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Assessment of in Vivo Oxidative Stress Biomarkers in Relation to Disease Progression and Cell Proliferation in Benign and Malignant Breast Diseases

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Abstract

The present study was aimed to evaluate the levels of oxidative stress markers in breast diseases by measuring the 8-hydoxy-2-deoxyguanosine (8-OHdG), vitamin A, vitamin C, vitamin E and total antioxidant status (TAS) alterations in relation to cell proliferation activity and disease progression. Significant increases in the level of oxidative damage marker 8-OHdG and cell proliferation activity were observed in breast carcinoma patients in comparison to benign and normal controls, which were accompanied by significant decrease in non enzymatic antioxidants and TAS concentrations. 8-OHdG and cell proliferation level were negatively correlated with non enzymatic antioxidants viz., Vitamin A, Vitamin C, vitamin E level and total antioxidant activity. Altered levels of biomarkers of oxidative stress and cell proliferation activity amongst the malignant, benign and controls suggest a correlation of increased oxidative stress and cell proliferation activity in the progression of disease in breast carcinoma patients. Among the oxidative stress markers and cell proliferation index, decreased level of vitamin A, vitamin C, vitamin E, TAS and increased level of 8-OHdG, cell proliferation index emerged as best predicted biomarkers for subjects with malignancy and benign breast disease.

Key words: 8-OHdG (8-hydroxy- 2-deoxyguanosine), vitamin A, vitamin C, vitamin E, ROS (reactive oxygen species), TAS (total antioxidant status);

Introduction

Breast cancer worldwide affects nearly one million women per year [1]. However with better investigation tools, better understanding of tumor biology and increasing public awareness more number of cases are being diagnosed at earlier stage still it accounts for the highest morbidity and mortality in female population. Benign breast diseases are at least ten times more common than breast carcinoma and

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account for 50% of all breast biopsies performed, hence their recognition, management and delineation from malignancy are probably as important as their malignant counterpart [2].

Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) contribute in different ways to carcinogenesis and now considered as a distinctive characteristic of cancer [3]. These species can cause base substitution, deletion, and strand fragmentation which may inactivate tumor suppressor genes or increase expression of proto-oncogenes within cells that are critical initial events in carcinogenesis. ROS can modify many intracellular signaling pathways via growth factor receptors, transcription factors, inducing inflammation/repair, angiogenesis and cell proliferation in the tumor microenvironment [4-8].

An attempt has been made to investigate the alterations in the biochemical parameters of pro-oxidant/antioxidant by using biochemical and cell based assays in the serum of breast disease patients with respect to controls to study *in vivo* DNA damage, non enzymatic antioxidants viz., vitamin A, vitamin C, vitamin E, total antioxidant capacity (TAS) and cell proliferation index to have a better understanding of the contributions of reactive species to breast carcinogenesis and also to identify biomarkers that classify subjects with risk of developing breast cancer.

2. Materials and methods

2.1. Selection of patients and control cases

For the present case control study, histo-pathologically confirmed 60 cases of benign breast disease and 60 consecutive female patients of breast carcinoma from the Department of General Surgery, University Hospital, Banaras Hindu University, Varanasi were involved. Benign breast disease patients namely fibroadenoma, fibrocystic disease, breast abscess and ductectasia were included in the study. The patients underwent through clinical breast examination, fine needle aspiration cytology and biopsy. Patients with breast cancer were classified using the TNM system [8]. Also 60 age and sex matched healthy volunteers having socio-economic status similar to that of patients served as controls. Specific exclusion criteria considered for the present study were the healthy controls having no acute or chronic diseases such as diabetes, parasitosis, immune dysfunction or any other malignancy and they were not under any pharmacological therapy. None of the study subjects were under oral contraceptives, hormonal therapy or antioxidant supplementation. The study protocol was approved by the ethical committee of the Institute of Medical Sciences, Banaras Hindu University. Informed consent from all participants was obtained for further analysis.

