700 microbial species and many intrinsic and extrinsic factors affect the composition, metabolic activity and pathogenicity of the highly diversified oral microflora (Samaranayake et al., 2002; Aas et al., 2005; Nejad et al., 2011). This fact has been correlated mainly to the use of broad-spectrum antibacterials, corticosteroids, anti-tumoral agents, oral contraceptives and increase in the number of immunocompromised patients (Eggimann et al., 2003; Nejad et al., 2011).

Yeasts, especially Candida spp. are the normal oral flora and their isolation from the mouth can be investigated in excessive consumption of fermentable carbohydrates (Samaranayake al., 1986; Nejad et al., 2011), dental caries risk and denture-wearing status (Beighton et al., 1991; Nejad et al., 2011). *Candida albicans* is the most common cause of oral fungal infection. *Candida albicans* is a diploid fungus (a form of yeast) and a causal agent of opportunistic oral and genital infections in human (Ryan and Ray 2004). Candida is not harmful in healthy hosts, but may cause opportunistic infections in immunocompromised hosts, such as patients suffering from AIDS, leukemia and diabetes (Nejad et al., 2011). Oral candidiasis, which is frequently caused by *Candida albicans*, is one of the most common fungal opportunistic infections in immunocompromised patients (Klein et al., 1984; Nejad et al., 2011).

Systemic fungal infections (fungaemias) have emerged as important causes of morbidity and mortality in immune compromised patients (e.g., AIDS, Cancer chemotherapy, organ or bone marrow transplantation). *Candida albicans* biofilms readily form on the surface of implantable medical devices. In addition, hospital-acquired infection in patients not previously considered at risk (e.g., patients in an intensive care unit) has become a cause of major health concern (Ryan and Ray, 2004). Importantly, many non-albicans Candida have decreased susceptibility to antifungal agents. Specifically, *Candida krusei* and *Candida glabrata* demonstrate decreased susceptibility to fluconazole

(Lynnet al., 2003; Nejad et al., 2011). Clinicians now depend on identification of Candida species for accurate selection of antifungal agent and to provide the best treatment possible to the patient (Nejad et al., 2011).

Staphylococcus aureus is also an inhabitant of the oral cavity. *Staphylococcus aureus* is a facultative anaerobic, gram positive cocci, which appears as grape like clusters, when viewed under the microscope, it has large, round golden yellow colonies, often with haemolysis, when grown on blood agar plate (Tolan, 2007). The term Staphylococcus is derived from Greek term stapyle, meaning a bunch of grapes. Staphylococci are non motile, non spore –forming and catalase - positive bacteria. The cell wall contains peptidoglycan and teichoic acid. The organisms are resistant to temperatures as high as 50^{o} c, to high salt concentrations, and to drying (Tolan, 2007). Colonies are usually large (6 – 8 mm in diameter), smooth and translucent. The colonies of most strains are pigmented, ranging from cream – yellow to orange (Tolan, 2007).

The ability to clot plasma continues to be the most widely used and generally accepted criterion for the identification of *Staphylococcus aureus*. One such factor, bound coagulase, also known as clumping factors, reacts with fibrinogen to cause organisms to aggregate (Tolan, 2007). Another factor extracellular staphylothrombin, which convert fibrinogen to fibrin. Approximately 97% of human *Staphylococcus aureus* isolates possess both of these forms of coagulase (Tolan, 2007).

A recent review has highlighted the paucity of both clinical and laboratory data on the role of *Staphylococcus aureus*, in the oral cavity in both healthy and disease people (Smith *et al*., 2001, 2003). Some oral infections are caused at least in part by *Staphylococcus aureus*, for example, angular cheilitis, parotitis and staphylococcal mucositis (Smith *et al*., 2003).

The aim of this study was to evaluate the prevalence of *Candida albicans* and *Staphylococcus aureus* among oral isolates from primary school pupils using different methods of oral hygiene.

2. MATERIALS AND METHODS

2.1. Study Area

The study was carried out among the pupils of Ngwu/Amankwo Community Primary School, Uzuakoli, Abia state. Nigeria

2.2. Study Population

Samples of oral swab were collected from pupils (8 to 11 years) of Ngwu/Amankwo community primary school, Uzuakoli, Abia state. Before the collection of the specimens, age, sex, educational background of parents, and what they use for oral hygiene. Specimens were collected by rubbing the sterile swab sticks on the surface of the tongue, below the tongue and on the surface of the gum behind the molars. A total of 100 specimens were collected from 100 pupils and information on the sources of specimens is given in Table 1.

2.3. Isolation and identification of isolates

Isolated colonies on Nutrient agar were subcultured on to Mannitol Salt Agar. The confirmation of the colonies as *Staphylococcus aureus* was made when growth occurred on the Mannitol salt Agar. The isolated colonies on Sabouraud Agar plates were also Subculture on Corn Meal Agar. Pure cultures were obtained by streaking and restreaking colony on to nutrient agar plates and Sabouraud Agar plate until single colonies were obtained after incubation. All tests were performed on pure culture. The smears of colonies were prepared and gram stained (Cheesbrough, 2006). Slide coagulase test was performed using the method of Cheesbrough (2006). A loopful of a pure colony was emulsified in saline of a clean microscopic slide to produce a thick milky suspension. A loopful of citrated plasma was then added to the suspension. Both were mixed using a sterile wire loop and examined microscopically for the presence of clumps which indicate a positive result. The *Staphylococcus aureus* was identified based on their biochemical reactions. *Candida albicans* was identified based on the Chlamydospore formation test and the biochemical reactions.

3. RESULTS ANALYSIS

3.1 Frequency of isolation of *Candida albicans* from different methods of oral hygiene

Table 1 shows the frequency of isolation of *Candida* isolates from pupils using different methods of oral hygiene. It showed that isolation of *Candida albicans* was most predominant 40(61.5%). This was followed by *C. krusei* 6(9.2%), *C. guilliermondii* 5(7.7%), *C. parapsilosis* 4(6.2%), *C. tropicalis* 4(6.2%), *C. stellatoidea* 3(4.6%) and *C. pseudotropicalis* 3(4.6%) as shown in Table 1.

Table 1: Frequency of isolation of *Candida* isolates from pupils using different methods of oral hygiene

Candida Isolates	No. (%)
Candida albicans	40(61.5)
C. krusei	6(9.2)
C. stellatoidea	3(4.6)
C. guilliermondii	5(7.7)
C. pseudotropicalis	3(4.6)
C. parapsilosis	4(6.2)
C. tropicalis	4(6.2)
Total	**65(100.0)**

3.2. Frequency of isolation of bacterial isolates (*Staphylococcus aureus* and *Streptococcus* sp) from different pupils using different methods of oral hygiene

Table 2 shows the frequency isolation of bacterial isolates (*Staphylococcus aureus* and *Streptococcus* sp) from different pupils using different methods of oral hygiene. It showed that the isolation of *Staphylococcus aureus* was most predominant 61(71.8%), followed by *Streptococcus* spp. 23(27.0%). Isolation of *S. aureus & Streptococcus spp.* coinfection was least predominant 1(1.2%) as shown in Table 2.

Table 2: Frequency isolation of bacterial isolates (*Staphylococcus aureus* and *Streptococcus* sp) from different pupils using different methods of oral hygiene

Bacterial isolates	No. (%)
Staphylococcus aureus	61(71.8)
Streptococcus spp.	23(27.0)
S. aureus & Streptococcus spp. Coinfection	1(1.2)
Total	**85(100.0)**

3.3. Coinfections of *S. aureus*, *Streptococcus* spp. and *Candida* species from different pupils using different methods of oral hygiene

Table 3 shows the coinfections of *Staphylococcus aureus*, *Streptococcus* sp. and *Candida albicans* from different pupils using different methods of oral hygiene. It showed that coinfections of *Staphylococcus aureus* and *Candida albicans* were most predominant 43(78.2%). This was followed by *Candida* species *& Streptococcus* spp. 10(18.2%). Coinfections of *S. aureus & Streptococcus* spp. and that of *S. aureus, Streptococcus* spp. and *Candida* species were least predominant 1(1.8%) as shown in Table 3.

Table 3: Coinfections of *S. aureus*, *Streptococcus* spp. and *Candida* species from different pupils using different methods of oral hygiene

Isolates	No. (%)
S. aureus & Streptococcus spp.	1(1.8)
S. aureus & Candida spp.	43(78.2)
Candida species *& Streptococcus* spp.	10(18.2)
S. aureus, Streptococcus spp. and *Candida* species	1(1.8)
Total	**55(100.0)**

3.4. Frequency of isolation of both *Candida albicans and Staphylococcus aureus* from different pupils using different methods of oral hygiene

Table 4 shows the frequency of isolation of *Staphylococcus aureus* and *Candida albicans* from different pupils using different methods of oral hygiene. The highest frequency of isolation of *Candida albicans* was obtained from pupils using charcoal and chewing stick 2(100.0%). Those that do infrequent tooth brushing had a frequency of 75.0%. Those that use chewing stick alone had 73.0%. Those that use both charcoal and tooth brush had 0.0%. Those that use tooth brush only had a frequency of 19.0%, while the pupils that use tooth brush and chewing stick had 48.0% isolation of *Candida albicans.* The highest frequency of isolation of *Staphylococcus aureus* was obtained from the mouth of pupils that use chewing stick and charcoal (100.0%), tooth brush and charcoal (100.0%), infrequent tooth brushing (100.0%). Those that use tooth brush had a frequency of 34.0% isolation of *S. aureus*. The pupils that use chewing stick had an isolation frequency of 80.0% for *S. aureus*, while those that use both toothbrush and chewing stick had 83.0% isolation for *S. aureus* (Table 2). The highest frequency of isolation of *S. aureus* and *C. albicans* was obtained from the mouth of pupils using charcoal and chewing stick (100.0%). Those that use chewing stick only had the frequency isolation of 67.0%. Those that do infrequent tooth brushing had isolation frequency of 75.0%. The pupils that use both tooth brush and chewing stick had 42.0%, while those that use tooth brush had isolation frequency of 4.0% (Table 4).

Table 4: Frequency of isolation of *Candida albicans and Staphylococcus aureus* from different pupils using different methods of oral hygiene

Method of oral hygiene	No. Examined (%)	No. Positive for *Candida* isolates (%)	No. Positive for bacterial isolates (%)	No. Positive for both *Candida* and bacterial isolates (%)
Toothbrush	47(47.0)	9(19.0)	16(34.0)	2(4.0)
Toothbrush and Chewing stick	31(31.0)	15(48.0)	26(83.0)	13(42.0)
Chewing stick	15(15.0)	11(73.0)	12(80.0)	10 (67.0)
Charcoal and Chewing stick	2(2.0)	2(100.0)	2(100.0)	2(100.0)
Charcoal and Toothbrush	1(1.0)	0(0.0)	1(100.0)	0(0.0)
Infrequent tooth brushing	4(4.0)	3(75.0)	4(100.0)	3(75.0)
Total	**100(100.0)**	**40(40.0)**	**61(61.0)**	**30(30.0)**

Table 5 shows frequency of isolation of *Candida* and bacterial isolates from different pupils using different methods of oral hygiene in relation to their demographic characteristics. It showed that males (96.4%) had higher carriage rate of the isolates compared to their female counterparts (91.1%). Pupils within ages 8 years old had higher carriage rate compared to those in ages 9 years old with lower carriage rate of 83.3%. Isolation of pathogens were more prevalent among pupils whose parents were illiterate (98.1%) than those who had educated parents (89.4%). Other details are shown in Table 5.

