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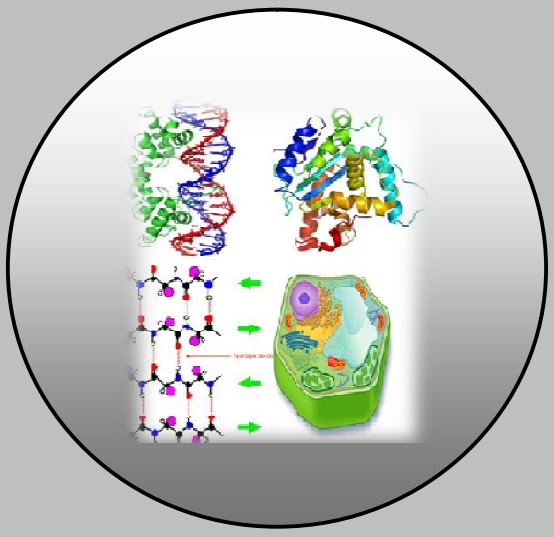
ISSN 0970-4973 (Print)
ISSN 2319-3077 (Online/Electronic)



J. Biol. Chem. Research
Volume 30 (2) 2013 Pages No. 724-733

Journal of Biological and Chemical Research
(An International Journal of Life Sciences and Chemistry)

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 30, No. 2: 724-733 (2013)
(An International Journal of Life Sciences and Chemistry)

ISSN 0970-4973 (Print)
ISSN 2319-3077 (Online/Electronic)
Published by Society for Advancement of Science®

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RESEARCH PAPER

Received: 20/06/2013 Revised: 22/08/2013 Accepted: 29/08/2013

Free Radical Induced Oxidative Stress in Cervical Dysplasia

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ABSTRACT

The present study was aimed to manage the disease far before time and delineate the status of free radicals and its scavenging enzymes in cervical dysplasia as compared to normal female. This study included 35 patients who attended OPD of Department of Obstetrics and Gynaecology, GSVM Medical College, Kanpur. Out of those patients 14 were having no disease, and were constituting control group while 21 having cervical dysplasia grade I-II, diagnosed colposcopically and histopathologically. Their blood was collected for estimation of malonaldhyde and for assessment of levels of superoxide dismutase and catalase. Study was done in collaboration with.the Department of Biochemistry, King George's Medical College, Lucknow. The levels of malonaldehyde were found to be significantly higher (P< .001) in test group than control. Levels of enzymes superoxide dismutase (P< .05) and catalase (P< .001) were found to be low in test group than in control group .The study patients showed maximum incidence of cervical intraepithelial neoplasia grade -1 in 3rd decade of life (33.3%) and more in multiparous females (66.6%). Correlation of Hb with MDA showed raised levels of MDA in anaemic patients (9.39± 0.15) as compared to non anaemic patients. It is established beyond doubt that free radicals in tissues and cells can damage DNA, proteins andcarbohydrates and proper balance between free radical and antioxidants are essential for the health. Present study is to generate ground information on role of free radical in etiology of cervical dysplasia was undertaken. It showed high level of MDA in case group which means greater exchange of end products of lipid peroxidation between dysplastic tissue and general circulation .Low levels of superoxide dismutase and Catalase enzymes in patients confirm disturbed oxidant/antioxidant balance in genital dysplastic lesion and also support that nutrition has a great role and its deficiency may aggravate free radical damage

Key words: Free Radical, Dysplasia, Antioxidant and Anemia.