2.2. Analytical methods

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All biochemical and hematological investigations were done in serum collected in sterile tubes, from venous blood of patients and healthy volunteers. Collected serum was stored at -20^{0}C for further examination.

2.2.1. Reagents

All reagents used in this study were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO) and Merck (Darmstadt, Germany).

2.2. 2.Estimation of 8-hydroxy-2-deoxy guanosine

Serum from the cases and control samples was used for the measurement of 8-OHdG levels using competitive *in-vitro* enzyme-linked immunosorbent assay (ELISA) kit obtained from Cayman Chemical Company (Ann Arbor, MI, USA) [10].

Assay of Non- Enzymatic antioxidants

Vitamin A: Vitamin A level in serum of the patients and controls was assayed by using the colorimetric method of Paterson and Wiggins [11].

Vitamin E: Vitamin E level in the serum was assayed by using the colorimetric method of Quaife *et. al* [12].The method is based on the Emmeric-Engel reaction which is based on the reduction by tocopherols of ferric to ferrous ions which then form a red complex with α,α'-dipyridyl.

Vitamin C: Vitamin C level in serum was assayed by using the colorimetric method of McMurray and Gowenlock [13].

2.2. 5.Assay of Total antioxidant status

The Total antioxidant status (TAS) in serum was determined by using Cayman's assay kit (Ann Arbor, MI, USA) [14].

2.2.6. Cell proliferation assay

The cell proliferation index was assayed by Cell Proliferation ELISA, BrdU kit (Roche Diagnostics, GmbH, Penzberg, Germany) and experiment was performed according to the manufacturer's protocol [15].

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2.3. Statistical analysis

The statistical analysis was conducted with the commercial SPSS 16.0 package for Windows (SPSS, Inc., Chicago, IL). All values were expressed as the mean ± standard error of the mean (SEM). Statistical significance was assessed by using Student's t-test (for 2-group comparison). Categorical variables were presented as absolute numbers (frequency percentages) and analyzed by Chi-square test. All statistical analyses were 2-tailed and p value < 0.05 was considered statistically significant. Correlation between levels of oxidative stress biomarkers and cell proliferation status was evaluated with Pearson's correlation coefficient. Odds ratios (ORs) and 95% confidence intervals (CIs) for breast cancer risk in relation to marker of oxidative damage, non enzymatic antioxidant, TAS and cell proliferation index were estimated by using a multinomial logistic regression analysis.

3. Results

A case control study was conducted on the 60 histo-pathologically confirmed cases of breast carcinoma and 60 cases of benign breast disease along with the 60 age- and sex-matched healthy volunteers. The present study was conducted to assess 8OH-dG, non enzymatic antioxidant viz., Vit A, Vit E, Vit C and total antioxidant status as biomarkers for oxidative stress, and cell proliferation activity in study subjects and controls.

3.1. Clinical symptoms of breast disease patients

Major clinical symptoms of carcinoma breast and benign breast patients are depicted in Table 1. Amongst the study subjects 90% (n=54) of benign breast disease patients were having fibroadenoma while 10% had other benign breast diseases such as fibrocystic disease (5%), duct ectasia (3.3%) and breast abscess (1.7%). Among the main symptoms of carcinoma breast and benign breast all the patients showed mixed symptoms (Table 1). The masses were all unilateral and equally distributed in the left and right breast.

Table 1: **Clinical symptoms of carcinoma breast and benign breast patients**

Clinical symptoms	Carcinoma Breast (n=60)	Benign Breast (n=60)
Lump		
No	8 (13.33%)	6(10%)
Yes	52(86.67%)	54(90%)
Ulceration of skin/nipple		
No	45 (75%)	46(76.7)
Yes	15 (25%)	14(23.3%)

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Breast pain No Yes	13 (21.67%) 47 (78.33%)	12(20%) 48(80%)
Nipple discharge No Yes	55(91.67%) 5 (8.33%)	58(96.7%) 2(3.3%)
Mass in axilla No Yes	56(93.33%) 4(6.67%)	57(95%) 3(5%)

3.2. Major clinical characteristics from patients and controls are depicted in Table 2. Results of chi-square test depicted that there were no significant differences between the studied groups in terms of age, menopausal status, parity, residence and diet.

