WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 9, 1213-1226. Research Article ISSN 2277– 7105

EVALUATION OF OXIDATIVE STRESS AND ANTIOXIDANTS IN PATIENTS WITH BENIGN AND MALIGNANT BREAST DISEASE

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Article Received on
24 June 2015,

Revised on 14 July 2015,
Accepted on 08 Aug 2015

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ABSTRACT

Increased formation of reactive oxygen species and decreased antioxidant defense can be defined as oxidative stress. Oxidative stress plays an important role in the pathogenesis of breast cancer and benign breast disease by inducing oxidative damage to DNA leading to mutagenesis. Hormones like Estradiol, Prolactin have been implicated in the development of breast cancer and benign breast disease respectively, as they play central role in breast development and differentiation. The present study comprised of 70 patients, 40 patients of benign breast disease, 30 untreated breast cancer patients and 40 healthy age and sex matched subjects served as controls. The subjects present in the study and control group were aged between 20 – 60 years. This study was undertaken to evaluate the oxidative stress and role of hormones in the pathogenesis of benign breast disease and breast cancer patients. The biochemical parameters evaluated were plasma levels of Vitamin C, serum Vitamin E, β carotene, Blood Glutathione, serum Malondialdehyde (MDA), serum Estradiol, Progesterone, Prolactin, Testosterone and Follicle stimulating hormone. It was found that MDA levels were significantly higher in breast cancer patients as compared to controls and levels of vitamin C, E, β carotene and glutathione were significantly low in breast cancer and benign breast disease patients as compared to controls. Serum estradiol was found to be increased in breast cancer patients and serum prolactin levels was found to be increased in benign breast disease patients as compared to controls respectively. This study was undertaken to evaluate the oxidative stress and the role of hormones in the etiology of benign and malignant breast disease patients.

KEY WORDS: Breast cancer, MDA, Estradiol, Antioxidants

INTRODUCTION

Breast carcinoma is the leading female malignancy with varying morbidity and mortality globally. Carcinoma of the breast is the cancer originating from the breast tissue. Prevalence of breast cancer cases.[1] is on the rise and estimated incidence rate in India is 22.9 per lac population. The rising incidence of breast cancer has been attributed to the westernisation of lifestyle and reproductive behaviour. However much concern is given to malignant lesions of breast but benign lesions are also more frequent. The aetiology of breast cancer is multifactorial. The major influence on breast cancer risk appears to be hormonal factors, dietary factors, environmental and genetic factors which interplay in the pathogenesis of breast cancer.[2] The risk factors may exert their effects via generation of reactive oxygen species (ROS) or free radicals which induces oxidative damage of DNA and leads to carcinogenesis.[3] In normal cells there is a steady balance between oxidative damage and anti-oxidative protection. Oxidative stress arises when there is imbalance between oxygen free radical generation and scavenging action by antioxidants.

Free radicals,[4] are highly reactive molecules and excess generation of these molecules can cause oxidative damage to biomolecules such as polyunsaturated fatty acids within the cell membrane forming peroxyl radicals which further attack the adjacent fatty acids within the membrane causing chain reaction of lipid peroxidation. Thereby, lipids in the cell membrane undergo degradation to form hydro-peroxides which decompose to form a variety of products including Malondialdehyde (MDA). This oxygen free radical induced lipid peroxidation has been implicated in the malignant transformation of cells. In order to combat the deleterious effects of oxidative stress, cells are endowed with an antioxidant defence system, consisting of variety of enzymatic and non – enzymatic antioxidants thereby protecting cellular macromolecules such as proteins, lipids, DNA from oxidative damage. Antioxidants are compounds that dispose, scavenge and suppress the formation of free radicals or oppose their Actions.[5] The two main categories of antioxidants are those whose role is to prevent the generation of free radicals and those that scavenge any free radicals that are generated.[6] In the present study the parameters which are assessed are non-enzymatic antioxidants i.e. vitamin E, vitamin C, β carotene and glutathione.