Table 5: Frequency of isolation of *Candida* and bacterial isolates from different pupils using different methods of oral hygiene in relation to their demographic characteristics

Demographic Characteristics	No. Tested (%)	No. positive (%)	*Candida* spp. (%)	*S. aureus* (%)	*Streptococcus* spp. (%)	Coinfections of *Candida* and bacterial isolates (%)
Sex						
Male	55	53(96.4)	4(7.5)	9(16.9)	10(18.9)	31(58.5)
Female	45	41(91.1)	8(19.5)	5(12.2)	3(7.3)	24(58.5)
Age (year)						
8	23	22(95.6)	4(18.2)	4(18.2)	4(18.2)	11(50.0)
9	12	10(83.3)	2(20.0)	1(10.0)	3(30.0)	5(50.0)
10	21	20(95.2)	1(5.0)	3(15.0)	2(10.0)	14(70.0)
11	44	42(95.4)	5(11.9)	6(14.3)	4(9.5)	25(59.5)
Educational background of parents						
Illiterate	53	52(98.1)	6(11.5)	9(17.3)	3(5.8)	32(61.5)
Educated	47	42(89.4)	6(14.3)	5(11.9)	10(23.8)	23(54.8)
Total	**100**	**94(94.0)**	**12(12.8)**	**14(14.9)**	**13(13.8)**	**55(58.5)**

4. DISCUSSION

This study demonstrates clearly that *Staphylococcus aureus* and *Candida albicans* are part of the normal oral flora in most humans. From the results, it is clear that out of the 100 samples examined, 65 samples contained yeast –like organisms. The various tests carried out confirmed and to differentiate the species of *Candida*. It was realised that majority of these yeast – like organisms were *Candida albican*s. This result agrees with that reported by Rosenthal and Blecham (1962) and Budtz- Jorgensen et al. (1975).

The role of *S. aureus* in some types of oral disease may be more important than previously recognized (Smith et al., 2003). Some oral infections are caused at least in part by *S. aureus*, for example, angular cheilitis (MacFarlane and Helnarska, 1976; Smith et al., 2003), parotitis (Goldberg, 1981) and staphylococcal mucositis (Bagg et al., 1995). Furthermore there is now a growing body of evidence to suggest that staphylococci can be frequently isolated from the oral cavity of particular patient groups such as children (Miyake et al., 1991), the elderly (Bagg et

al., 1995) and some groups with systemic disease, such as the terminally ill (Jobbins et al., 1992), rheumatoid arthritis (Jacobson et al., 1997) and patients with haematological malignancies (Jackson et al., 2000).

This study highlights the potential role of *S. aureus* in a number of oral diseases. However, it is difficult from this study to ascribe a pathogenic role to the *S. aureus* isolates, which may have been colonizing rather than infecting the oral cavity. *S. aureus* infection is commonly associated with oral diseases and the findings of this study have confirmed those of earlier workers, suggesting a *S. aureus* isolation rate of 71.8% from oral cavity of primary school pupils.

There was no particular trend to increased recovery of *S. aureus* isolates from younger or older children in agreement with previous work (Percival et al., 1991) which found no age related trend for the recovery of *S. aureus* from a healthy population. This finding is in contrast to that of Smith et al. (2003). It is unclear whether this reflects changes in the oral flora associated with increasing age, medication, increased incidence of prosthetic oral devices or referral patterns (Smith et al., 2003). The percentage of isolation of *Staphylococcus aureus* revealed that the pupils that use charcoal and chewing stick, charcoal and toothbrush, and infrequent tooth brushing had the frequency percentage of 100.0%. Those that use Toothbrush and Chewing stick had frequency percentage of 83.0%, while pupils that use Chewing stick had frequency percentage of 80.0% and pupils that use Toothbrush had the least percentage (34.0%).

This study showed that the isolation of *Staphylococcus aureus* was most predominant (71.8%), followed by *Streptococcus* spp. (27.0%) and isolation of *S. aureus & Streptococcus spp.* coinfection was least predominant (1.2%) from oral swab of pupils using different methods of oral hygiene. In a study of 110 patients attending a dental hospital with a range of oral diseases there was an observed prevalence of *S. aureus* in saliva of 21.0% and from gingival swabs of 11.0% (Kondell et al., 1984; Smith et al., 2003). Salivary carriage of *S. aureus* in a cohort of patients with reduced salivary flow rates attending an oral medicine clinic was found in 41.0% of patients (Samaranayake et al., 1986; Smith et al., 2003).

Isolates of *S. aureus* are capable of producing a wide range of exotoxins which has been noted in oral isolates. A study of staphylococcal carriage in children attending a paedodontic department found that 19.0% of the *S. aureus* isolates produced exfoilotive toxin and 40.0% produced enterotoxin (Miyake et al., 1991; Smith et al., 2003). In line with more recent surveys (Smith et al., 2001, 2003), this study suggests that *S. aureus* may be a more frequent isolate from the oral cavity than hitherto suspected.

During the past two decades, there has been a significant increase in the prevalence of fungal infections caused by *Candida* species (Nejad et al., 2011). Oral candidiasis is a common opportunistic infection of the oral cavity caused by yeast fungi of the genus Candida on the mucous membranes of the mouth (Nejad et al., 2011). The study showed that isolation of *Candida albicans* was most predominant 40(61.5%) among pupils using different methods of oral hygiene. In agreement with findings of others (Back-Brito et al., 2009; Nejad et al., 2011), the majority of yeast isolates from oral cavity swabs were *C. albicans* (61.5%), but it was often recovered in association with other yeasts. This was followed by *C. krusei* 6(9.2%), *C. guilliermondii* 5(7.7%), *C. parapsilosis* 4(6.2%), *C. tropicalis* 4(6.2%), *C. stellatoidea* 3(4.6%) and *C. pseudotropicalis* 3(4.6%). These values for *Candida* species is comparable to what was reported by Donbraye-Emmanuel et al. (2010), Alli et al. (2011), and Nejad et al. (2011). *Candida* species that cause vaginitis most often are C. *albicans,* C. *g/abrata* and C. *tropicalis*. *Candida* species that rarely causes infection includes C. *parapsilosis,* C. *pseudotropicalis,* C. *krusei,* C. *guilliermondi* and C. *stellatoidea* (Alli et al., 2011).

In agreement with findings of others (Nejad et al., 2011), the most common mixtures observed in the present study were *C. albicans* plus *C. krusei* or *C. albicans* plus *C. tropicalis*. Although, *Candida* species been less common than bacterial infections, serious fungal infections occur in the immunocompromised patient both as new infection and as reactivation of latent disease (Donbraye-Emmanuel et al., 2010; Alli et al., 2011). The percentage reported for *Candida albicans* (61.5%) in this study is lower than the 75.0% reported by Nejad et al. (2011).

The result also showed that out of the 100 samples examined, *Staphylococcus aureus* was isolated from 61 samples. Both *Staphylococcus aureus and Candida albicans* were isolated from 30 samples. The comparison of samples made revealed that the percentage isolate of *Staphylococcus aureus and Candida albicans* is higher in the pupils that use charcoal and chewing stick for their oral hygiene. In the pupils that use charcoal and chewing stick for their oral hygiene had a

frequency percentage of 100.0%. The pupils that practice infrequent tooth brushing had frequency percentage of 75.0%. The pupils that use chewing stick had frequency percentage of 67.0%, the pupils that use tooth brush and chewing stick had frequency percentage of 42.0% while the pupils that use tooth brush only as their source of oral hygiene had frequency percentage of 4.0% and the pupils that use charcoal and Tooth brush had frequency percentage of 0.0%.

The percentage of isolation of *Candida albicans* revealed pupils that use charcoal and chewing stick had the highest frequency percentage (100.0%). The pupils that practice infrequent tooth brushing had a frequency percentage of 75.0%. Those that use Chewing stick had frequency percentage of 73.0%. Those that use Tooth brush and chewing stick had frequency percentage of 48%. While those that use Tooth brush had 19.0% and pupils that use Toothbrush and Charcoal had 0.0%.

From the percentage isolations of *Candida albicans*, the pupils that use charcoal and chewing stick as their method of oral hygiene had the highest frequency percentage (100.0%) in all the isolations, followed by pupils that practice infrequent tooth brushing. The pupils that use toothbrush had the least frequency percentage of organisms in all isolations. This might have been because toothbrushes are used with toothpastes. Toothpastes are a pastes or gel dentifrices used with a tooth brushes as accessories to clean and maintain the aesthetics and health of the teeth. They are also used to promote oral hygiene. There were no growths seen in a few Nutrient and Sabouraud's agar plates. This may be as a result of the absence these organisms as part of the normal oral flora of some of the pupils.

The ages of the subjects used in this study ranged from 8 to 11 years. This conforms to the findings of previous studies. Konje et al. (1991) showed that the infections were almost uniformly distributed in all age groups studied. In this study, 55.0% of the subjects were males while 45.0% were females. Isolation of *Candida* species were higher among females (19.5%) than the males (7.5%), however, there was no association with any of the demographic characteristics studied. Klufio et al. (1995) also reported that infections by *C. albicans* had no association with any of the sociodemographic characteristics studied. According to Adad et al. (2001), infection by *Candida sp* were most frequent among younger patients, especially those ages under 20 years, in all decades. Alli et al. (2011) also reported that infections by *C. albicans* had no association with any of the sociodemographic characteristics studied.

Toothpastes are derived from a variety of components, including three main ones: abrasives, fluoride and detergents or surfactants. Abrasives constitute at least 50.0% of typical toothpaste. These insoluble particles help remove plaque from the teeth. The removal of plaque and calculus prevents cavities and periodontal disease. Representative abrasives include Aluminium hydroxide ($Al(OH)_3$,Calcium carbonate ($CaCO_3$) etc. Fluoride in various forms is the most popular active ingredient in toothpaste to prevent cavities. It has beneficial effects on the formation of dental enamel and bones. Sodium fluoride (NaF) is the most common source of fluoride but Stannous fluoride (SnF_2), Olaflur (an organic salt of Fluoride), and Sodium monofluorophosphate (Na_3PO_3F) are also used. Many, although not all, toothpastes contain Sodium Lauryl sulphate (SLS) or related surfactants (detergents).SLS is mainly a foaming agent, which enables uniform distribution of toothpaste, improving its cleansing power other components include antibacterial agents which prevent gingivitis (Wolfgang, 2005). Other components of toothpaste include antibacterial agents which prevent gingivitis.

Candida albicans and *Staphylococcus aureus* had been isolated from several clinical specimens from different part of Nigeria (Donbraye-Emmanuel et al., 2010) and different parts of the world (Smith et al., 2003; Nejad et al., 2011). The differences in the frequency of isolation of these organisms in our study and that reported by other workers could be due to geographic, ethnic, and socioeconomic factors, as well as differences in sampling and culturing techniques. Variations may also reflect differences in sexual practice and environmental factors such as hygiene and nutrition (Donbraye-Emmanuel et al., 2010; Alli et al., 2011).

5. CONCLUSION

The result of this study shows that the method of oral hygiene affects the percentage isolation of *Staphylococcus aureus* and *Candida albicans*. The highest percentage of isolation was recorded from pupils that use charcoal and chewing stick as their method of oral hygiene, which is a poor method of oral hygiene. The least percentage of isolation was recorded from pupils that use tooth brush only as their method of oral hygiene which is a good method of oral hygiene. This study suggests that oral carriage of *S. aureus* may be more common

than previously recognized and the data collected suggests a reappraisal of the role of *S. aureus* in the health and disease of the oral cavity. The use of Toothbrush is the best method of oral hygiene, as confirmed by this study. Parents both illiterate and educated should therefore provide their children, with toothbrush and toothpaste and also teach them how to brush their teeth. This is because most of the cleaning is achieved by the mechanical action of the toothbrush, and not by the paste. Quality tooth paste should be used and also brushing regularly is highly recommended.

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On mammograms and CAD for breast cancer

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Abstract: Breast cancer is the most common type of cancer in women with approximately one in nine women developing the disease in her life time. Breast cancer is a disease in which cancerous cells are found in the tissues of the breast. These cancerous cells divide and grow continuously and eventually form a lump known as tumor. Although breast is the leading causes of cancer deaths in women, the causes of breast cancer are unknown. However, heredity does play a vital role in the development of breast cancer. The DNA in your cells carry the genetic information that you receive from your parents. Mammography is a specific type of imaging that uses a low dose x-ray for examination of the breast. The images can be viewed on a film at a view box. Most experts agree that successful treatment of breast cancer often is linked to early diagnosis. Mammography plays a major part in early detection of breast cancer, because it can show changes in the breast up to two years before a patient or a physician can feel them. In this work various aspects of mammography and computer aided detections have been analyzed.
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Keywords: Breast; cancer; women; disease; life; time; cell; tissue; vital role; genetic information; x-ray; examination; diagnosis; patient; physician; mammography

Introduction

Numerous fields, which range from military to commercial applications, are in need of paced and efficacious analysis of multimedia data. In the area of multimedia data, images get the place of a fortress. A plenty of knowledge can be hidden in image data and the knowledge discovery in image databases is known as image mining [3]. The tasks of image mining are mostly concerned with classification problems such as "labeling" regions of an image based on presence or absence of some characteristic patterns, and with image retrieval problems where "similar" images are identified [1]. Image mining is more than just an extension of data mining to the image domain [2]. Applications of the technology include, beyond remote sensing, geographic information systems, medical imaging, geomarketing, navigation, traffic control, environmental studies, and many other areas where spatial data are used [4].