Table 2: Patient Characteristics

Parameters	Malignant (n= 60)	Benign (n= 60)	Control (n= 60)	χ^2 value	p value
Age					
≤45 yrs	37(61.67%)	41(68.33%)	40(66.67%)	0.640	0.726*
>45 yrs	23(38.33%)	19(31.67%)	20(33.33%)		
Menopausal status					
Pre-menopausal	21(35%)	15(25%)	18(30%)	1.429	0.490*
Post-menopausal	39 (65%)	45(75%)	42 (70%)		
Parity					
≤3	46(76.67%)	48(80%)	48(80%)	0.265	0.875*
>3	14(23.33%)	12(20%)	12(20%)		
Residence					
Urban	10(16.67%)	10(16.67%)	11(18.33%)	0.078	0.962*
Rural	50(83.33%)	50(83.33%)	49(81.67%)		
Diet					
Vegetarian	52 (86.67%)	48(80%)	51(85%)	1.069	0.586*
Non-vegetarian	8(13.33%)	12(20%)	9(15%)		

Categorical variables were presented as absolute numbers (frequency percentages) and analyzed by Chi-square test. P <0.05 was considered statistically significant.

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3.3. Level of Oxidative stress markers and cell proliferation index in cases (benign and malignant breast disease) and controls

The mean ± SEM value of 8-OHdG and cell proliferation index for those with malignant tumor was significantly higher than that for those with benign and the normal controls [Table 3]. The increase in oxidative damage and cell proliferation activity of malignant group compared to benign and control group was accompanied by diminished antioxidant protection. However a similar and significant ($p<0.05$) pattern of changes were seen in benign group patients as compared to their corresponding control subjects.

Table 3: Levels of non- enzymatic antioxidants viz. Vitamin A, Vitamin C and Vitamin E in carcinoma breast patients, benign breast patients and controls.

Parameter	Serum value			p value		
	Malignant Group I	Benign Group II	Control Group III	I/II	I/III	II/III
8-OHdG (pg/ml)	432.14±15.64	242.188±5.980	222.934±6.430	p=0.000	p=0.000	p=0.037
Vit A (µg/dL)	55.567±2.906	77.687±6.786	99.628±8.57	p=0.003	p=0.000	p=0.047
VitC (mg/dL)	1.654± 0.076	3.233± 0.676	5.412±0.741	p=0.022	p=0.000	p=0.032
Vit E (mg/L)	10.851±0.621	15.298± 0.563	17.754± 0.975	p=0.000	p=0.000	p=0.031
Cell proliferation index (ng/ml)	1.377±0.066	0.882±0.046	0.735±0.025	p= 0.000	p= 0.000	p=0.006

**Data are presented as mean ± S.E.M. Statistical analysis was done by independent Student t- Test. p value <0.05 was considered statistically significant.*

3.4. Levels of oxidative stress markers and cell proliferation index in breast carcinoma patient in relation to their pathological stages

Levels of Oxidative damage markers 8 OHdG and cell proliferation activity level were found to increase significantly with the progression of the disease while vitamin A, vitamin C, vitamin E and total antioxidant levels significantly decreased with disease progression [Table 4]. A significant association was observed among serum cell proliferation index and levels of oxidative stress biomarkers on the basis of clinico-pathologic stages where half of the breast carcinoma patients were in stage I/II, 50% had tumour with diameter between 2 to 5 cm and an equal no. of cases had tumour with diameter more than 5 cm, 39 patients have clinically palpable lymph node and 10% of patients were having distant metastasis.

