

Synergistic effect of accumulated chlorpyrifos and raised levels of MDA and oestrogen induced ovarian cancer progression

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Abstract: Lethality of ovarian cancer (Oca) escalates due to lack of sufficient techniques for authentic prognosis. Many factors are responsible for the progression of cancer. Oca patients from Gangetic and Non-Gangetic were categorized to evaluate the effects of chlorpyrifos (organophosphate) on Oca patients. Oestrogen and several xenoestrogens are believed to be the primary causative agents for female gynaecological cancers. Mutagenicity caused by malondialdehyde (MDA), a by-product of lipid peroxidation (LPO) is also suggested to be a major source of many diseases, including cancers. Blood was sampled from Oca patients at Dept of Pathology, Mahavir Cancer Institute and research centre, India. A standard protocol was followed for Thiobarbituric acid reactive substance assay. Standard protocols were followed for the evaluation of various haematological parameters, estrogen level (estrogen ELISA kit), blood chlorpyrifos detection (reversephase HPLC),

MDA level was significantly ($P < 0.001$) higher in Oca patients from Gangetic and non-Gangetic zones than the normal healthy persons. WBC values showed no significant ($P > 0.05$) difference between Gangetic and non-Gangetic Oca patients. However, the WBC values of Oca patients were significantly ($P < 0.05$) higher than those of healthy patients. Detection of chlorpyrifos in Oca patients suggests its genotoxic effect. Higher estrogen level in postmenopausal patients from both the zones indicates increased aromatase activity. Simultaneous effects of xenoestrogen and estrogen could be the possible cause of Oca development. Histopathological observations corroborates haematological and biochemical values. Since Gangetic and non-Gangetic parts of Bihar are pesticide-prone zones, the use of chlorpyrifos should be discouraged to avoid its genotoxic-gynecological effects leading to cancer. More studies are warranted to confirm the role of simultaneous effects of estrogen, xenoestrogens and MDA in the initiation and progression of gynaecological cancers in women.

Keywords: MDA, chlorpyrifos, estrogen, ovarian cancer.

I. Introduction

Ovarian cancer is among four commonest cancers in women and one of the most leading causes of deaths from gynaecological diseases. Fatality from ovarian cancer can be attributed to numerous factors, including inadequate prognosis leading to unreliable screening procedure. Oca patients at an advanced stage show greater mortality rate. Due to improper diagnosis, the ovarian cancer turns out to be non-symptomatic and difficult to detect the origin, growth and metastasis for a long time [1]. The aetiology of ovarian cancer is poorly understood but the risk factors are related to cause hormonal imbalance. "Incessant ovulation" triggers cell proliferation resulting in malignant ovarian epithelium [2]. Female sex hormones are also indicated to be stimulants of ovarian cancer [3].

Environmental factors and epigenetic changes are also pointed to cause reproductive cancer. Wetlands play a pivotal role in the history of mankind. They were "biological supermarkets" as they possess extensive food chain and rich biodiversity. But with the change of time, wetlands became "kidneys of the landscapes" as they can process hydrological and chemical cycles [4]. Gangetic zone of Bihar is also wetland with nutrient rich soil and fertile land around river Ganga. Gangatic zone sustains highly dense and agriculture based population dependent on the fertility of their land. Unrestrained use of pesticides is a common activity in Indo-agricultural methodology. Agriculture based health study is considered to be a perspective cohort study designed to evaluate the relationship between variety of occupational exposure, pesticide application and cancer and other diseases [5]. Organophosphates are the pesticides which contaminate soil, food and ground water and enter our food chain. Pesticides are well established xenoestrogen, which are able to escape degradation by chemical or enzymatic action and excretion and get stored in adipose tissues [6]. Chlorpyrifos is one of the commonly used organophosphate insecticides in India. Chlorpyrifos is metabolized in our liver to form chlorpyrifos oxon which is supposed to cause neurotoxicity by inhibiting cholinesterase in CNS [7]. Few reports have confirmed chlorpyrifos-induced mutagenesis [8 & 9], sister chromatid exchanges [10 & 11], chromosomal aberration