Besides the other applications, medical imaging plays a significant role in the human's life. In medical imaging, abnormality detection in images is predicted to play an important role in many real-life applications [5]. In fact, the New England Journal of Medicine regards medical imaging as one of the most important medical developments of the past thousand years, basically du e to the fact that it provides physicians with physiology and functionality of organs and cells inside human bodies. The medical image is utilized to detect the abnormalities of tissues; the one among them is the breast cancer tissue which is the major death cause of women. Among different imaging modalities used for breast cancer detection, mammography remains the key screening tool for the detection of breast abnormalities [6]. Breast cancer is considered as one of the primary causes of women mortality [7] [10]. The mortality rate in asymptotic women can be brought down with the aid of premature diagnosis. Despite the increasing number of cancers being diagnosed, the death rate has been reduced remarkably in the past decade due to the screening programs [8].

Premature detection of breast cancer increases the prospect of survival whereas delayed diagnosis frequently confronts the patient to an unrecoverable stage and results in death [9]. The most effective way to reduce breast cancer deaths is detect it earlier. However, earlier treatment requires the ability to detect breast cancer in early stages. Early diagnosis requires an accurate and reliable diagnosis procedure that allows physicians to distinguish benign breast tumors from malignant ones [11]. Mammography is the most contemporary option for the premature detection of breast cancer in women [6].

Mammography [13], [14] is the single most effective, reliable, low cost and highly sensitive method for early detection of breast cancerous. Mammography offers high quality images at low radiation doses and is the only widely accepted imaging metho d for routine breast cancer screening. It is reco mmended that women at the ages of 40 or above should have a mammogram

every one to two years. [12]. Detecting breast cancer in mammograms is challenging because the cancerous structures have many features in common with normal breast tissue [15].

Review on Related Researches

Some of the recent research works are briefly reviewed here. **Muthu Rama Krishnan et al.** [16] have aimed at designing a support vector machine (SVM)-based classifier for breast cancer detection with higher degree of accuracy. It has been introduced a best possible training scheme of the features ex tracted from the mammogram, by first selecting the kernel function and then choosing a suitable training-test partition. Prior to classification, detailed statistical analysis viz., test of significance, density estimation have been performed for identifying discriminating power of the features in between malign ant and benign classes. A comparative stud y has been performed in respect to diagnostic measures viz., confusion matrix, sensitivity and specificity. They have considered two data sets from UCI machine learning database having nine and ten dimensional feature spaces for classification. In addition, the ov erall classification accur acy obtained by using the proposed classification strategy is 99.385% f or dataset-I and 93.726% for dataset-II,respectively.

Subashini et al. [17] have compared the use of polynomial kernel of SVM and RBFNN in ascertaining the diagnostic accuracy of cytological data obtained from the Wisconsin breast cancer database. The data set has been included nine different attributes and two categories of tumors namely benign and malignant. Known sets of cytologically proven tumor data was used to train the models to categorize cancer patients according to their diagnosis. Performan ce measures such as accuracy, specificity, sensitivity, F-score and other metrics used in medical diagnosis such as Youden's index and discriminant power were evaluated to convey and compare the qualities of the classifiers. Their research has demonstrated that RBFNN outperformed the polynomial kernel of SVM for correctly classifying the tumors.

Jihene Malek e al. [18] have proposed a design of automated detection, segmentation, and classification of breast cancer nuclei using a fuzzy logic. The first step was based on segmentation using an active contour for cell tracking and isolating of the nucleus in the cytological image. Some of the textural features have been extracted from this nucleus, using the wavelet transforms to characterize image using its texture, so that malign texture could be differentiated from benign on e with the assumption that tumoral texture was different from the texture of other kinds of tissues. Finally, the obtained features will be introduced as the input vector of a fuzzy C -means (FCM) clustering algorithm to classify the images into malign and benign ones. The implementation of such algorithm has been done using a methodology based on very high speed integrated circuit, hardware description language (VHDL). The design of the circuit has been performed b y using a CMOS 0.35 μm technology.

Mehmet Faith Akay [19] has been proposed breast cancer diagnosis based on a SVM-based method combined with feature selection. Experiments have been conducted on different training-test partitions of the Wisconsin breast cancer dataset (WBCD), which has been commonly used among researchers who used machine learning methods for breast cancer diagnosis. The performance of the method has been evaluated using classification accuracy, sensitivity, specificity, positive and negative predictive values, receiver operating characteristic (ROC) curves and confusion matrix. The results have been showed that the highest classification accuracy (99.51%) has been obtained for the SVM model that contains five features, and that has been very promising when compared to the previously reported results.

Lukasz Jelen et al. [20] have presented a framework for automatic malignancy grading of fine needle aspiration biopsy tissue. The malignan cy grade was one of the most important factors taken into consideration during the prediction of cancer behavior after the treatment. Their framework was based on a classification using Support Vector Machines (SVM). The SVMs were able to assign a malignancy grade based on pre-extracted features with the accuracy up to 94.24%. They have also showed that the SVMs performed best out of four tested classifiers.

Inspiration of the Research

Recently, the image processing is an upcoming research field for the researchers. The image mining is used to extract the images from the large image database and it has been utilized in many fields. Apart from other applications, the medical image mining plays a vital role in human's life, where the abnormality has been detected. The medical image mining is utilized for the detection of a plenty of diseases. The breast cancer is one of the diseases, which has been detected with the aid of medical image mining. The breast cancer is the primary cau se for the women mortality.

The earlier detection of the breast can cer may diminish the mortality rate. For the earlier detection of breast cancer, the mammography is used. Mammography is supposed to minimize mortality from breast cancer. Early detection of breast cancer is the main objective of mammography and in general this is achieved through detection of characteristic masses and/or microcalcifications. Yet, detecting breast cancer in mammograms seems to be on demand

since the cancerous structures posses numerous characteristics in common with normal breast tissue. The deadly nature of the breast cancer and the difficulties in detecting the cancer in mammograms necessitates an efficient technique for the detection of breast cancer cells. This has motivated to do the research wo rk in identifying an efficient technique.

The Anticipated Solution

The primary intent of my work is to detect the anomaly of the breast cancer tissue. To accomplish this, the digital mammography will be segmented using contour-based segmentation and the segmented mammography will be subjected under clustering so as to identify the cancer affected breast tissue locations. The cancer cells will be classified whether the cancer is benign or malign ant. To accomplish this, an extensive feature set will be extracted from the clustered breast cancer cells. The extensive feature set is comprised of shape, texture and gray intensity features. The shape features plays major role in classification of the cancer to benign or malignant. Owing to its foremost role in the identification and for the further differentiation of lesion throughout the diagnosis process, the texture feature shall thus be extracted. The ex tracted features will be used to train the Support Vector Machine (SVM). The SVM is chosen because of its capability of learning with very little samples. The well-trained SVM can effectively detect and classify the breast cancer, when a mammography is given. This paves the way for effective clinical diagnosis of the breast cancer.

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On mammograms and current technical classifications

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Abstract: Breast cancer has become a common health problem in developed and developing countries during the last decades and also the leading cause of mortality in women each year. Mammogram is a special x-ray examination of the breast made with specific x-ray equipment that can often find tumors too small to be felt. In this paper, the classification of microcalcification in digital mammogram is achieved by using Stochastic Neighbor Embedding (SNE) for reducing high dimensionality data into relatively low dimensional data and K-Nearest Neighbor (KNN) Classifier. This system classifies the mammogram images into normal or abnormal, and the abnormal severity into benign or malignant. Mammography Image Analysis society (MIAS) database is used to evaluate the proposed system. The experiments demonstrate that the proposed method can provide better classification rate.
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Key words: Stochastic Neighbor Embedding, K-Nearest Neighbor, Digital mammograms, microcalcifications

1. Introduction

Today breast cancer is the most frequent form of cancer in women above 40. The World Health Organization's International Agency for Research on Cancer estimates that more than 150,000 women worldwide die of breast cancer each year. A computer-aided diagnosis (CAD) system for the automatic detection of clustered microcalcifications in digitized mammograms is presented in [1]. It consists of two main steps. First, potential microcalcification pixels in the mammograms are segmented out by using mixed features consisting of wavelet features and gray level statistical features, and labeled into potential individual microcalcification objects by their spatial connectivity. Second, individual microcalcifications are detected by using a set of 31 features extracted from the potential individual microcalcification objects.

A computerized scheme for detecting early-stage microcalcification clusters in mammograms is proposed [2]. It developed a novel filter bank based on the concept of the Hessian matrix for classifying nodular structures and linear structures. The mammogram images were decomposed into several sub images for second difference at scales from 1 to 4 by this filter bank. The sub images for the nodular component (NC) and the sub images for the nodular and linear component (NLC) were then obtained from analysis of the Hessian matrix.

A computer aided decision support system for an automated diagnosis and classification of breast tumor using mammogram is presented in [3]. It differentiates two breast diseases namely benign masses and malignant tumors. From the preprocessed mammogram image, texture and shape features are extracted. The optimal features can be extracted by using a feature selection scheme based on the Multi Objectives Genetic Algorithm (MOGA).

A new method of feature extraction from Wavelet coefficients for classification of digital mammograms is proposed in [4]. A matrix is constructed by putting Wavelet coefficients of each image of a building set as a row vector. It consists then on selecting by threshold, the columns which will maximize the Euclidian distances between the different class representatives. The selected columns are then used as features for classification. A novel methodology for the classification of suspicious areas in digital mammograms is presented in [5]. It is based on the fusion of clustered sub classes with various intelligent classifiers. A number of classifiers have been incorporated into the methodology and evaluated on the well known benchmark digital database of screening mammography (DDSM).

Detecting the abnormalities in mammogram by using local contrast thresholoding and rule based classification is presented in [6]. Classification of Microcalcification Using Dual-Tree Complex Wavelet Transform and Support Vector Machine is proposed in [7]. It consists of two phases. At the offline phase, training for the SVM is conducted using some training data to find the support vectors. At the online phase, a mammogram to be classified inputted into the system and then classified by the SVM.

A novel opposition-based classifier has been developed [8] which classifies breast masses into

benign and malignant categories. An MLP network with a novel learning rule, called Opposite Weighted Back Propagation (OWBP), has been utilized as the classifier. The features include circularity, Zernike moments, contrast, average gray level, NRL derivatives and SP. It evaluated the classifier has been trained with both traditional BP and OWBP learning rules.

The fractal modeling of the mammographic images and their background morphology is presented in [9]. For fractal modeling, the original image is first segmented into appropriate fractal boxes followed by identifying the fractal dimension of each windowed section. Then used two dimensional box counting algorithm after which based on the order of the computations; they are placed in an appropriate matrix to facilitate the required computations. Finally using eight features identified as characteristic features of tumors extracted from mammogram images.

A novel semi-supervised k-means clustering is proposed for outlier detection in mammogram classification is proposed in [10]. The shape features are extracted from the digital mammograms, and k-means clustering is applied to cluster the features, the number of clusters is equal with the number of classes. A novel Genetic Association Rule Miner (GARM) is applied with this reduced feature set to construct the association rules for classification. The performance is analyzed with rough set using Receiver Operating Characteristic (ROC) curve analysis.

Texture analysis based on curvelet transform for the classification of mammogram tissues is presented in [11]. The most discriminative texture features of regions of interest are extracted. Then, a nearest neighbor classifier based on Euclidian distance is constructed. The obtained results calculated using 5-fold cross validation. The approach consists of two steps, detecting the abnormalities and then classifies the abnormalities into benign and malignant tumors.

A new classification approach using Support Vector Machines (SVM) for detection of microcalcification clusters in digital mammograms is proposed in [12]. Classifying data is a common task in machine learning. The MC (Microcalcification) detection is formulated as a supervised learning problem and apply SVM as a classifier to determine at each pixel location in the mammogram if the MC is present or not.