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But serum level of vitamin A, vitamin E and total antioxidant levels were not associated significantly with the histology of breast carcinoma where 81.67% (n= 49) of the carcinoma patients were having ductal carcinoma while 18.33% (n=11) had lobular carcinoma and medullary carcinoma.

Table 4: Levels of non-enzymatic antioxidant in carcinoma breast patients in relation to their clinico -pathological stage.

	8-OHdG (pg/ml)	Vit A (µg/dL)	Vit C (mg/dL)	Vit E (mg/L)	TAS (mmol/L)	Cell proliferation (ng/ml)
Stages						
I/II (n=30)	339.898±7.772	66.202±3.999	2.020±0.083	13.647±0.723	0.234±0.010	1.000±0.039
III/IV(n=30)	511.970±19.503	47.679±3.351	1.250±0.087	7.821±0.651	0.139±0.010	1.775±0.079
p value	0.000	0.001	0.000	0.000	0.000	0.000
Tumour extension						
1-2 (n=30)	346.085±8.476	65.818±4.077	1.969±0.091	12.894±0.829	0.241±0.011	1.056±0.057
3-4 (n=30)	513.647±21.018	43.001±4.199	1.300±0.093	8.574±0.723	0.141±0.012	1.719±0.085
p value	0.000	0.000	0.000	0.000	0.000	0.000
Lymph node metastasis						
0 (n=39)	352.665±15.255	69.076±4.739	2.083±0.105	13.265±0.812	0.206±0.014	1.040±0.069
1(n=21)	469.309±20.070	50.437±3.142	1.393±0.083	9.551±0.779	0.156±0.010	1.558±0.082
p value	0.000	0.001	0.000	0.003	0.010	0.000
Distant Metastasis						
0(n=54)	402.756±13.525	59.234±3.005	1.730±0.075	11.384±0.617	0.189±0.009	1.290±0.056
1 (n=6)	634.537±21.582	36.302±2.148	0.776±0.074	4.881±0.666	0.085±0.004	2.249±0.246
p value	0.000	0.015	0.000	0.001	0.001	0.000
Histology						
Ductal(n=49)	447.112±18.007	63.718±6.966	2.077±0.094	13.227±1.067	0.241±0.016	1.449±0.076
Others(11)	353.587±15.257	55.664±3.118	1.535±0.087	10.331±0.705	0.189±0.012	1.050±0.087
p value	0.019	0.278*	0.006	0.071*	0.067*	0.020

**not significant*

Data are presented as mean ± S.E.M. Statistical analysis was done by independent Student t- Test. p value <0.05 was considered statistically significant.

3.5. Interrelationship between cell proliferation activity and oxidative stress markers

Correlation analysis revealed a significant association between all examined indices. Significantly negative correlation of TAS and non enzymatic antioxidants was found with 8-OHdG and cell proliferation index. However a significantly positive correlation was found between 8-OHdG, MDA and cell proliferation level.

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Table 5: Interrelationship between cell proliferation activity and oxidative stress markers

	Cell Proliferation	8-OHdG
8-OHdG	r = 0.529 (p=0.000)	1
Vit A	r=-0.517 (p=0.001)	r=-0.249 p=0.055
Vit C	r =-0.369 (p=0.019)	r=-0.634 p=.000
Vit E	r = 0-.556 (p=0.000)	r=-0.603 p=0.000
TAS	r=-0.727 (p=0.000)	r=-0.536 p=0.000

p value <0.05 was considered statistically significant.

3.6. Level of 8-OHdG, non enzymatic antioxidants and cell proliferation index and assessment of risk of breast cancer

We used multinomial logistic regression analysis to identify biomarkers that classify subjects with risk of developing breast cancer. Table 6 shows the results of the multinomial logistic regression analyses, performed to evaluate the adjusted odd ratios of oxidative stress biomarkers, trace elements and cell proliferation index in malignant benign and control groups. Among the oxidative stress markers and cell proliferation index, decreased level of non enzymatic profile, TAS and increased level of MDA, cell proliferation index emerged as best predicted biomarkers for subjects with malignancy and benign breast disease. Similar results were observed for subjects with malignancy and controls *i.e.* for group B.