Caretenoids like β carotene is an effective antioxidant as it is most powerful singlet oxygen quencher. It may augment tocopherol in scavenging peroxy radical therefore act as chain

terminator and has obvious implication for controlling cancer growth. Vitamin C has antioxidant property. It protects critical macromolecules from oxidative damage. It may reduce carcinogenesis through stimulation of immune system where cytotoxic T lymphocytes, macrophages and natural killer cells stimulated to lyse tumor cells. Vitamin E is a major chain breaking antioxidant which inhibit carcinogenesis primarily through its antioxidant activity (Helliwell 1994).[7] by trapping peroxyl radicals thereby inhibiting propagation of chain reaction of lipid peroxidation within polyunsaturated fatty acids of membrane phospholipids. Vitamin C also plays a role in recycling of tocopherol and indirectly involved in neutralisation of reactive oxygen species.[8] Thus vitamin E and vitamin C acts synergistically to reduce lipid peroxidation and protects cell membrane from oxidative damage. Vitamin E also protects the double bond of β carotene from oxidation and thus exhibits sparing effect.

Reduced glutathione is the major endogenous antioxidant produced by cells and participates directly in neutralisation of free radicals as well as maintaining vitamin C and E in reduced form. Thus identification of various oxidant and antioxidant may help to determine their possible role in the etiology of breast cancer and benign breast disease.

Along with the dietary factors, hormones also play vital role in the pathogenesis of breast cancer. The role of hormones was first observed in 1895, when recurrent cancer of breast remitted following removal of ovaries,[9] It is likely that high concentration of estrogen in post - menopausal women increase breast cancer risk by increasing the mitotic rate of breast epithelial cells. Observations reveal that mitotic rate of human breast epithelial cells is greatest during the luteal phase of menstrual cycle which led to the hypothesis that progesterone may augment the mitotic action of estradiol.[10] Besides hormone estrogen and progesterone, there is growing evidences that supporting association between serum prolactin levels and breast cancer risk[11].Studies also suggest that interventions to reduce serum hormonal concentration may reduce the risk of breast cancer.

This study is thus designed to evaluate the oxidative stress, hormonal status and the effectiveness of antioxidants in breast cancer patients and benign breast disease patients as compared to healthy subjects, inorder to derive their possible role in the etiology or prevention of breast cancer and benign breast disease.

MATERIALS AND METHODS

Selection of Subjects

The present study comprises of 70 patients, 30 newly diagnosed breast cancer patients, 40 untreated benign breast disease patients seeking medical care in Goa Medical College, Hospital, Bambolim – Goa during the period of 2011-2012. The control group included 40 healthy age and sex matched subjects. The patients in the study and control group were aged between 20 to 60 years. The consent was obtained from the institution's ethical committee and the patient consent for the test was also obtained in both the groups. A detailed history was obtained and a thorough clinical examination was carried out. The special investigation like FNAC and biopsy were done in all patients and clinically or histologically diagnosed subjects for benign and malignant lesions were taken for the study.

Collection of Blood Samples

10ml fasting samples were collected in plain and EDTA bulbs under aseptic conditions of the above mentioned subjects and controls. Thereafter serum and plasma was separated by centrifugation at 3000 rpm in clinical centrifuge for 10 minutes.

Serum was used for measurement of MDA, vitamin E, β carotene,estrogen, progesterone, prolactin. Plasma was used for measurement of vitamin C and whole blood was used for measurement of blood glutathione. All the estimations were done within 24 to 48 hours after specimen collection.

VITAMIN C

DETERMINATION OF PLASMA ASCORBATE BY 2, 6 DICHLOROPHENOLINDOPHENOL TITRATION METHOD[12]

Titration with 2, 6 Dichlorophenolindophenol in acid solution. This blue coloured compound is red in acidic solution and on titration with ascorbic acid is oxidised to D- L ascorbic acid.

VITAMIN E

DETERMINATION OF SERUM TOCOPHEROL (BAKER & FRANK, 1968)[13]

Serum tocopherol can be measured by reducing of ferric to ferrous ions by tocopherol which then forms a red complex with α α' dipyridyl. Tocopherol and carotenes are first extracted into xylene and the extinction read at 460 nm to measure the carotenes. A correction is made for these after adding ferric chloride and read at 520 nm.