INTRODUCTION

The oxygen *paradox* underpins the biology of the whole free radical system. The role of free radicals can be traced back to the origin of life on earth. About 3-5 billion years ago the basic chemical components of life were produced by free radicals reaction with the help of solar radiation. Free radicals are *chemical species* with an unpaired electron in the outer most orbital. The unpaired electron makes them *paramagnetic* and relatively reactive. It is now widely accepted that the degree of cellular damage produced is determined by the balance between the rate at which the reactive oxygen species are produced and the rate of removal which in turn depends upon the activity of total *antioxidant* defense mechanism (the antioxidant enzymes SOD, Catalase, Glutathione peroxidase etc.). At present reactive oxygen species and lipid peroxidation have been implicated in the pathogenesis of large number of diseases. There have been reports of study of free radical injury in relation to oral cancer and colon cancer but literature is scanty on study of free radical injury in relation to *genital cancer*. It is believed that the natural history of cervical cancer starts as CIN-I and passes through stages of moderate and the severe dysplasia referred to as CIN-II and CIN-III and then carcinoma in situ. The current hypothesis of natural history of cervical cancer postulates that unknown carcinogens as well as exogenous stimuli act over extended period of time frame. If action of these stimuli could be prevented, progression of neoplasia could be stopped. With the aim to manage the disease far before time and to establish a correlation, or to critically evaluate indirect evidence of free radical injury to epithelial tissue in cervical cancer, this study has been planned. In this present study *Oxidative stress* is evaluated by: (1) measuring the generation of free radicals or their effect on cellular membranes particularly the end product of lipid peroxidation such as *malonaldehyde* and (2) measuring the levels of scavenging enzymes namely superoxide dismutase and catalase. Present work is aimed to establish a co-relation between status of antioxidant enzymes, lipid peroxidation product and severity of cervical dysplasia.

MATERIAL AND METHODS

Aims of study: To see co-relation between the free radical induced oxidative stress and cervical dysplasia.

Present work on role of the free radical induced oxidative stress in cervical dysplasia was carried out in the Department of Pathology, G.S.V.M Medical College, Kanpur in collaboration with the Department of Bio chemistry, K.G.M.C, Lucknow and Department of obstetrics/gynecology, G.S.V.M Medical College, Kanpur .

The study included 35 patients who attended outpatient department out of whom 14 were without any disease and 21 were having cervical dysplasia grade I –II

The study included 2 groups:

The control group - Patients more than 20 years of age and having 2 or more living children and having no inflammatory lesion and dysplasia of cervix

The test groups

- patients more than 20 yrs of age
- With two or more living children
- With only one sexual partner
- Number of coitus 3 or more per month
-Having dysplasia Grade-I-II diagnosed by colposcopically guided biopsy.

Exclusion criteria

In study group the illnesses like anaemia, diabetes mellitus, essential hypertension, renal insufficiency and cardiovascular disease which themselves are known to alter free radial status were excluded from the study.

History:

History of paitents included age parity, occupation, presenting complaints- discharge, chronic PID, exposure to carcinogens, menstrual history, obstetrical history, and socio economic history, use of contraceptive and urinary complaints.

Examination

General: - Pulse
B.P
Pallor
Icterus

Local examination: - inspection of genetalia for any obvious lesion

Per speculum- for cervicitis, vaginitis, erosion

Colposcopic Examination of Cervix (atypical findings which indicated cervical dysplasia)

- looked for acetowhite area
- atypical vascular pattern
- Leukoplakia
- Mosaic or punctuation pattern of small blood vessels in cervical epithelium

Other investigations:

Blood - - Hb
- PCV

Enzyme investigation:

- Lipid peroxidation product (MDA)
- Superoxide dismutase
- Catalase

Biopsy of lesion present in cervix was also done to know the type of lesion and grading of cervical dysplasia by means of histopathololgical examination.

Specific Investigation: Enzymes analysis:-

Collection of blood for enzymes analysis

Blood was collected with informed consent of all patients. It was taken from both control as well as study groups for determination of MDA and for assessment of antioxidant status by determining levels of SOD and catalase. Plasma thus obtained was used for the estimation of lipid peroxidation.

Packed RBCs were washed with cold isotonic saline (0.9% NaCl) which was added to the packed RBCs and mixed by inverting the tube gently. The tubes were chilled with 0^0 C for 1 hour and then centrifuged for 10 min. at 200 r p m .The process was repeated twice more to ensure proper washing.

The RBCs were ruptured by adding thrice their volume of water to the packed mass. The resulting hemolysate was centrifuged at 1000 x g for 20 min. To remove the ghost cells or RBCs membranes. Supernatant was then diluted suitably with water for the assay of catalase and superoxide dismutase.

All the reagents used were purchased from sigma Co. Ltd. St. Louis, USA and were purest analytical grade.