[12]. It is extracted 200-300 times more in fat tissue than in serum [13]. Best studied organochlorine is o,p'-DDT, which has been shown to bind and activate estrogen receptor altering gene expression of certain transcription factor [14-16]. Xenoestrogens have been demonstrated to promote tumorigenesis in animal models [17], but still is controversial due to their lesser potency than indigenous estrogen. As such it has been proposed that xenoestrogen with synergistic effect of indigenous estrogen can expedite cancer development [18].

Biosynthesis of estrogen in body is catalyzed by aromatase enzyme in postmenopausal women and major site for its production is adipose tissues which increase with age and body weight [19 & 20]. In uterus, estrogen induces cell division of endometrium every month, followed by cell death during menstruation until menopause is achieved naturally. Such repeated cycles of estrogen induced division of cells that already have mutation from carcinogens increasing the risk of development of cancer [21 & 22]. According to a report, estrogen level in breast tumour area is significantly higher than serum. This might be due to certain factor produced by tumour cells which stimulates the expression of aromatase leading to aggrandized production of estrogen [23].

Lipid peroxidation is a consequence of ROS action on PUFA (Polyunsaturated fatty acid) and its degradation to MDA. ROS is produced during normal cellular metabolism from mitochondria and monooxygenase. ROS can produce damaged base or strand by interacting with DNA and other biomolecules like proteins and lipids whose intermediates have potential to form adducts with DNA [24 & 25]. Such an intermediate is MDA which forms DNA-MDA adduct leading to cause mutation by forming M₁A, M₁G and M₁C with dA, dG and dC respectively. There are several antioxidant enzymes and compounds that are responsible to balance free radicals produced such as catalase, superoxide dismutase, glutathione reductase and oxidase, ascorbic acid, tocopherol etc. Imbalance in free radicals generation and antioxidants leads to oxidative stress.

CA-125 is a high molecular mass membrane bound glycoprotein whose size ranges from 200-2000 KDa (26-28). It has been indicated that CA-125 retains high carbohydrate content and a preponderance of serine and threonine (O-linked) glycan chains (29,30). MUC16 is also reported to induce metastasis of tumour cells. MUC16 specifically binds with mesothelin, an integral glycoprotein normally expressed by mesothelial cells of the peritoneum (31). Such an interaction of MUC16 and mesothelin is supposed to promote metastasis tumour cell invasion (32).

The present endeavour is aimed to reveal the role of chlorpyrifos, MDA, and Oestrogen on initiation and progression of ovarian cancer.

II. Materials and Method

With the consent of 140 ovarian cancer patients and 48 normal women, the blood was sampled from Department of Pathology, Mahavir Cancer Institute and research centre, Patna, India. Part of blood was used for RBC count and haemoglobin level and serum was prepared and used for LPO assay, and estrogen test. Tissues collected from operation theatre, paraffin blocks were prepared for histopathological study.

2.1 Hematological Parameter

RBC count, WBC count, platelet count and Haemoglobin level were estimated by standard procedures using Cell Counter (Medonic M- Series) in the Department of Haematology, Mahavir Cancer Institute, Patna.

2.2 Assessment of MDA

The blood was centrifuged at 3000RPM for 10 minutes and serum was collected and stored at -80°C. TBARS level in each ovarian cancer patient was estimated by standard procedure with slight modifications [33]. 10% TCA and 0.675% TBA were prepared as stock for the assay. 2.5 ml of TCA and 500µl serum was added to the test tube. It was kept for incubation for 15minutes at 95°C, followed by centrifugation at 3000rpm for 10 minutes. 2ml of supernatant was transferred to another test tube and 1ml of stock TBA was added to it. The test tube was again incubated at 95 °C for 15 minutes. With the use of UV spectrophotometer, optical density was taken and concentration was analysed from standard prepared.