In this paper, an automatic classification of microcalcification in digital mammograms based on SNE and KNN classifier is presented. The remainder of this paper is organized as follows: The methodologies and proposed method used for the proposed system is described in sections 3 and 4. The experimental results are given in section 5.

2. Methodology

The proposed system for the classification of microcalcification in digital mammograms is built based on SNE and by applying KNN for building the classifiers. In this following section the theoretical background of all the approaches are introduced.

2.1 Stochastic Neighbor Embedding

SNE is a probabilistic approach to the task of placing objects, described by high-dimensional vectors or by pair-wise dissimilarities, in a low-dimensional space in a way that preserves neighbor identities. A Gaussian is centered on each object in the high-dimensional space and the densities under this Gaussian (or the given dissimilarities) are used to define a probability distribution over all the potential neighbors of the object. The aim of the embedding is to approximate this distribution as well as possible when the same operation is performed on the low-dimensional "images" of the objects. A natural cost function is a sum of Kullback-Leibler divergences, one per object, which leads to a simple gradient for adjusting the positions of the low-dimensional images.

For each object, i and each potential neighbor,j the asymmetric probability is calculated by the formula (1) that i would pick j as its neighbor is given by

$$pij = \frac{\exp\left(-d_{ij}^2\right)}{\sum_{k\neq i} exp(-d_{ik}^2)} \qquad (1)$$

The dissimilarities, d_{ij}^2 , may be given as part of the problem definition (and need not be symmetric), or they may be computed using the scaled squared Euclidean distance ("affinity") between two high-dimensional points,X_i; X_j

$$d_{ij}^2 = \frac{\left\|X_i - X_j\right\|^2}{2\sigma_i^2} \qquad (2)$$

where σ_i is either set by hand or found by a binary search for the value of σ_i that makes the entropy of the distribution over neighbors equal to $\log k$. Here, k is the effective number of local neighbors or "perplexity" and is chosen by hand. In the low-dimensional space, the Gaussian neighborhoods are used with a fixed variance so the *induced* probability qij that point i picks point j as its neighbor is a function of the low-dimensional *images* y_i of all the objects and is given by the expre

$$qij = \frac{exp\left(-\left\|y_i - y_j\right\|^2\right)}{\sum_{k \neq i} exp\left(-\left\|y_i - y_j\right\|^2\right)} \quad (3)$$

The aim of the embedding is to match these two distributions as well as possible. This is achieved by minimizing a cost function which is a sum of Kullback-Leibler divergences between the original (pij) and induced (qij) distributions over neighbors for each object is given by

$C = \sum_i \sum_j pij\ log \frac{pij}{qij} = \sum_i KL(P_i||Q_i)$ (4)

The minimization of the cost function in Equation 4 is performed using gradient method. The gradient has the simple form as

$$\frac{\partial C}{\partial Y_i} = 2\sum_j \left(y_i - y_j\right)\left(p_{ij} - q_{ij} + p_{ij} - q_{ij}\right) \quad (5)$$

The gradient descent is initialized by sampling map points randomly from an isotropic Gaussian with small variance that is center around the origin. For speed up the optimization and avoid been stuck in local optima, a momentum term is added to the gradient [4]. The current gradient is added to an exponentially decay sum of previous gradients in order to determine the changes in the coordinates of the map points at each iteration of gradient search. Mathematically, the gradient with a momentum term is given by [4]

$y^{(t)} = y^{(t-1)}\eta \frac{\partial J}{\partial y_i} + \alpha(t)\left(y^{(t-1)} - y^{(t-2)}\right)$ (6)

Where $y^{(t)}$ indicate the solution at iteration$t$, η indicates the learning rate, and $\alpha(t)$ represents the momentum at iteration t. In the early stages of the optimization, after the each iteration, a random jitter is added to the map points. Then gradually reducing the variance of this noise performs a type of simulated annealing that helps the optimization to escape local minima in the cost function.

2.2 K-NN Classifier

The k-nearest neighbor algorithm (K-NN) is a method for classifying objects based on closest training examples in the feature space. K-NN is a type of instance-based learning where the function is only approximated locally and all computation is deferred until classification. In K-NN, an object is classified by a majority vote of its neighbors, with the object being assigned to the class most common amongst its k nearest neighbors (k is a positive integer, typically small). If k = 1, then the object is simply assigned to the class of its nearest neighbor. The neighbors are taken from a set of objects for which the correct classification is known. This can be thought of as the training set for the algorithm, though no explicit training step is required.

3. Proposed Method

The proposed system for the classification of microcalcification in digital mammograms mainly consists of two different stages which include the feature extraction stage and classification stage. All the stages are explained in detail in the following sub sections.

3.1 Feature Extraction Stage

Feature extraction involves simplifying the amount of resources required to describe a large set of data accurately. Analysis with a large number of variables generally requires a large amount of memory and computation power or a classification algorithm which over fits the training sample and generalizes poorly to new samples. Feature extraction is a general term for methods of constructing combinations of the variables to get around these problems while still describing the data with sufficient accuracy. Figure 1 shows the block diagram of feature extraction stage of the proposed system based on SNE.

The well known microcalcification area in the MIAS mammogram images are given to the feature extraction stage. The known microcalcification area which was given by the MIAS database is separated from the whole image. The size of the extracted ROI is 256 x 256. This high dimensional data is reduced into a relatively low dimensional data by using SNE and this reduced data set is stored in the database as feature. Database-I is constructed by using the training images of normal and abnormal images and used in the initial stage classifier. Database-II is constructed by using the training images of benign and malignant images and used in the final stage classifier.

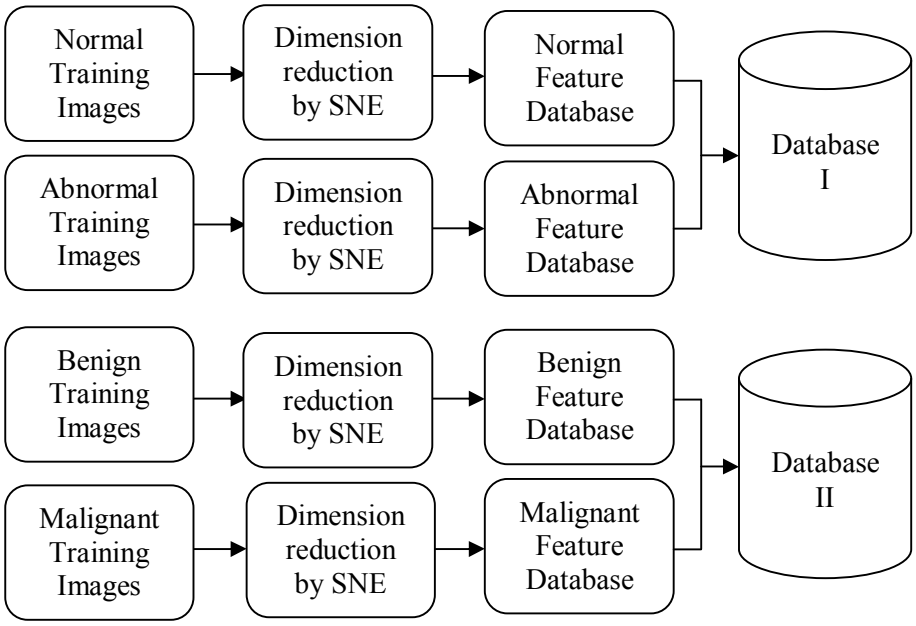

Fig 1 Block diagram of the feature extraction stage of the proposed system

3.2 Classification Stage

Classification phase executes two phases. In the first one, the classifier is applied to classify mammograms into normal and abnormal cases. Then the mammogram is considered abnormal if it contains tumor (microcalcification).

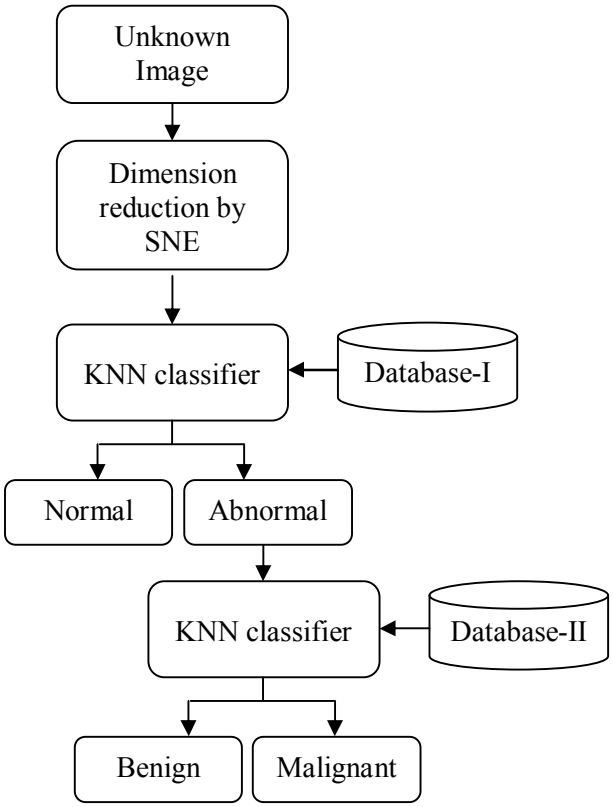

Fig 2 Block diagram of the classification stage of the proposed system

Finally, the abnormal mammogram is classified into malignant or benign in the final stage. In this classification stage, KNN classifier in every phase is trained at specific number of training set in each category. The block diagram of the classification stage of the proposed system based on KNN classifier is shown in Figure 2.

3.2.1 Initial Stage Classifier

In the initial stage classifier, the given unknown ROI from the digital mammogram image is tested for normal or abnormal category. The given high dimensional unknown ROI image is reduced into a relatively low dimensional dataset by using the SNE. This reduced dataset is initially tested with the trained KNN classifier which uses DATABASE-I. Table 1 shows the number of training and testing images used for the initial stage classifier.

Table 1: Number of training set and testing set for initial stage classifier

Type of image	No of training Images	No of Testing Images
Normal	66	99
Abnormal	17	25

3.2.2 Final Stage Classifier

In the final stage classifier, the abnormal ROI image from the initial stage classifier is further classified into Benign or Malignant. The reduced dataset of unknown ROI image is again tested with the trained KNN classifier which uses DATABASE-II. Table 2 shows the number of training and testing images used for the final stage classifier.

Table 2: Number of training set and testing set for final stage classifier

Type of image	No of training set	No of Testing set
Benign	8	12
Malignant	9	13

4. Experimental Results

To assess the performance of the proposed system, many computer simulations and experiments with MIAS database images were performed. The performance of the proposed system is carried on 99 normal images and 25 microcalcification images. Among the 25 abnormal images, there are 12 benign and 13 malignant images available. All the images are considered for the classification test. The classification rate obtained using the SNE data sets are show in Table 3. From the table 3, it is clearly found that all the normal images and malignant images are classified with no error while the abnormal and benign category, over 80 % classification result is achieved.

Table 3: Classification results of proposed method based on SNE

Mammogram Type	Classification Rate (%)
Normal	100
Abnormal	84
Benign	83.33
Malignant	100

5. Conclusion

In this paper, the classification of microcalcification in digital mammogram based on SNE and KNN classifier is proposed. The high dimensional data from the ROI image is relatively reduced into low dimensional data set by using the SNE and the reduced data set is used as features to classify the given mammogram images into normal or abnormal as well as benign or malignant. The proposed classification scheme is carried on MIAS database image.

Experimental results show that the proposed system achieves 100% classification rate for normal and malignant cases and over 80% classification rate for benign and abnormal cases. Still, the work is going on to get the better result for abnormal and benign cases.

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An easy new technique for computer aided detection (CAD)mammograms

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Abstract: Mammograms screenings, mammogram diagnosis, computer aided detection (CAD) technique for mammography, Magnetic resonance image (MRI) and several other algorithms are currently practiced by doctors, surgeons and radiologists to test breast cancer. All these mode of breast examinations have many drawbacks. The main problem with these methods is that the tests do not give us the real picture of the breast cancer. Even highly qualified and well experienced experts in this field miserably fail in the analysis of breast cancer. Consequently this leads to the death of the breast cancer patients. To over come all these burning issues a new physiotherapy practice have been proposed in this work.
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Key Words: Mammography, Cancer, CAD, MRI, physiotherapy.