Precautions:

1. Haemolysed blood was not used for estimation.
2. For all laboratory procedures high quality plastic ware and only siliconised glassware were used. Ordinary glassware was totally avoided for all estimation.
3. Centrifugation of samples was done at desired temperature and speed for the required period only.

ESTIMATION OF LEVELS OF LIPID PEROXIDES / MALONALDEHYDE (MDA)

MDA is the most abundant individual aldehyde resulting from lipid peroxidation **(tappel, 1973)** and its determination by thiobarbitaric acid (TBA) is the most common method of estimating lipid peroxidation .

Principle

In the TBA test reaction one molecule of malonaldehyde reacts with two molecules of thiobarbuturic acid with the production of pink pigment having an absorption maximum at 532 – 535 nm.

Unit of malonaldehyde

Levels of peroxidation products were expressed as the amount of MDA per ml of plasma and were calculated using an extinction coefficient value of 1, 53, 000 for MDA –TBA adduct.

Activity of Superoxide dismutase (SOD)

SOD was determined by using the spectro-photometric method (**Misra and Fridovich, 1972).**

Indirect assay method for estimation of SOD activity is generally used because the substrate for its enzymatic activity is a free radical in nature (superoxide anion) which is generated by some mechanisms and is allowed to react with a detector molecule SOD. Removal o2 inhibits the reaction with the detector molecule thus decreases the color intensity.

Principle

Auto-oxidation of epinephrine in solution at alkaline pH values produces 02**.** O2 once formed participates in the oxidation of further molecules in a chain reaction to give rise to adrenochrome. Adrenochrome exhibits an absorption maximum at 480 nm. The addition of SOD greatly slows down the rate of oxidation of epinephrine because of the simultaneous and rapid utilization of O2 by SOD. The retardation in the production of adrenochrome from epinephrine in the presence of SOD provides a very convenient method for the assay of enzymes. Here epinephrine acts both as producer of O2 as well as the detector molecule. The rate of oxidation of epinephrine to adrenochrome is measured by the change in absorbance at 480 nm.

Unit of superoxide dismutase activity

The unit of enzyme activity is defined here as the amount of enzyme required inhibiting the rate of auto –oxidation of 5mu moles of epinephrine by 50% under the condition of the experiment.

ASSAY OF CATALASE

Catalase was assayed at 25 degree centigrade by modification of spectrophotometric method of **Beers and Sizer (1952**). The uncontrolled production of superoxide anion and hydrogen peroxide (H_2 O_2) is associated with erythrocyte hemolysis. The accumulation of H_2O_2 in cells is controlled mainly by two enzymes namely catalase and peroxidase.

Principle

The maximum absorption of H_2O_2 is at 240nm. H_2O_2 decomposes to water and oxygen by the catalytic action of catalase .On decomposition the absorption at 240nm decreases with time and these decreases is used as a measure of enzyme activity.

Unit of the catalase activity

One unit of catalase is defined here as the amount of enzyme required for the decomposition of one micro mole of hydrogen peroxide in one minute under the experimental conditions Specific activity was expressed as units per mg protein.

STATISTICAL ANALYSIS

Results were presented as mean SD. The statistical significance of observed difference between control and study group was determined by student "t"test devised by W.Scorset.

P > 0.05 -- not significant

P value between 0.05 and 0.01--- significant

P < 0.001 --- highly significant

RESULTS

The present study is conducted on 35 females consisting of 21 cases of CIN grade 1 diagnosed colposcopically and histologically. Amongst them 14 were constituting control group who don't have any dysplasic lesion in cervix.

Table 1. AGE DISTRIBUTION OF STUDY PATIENTS.

Age group	Test		Control	
	No	%age	No	%age
20-25	4	19.05	4	28.57
25-30	4	19.05	2	14.28
30-35	6	28.57	3	21.42
35-40	7	33.30	5	35.71

Out of 21 cases, 7 cases are in age group of 35-40 (33.3%) where as only 4 patients are in range of 20-25 (19.0 %)

Table 2. PERCENTAGE DISTRIBUTION OF SOCIOECONOMIC STATUS IN STUDY GROUP.