DETERMINATION OF β CAROTENE IN SERUM USING CARR PRICE REACTION.[14]

Proteins are precipitated with ethanol and the retinol and carotene are extracted into light petroleum. The intensity of yellow colour due to carotenes is measured in colorimeter at 440 nm using light petroleum as blank.

DETERMINATION OF SERUM MDA USING SATOH KEI'S METHOD[15]

Lipoproteins were precipitated from serum by adding 20% trichloroacetic acid, specimen was treated with Thiobarbituric acid in sodium sulphate to form chromogen. This chromogen is allowed to form a pink coloured complex in boiling water bath and extracted with butanol which is then measured at 530 nm filter.

ESTIMATION OF BLOOD GLUTATHIONE (BEUTLER'S METHOD, 1963)[16]

Glutathione (GSH) in the whole blood or red blood cells is maintained in reduced state through reduced nicotinamide adenine dinucleotide phosphate and glutathione reductase. The function of reduced glutathione is to be to keep sulfhydryl groups in their active reduced state and through glutathione peroxidase to remove peroxide. Photometric method adapted by Beutler using 5 5' Dithiobis 2'Bitro benzoic acid (DTNB) was used for the assay of blood glutathione levels. The method is based upon the development of a relatively stable yellow colour when DTNB is added to sulfhydryl compound.

DETERMINATION OF SERUM HORMONES: ESTRADIOL, PROGESTERONE, TESTOSTERONE AND PROLACTIN

Serum separated was used for quantitative determination of hormones estradiol, progesterone and prolactin by Chemiluminescent Microparticle Immunoassay (CMIA) on Ci2000 autoanalyser. The quality control was maintained for the above parameters.

Table 1: Distribution of Total Subjects

Group	Number of cases
Benign breast disease	40
Breast cancer	30
Controls	40
Total	110

Table 2: Table showing mean levels of Several parameters between benign breast disease, breast cancer patients and control group

Group	No. of cases	VITC (mg/l)	VIT E (mg/l)	β CAROTENE (mg/l)	MDA (nmol/ml)	GSH (mg %)
Benign breast disease	40	8.24±1.70	7.79±3.03	1.22±0.82	9.84±2.05	46.09±21.44
Breast cancer	30	6.22±1.13	6.01±2.90	1.10±0.72	14.95±2.07	29.88±10.80
Controls	40	9.52±1.67	10.80±1.41	1.58±0.77	1.35±0.63	52.06±18.69