2.3 Pesticide extraction

Chlorpyrifos was extracted from 45 human blood serum based on the method Mathur et al [34] and Darko and Acquah [35]. The blood sample was allowed to clot at room temperature and left for incubation for 30 minutes. Blood samples were centrifuged at 3,000 rpm for 15 minutes. Serum was collected and stored at -20 C. 1 ml serum was vigorously shaken with 5 ml of hexane in a cyclomixer. The hexane layer was concentrated under vacuum. The obtained pesticides extract was subjected to HPLC.

2.4 Oestrogens level

Estimation of oestrogen in blood of 54 ovarian cancer patients was conducted with ELISA kit. 25µl of test serum was added in the wells, followed by 100 µl of enzyme conjugate solution and was incubated for 1hr. The wells were washed 3 times with washing buffer and soaked on absorbent paper. 100µl of TMB solution was dispensed in wells and kept for incubation for 30 minutes after shaking gently for 20 seconds. 50µl of stop solution was added to stop the reaction, followed by shaking gently. Yellow colour reaction was taken for optical density reading and concentration was analysed from the standard prepared.

2.5 Histopathological procedure

Histological parameters were studied by collecting tissues of ovarian cancer patients from operation theatre. Tissues were fixed in 10% formalin and dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin wax and blocks were prepared. Sections (6µm) were cut and fixed on slide with the help of Mayer's albumin. Double staining was performed and the sections were dehydrated in ascending concentrations of ethanol, cleared in xylene, mounted with DPX and examined under light microscope.

2.6 Statistical analysis

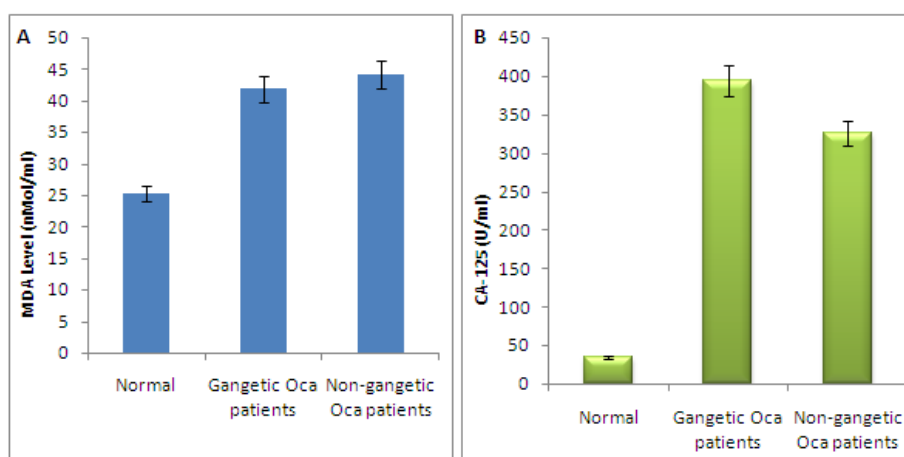
Mean and standard deviations were calculated using MS Excel 2010. Statistical analysis was performed using One way ANOVA and SPSS software package 11.5.