SOCIOECONOMIC GROUP	TEST GROUP	%AGE
Low income group	18	85.71
Middle income group	3	14.28

P< 0 .001

In present study maximum incidence of CIN-1 is in low socioeconomic status. There is highly significant difference (P<.001) in percentage distribution among two groups.

Table 3. PERCENTAGE DISTRIBUTION OF PARITY IN THE STUDY GROUPS.

PARITY	NUMBER	PERCENTAGE
P 0	0	--
P 1 – 2	7	33.3
P > 3	14	66.6

The incidence of cervical dysplasia is more in multiparous female patients 33.3% of cases belong to women with one or two children and 66.6% cases of CIN gr 1 found in female with children more than three. While no cervical lesion was found in nulliparous women

Table 4. MDA LEVELS IN STUDY PATIENTS.

PATIENTS	NO	RANGE	MEAN with SD
			Nmoles /ml of plasma
Test	21	6.62--------10.26	8.31±1.25
Control	14	3.23--------5.43	4.31± 0.95

T = 10.25 P<0.001

MDA value is raised in cases as compared to mean value of MDA in case and is 8.31±1.25 Nmoles/ ml of plasma whereas in control, it is 4.31± 0.95 Nmoles/ml of plasma. The difference is highly significant (P< 0.001).

Table 5. SOD LEVEL IN STUDY PATIENTS.

PATIENTS	NO	RANGE	MEAN with SD
		Unit/mg of protein	Unit/mg of protein
Test	21	0.172 ----0.375	0.319±0.78
Control	14	0.524-----0.842	0.723±0.12

t =2.0 P< 0.05

The superoxide dismutase value is low in test group as compared to control group. The mean value of superoxide dismutase in test is 0.319± 0.78 unit/mg of protein whereas in control group. It is 0 .723±0.12 unit/mg of protein. The difference is significant (P <0 .05).

Table 6. CATALASE LEVEL IN STUDY PATIENTS.

PATIENTS	**NO**	**RANGE**	**MEAN with SD**
		Unit/mg of protein	Unit/mg of protein
Test	21	0.295 ----0.369	0.323±0.03
Control	14	0.058-----0.162	0.098±0.02

t =22.46 P<0.001

The catalase level in test group is very much low as compared to control group. The mean value in test group is 0.323±.03 unit/mg of protein whereas in control group, it is 0.098 ±0 .02 unit/mg of protein. The difference is highly significant. (P < 0 .001).

Table 7. CORRELATION OF HB% TO FREE RADICAL LEVELS.

HB OF TEST GROUP MDA LEVELS IN STUDY PATIENT

< 8 gm%	**9.39± 0.15 Nmol/ml of plasma**
8.1---9 **gm%**	7.51±0.16 **Nmol/ml of plasma**
9.1---10 **gm%**	6.43±0.13 **Nmol/ml of plasma**
> 10 gm%	**5.42± .007 Nmol/ml of plasma**

The present study shows higher MDA levels in patients with Hb % less than 8 gm (9.39 ± 0.15) as compared to non-anaemic patient.

DISCUSSION

Free radicals interactions have been implicated in large number of disease states which include inflammation, infection and cancer **(Maeda, 1998)**. Deleterious effects of these free radicals are counteracted by antioxidants such as superoxide dismutase (SOD), Glutathione per oxidase (GPX). **(Snezzana et al 2006)**. Studies indicate that the level of these antioxidants in the body decrease in case of carcinogenesis. The levels of vitamin E were found to vary in a study of cervical carcinogenesis (**Khanna et al 2002)**. The relationship of individual free radical species to the biomolecular and tissue injury may not only aid in the understanding of the processes in disease states but also help in their control.

The present work is aimed to delineate the status of free radical and its scavenging enzymes. The membranes of mammalian cell contain large amount of **polyunsaturated** fatty acids **(Rouser et al., 1968)** which can undergo peroxidative injury. Lipid peroxidative chain reactions with subsequent disruption of both liposomal and cellular membranes may be induced enzymatically generated free radicals (**Kellogg and Fridovich, 1977)**

Which may react with the unsaturated lipids of biomembranes resulting in the generation of lipid peroxide radicals, lipid hydroperoxides and fragmentation products such as malonaldehyde (MDA). Damage caused by LPO impairs the functioning of the biological membrane and the continued damage leads to loss of membrane integrity. **Beevi et al 2002** observed increased plasma as well as erytherocyte MDA in patients with cervical cancer. **Ahmed et al 1999** in their study demonstrated an overall progressive impaired status of **antioxidants** in Ca cervix.