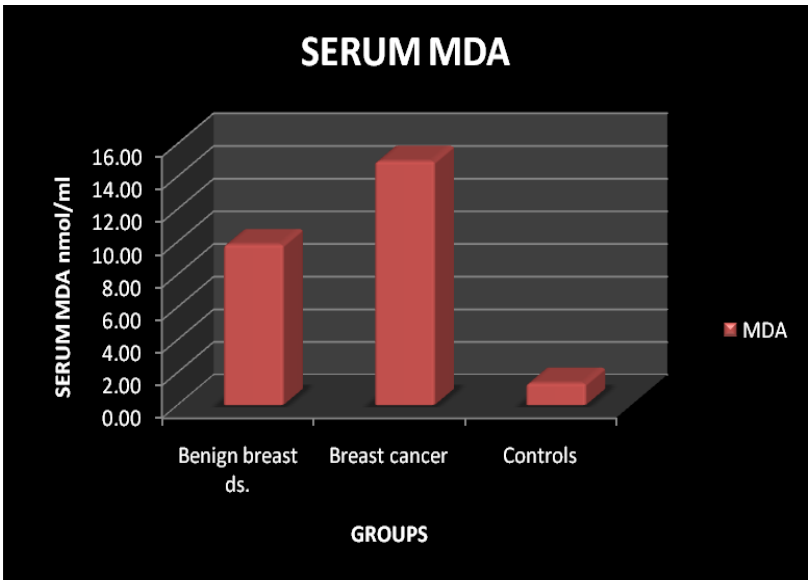

Figure 1: Diagramatic representation of mean serum MDA levels among groups

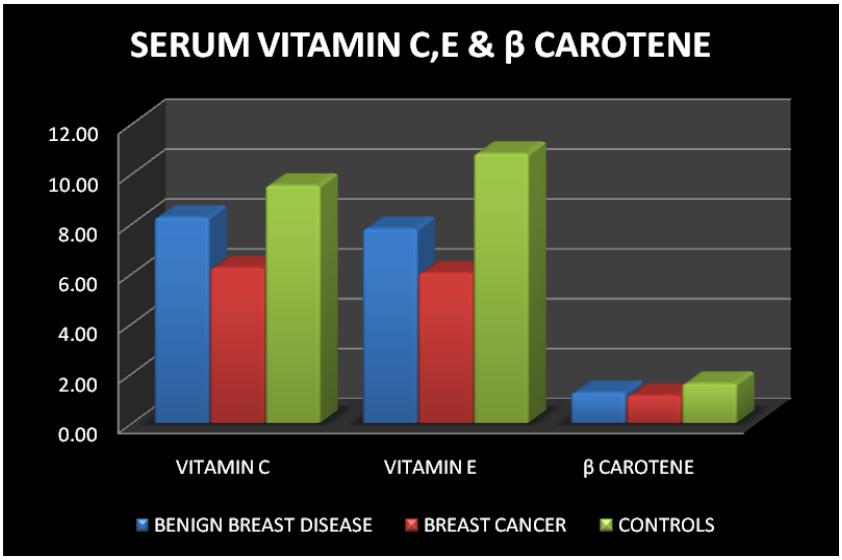

Figure 2: Diagramatic representation of mean serum vitamin C, vitamin E and β carotene among groups.

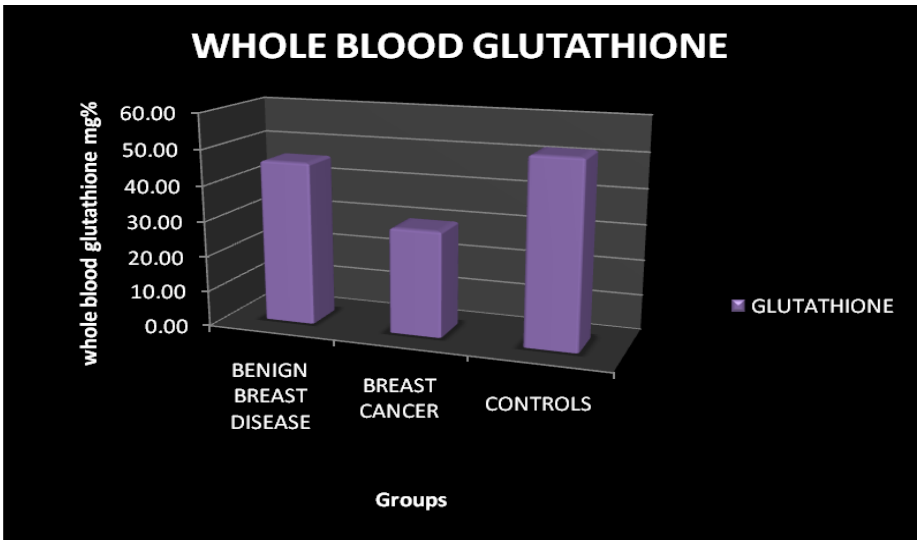

Figure 3: Diagramatic representation of mean levels of whole blood glutathione among groups

Table 3: Serum levels of prolactin , progesterone, testosterone, estradiol and follicle stimulating hormone in benign breast disease patients and controls.

Group	No of cases	PROL (ng/ml)	PROGEST (ng/ml)	TEST (ng/ml)	ESTRADIOL (pg/ml)	FSH (mIU/ml)
Benign breast disease	40	26.57±14.2	3.14±5.73	0.38±0.18	137.5±58.71	7.50±10.27
Pre menopausal controls	20	8.98±7.93	5.63±6.37	0.31±0.11	131.86±52.48	11.91±16.89
Breast cancer	30	19.97±8.21	1.05±2.77	0.92±0.26	40.40±11.13	36.57±30.81
Post menopausal controls	20	21.16±12.3	0.14±0.05	0.37±0.18	16.68±4.41	57.18±38.11