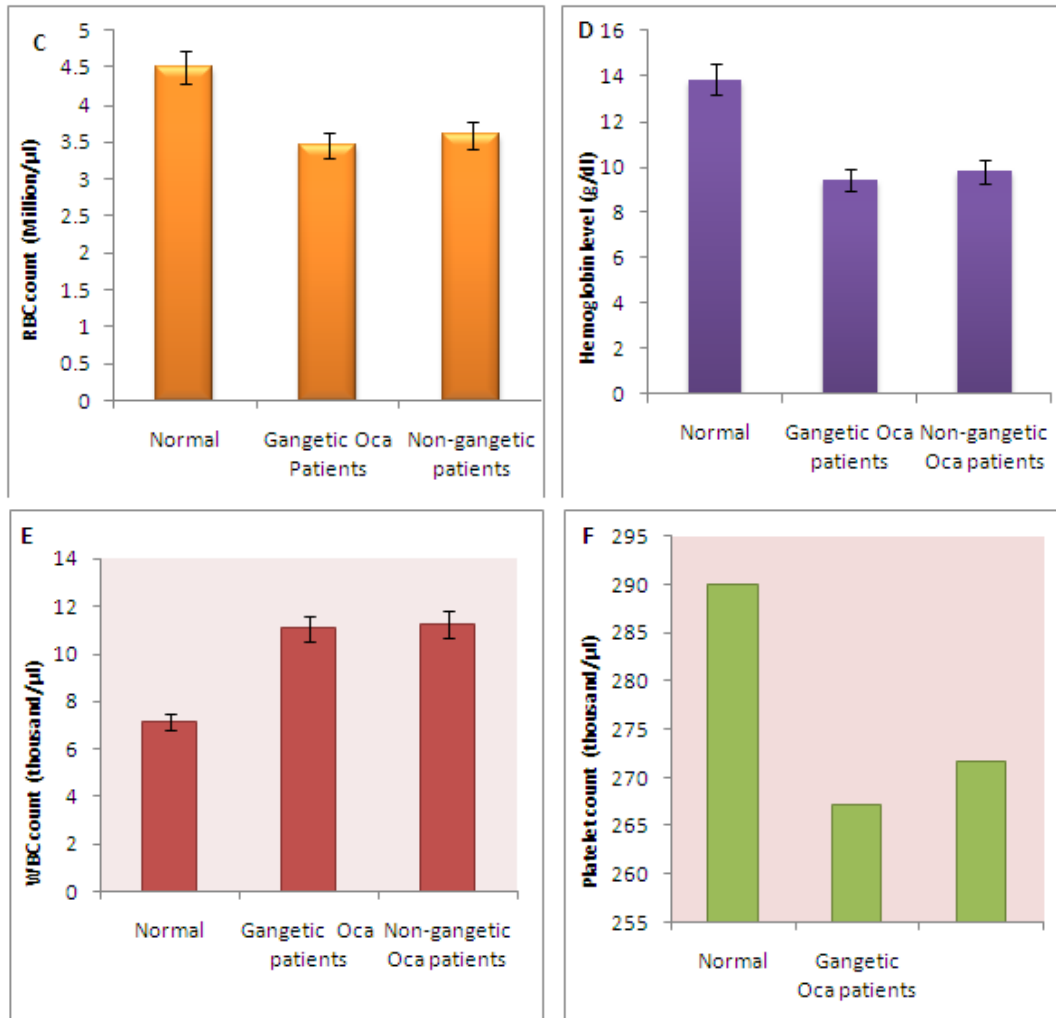
III. Results

Ovarian cancer is more prevalent in Gangetic (74.24%) than in non-gangetic zone (25.76%). Out of total Oca patients (88), greater percentage (55.68%) was recorded in the age group of 30-50 yrs, followed by 39.77% and 4.54% in 51-70 and 71-90 yrs age groups of women respectively. To the contrary higher percentage of Oca patients (67.31%) was recorded in the age group of 51- 70 yrs than the 30-50 yrs age group of women in the non-gangetic zone (Table 1).