Manoharan et al have demonstrated increased erythrocyte LPO and impaired antioxidant enzyme activities, suggesting damage to the red cell membrane and function in cervical cancer.

Increased LPO and reduced antioxidant levels may be taken as associated productive marker thus suggesting Ca cervix cases should get nutritive supplement to contain the blood LPO level and maintain a positive balance of antioxidants for better outcome in terms of better quality of life.

In one study sources of two major antioxidant beta carotene and alpha tocopherol were measured in plasma of women with histopathologically diagnosed cervical dysplasia or cancer, and results shows significantly reduced plasma level of both these compounds. The groups with advanced dysplasia show significantly decreased level of these two antioxidants **(Palan, et al, 1991).**

So the study to generate ground information on role of free radical in etiology of dysplasia was undertaken. We measured serum MDA, a stable end product of lipid peroxidation, and antioxidant system was assessed by measuring two enzymes i.e. SOD & catalase. The presents study was conducted on 35 females with 21 cases of cervical intra epithelial neoplasia grade 1 while 14 females having no dysplastic lesion in cervix, taken as control group.

The maximum number of cases of cervical dysplasia are found in age 35-40 yrs (33.3%), whereas slightly lower no of cases were recorded in age group 20-25 (19.05%). Similar results were found in study of **Iyer and Shalu, 1981.**

Distribution of study group according to socio- economic status showed that 85.71% of cases were from low income group as compared to middle income group (14.28%). This study is in accordance with **Christopherson, 1965.**

In present study, most of the cases of cervical intra epithelial neoplasia group I were found in multiparous women (66.6), as compared to primiparous women (33.3%). The nulliparous women were found to bedisease free. This is in accordance with study of **Wahi et al.1969.**

In present study, serum MDA level was found raised in test group as compared to control group. Range of MDA was found 3.23- 5.43 with mean of 4.31± 0.95 Nmoles/ml of Plasma in control group. Whereas it was found in range of 6.62-10.26 with mean 8.31± 1.25 Nmoles/ml of plasma in test group. Difference was highly significant (t=10.25 P< .001.). This study is in accordance with the study done by **Balasubramaniyam, 1994 and Shail Khanna, et al, 2002.** These MDA value further suggest that free redical injury is increased in dysplastic lesions and there is great exchange of end products of lipid peroxidation due to broken barrier between dysplastic tissue and general circulation.

In present study, blood level of enzyme catalase was found to be low in test groups as compared to control group. The mean value of catalase was 0.098±0.02 U/mg of protein in test group, Where as it was 0.323±0.03 U/mg of protein in control group. The difference was highly significant ($p< 0.001$).

In present study, blood level of enzyme superoxide dismutase was found to be low in test group (0.319±0.78 U/mg of protein) as compared to control group (0.723± 0.12 U/mg of protein). The difference between mean values of SOD found to be highly significant.

The present study showed higher MDA level in blood of anaemic patients (9.39±0.15 Nmol/ml of plasma) as compared to nonaneamic patients (5.42±0.007Nmol/ml of plasma). Thus this finding is in accordance with study of **Shail Khanna et al., 2002.** This confirms the evidence of disturbed oxidant/antioxidant balance in genital dysplastic lesion and supports the view that nutrition has a great role and its deficiency may aggravate free radical damage.

So this study gives a convincing support in favor of etiological role of free radical injury in cervical dysplasia.

ACKNOWLEDGEMENTS

Authors acknowledge the immense help received from the scholars whose articles are cited and included in references of this manuscript. The authors are also grateful to authors / editors /publishers of all those articles, journals and books from where the literature for this article has been reviewed and discussed.

Authors are also grateful to Prof S.N Singh, Head of Department of Pathology, G.S.V.M Medical College Kanpur for providing necessary facilities to complete this manuscript. The compiling and typing by Kushagra Pathak is also duly acknowledged.

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