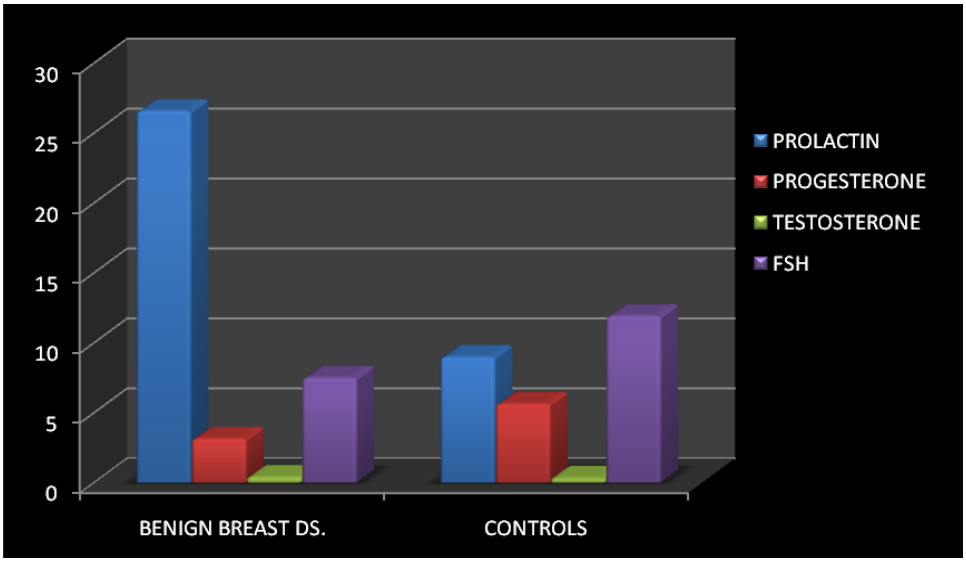

Figure 4: Diagramatic representation of mean hormone levels in benign breast disease and controls.

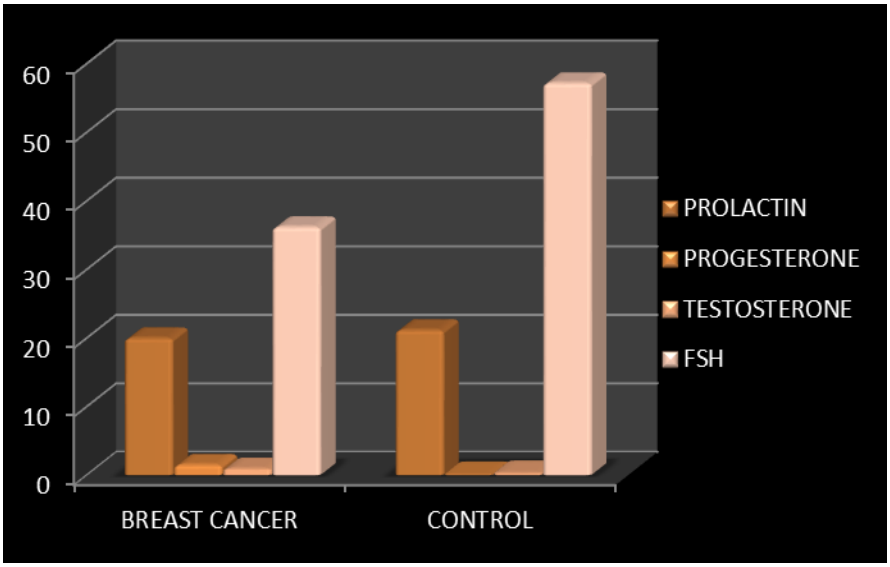

Figure 5: Diagrammatic representation of mean serum hormone levels in breast cancer and controls.