Table 6: Multinomial regression analysis of cell proliferation, Oxidative stress markers, in relation to carcinoma breast risk

Odds ratio and 95% CI for oxidative stress markers and cell proliferation index for subjects:

(A) With malignancy and without any breast disease and

(B) With malignancy and benign breast disease

	With malignancy and without any breast disease (A)		With malignancy and benign breast disease (B)	
8-OHdG	22.208 (3.33-1.482)	0.000	150.737 (23.472-968)	0.000
Vit A	0.988 (0.980-0.997)	0.025	0.981 (0.973-0.990)	0.000
Vit C	0.794 (0.679-0.928)	0.034	0.720 (0.617-0.842)	0.000
Vit E	0.776 (0.697-0.864)	0.000	0.727 (0.651-0.813)	0.000

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TAS	1.59 (1.79-1.41)	0.000	1.32 (1.57-1.39	0.000
Cell proliferation	20.784 (5.907-73.136)	0.000	199.698 (34.559-1153.950)	0.000

**Not significant. p value <0.05 was considered statistically significant.*

4. Discussion

ROS- induced DNA damage can result in single or double strand breakage, base modifications and DNA cross-linking which induce cell death, replication errors, inhibition of apoptosis, increased proliferation, angiogenesis and genomic instability resulting in onset of variety of diseases including breast cancer [16, 19]. The most abundant oxidative DNA lesion produced is 8-hydroxydeoxy guanosine (8-OHdG) and measurement of its level may be applied to evaluate the load of oxidative stress that could be important to understand the role of oxidative stress in breast cancer development and disease intervention. In the present study, we have observed a significantly higher serum 8-OHdG levels in the cancer patients in comparison to benign and controls. Our previous studies also reported an elevated level of this oxidatively modified biomolecule in breast carcinogenesis and benign breast diseases and might be involved in the pathophysiology of breast cancer formation [19-20]. Our study also indicates the increased level of 8-OHdG in breast cancer patients was associated with the disease progression and advanced stage of carcinogenesis [20-21].

Free radical generation is controlled by a large number of antioxidant systems that act as protection against free radicals. In the present study, significant reduction in total antioxidant status and non enzymatic antioxidant profile in malignant group in comparison to benign and control suggest increased consumption of serum antioxidants in response to enhanced oxidants production in malignant group patients. A similar and significant ($p< 0.05$) pattern of changes was followed by benign group patients as compared to their corresponding control subjects. Total antioxidant activity is a measure of scavenging capacity of the cells defense system. Depleted level of antioxidant defence mechanism has been documented in a wide variety of malignancies including breast cancer [22-25].

Vitamin A, well known as an important natural antioxidant, plays an important role in cellular function, development, and maintenance of normal visual acuity [26]. It acts with vitamin C and vitamin E in order to protect cells against ROS [27]. Vitamin C, ascorbic acid, is a water-soluble chain-breaking antioxidant [27] which strongly inhibits lipid peroxidation, oxidation of glutathione and other enzymes [28, 29]. It can react directly with superoxide, hydroxyl radicals and singlet oxygen and is important in recycling the tocopherol radical of vitamin E to an active reduced state [30]. Vitamin E is the major lipid-soluble antioxidant present in lipid membranes and human plasma lipoproteins [31]. It exists in 8 different

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isoforms, of which alpha-tocopherol is biologically the most important. Vitamin E is a strong inhibitor of apoptosis and a stabilizer of biological membranes and is known to act on all steps of membrane oxidative damage [32]. Alpha-tocopherol also functions in vivo as a strong protector against lipid peroxidation and also blocks nitrosamine formation [30, 33]. In our study a strong negative correlation was found between increased level of oxidative stress and depleted level of non-enzymatic antioxidants, which results in redox imbalance and may be associated with advancement of breast diseases.