1. On Mammograms

Cancer is an abnormal growth of cells which tend to proliferate in an uncontrolled way and, in some cases, to metastasize (spread). Cancer is not one disease. It is a group of more than 100 different and distinctive diseases. Cancer can involve any tissue of the body and have many different forms in each body area. Most cancers are named for the type of cell or organ in which they start. If a cancer spreads (metastasizes), the new tumor bears the same name as the original (primary) tumor. Cancer is the Latin word for crab. The ancients used the word to mean a malignancy, doubtless because of the crab-like tenacity a malignant tumor sometimes seems to show in grasping the tissues it invades. Cancer may also be called malignancy, a malignant tumor, or a neoplasm, literally, a new growth.

A mammogram is a safe test used to look for any problems with a woman's breasts. The test uses a special, low-dose x-ray machine to take pictures of both breasts. The results are recorded on x-ray film or directly onto a computer for a radiologist to examine.

Mammograms allow the doctor to have a closer look for breast lumps and changes in breast tissue. They can show small lumps or growths that a doctor or woman may not be able to feel when doing a clinical breast exam. "Mammography" is the best screening tool that doctors have for finding breast cancer.

If a lump is found, your doctor may order other tests, such as ultrasound or a biopsy--a test where a small amount of tissue is taken from the lump and area around the lump. The tissue is sent to a lab to look for cancer or changes that may mean cancer is likely to develop. Breast lumps or growths can be benign (not cancer) or malignant (cancer). Finding breast cancer early means that a woman has a better chance of surviving the disease. There are also more choices for treatment when breast cancer is found early.

Screening mammograms are done for women who have no symptoms of breast cancer. When you reach age 40, you should have a mammogram every one to two years.

Diagnostic mammograms are done when a woman has symptoms of breast cancer or a breast lump. This mammogram takes longer than screening mammograms because more pictures of the breast are taken.

Digital mammograms take an electronic image of the breast and store it directly in a computer. Current research has not shown that digital images are better at finding cancer than x-ray film images.

2. Drawbacks of mammograms

- Breast screening cannot prevent cancer
- Having a mammogram is uncomfortable
- Having a mammogram involves x-rays
- False-positive results may cause unnecessary worry
- Mammograms sometimes need to be repeated
- Breast screening occasionally misses a cancer
- Cancer may occur even in women having screening
- Can diagnose a cancer which never needed treating

3. Breast MRI (Magnetic Resonance Image)

MRI (magnetic resonance imaging) allows doctors to see inside the body without cutting anything open. MRI uses a large magnet and radio waves to look at organs and structures inside the body. It does not use radiation like an x-ray. An MRI can help doctors diagnose many types of medical conditions,

especially problems with the brain and spinal cord, the heart, and other organs deep inside the body. It is particularly effective at distinguishing the body's soft tissues.

3.1 Breast MRI advances

- More sensitive than mammograms, ultrasounds, and clinical breast exams
- Useful for women at high risk for breast cancer
- Finds invasive breast cancer well
- Excellent at imaging around breast implants
- Accurately images implant ruptures and leaked material
- No compression of breast tissue
- Effectively images dense breast tissue
- Helps evaluate inverted nipples
- Finds primary tumor if cancer has spread to lymph nodes in armpit
- Detects any remaining cancer after lumpectomy
- Helps determine whether lumpectomy or mastectomy will be best treatment
- Images both breasts simultaneously (useful for symmetrical comparison)

3.2 Breast MRI disadvantages:

- Not good at detecting DCIS
- Leads to many false-positive findings
- Additional follow-up examinations and biopsies
- May not show all calcifications
- May cause claustrophobia
- Requires use of injected contrast agent (Gadolinium)
- More expensive than mammogram ($100 vs $1000)
- Not widely available
- Slower than mammogram (30 – 60 minutes)

4. Breath technique for accurate computer aided detection of breast cancer

Dense breasts have less fatty tissue and more non-fatty tissue compared to breasts that aren't dense. One way to measure breast density is the thickness of tissue on a mammogram. Another categorizes breast patterns into four types depending on which type of tissue makes up most of the breast. Still, no one method of measuring breast density has been agreed upon by doctors. Breast density is not based on how your breasts feel during your self-exam or your doctor's physical exam. Dense breasts have more gland tissue that makes and drains milk and supportive tissue (also called stroma) that surrounds the gland. Breast density can be inherited, so if your mother has dense breasts, it's likely you will, too.

Research has shown that dense breasts can be 6 times more likely to develop cancer. Dense breast scan make it harder for mammograms to detect breast cancer; breast cancers (which look white like breast gland tissue) are easier to see on a mammogram when they're surrounded by fatty tissue (which looks dark).

The term physiotherapy has been added to medical dictionary only recently. The allopathic medical studies and research have no traditional or heritage values. Its history is only 100 to 150 years. But on the other hand, the Ayurveda, unani, siddha and herbal means of treatments began several thousands of years ago. The first ancient medical treatment is the application of herbals. The ayurveda and siddha have more than 10000 years history. This is followed by the unani branch. Yoga was firstly introduced by both ayurveda and siddha school of thoughts. Initially the allopathic did not admit the inclusion of yoga science. It cleverly renamed the yoga as physiotherapy. Now a days, each and every allopathic doctor on earth prescribe physiotherapy along with drugs. Particularly for ortho patients, physiotherapy is strongly recommended.And knowingly or unknowingly, many current examinations such as to take blood, to test the functioning of the heart, the optical test of eyes and all other intestines examinations involve physiotherapy.

Sennimalai Kalimuthu, research scientist in mathematics & yoga has come across a yoga posture for clear detection of breast cancer in women. He has not revealed this technique to anyone yet.If one wants more details, Kalimuthu can be caught at math.kalimuthu@gmail.com.

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Cancer Stem Cells and Differentiation Therapy: An Innovative Therapeutic Approach

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Abstract: Cancer stem cells are closely related to and may often originate with adult stem cells. Under normal circumstances, the regular turnover of cells in developed tissues is offset by the work of adult stem cells, which can divide to make more stem cells or progenitor (immature) cells. The progenitors then differentiate into the mature cells needed to maintain the organ or to respond to an injury, hormones, or other external signals. Cancer stem cells can produce more of their kind or progenitors that multiply and differentiate to become the malignant cells that make up the bulk of a cancer. Nowadays it is reported that, similarly to other solid tumors, colorectal cancer is sustained by a rare subset of cancer stem–like cells (CSCs) which survive conventional anticancer treatments, thanks to efficient mechanisms allowing escape from apoptosis, triggering tumor recurrence. To improve patient outcomes, conventional anticancer therapies have to be replaced with specific approaches targeting CSCs. In this review we provide strong support that BMP4 is an innovative therapeutic approach to prevent colon cancer growth increasing differentiation markers expression and apoptosis.

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Keywords: cancer stem cells; apoptosis; BMP4.

1. INTRODUCTION

Stem cells are defined as undifferentiated cells that are capable of self-renewing and differentiating into a large number of diverse mature progeny. Amongst the various categories of stem cells, the embryonic stem (ES) cells are totipotent and able to differentiate into many cell types under appropriate conditions in vitro and contribute to all different tissues in vivo (1–3), making them a very promising foundation for stem cell-based therapeutics. Somatic stem cells from different organs, on the other hand, are pluripotent and responsible for tissue regeneration and repair. Adult stem cells have been identified in several organs, such as the hematopoietic system, brain, skin, mammary gland and lung, but it is not yet clear whether they are present in all other adult organs (4, 5). The best-studied somatic stem cells are hematopoietic stem cells (HSC). HSCs in mice and humans have been positively identified and successfully isolated by Weissman and colleagues (5, 6). HSCs are known to be responsible for the generation of all cell types in the blood, although their potential for giving rise to other tissues (or plasticity) is still controversial (4, 5). Dick et al. have recently revealed that, like the normal hematopoietic system, leukemia is organized as a hierarchy in which only a rare population retains a clonogenic capacity upon transplantation (7). Similarly, a solid tumor can be likened to an organ developed in an aberrant way, as it contains a heterogeneous mixture of cell types and abnormal tissue structures. More importantly, such an aberrant organ can be maintained and even formed at remote sites if no therapeutic intervention is performed. It is well established that tumor engraftment, although requiring a large number of cells, results in the formation of secondary tumors that recapitulate primary ones. The clonogenic and heterogenic nature of tumors suggests that a rare cell population in cancer, which acts like stem cells, is responsible for tumor growth and metastasis. These rare cells are named cancer stem cells (CSC) after normal stem cells, as both have similar abilities to self-renew and to give rise to heterogeneous differentiated cell types (8). Recent advances have begun to disclose the biologic identity and origin of CSC in several types of cancers and to elucidate the mechanisms underlying the transformation of normal cells into CSC.

1.1 Cancer Stem Cells (CSCs) Hypothesis

During the past years the process of tumorigenesis was explained by cancer biologists through the stochastic model, according to which all tumor cells share common genetic and epigenetic mutations, reflective of their clonal origin (9). In addition to the genomic instability, intrinsic factors (levels of transcription factors, signaling pathways) and extrinsic ones (host factors, microenvironment, immune response) influence tumor cells behavior leading to significant heterogeneity in terms of features, surface markers expression, proliferation kinetics and tumor

initiation capacity (10). More recently, the hierarchy model has been proposed, according to which cancer consists of a heterogeneous population characterized by various stages of differentiation. Accumulating evidence has posited that tumor mass is characterized by the presence of a small population of cells, necessary and sufficient to initiate and sustain indefinitely tumor growth and subsequent progression. These "tumor-initiating cells" are also called cancer stem cells (CSCs) since they share the hallmarks of normal stem cells (e.g., unlimited self-renewal, quiescence, multipotentiality and expression of drug and apoptosis resistance genes), expand the stem cell compartment undergoing symmetric division and differentiate into the multiple lineage via asymmetric division (11) (Figure 1).

Dick and colleagues demonstrated that only a small minority of acute myeloid leukemia (AML) cells were able to produce leukemia in NOD/SCID murine model (11). From the initial studies in hematological malignancies, CSCs have been identified in a variety of solid tumors including breast, prostate, brain colon, pancreas, ovary, lung, and, recently in thyroid, as assayed either by their in vitro clonogenity or by their ability to initiate new tumor growth after xenotransplantation into immunocompromised mice which recapitulate the phenotypic heterogeneity of the primary tumor (12-19). The emergence of CSCs and subsequent cancer development may arise from deregulation of the processes that regulate self-renewal, cell fate and differentiation of normal stem or progenitor cells (20), but moreover CSCs may originate from mutations in differentiated cells favoring timeless proliferative potential (21). Several signaling pathways such as Wnt, Notch and Sonic Hedgehog (Shh) have been found to regulate the self-renewal of normal stem cells in a variety of cancers.The importance of a self-renewal pathway in maintaining Leukemia Stem cells (LSCs) has been first underlined by Jamieson group. Their results showed that an aberrant activation of Wnt pathway is implicated in human blast crisis LSCs propagation. They also identified an increased activation of Wnt signaling in breast CSCs growth. Shh signaling pathway is also known to play a critical role in maintaining human LSCs, breast, glioblastoma and colon CSCs. Finally, Notch pathway has been shown to be activated in colon CSCs subset but also in breast and glioblastoma CSCs (22).

1-2 Colorectal Cancer Stem Cells

Normal colonic stem cells (NCSCs) are localized at the base of the crypts surrounded by intestinal subepithelial myofibroblasts (ISEMFs). Defined by properties of self-renewal and multilineage differentiation, they ensure a high rate of tissue renewal: by asymmetric division NCSCs generate another SC and a progenitor cell also known as a transit-amplifying cell (TAC) which, in turn, generates more mature cells of colonic epithelium. It has been suggested that ISEMFs play a critical role in the regulation of a correct balance between SCs self-renewal and differentiation, by paracrine secretion of growth factors and cytokines (21, 23).