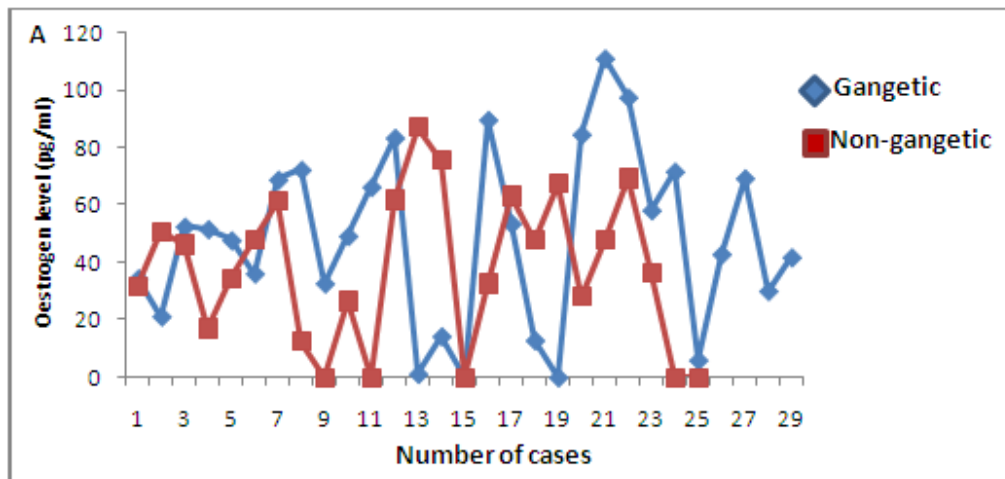
Age	Ovarian cancer cases				Total	Percentage	
	Gangetic		Non-Gangetic			Gangetic	Non-gangetic
	No. of cases	%age	No. of cases	%age			
30-50	49	55.68	17	32.69	66	74.24	25.76
51-70	35	39.77	35	67.31	70	50	50
71-90	4	4.54	0	0	4	100	0
Total	88		52		140		
Percentage	62.86%		37.14%				

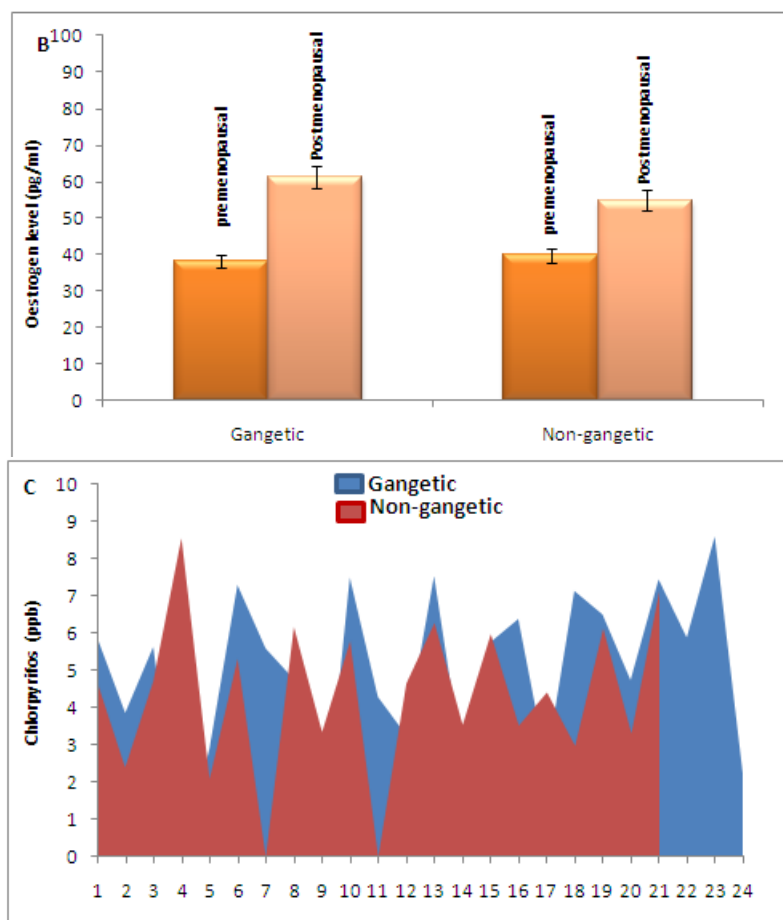
Table 1- number and percentage of ovarian cancer patients coming from Gangetic and non gangetic zone with different age groups.