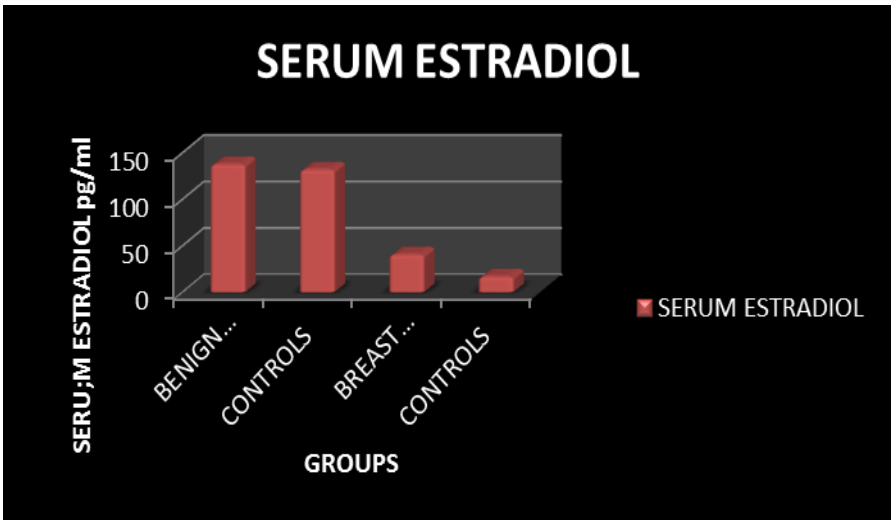

Figure 6: Comparison of mean serum estradiol levels between benign breast disease and breast cancer patients as compared to controls respectively.

RESULTS AND DISCUSSION

In the present study, marker of oxidative stress i.e. MDA, antioxidants such as vitamin E , vitamin C, β carotene, glutathione and hormonal levels were evaluated in patients with benign and malignant breast disease. The average age of breast cancer was 48 years while benign breast disease was 30 years. These figures depict that relatively younger female population were exposed to benign disease and old females were exposed to malignant breast disease. More than half of breast cancer patients were postmenopausal. Histologically almost

all cases of breast cancer were infiltrating ductal carcinoma II, III whereas benign breast disease revealed fibroadenoma in 28 patients.

Increasing epidemiological studies have shown involvement of reactive oxygen species in the development of cancer in human,[17] Low levels of oxygen free radicals have been reported to stimulate cell proliferation whereas high levels induce mutagenicity, cytotoxicity and cell death.[18] MDA is considered as a marker of oxidative stress and is one of the frequently used parameter for measurement of free radical induced cellular damage.. Among the study group, MDA levels were increased in breast cancer and benign breast disease patient as compared to control group,but breast cancer has highest level of serum MDA levels. This difference was statistically significantThe damage induced by lipid peroxidation renders the cell unstable, compromises fluidity,permeability of membranes, signal transduction, nuclear alteration.[19] Thus ROS have role in pathogenesis of breast cancer.

This study was similar to study done be S Khanna et al,[20] which showed increased MDA levels in benign and breast cancer patients. Studies have shown lipid peroxidation increases in plasma in solid tumor.[21] Kumar et al,[22] Hristosov et al.[23] has found significant difference between plasma levels of MDA in breast cancer and controls.

Oxidative stress is defined as the imbalance between pro oxidants and antioxidants in the body. It arises either when there is increased production of reactive oxygen species or there is deficiency of antioxidants in the body. Antioxidants play a role in primary prevention of cancer by reducing oxidative modification of DNA. Various antioxidants which were analysed in the study group are vitamin E, vitamin C, β carotene and glutathione. In this study statistically significant decrease was seen in patients with breast cancer as compared to benign breast disease patients. Studies by Kumaraguruparam et al.[24] Ray G et al.[25] showed decreased vitamin E levels in breast cancer as compared to controls, while Geiber et al have observed increase in plasma Vitamin E especially in young breast cancer patients. Franky D et al.[26] showed lower levels of Vitamin E in both benign and malignant breast disease patients. Vitamin E contributes to anti carcinogenic effect by inhibiting DNA damage by reactive oxygen species.

There was statistically significant decrease in vitamin C levels in breast cancer and benign breast disease patients. Vitamin C levels were lower in breast cancer than benign breast disease patients. This was similar to study by Frank D et al, the odd's ratio in this study

revealed higher quartile with increasing levels of serum Vitamin E, Vitamin C and β carotene were significantly associated with decreased breast cancer risk. Various studies have shown decreased levels of serum Vitamin E and Vitamin C in breast cancer and benign breast disease patients.[27] The mean levels of β carotene showed decrease in breast cancer and benign breast disease patients as compared to controls, but there was no statistical difference between benign and breast cancer patients. Study by Frank D et al showed similar finding and decreased levels of β carotene may be mainly due to its ability to quench singlet oxygen radicals. Ito et al.[28] showed inverse association between serum β carotene and breast cancer risk. Toniolo et al,[29] also documented increased risk of breast cancer for decreased concentration of β carotene.