In addition to ISEMFs, maintenance of colonic epithelial SCs niche is modulated by high Wnt activity in the lower region of the crypt which induces the expression of EphB receptors and the subsequent interaction with ephrin ligands located in the higher position of the crypt (21, 24). Another signaling pathway identified as a key regulator of the SCs niche is that mediated by Bone Morphogenetic Proteins (BMPs). As a consequence of the high expression of BMP antagonists in the colon bottom, the BMP activity is higher in the upper region of the crypt inducing differentiation of colonic epithelial cells (21, 23). In 1990, Fearon and Volgestein suggested a genetic model for colorectal tumorigenesis in which gene mutations occurred with a specific time defining a particular stage of tumor development (25). In patients with familial adenomatous polyposis, mutations in the Adenomatous Polyposis Coli (APC) gene are reported as the initiating gatekeeper regulating positively Wnt machinery and causing hyperproliferation and early adenoma formation (26).

The stage of intermediate adenoma is promoted by B-RAF and K-RAS mutations. Late adenoma results from loss of heterozygosis involving the chromosome 18q, mutations in Small Mother against DPP homolog 4 (Smad4), Cell Division Cycle 4 (CDC4) and Delected in Colorectal Cancer (DCC) or alternatively mismatch repair deficiency. P53, Bax and insulin-like growth factor receptor2 mutations are responsible for invasive cancer; lastly, unknown factors lead to metastatic cancer (21, 27). Even in cancers caused by alterations in genomic integrity, neoplastic change might initiate through subsequent mutations in morphogenetic pathways regulating normal proliferation of intestinal epithelium, such as Akt/ PKB, Wnt, Shh, Notch and BMPs (26).

These multiple genetic mutations, restricted to TACs, would be acquired by their progeny resulting in increased proliferative potential, independence of extrinsic growth control signals and autonomous control over all metabolic activities that feed tumor progression (28). Although it has long been assumed that neoplastic formation derives from alterations within adult colonic stem cells, the existence of colorectal cancer stem cells (CR-CSCs) has been demonstrated through the finding that colon CD133+ cells are able to grow exponentially in vitro as undifferentiated tumor spheres, when cultivated in serum-free medium, and initiate tumor growth in

mouse models, thus reproducing the same morphological and antigenic pattern of the original human tumor (29-31).

Many studies have provided proof that, within the CD133+ subpopulation, there exists a minority of cells possessing tumor-initiating ability. Dalerba et al. (15) suggest cell surface glycoprotein CD44 and Epithelial Cell Adhesion Molecule (EpCAM) as specific markers of CR-CSCs: in the context of CD133+ tumor population, they have identified a subset of stem-like CD44+/EpCAMhigh cells able to generate tumor xenografts upon serial transplantation into NOD/SCID mice. A further isolation of colon cancer cells using the mesenchymal stem cell marker CD166 enhanced the success of tumor xenograft. A recent study performed by Huang et al. (32) showed that enzymatic activity of ALDH1 can be used as a potential CR-CSCs marker being expressed by cells positive for CD44+ or CD133+ located at the base of normal crypts. It has been reported by the same group that during tumor progression the selection of CD44+, CD133+ cells with ALDH activity increases in number and reaches the crypt axis.

2. Clinical Implications of CSCs

The discovery of CSCs in a variety of tumors has changed the view of carcinogenesis and therapeutic strategies. According to the stochastic model, the tumor chemoresistance is due to preexisting clones with mutations that confer drug-resistance. The CSCs model postulates that CSCs evade death signals induced by current therapeutic drugs through a variety of strategies including upregulation of multidrug-efflux pumps able to exclude exogenous substances, alterations in DNA-repair mechanisms, altered cell cycle checkpoint controls and impaired apoptosis machinery. In addition, CSCs survive to current treatments, evaluated for the ability to kill only more differentiated and highly proliferating cells, because CSCs are proliferatively quiescent, less differentiated and overcome apoptosis resistance evading the control mechanisms. A combination of 5-FU, oxaliplatin and leucovorin (referred to as FOLFOX) and a combination of 5-FU, oxaliplatin and irinotecan (referred to as FOLFIRI) are the current therapy for colon cancer patients. Actually, the therapeutic approach for CRC includes anti-VEGF or EGFR monoclonal antibodies which improve positive outcomes in patients suffering from metastatic colon cancer and severe hepatic dysfunction (21). However, none of these anticancer therapies is curative in most patients with metastatic disease due to failure to eradicate the CSCs compartment. The development of targeted therapies for this cancer type would therefore require a better knowledge of the different aspects of stem cell biology in the context of CRC such as complex network of mechanisms that regulate tumor development and resistance to chemotherapy. It is therefore evident that a therapeutic approach to selectively target CSC pool bypassing their chemoresistance could be more effective to eradicate bulk tumor. Thus, the purpose of new therapeutic regiments is to eliminate the self-renewal compartment of tumor mass by:

- Targeting stem cell properties inducing the inactivation of survival pathways in CSCs.
- Forcing CSCs to differentiate [1] (Figure 2).

3. BMPs: An Example of Differentiation Therapy

Considerin the role of Bone Morphogenetic proteins (BMPs) in development and differentiation stages, these molecules have been studied over the past decade in tumorigenesis and metastasis formation.

BMPs belong to a subgroup of the transforming growth factor-beta (TGF-β) super-family; so far 20 BMPs have been discovered (33). According to current models, BMPs bind two distinct serine/threonine kinase receptors; different combination of type I and type II receptors determine the specificity for the ligands. Upon ligand binding, the type II receptor trans-phosphorylates type I receptor in its GS domain; initiating the signal transduction by phosphorylating Smad1/5/8 proteins (RSmads). Then RSmads form a complex with Smad4 (CoSmad) and translocate into the nucleus, where this complex could bind directly to gene regulatory elements or interact with other transcription factors regulating target gene expression (34). In addition to the Smad pathway, BMPs activate an alternative pathway, which includes p38 and ERK MAP kinases (35). Moreover, BMPs activation is tightly regulatregulated by the presence or the absence of antagonists, such as Gremlin, Chordin and Noggin (36).

Originally these proteins have been studied and characterized for their chondrogenic and osteogenic abilities, as they are able to induce ectopically bone formation in rodents (37). Afterwards, BMPs were analyzed for their role in cell growth, differentiation and apoptosis.

It is well established that several BMPs have a function in multiple developmental processes. Studies in Drosophila melanogaster and Xenopus laevis established that BMPs are required for correct dorsal-ventral axis formation and mesoderm induction in embryos (38, 39). Since these data prove that BMPs pathway is essential for the development of embryos invertebrates, further studies were carried out on murine models to strengthen the hypothesis that BMPs are important during vertebrate embryogenesis. Many knockout mice were generated for BMPs, BMPs receptors and molecules involved in the signaling pathway. Most of these models (BMP2, BMP4, BMPR

I and II, Smad 4 and 5 KO mice) are lethal, as mutant embryos, exhibiting multiple gastrulation defects, among which include lack of mesoderm formation and incorrect left-right axis asymmetry, morphogenesis and organs positioning (38-43). A different phenotype was observed in BMP7 null mice: these mice present postnatal lethal mutations with various developmental skeleton-kidney and eye defects. Given that BMPs have a role in embryonic development; it was supposed that these proteins may play a role during SCs differentiation. Pera et al. (44) demonstrate that human embryonic SCs treatments with Noggin impair their spontaneous differentiation, suggesting that in these cells BMPs pathway activation induces differentiation.

In the colon crypts ISEMF cells contribute to stem cells niche maintenance balancing different and opposite signals that promote self-renewal (Wnt and Notch pathways) and differentiation (BMPs pathway). The understanding of these mechanisms is important because it is hypothesized that the existence of a CSCs niche may have a role in maintaining and increasing the CSCs pool (45). In CRCs, an abnormal activation of Wnt signaling pathway leads to nuclear β-catenin accumulation and subsequent abnormal CSCs proliferation; moreover, BMPs signaling inhibition promotes nuclear β-catenin activity through PTEN inactivation and activation of PI3K-Akt pathway (46). A subsequent microarray study identifies a list of genes ifferentially expressed in colon bottom crypts and in the tops: the first group includes genes involved in Wnt and Notch pathways, but also BMPs inhibitors, such as Gremlin 1, Gremlin2 and Chordin-like 1; the second genes involved in BMPs and apoptosis pathway and cell cycle inhibitors (23). These data suggest that in the colon bottom crypts a balance between Wnt/Notch and BMPs pathways is necessary in order to maintain and regulate CSCs niches.

More evidence for their putative role in CRC was provided by genetic studies and transgenic mice models. Germline mutations in genes encoding SMAD4, BMPRIA and BMP4 are found in up to 50% of individuals with juvenile polyposis, an autosomal dominant syndrome with a high risk for CRC (47-49).

Furthermore, Noggin transgenic mice phenocopy the intestinal histopathology of patients with this syndrome (50); subsequently it was described that mice with an inducible mutation of BMPRIa develop intestinal polyps (46). This body of data argues that BMPs signaling disruption leads to precancerous lesions (51).

CRC develops as a result of increasing proliferation and apoptosis deregulation and TGF-β signaling inactivation have a key role in this pathology (52). It has been reported that SMAD4 is frequently deleted in CRC and that BMPs pathway is inactivated in the majority of colorectal tumors (53,54). Indeed, BMP2, BMP3, BMP4 and BMP7 inhibit proliferation and induce apoptosis and differentiation in colon cancer cells that do not have Smad4 mutation and loss of PTEN [51, 55-57].

Considering BMPs' role in regulating SCs differentiation and inducing apoptosis and differentiation, it is possible to suppose that CSCs treatment with these molecules could induce differentiation and following chemotherapies sensitization. Some preliminary studies have been performed on both CSCs of glioblastoma and CRCs. Preliminary studies in glioblastoma demonstrate that BMP2, BMP4 and BMP7 treatment inhibits sphere forming and induces differentiation of CD133+ cells; moreover CD133+ cells pre-treatment with these cytokines attenuates tumor formation in mice (58-60). Recently, the same results are obtained in CR-CSCs. The treatment of CD133+ CR-CSCs with BMP4 induces in vitro differentiation and reduces their tumorigenic potential; moreover in vivo the combined treatment with BMP4 and conventional chemotherapeutics reduces the tumor size (61). These data open the possibility to use BMPs or analogue-drug to induce the differentiation of CSCs and to make them more sensitive to conventional chemotherapy.

4. PROSPECTIVE

Despite recent progress in CSC research, our knowledge of these rare populations is still limited and many questions remain to be answered. Certain types of cancer are known to be multi-stage diseases, which generally progress into more malignant forms with the sequential accumulation of genetic and molecular alterations. For instance, hematological malignancies,such as CML, are often found to have two distinct phases: chronic phase and blast crisis (or leukemia). Similarly, some epithelial tumors, e.g. colon tumors, are thought to progress through at least five stages: pretumor patches/fields, hyperplasia, carcinoma in situ, invasive carcinoma and metastasis. One of the central questions in the CSC research is: how to link CSC to cancer progression in these tumors? Given sequential requirements of genetic and molecular alterations and distinct pathologic abnormalities associated with different stages of cancer progression, one may postulate that there could be multiple CSC populations, either intrinsically linked or generated independently, responsible for different stages of cancer progression.

To advance CSC research, we need to first understand the normal stem cells and critical pathways controlling stem cell properties. For this, identification of cell surface molecules for prospective stem cell isolation and biologically relevant stem cell assays are essential. In addition, technical improvement will expedite the studies of these rare and heterogeneous

population(s). We should investigate the molecular mechanisms for the CSC formation and maintenance, especially their self-renewal regulation, which holds the key for the development of effective therapeutic strategies against CSC. Although stem cell niches have been shown to play an instructive and pivotal role in the regulation of stem cells, their implication in the CSC formation remains to be elucidated. Ultimately, with further improvements in our understanding of CSC, we will be able to develop better diagnostic and therapeutic methodologies, with which to classify, treat, and cure cancer.