Text fig. 1- Values of MDA level (A), CA-125 (B), RBC count (C), Haemoglobin level (D), WBC count (E), and Platelet count (f) of normal, Gangetic and Non-gangetic Oca patients.





Text fig. 2- Values of Oestrogen in various Gangetic and non-gangetic cases (A), average pre- and postmenopausal oestrogen levels in Gangetic and non-gangetic cases (B) and chlorpyrifos in Gangetic and non-gangetic cases (C).

MDA was significantly ($p < 0.001$) higher in ovarian cancer patients than healthy ones which marks a burden of oxidative stress on the cell. Mean MDA levels (nmol/ml) in ovarian cancer patients of Gangetic zone (41.87 ± 3.75) and Non-gangetic zone (44.01 ± 6.85) were higher than that of healthy persons (25.3 ± 6.35).

Mean CA-125 values in healthy persons and ovarian cancer patients presented a significant ($p < 0.0022$) difference in text fig 1B. There was also substantial difference in mean CA-125 levels in ovarian cancer patients belonging to Gangetic or Non-gangetic zone of Bihar as compared to normal persons. Mean values of CA-125 (U/ml) in patients of Gangetic zone was higher (394.09 ± 384.20) than patients from Non-gangetic zone (315.04 ± 427.62). the CA-125 values of both the zones were significantly ($p < 0.001$) higher than those of healthy ones (35 ± 6.76).

	Normal persons mean±S.D	Ovarian cancer cases		p-value
		Gangetic (mean±S.D)	Non-gangetic (mean±S.D)	
MDA level (nMol/ml)	25.3±6.35	41.87±3.75	44.01±6.85	<0.001
CA-125 (U/ml)	35±6.76	394.09±384.20	315.04±427.62	<0.0022
RBC count (Million/ μ l)	4.5±0.68	3.45±0.9	3.59±1.14	<0.0065
Hemoglobin level (g/dl)	13.85±1.27	9.468±2.14	9.68±1.86	<0.0108
WBC count (thousand/ μ l)	7.15±2.55	11.06±2.64	11.24±3.07	<0.0001
Platelet count (thousand/ μ l)	290±100.54	267.2±96.11	271.73±120.49	<0.0265

Table 2- Avarage values of MDA, CA-125, RBC, haemoglobin, WBC, and Platelets of normal women, gangetic and non-gangetic Oca patients.

Complete blood picture provides enormous information on the effects of cancer and role played by oxidative stress. Erythrocyte count depreciated heavily in cancer patients in comparison to that of normal ones fig. 1C. Mean RBC count (million/ μ l) of healthy people was higher (4.5 ± 0.68) than those of Gangetic zone (3.45 ± 0.90) and non-gangetic (3.59 ± 1.14) Oca patients fig1C. Mean haemoglobin level (g/dl) of normal persons (13.85 ± 1.27) was higher than those from Gangetic (9.468 ± 2.14) and Non-gangetic zone (9.8 ± 1.86) (fig1D). Mean WBC count (thousand/ μ l) in patients from Gangetic (11.06 ± 2.64) and Non-gangetic zone (11.24 ± 3.07) was significantly ($p<0.001$) higher than normal persons (7.15 ± 2.55) (fig1E). Mean platelet count (thousand/ μ l) in Oca patients from Gangetic (267.2 ± 96.11) and Non-gangetic (271.73 ± 120.49) was lower than that of normal ones (290 ± 100.54) fig 1F.

Mean oestrogen levels (pg/ml) of Gangetic zone patients and Non-gangetic zone Oca patients were 48.31 ± 30.4 and 37.86 ± 26.26 pg/ml respectively fig2A & B. The oestrogen level in patients from Gangetic zone was significantly ($p<0.05$) higher than Non-gangetic patients. However, the mean oestrogen values postmenopausal patients from gangetic (61.15) and non gangetic (44.82) zones were higher than those of premenopausal patients from Gangetic (38.15) and Non-gangetic (39.85) zones (fig 2B). Chlorpyrifos (ppb) values of Gangetic zone was higher than those of Non-gangetic zone (fig2C).