In this study, statistically significant decrease is seen in glutathione levels in both benign and breast cancer patients as compared to controls. The thiol group of cysteine is responsible for antioxidant activity of glutathione. Vitamin E and Vitamin C both require GSH. Vitamin C spares GSH and together with Vitamin E prevents oxidation of GSH and regeneration of both. Thus antioxidant depletion in plasma may be due to increased scavenging of lipid peroxides by antioxidant as well as sequestration by tumor cells.[30] Thus significant lower levels of antioxidants indicate higher oxidative stress which may be the cause of lipid peroxidation, DNA damage and mutation leading to higher risk of breast cancer.

Hormones,[31] have been implicated as a risk factor for developing breast cancer. Benign breast disease also results from imbalance or inappropriate target gland response to changing tide of hormonal stimulation.[32] In this study serum estradiol showed statistically significant increase in breast cancer patients as compared to controls. There is no significant difference between benign breast disease and control group. This suggests that estradiol levels are increased in breast cancer patients and androgens are the major source of estrogen in postmenopausal females.Experimental evidences shows carcinogenic effect of estradiol is due to its cell proliferate, anti apoptopic function.[33] Clemon M et al.[34] suggest that estradiol is related to initiation and promotion of breast cancer. Thomas et al.[35] Hankinson et al.[36] have shown increased risk of breast cancer with increased level of serum estradiol. Estrogen stimulates mitosis because its metabolite causes direct damage to DNA through formation of free radicals.It is tempting to conclude based on the model that lipid peroxidation or oxidative stress may be one of the mechanisms whereby estrogen increases breast cancer risk.[37] Serum

progesterone levels were within normal range in both breast cancer and benign breast disease patients as compared to controls.

Prolactin plays a central role in breast development, differentiation and lactation. Prolactin is equally implicated in the development of breast cancer. In this study statistically significant increase was seen in serum prolactin levels in benign breast disease as compared to premenopausal control group. There was no statistical significant difference between breast cancer patients and postmenopausal controls. This suggests possible etiological role of prolactin in benign breast disease. Studies done by Nicol et al.[38] Strollo et al,[39] showed hyperprolactemia in benign breast disease patients,it was hypothesised that raised serum prolactin interact with estrogen to give mammary gland hypertrophy. Studies have shown that prolactin levels may be increased in patients with benign breast disease and these patients responded to treatment with antiprolactin bromocriptine.[40] It is suggested that patients with benign breast disease should be screened for hyperprolactinemia. Studies have also shown the possible role of testosterone in the development of breast cancer.[41] Testosterone could influence the risk directly by increased secretion from ovary or by aromatization of androstenedione by aromatase enzyme to estradiol. In this study statistical significant increase was seen in breast cancer patients as compared to control group.this suggest possible role of testosterone in the etiology of breast cancer. The prospective study done by Jane et al .[42] showed direct association between high serum free testosterone levels and breast cancer risk.

CONCLUSION

In the current study, the significant lower levels of plasma antioxidants and increase in lipid peroxide levels strongly suggest higher oxidative stress in breast cancer patients. Antioxidants depletion in plasma may be due to increased scavenging of lipid peroxides by antioxidants as well as sequestration by tumor cells. The present study provides interesting clues to the modifiable etiology of breast cancer to suggest that by advising increased dietary intake of antioxidants such as vitamin E, vitamin C and β carotene in the initial stages of the disease may prevent further oxidative damage in breast cancer patients and also prevent malignant transformation in benign breast disease patients. These results suggest necessity for therapeutic co administration of antioxidants and nutritional supplements along with conventional therapy for treatment of such patients. Serum estradiol levels were increased in breast cancer as compared to control group and benign breast disease patients. Such patients

may respond to anti-estrogenic therapy based on their receptor status. Serum prolactin levels were higher in benign breast disease patients as compared to breast cancer patients and healthy controls. These patients might respond to anti prolactin therapy. Further studies are required to evaluate its significance in the etiology of benign breast disease.

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