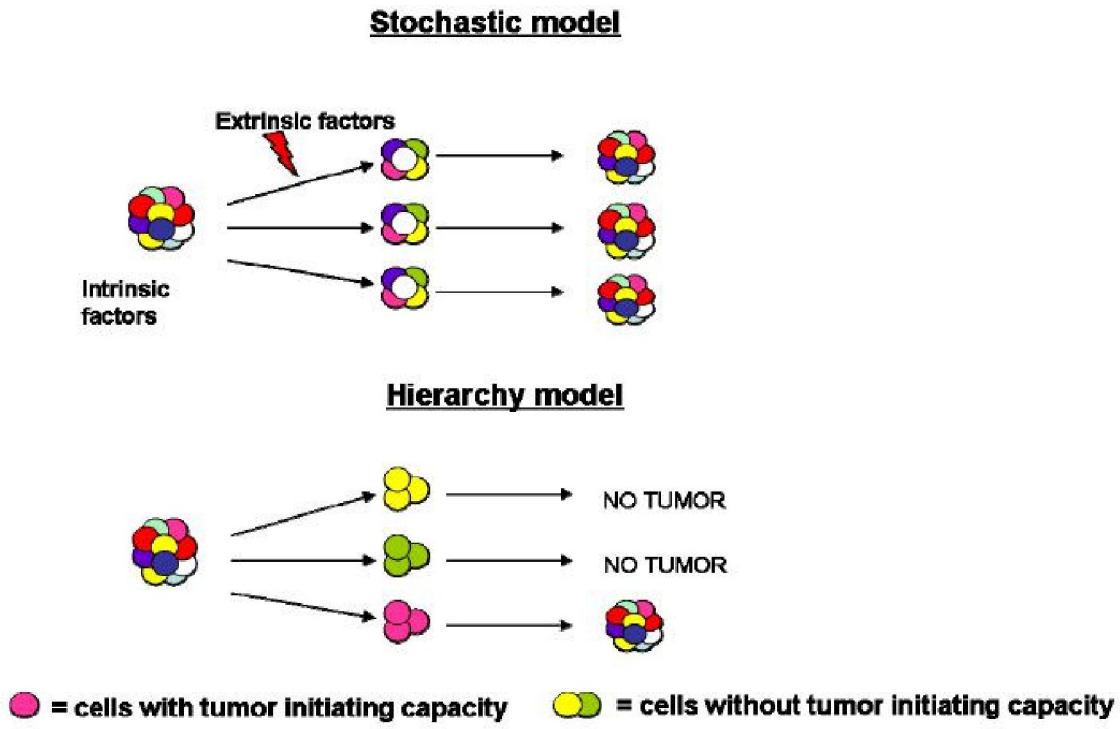

Figure 1. Models of tumor heterogeneity. Tumor heterogeneity has been explained by two theories: according to the stochastic model, tumor cells are influenced by intrinsic and extrinsic factors; by contrast, in the hierarchy model, tumor cells have different functional abilities and only a subset can initiate tumor growth

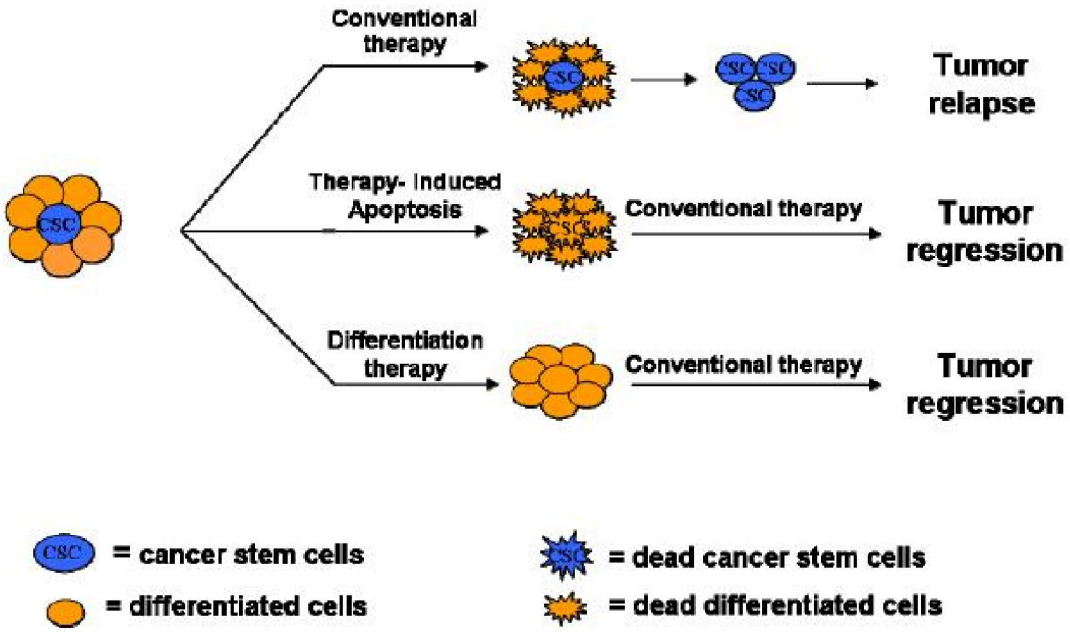

Figure 2. Therapeutic Strategies for CSCs sensitization.

5. CONCLUSIONS

CSCs are believed to play a critical role in tumor initiation and recurrence. Current chemotherapeutic regiments target the most actively cycling cells, which represent the tumor bulk, sparing the CSC compartment. Thus, novel and more efficient stem cell-based therapies, able to kill this chemotherapy-refractory population, are needed to improve patients' survival. In this scope, the identification of agents that can inhibit the CSCs survival machinery forcing apoptosis or induce their differentiation represents the first step to achieve in the near future, providing important advances for cancer treatment.

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Molecular study of the retinoblastoma in western Algerian population. Research of gene Rb mutations at the constitutional level.

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Background: Retinoblastoma is a malignant intraocular tumour which generally reaches the child with a frequency from 1/15000 to 1/20000. Antioncogene Rb changes (chromosome 13q14.2), are at the origin of this cancer. Two allele's mutations of this gene are required for retinoblastoma development. The aim of this study is research and identification of mutations able to affect the gene Rb at the constitutional level. **Methods:** Study concern 61 patients. The twenty-seven exons and promoter of Rb gene were amplified by PCR, with 15 exons studied by DGGE (Denaturing Gel Gradient Electrophoresis) and 12 by SSCP (Single Strand Conformation Polymorphism). These techniques allow selecting cases for sequencing. **Results:** Sequencing results gave nineteen different variations bases, including seven exonic changes: five nonsens mutations located in exons 1,7,8,18 and 23 and two misense mutations in exons 19 and 20. These changes remain minority compared to the twelve intronic changes whose possible implication remains to be elucidated. Polymorphisms found in exons 2,3,4,11 and 17 were already described in the literature. **Conclusion:** Neomutations detection is important because it allows both the early treatment of children with the mutated gene that screening asymptomatic carriers at risk of transmitting the disease to their offspring.
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Introduction

Retinoblastoma is a malignant neuro-epithelial origin, with an average incidence of one case every 15.000-20.000 live births (1). This cancer usually affects children in the first months of life. There are two forms, the hereditary form in which the first event is germinal and second somatic. For the sporadic form, the two events are somatic. Mutations affecting antioncogene Rb (chromosome 13q14.2) (2), are the cause of this cancer. The mutations of both alleles of this gene are required for the survey of retinoblastoma.

The Rb gene is part of the Rb/E2F pathway that plays a role in the development of retinoblastoma. It is at the middle of the cell cycle in the transition G1 / S. The protein encoded by Rb (*p*Rb) exists in two forms: phosphorylated, inactive form and a non-phosphorylated, active form (3). The last form has the essential property to bind E2F transcription factor and thus block the progression of S phase (4).

The aim of the present study is to identify mutations that may affect the Rb gene at the constitutional level. This would help in the first to understand the molecular pathology of retinoblastoma and second to the early identification of a person witch present a risk, asymptomatic carriers and possibly prenatal diagnosis.

Material and methods

Material

Study included 61 unrelated patients with unilateral or bilateral sporadic retinoblastoma, recruited at the ophthalmology clinic of "front de mer" in Oran and pediatric ophthalmology department of the EHS Canastel. Oran. The DNA of patients were designated by Ru or Rb letters, respectively, for unilateral and bilateral forms followed by a number

Methods

Extraction of genomic DNA was performed from whole blood using the NaCl technique (5). The 27 exons of the RB gene were amplified by PCR (Lille, France) (6). Twenty-seven primer pairs were used for amplification of the promoter and 27 exons Rb gene components. Exons 15 and 16 were amplified together. The choice of primers (7) was done in intronic parts flanking each exon to cover with all primers, most of the RB gene (Table 1).

Analysis of the Rb gene at the constitutional level was performed by DGGE (Denaturant Gradient Gel Electrophoresis) (8) for 15 exons and SSCP (Single Strand Conformation Polymorphism) (9) for the remaining 12 exons. Both techniques enable screening of the index case to sequence (ABI 3130, Applied Biosystems Foster City, Californie USA) (10). Sequence analyzes were performed using the Seqscanner of applied biosystem (http://marketing.appliedbiosystems.com/mk/get/SSS_login?isource=fr_E_Pg_Prod_AB_Gbl_SeqScan_20050920) and Multalin (http://multalin.toulouse.inra.fr/multalin/).

Results and Discussion

Constitutional analysis of 61 DNA patients after study by DGGE (Figure 1) and SSCP (Figure2), revealed 19 different variations bases, in 14 exons: 1, 2, 3, 4, 7, 8, 9, 11, 12, 17, 18, 19, 20 and 23. The result of sequencing followed by "Multalin" analysis (Figure 3) gave: **exon 1:** G→ A transition in position 91pb of exon 1, Glu31Stop, **exon 2:** C → T transition 76 bp intron2, **exon 3 :** C → T transition 12pb, intron3 ; A → G transition 37 bp, intron3, C → T transition 45pb, intron3; **exon 4:** G → T transversion 23pb, intron4; **exon7:** deletion of 4pb position 24 between codons 7 and 10 in exon7 (Patient Ru14 given as example in Figures 2 and 3) transition G → A 78pb, intron7; **exon 8:** C → T transition 45pb, exon 8, arg225stop; **exon 9:** transition T → C-29pb, intron 8, C → A transversion -15 bp intron 8 (RB7 Patients and Ru42 given as example in Figure 1), **exon 11:** A → C transversion 41pb, intron11; **exon 12:** G → A transition 1pb, intron 12 ; **exon 17:** del A-56, intron16; **exon 18:** C → T transition 40bp, exon 18, arg578stop; **exon 19:** C → T transition 61pb, intron19; G →C transversion 80pb, exon19, Ala635Pro; **exon 20:** T →A transversion 1pb, exon20, Val654Glu; **exon 23:** deletion of 7 bp in exon 23 stop codon position 809.

Identified mutations distribution along the gene shows that there is no preferential region of mutations. Among the 7 exonic mutations found: 5 are responsible in the appearance of nonsense mutations and 2 are missense (Table 2). These mutations are minority compared to intronic mutations witch are the most numerous and whose possible involvement remains to be elucidated (Table 3).

Exonic mutations found are causal, since they all lead to the appearance of inactive truncated protein, due to the absence of the region between exons 12 and 27. Indeed, this region encodes a structure called the "binding pocket of oncoproteins." This pocket has two domains, 179 amino acids (codon 393 to codon 571) and 125 amino acids (codon 649-773), separated by an open area of 76 amino acids called "gap" (codon 572 to 648). It serves to hold the transcription factor E2F (11). This region is required for *p*Rb protein in cell cycle regulatory function, and any modification or mutation occurring at this level will cause retinoblastoma. The intron 12 variation base cause an abnormal splicing, it abolishes the splice donor site by change the sequence GT to AT (13). This leads to the production of a longer protein due to intron 12 translate. This variation can affect the protein conformation witch will have impact in its normal regulatory function (14). A study of functional and structural protein *p*Rb is required to confirm the role of this mutation.

Polymorphisms already described in the literature were found in exons 2, 3, 4, 11and 17.

For this study, which involves only sporadic forms of retinoblastoma, we were able to identify mutations in constitutional level that are potentially transferable. This type of event is generally ignored because there is no history of disease in families of index cases. Beyond that, we are well aware of the need to sequence the entire Rb gene to detect mutations that are in the range of from 10 to 15 percent. Our results confirm that because we were able to detect seven mutations in a population of 61 patients with sporadic form of retinoblastoma representing 11.47%.

Germinal neomutations detection is important because it allows the prevention and early management of the patient if the disease were to break out. This detection is also important for genetic counseling.

It does allow reassuring branch of family members showing no mutations.

For subjects did not show any molecular defect, it is necessary to explore other signaling pathways may cause retinoblastoma in these cases (15).