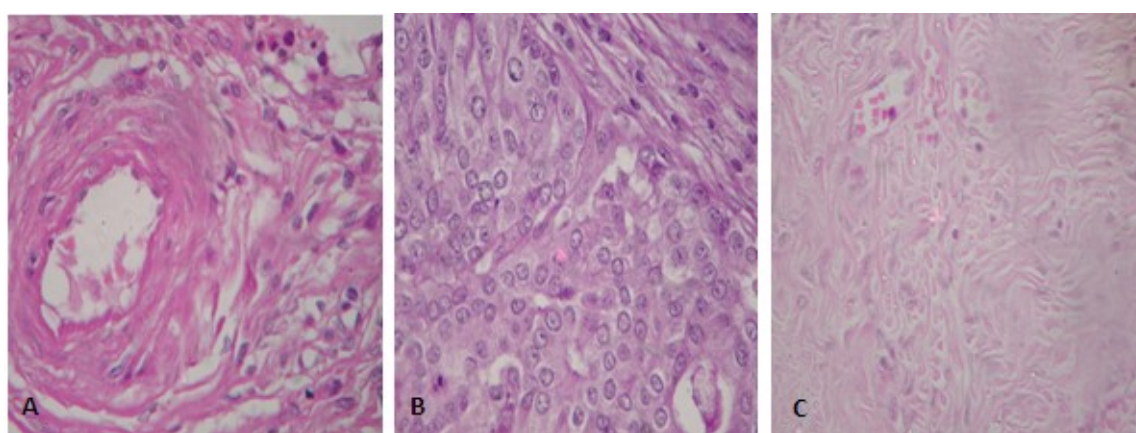


Fig 3- photomicrograph (X40) of section of (A) normal tissue of ovary, (B) endometrioid ovarian cancer, (C) serous papillary ovarian cancer.

Normal ovarian histology is characterized by glandular epithelium with regular shaped nuclei (fig 3A). However, endometrioid carcinoma closely resembles to its uterine counterpart, well differentiated with several well formed glands (fig 3B). In high grade serous adenocarcinoma, atypical nuclei are observed in fig. 3C. High mitotic count with active stratification and branching papillary formation along with stromal invasion can also be observed in serous papillary adenocarcinoma.

IV. Discussion

Since ovarian cancer is caused by more than one factor including oxidative stress. ROS is the initiative agent for lipid peroxidation and generated during electron transport chain in mitochondria. Its amount can increase due to the presence of exogenous or endogenous stimuli. During infection, malfunction or increased toxicity of the cell, oxygen free radicals are produced in myriad, leading to apoptosis. Due to unconstrained use of pesticide and its deadly penetration into our food web escalates the probability of fatality of diseases like cancer many fold. Pesticide applicators and consumers of such foods possess risk of developing increased toxicity level in their blood and tissue. Genotoxic and cytotoxic effects of chlorpyrifos on plant tissue have been affirmed [36]. Higher toxicity level is directly correlated with greater lipid peroxidation, due to malfunction developed by chlorpyrifos in the cells. In current study, presence of chlorpyrifos in tissues and serum of Oca patients signifies higher level of MDA in patients belonging to Gangetic and Non-gangetic zones. As it can be assumed that Non-gangetic zone of Bihar is also very fertile and use of pesticides and insecticides is common. In addition, some parts of Non-gangetic zone of Bihar are situated along the bank of Kosi, Gandak, Baghmati, Sone, Kamalabagan etc (rivers other than Ganga in Bihar which have not been taken separately in this paper).