Proliferation rates can provide useful information on prognosis and aggressiveness of individual cancers and can be used to guide treatment protocols in clinical practice. Carcinogenesis is associated with various epigenetic mechanisms which can alter cellular communication and gene expression effecting cell proliferation, differentiation and apoptosis. Increased proliferation correlates strongly with poor prognosis. In our study also the development and continued growth of breast cancer involves altered rates of cell proliferation index. 8-OHdG have been suggested as modulators of signal pathways related to cell proliferation and apoptosis that leads to breast cancer initiation and progression[34]. Yano et al also found that vitamin E suppresses lung tumorigenesis by inhibiting cell proliferation at the promotion stage [35]. Phenobarbital (PB) activates NF-κB and dietary vitamin E is effective in decreasing PB-induced NF-κB DNA binding. It showed that dietary vitamin E influences PB-induced changes in cell proliferation and apoptosis through its action on NF-κB [36]. Vitamin C can inhibit the proliferation of A549 cells (adenocarcinomic human alveolar basal epithelial cells) in G0/G1 and S phase, and induce apoptosis of A549 cells [37]. Vitamin A intake has a complex relationship with cancer cell proliferation while small doses of vitamin A or beta-carotene appear prevent cancer, higher doses seem to have the reverse effect. The anti-cancer effects of beta-carotene appear from its anti-oxidative ability to scavenge for reactive oxygen species, as well as through its conversion to vitamin A, which can improve immune function in addition to eliciting an anti-proliferative effect through the RAR (retinoid acid receptor) and RXR retinoid X receptor) receptors, thereby acting to block certain carcinogenic processes and inhibit tumour cell growth. Our study also observed a significant positive correlation between levels of 8-OHdG and cell proliferation activity. Diminished levels of antioxidants showed a significant negative correlation with cell proliferation activity which are in agreement with previous studies where antioxidants were found to be inhibit modulation of gene expression and cell proliferation [23,38].

Multinomial Logistic Regression used to identify the contribution of the selected biomarkers using odds ratio and associated confidence interval reflects subjects with increased levels of oxidative DNA damage and cell proliferation activity or reduced level of antioxidant defense might be at higher risk of developing breast cancer.

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Taken together, our results reveal an alteration in the redox homeostasis in benign and malignant breast disease patients. The oxidative damage markers appear to be an important component in the complex pathophysiology of the breast cancer and likely to be associated with progression of breast diseases. Present study not only establishes the role of these parameters in breast carcinoma but also provide evidences of altered level of these indices/profiles in benign breast disease patients placing them more vulnerable to breast cancer development. Despite the exciting advances in the field of free radical research, such applications, however, needs to be further investigated on a larger sample size and follow-up cases. Besides that involvement of oxidative stress in the pathogenesis of plethora of human disorders including various malignancies limits its usefulness as a screening tool. One of the various challenges in this field is how oxidative stress induced cancer-related signaling pathways be targeted for drug development.

5. Conclusion

These findings suggest an important role of intermittent oxidative damage in the initiation and development of malignant and benign breast disease and demonstrate an important role of oxidant-antioxidant status in mediating this process. Present study not only established the role of these parameters in malignancy, also provided insight into the altered level of parameter profiles in benign breast disease patients placing them in a "high-risk" category. Thus, determination of markers of oxidative stress, inflammation and cell proliferation index may be useful not only in evaluating patients with benign and malignant tumours of the breast but also in establishing pathogenic stages of breast cancer. Therefore, these tests should be employed to inform the design and outcome measures of clinical trials.

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7. Conflict of Interest

None of the author has any financial or personal relationship with organizations that could potentially be perceived as influencing the described research .All authors have read the journal's policy on disclosure of potential conflicts of interest.

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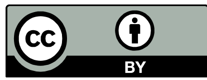

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