Indeed, recent research, demonstrate the existence of genes other than Rb, which may play role in the occurring of retinoblastoma. This work is still ongoing, will in the future a better understanding of the molecular mechanisms that could be causing retinoblastoma (16).

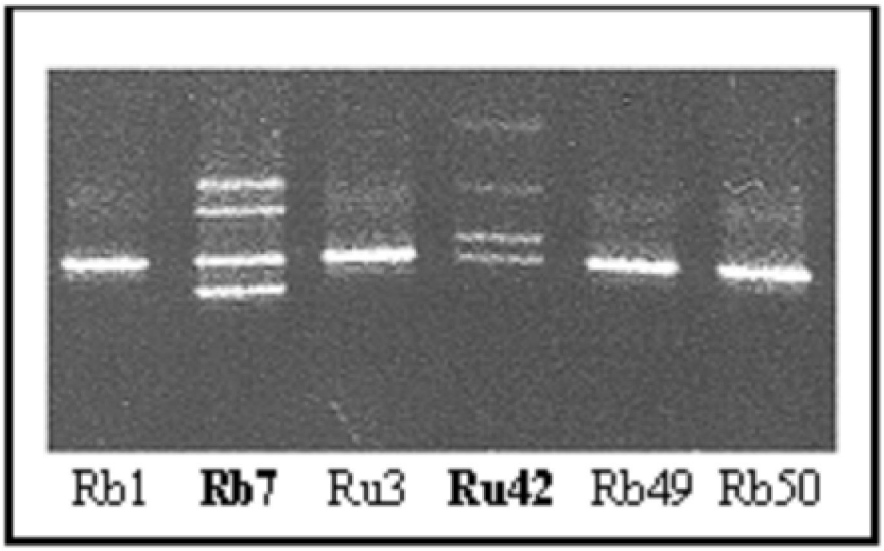

Figure 1: Identification of two sequences variations in exon 9 by DNA DGGE electrophoresis. Patients RB7 and Ru42 shows four bands different profiles.

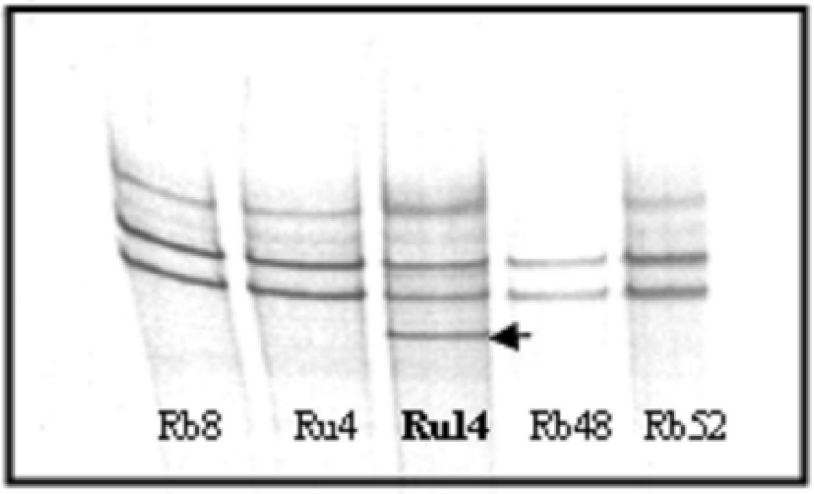

Figure 2 : Identification of variation sequence in exon 7 by SSCP DNA electrophoresis. The patient Rus14 shows additional band profile (arrow)

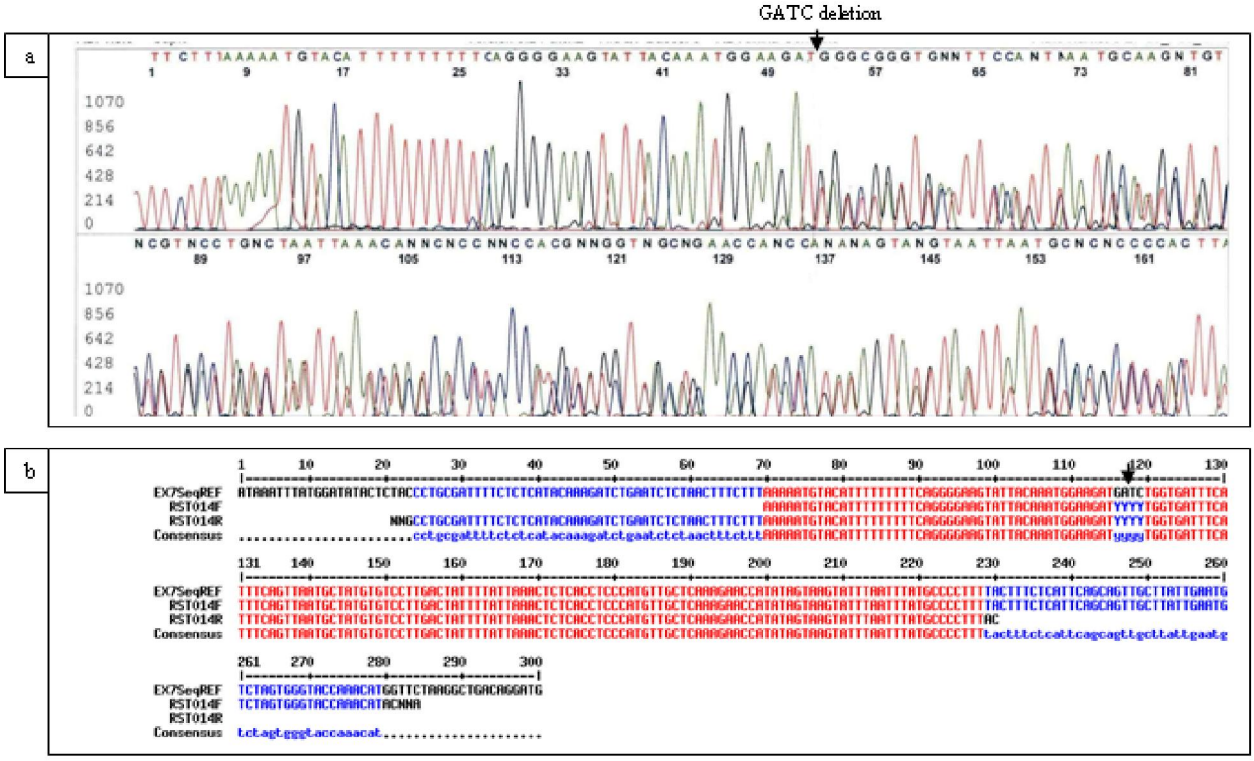

Figure 3: Patient Ru14 DNA sequencing shows a heterozygous deletion in exon7 of GATC bases in codons 7-10 (arrow).

a) Frame shift mutation. Heterozygous deletion revealed by peaks overlapping.

b) Deletion confirmed by the two strands alignment (arrow).

Table 1: List of sequence amorce of Rb gene

Exon/ Promoter	Sequence
Promoteur	CTGGACCCACGCCAGGTTTC
	GTTTTGGGCGGCATGACGCCTT
Exon1	CCGGTTTTTCTCAGGGGACGTTG
	TTGCGCCCGCCCTACGCACAC
Exon 2	(35GC)TTTCACAGTAGTGTTATGTG
	ATTTCCTCTGGGTAATGG
Exon 3	(40GC)CAGTTTTAACATAGTATCCA
	ATACACTTTCATAACGGCTC
Exon 4	(35GC)AGTAGTGATTTGATGTAGAG
	TGAGCTAACATTAAAAGGGA
Exon 5	(35GC)CTACTATGACTTCTAAATTACG
	CAAGATGTTTGAGATTATTCC
Exon6	TCTATTATGCATTTAACTAAGG
	(30GC)GAGTACCAGAATTATAGGAAC
Exon 7	(25GC)ATACTCTACCCTGCGATTTT
	CATTTGTTGTATTTTGACCC
Exon 8	TATCCTTCTAATGAAACCTA
	GCTCATAACAAAAGAAGTAA
Exon 9	TTGACACCTCTAACTTACCC
	(40GC)ACAATTATCCTCCCTCCACA
Exon 10	ACCTCACTTTTAGATAGACC
	(45GC)GTTATAGGACACACAATTCACA
Exon 11	(40GC)GCTGGGTCATCTATTTTCTA
	ACCTGGCCTTCAATATATA
Exon 12	GAGGCAGTGTATTTGAAGAT
	AATGGATAAACGGAACGAGT
Exon 13	(50GC)CTTATGTTCAGTAGTTGTG
	CCTATGTGTTCCTTTATTAC
	(50GC)CTTATGTTCAGTAGTTGTG
Exon 14	CCATATTTGTAAGAAGGGT
	(45GC)GATGATCTTGATGCCTTGAC
Exon 15-16	ATTCAATGCTGACACAAATAAGGTT
	TTCTCCTTAACCTCACACTATCC
Exon 17	GCTATTTCCTATGAGTCCGT
	GAGTTCTTGTTTATCCCTTA
Exon 18	(50GC)GACTTTTAAATTGCCACTGT
	ATTCCCTACAGTTTCTTTAT
Exon 19	AGGCAGTAATCCCCAGGAAAAGCCA
	CACAGAGATATTAAGTGACTTGCCC
Exon 20	TTCTCTGGGGGAAAGAAAAGAGTGG
	AGTTAACAAGTAAGTAGGGAGGAGA
Exon 21	TCCTGGATAATTGAGCCTTG
	(45GC)CCTTATCTTTCCAATTCTAT
Exon 22	(50GC)TAATCCAAGCCTAAGAAGTA
	GATACTTTTGACCTACCCTG
Exon 23	TCTAATGTAATGGGTCCACCAAAAC
	CATCTTGCGTTGCTTAAGTCGTAAA
Exon 24	TAAAACTAAGAGACTAGGTGAGTAT
	TAGATTTGGGTAGGAAAAAAATCTC
Exon 25	ATATTTGGTCCAATGAAGCAGAAAATT
	TGATGCTATGTATTTTTTCAGTGGT
Exon 26	AACCACTGTATTTTGTGAGAACCAC
	TGAATGTGGTCAAGCAATGT
Exon 27	TCTAGCTATTTGAATATGCA
	(46GC)AAATCTTGTGTAAATCCTGCC

Table 2: Exonic mutations of Rb gene

Exon n°	Position of the mutation	Consequences
1	Transition G→A, 91	Glu 31 Stop
7	del 4pb codons 7-10, 24pb	Codon Stop 212
8	Transition C→T, 45pb	Arg225Stop
18	Transition C→T, 40pb	Arg578Stop
19	Transversion G→C, 80pb	Ala635Pro
20	Transversion T→A, 1pb	Val654Glu
23	del 7pb, codon 23 et 27	Stop 809

Table 3: Intronic mutations of Rb gene

Amplified region exon n°	Position of the mutation
Exon 2	transition C→T, 76pb
Exon 3	transition T→C, 12pb intron3
Exon3	transition A→G, 37pb intron3
Exon 3	transition C→T, 45pb intron3
Exon 4	transversion G→T, 23pb intron4
Exon 7	transition G→A, 78pb intron7
Exon 9	transition T→C, -29pb intron8
Exon 9	transversion C→A, -15pb intron8
Exon 11	transversion A→C, 41pb intron11
Exon 17	delA -56pb intron16
Exon 12	transition G→A, 1pb intron12
Exon 19	transition T→C 61pb

Inactivation of both alleles of Rb gene represents the tip of an iceberg of event that determines retinoblastoma development, progression and severity. It is well established that mutations in the Rb gene alone are not sufficient to trigger retinoblastoma. An increasing number of studies have suggested that the epigenetic modifications are associated with retinoblastoma development (17, 18).

Conclusion

The situation is different depending on whether a sporadic case with a first event or a germinal case with only somatic mutations.

For patients with a constitutional anomaly Rb gene, an investigation is crucial to distinguish whether they are carriers of the deleterious allele but are asymptomatic or whether it simply a neomutations. For individuals with no constitutional mutation, we can confirm a non- transmissible retinoblastoma and the investigation of non affected family members is not need.

In retinoblastoma, as in most cancers, the prognosis depends on early diagnosis. The early identification of mutation allows a rapid management, continuous monitoring and thereby contribute to the prevention of disease.

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