Oestrogen is synthesized by aromatase which is a member of cytochrome P450 superfamily, a product of cyp19 gene [37]. Aromatase is expressed at various sites, including granulosa cells and corpus luteum of ovary in females [38, 39], the syncytiotrophoblast of the placenta [38]; adipose tissue of the breast, abdomen, thighs, and buttocks (20 & 40); and osteoblasts of bone (41 & 42), the Leydig cells and germ cells of the testis [43 & 44] and various sites in the brain, including the hypothalamus and hippocampus [45 & 46]. Aromatase presence and activity in granulosa cells, corpus luteum of ovary and adipose tissue are of prime importance in

pertinence to postmenopausal ovarian cancer patients. The activity of aromatase is believed to show 10 times more in preadipocytes than mature adipocytes and aromatase activity also has site specificity with greater activity in preadipocytes. It has been confirmed that estradiol helps in the growth and proliferation of mature adipose tissue in postmenopausal women and older men [47]. Oestrogen levels of postmenopausal patients from either zones of Bihar were higher than premenopausal patients and normal postmenopausal women which should be (0-40pg/ml). A report has proposed that prostaglandin (PG) E₂ is an important factor for stimulation of aromatase expression via cAMP and promotorII. PGE₂ has been shown to interact with EP1 and EP2 receptor stimulating both PKC and PKA pathways, hence combined stimulation of both pathways produce greater expression of promotorII-specific aromatase [48].

Haemoglobin level is maintained throughout life due to bone marrow stem cells. RBCs are derived from committed stem cells of erythroblastic stages. Kidney increases erythropoietin production in response to hypoxia sensed by nephron. Anaemia in cancer can be treated with re-oxygenation or erythropoietin doses which are linked with improved survival in patients with various malignancies. [49 & 50]. When RBC count and haemoglobin levels provide similar pattern. RBC count, haemoglobin level and MDA level of Oca cancer patients only reflected similar pattern when compared. Superoxides are produced in reperfused (artificial restoration of blood supply in ischemia or in cancer) tissues due to incomplete electron transfer or reduction of oxygen by damaged mitochondria [51] and cellular antioxidant defence mechanism is also crippled. RBC count and haemoglobin levels show similar pattern in text figure 1C and D, which explicit that RBC count and haemoglobin are indubitably correlated. Leukocytes participate in defending body from foreign particles which include Neutrophils, eosinophil, basophil, lymphocytes and monocytes. Leukocytes are derived from hematopoietic stem cells in bone marrow. WBC count in Oca delineates from the normal range in text figure 1E. Levels of WBC count in Gangetic and Nongangetic patients are higher normal. Due to production of tumour antigens, it is anticipated that WBC count increases significantly. There are two types of Tumour antigens on tumour cells- tumour specific transplantation antigens (TSTAS) and Tumour associated transplantation antigens (TATA) have been recognized. Tumour specific antigens are unique to tumour cells and do not occur on normal cells in the body. They may result due to mutations in tumour cells that generate altered cellular proteins [52]. Platelets count in ovarian cancer is observed to be normal and does not provide any significant relationship to Oca (text fig. 1F).

On histopathological examination, several anatomical changes in cells can be observed. Atypical cellular architectures are prominent as clearly manifested by irregular shape. Cytoplasm can be seen to be pervading throughout the tumour area and apparent nucleoli are eminent in endometrioid ovarian cancer (fig. 1 B). Mitotic count can be seen greater in number. When fig. 1 B & c observed, there is a heavy diffusion and irregularities of cytoplasm which solves our problem. Due to attack by ROS, PUFA gets degenerated and plasma membrane becomes weakened and readily ruptured. Cytoplasm indicated that plasma membrane no longer provides support and is lost. Moreover, other organelles are damaged too.

V. Conclusion

Effects of pesticide, oestrogen, and oxidative stress on ovarian cancer should be taken seriously in Gangetic and Non-gangetic zone of Bihar and worked more to mitigate their disastrous effects. Erythropoietin dosages could minimize the number of cancer related anaemia cases and yet, consumption of more antioxidants could alleviate oxidative stress. Refraining pesticide usage or developing method to degrade it in the body must be promoted blindly to curb the disaster of cancer.

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