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Research Article

IMPACT OF RADIOTHERAPY ON OXIDATIVE STRESS IN NEUTROPHILS OF CERVICAL CANCER PATIENTS

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ABSTRACT

Cervical carcinoma is the second most common malignancy seen among women in the world. Neutrophils constitute the "first line of defense" against any infectious agents that penetrate the body's physical barriers. The present study was conducted to investigate the effect of radiotherapy on oxidative stress in neutrophils of cervical cancer patients. Blood samples were collected from 30 cervical cancer patients of stage II (b) before and after radiotherapy. Neutrophils were isolated from blood by density gradient centrifugation. Lipid peroxides, conjugated dienes , hydroperoxides , nitrites, Myeloperoxidase (MPO) and antioxidants like Superoxide dismutase(SOD), catalase , Glutathione reduced (GSH), Glutathione peroxidase (GSH-PX), ascorbate were estimated in neutrophils of cervical cancer patients before and after radiotherapy. They were compared with age and sex matched healthy volunteers. Level of lipid peroxides, conjugated dienes, hydroperoxides and nitrites increased significantly in cervical cancer patients. Ascorbic acid and GSH levels were decreased significantly in neutrophils of cervical cancer patients .The activities of SOD, catalase, GSH -PX decreased significantly ($P< 0.001$) in neutrophils of cancer patients as compared to control subjects. Decreased activity of myeloperoxidase ($P<0.001$) was observed in cervical cancer patients as compared to controls. Upon radiotherapy, the level of oxidation products and myeloperoxidase were decreased and the antioxidants levels were increased significantly in neutrophils of cervical cancer subjects. Thus, it may be concluded that radiotherapy decreases the oxidative stress in neutrophils of cervical cancer patients.

Keywords: cervical cancer, radiotherapy, neutrophils, oxidative stress, myeloperoxidase.

INTRODUCTION

Cervical cancer is one of the most common malignancies among women [1]. In India, cervical cancer ranks first among women cancers. The incidence of cervical cancer is very high among rural population and this could be due to changes in life style, personal hygiene and health care. Cervical cancer is generally associated with HPV infection [2]. Various authors suggest that cervical neoplasia is associated with various microbial infections.

Neutrophils constitute the first line of defense against infectious agents or "non-self" substance that penetrates the body's physical barriers. Since cervical cancer could arise due to microbial infections (mostly HPV/HIV infection) and as neutrophils are the defense cells involved to counteract the infection, the ANC and biochemical changes in neutrophils during cervical cancer and the effect of ^{60}Co radiotherapy was assessed in our previous study. As we observed decreased ANC and some biochemical changes in neutrophils during cervical cancer [3], we intend to study the oxidative stress in neutrophils of cervical cancer patients. The present study focuses on the effect of ^{60}Co radiotherapy on oxidative stress in neutrophil, the professional phagocytic cell during cervical cancer.

Free radicals are produced continuously in cells either as by-products of metabolism or deliberately as in phagocytosis. Two types of free radicals are produced by neutrophils and they are mainly reactive oxygen species (ROS) and reactive nitrogen species (RNS).Free radicals have been known to play an important role in the initiation and promotion of multistep carcinogenesis. Oxidative damage has been implicated in carcinogenesis in human cancers and in cancer models for other animals[4] .

ROS in neutrophils are produced by the activity of NADPH oxidase and myeloperoxidase. NADPH oxidase catalyses the production of superoxidase(O_2^-) anion. By the reduction of O_2^- , H_2O_2 is produced. Myeloperoxidase (MPO) is the most abundant protein of neutrophils [5]. MPO catalyses the oxidation of halide ions (cl-, Br-, I -) to hypohalous acids at the expense of H_2O_2. Most of the hydrogen peroxide generated by superoxide dismutase in neutrophils is consumed by MPO [6]. Many species of bacteria are killed readily by a MPO/ Hydrogen peroxide /chloride system [7]. HOCl is the most potent bactericidal oxidant known to be produced by the neutrophils [8] and MPO is the only enzyme that produces HOCl under physiological conditions.

Neutrophils contain large reserves of endogenous antioxidants such as glutathione (GSH), ascorbate , catalase, superoxide dismutase(SOD),Glutathione peroxidase (GSH-PX),etc,. Their ablity to maintain these antioxidants in the reduced state during phagocytosis may prevent their death from oxidative suicide. In Neutrophils, ascorbate is present in millimolar concentration and they prevent the deleterious effects of hypochlorous acid (HOCl) [9].

Neutrophils contains large amount of GSH predominantly in the reduced form [10] as well as GSH-Px and GSSG-reductase activity . The function of this redox system in phagocytic leukocytes might be to protect the cells against oxidative damage and/or to transfer the reducing equivalents for the generation of bactericidal products [11]. Neutrophils also contain SOD and catalase which plays an important role in protection against lipid peroxidation.

The improper balance between ROS production and antioxidant defense results in oxidative stress which deregulates the cellular functions leading to various pathological conditions including cancer [12].The aim of this study is to evaluate the effect of radiotherapy on oxidative and nitrosative stress present in neutrophils during cervical cancer.

MATERIALS AND METHODS

This prospective study was conducted on 30 women with biopsy proven squamous cell carcinoma of the cervix with clinical stage II (b) (n=30) registered at the Department of Radiation Oncology, Government Royapettah hospital, Chennai. The details about the age, family history, height , weight, diet, etc,. of the patients were registered as shown in the proforma (Table 1). The age range was 45-55 yrs and these women had a weight of about 55 ± 2 Kg. The control group consists of 30 age , weight and sex matched healthy volunteers .The patients were given external radiation using cobalt-60 at a dosage of 50 Gy in 25 sittings for a period of 3 months.

The study was approved by the ethical committee of the hospital and all the women gave their written consents for providing blood samples.

5 ml of blood was collected before and after 3 months of radiation treatment and the following analysis were done.

Lipid peroxidation and its end products analysis.

Neutrophils were isolated by the method of Boyum A [13] using dextran and ficoll paque solution and suspended in PBS buffer at a concentration of 10^7 cells/ml . Neutrophils were freeze thawed three times and centrifuged. The particle free supernatant was used for

the estimation of malondialdehyde (MDA)[14] , conjugated dienes [15] and hydroperoxides [16].

Nitrite analysis

Neutrophils were isolated by the above said method and they were freeze thawed three times and centrifuged. The particle free supernatant was used for the estimation of nitrite [17].

Assay of Myeloperoxidase.

The activity of Myeloperoxidase in neutrophils was assayed by the method of William M. Nauseef *et al.* [18] after lysing the cells with 0.2% Triton X-100.

Antioxidants analysis.

Glutathione [19] and Glutathione peroxidase activity [20] was measured after pre incubating the cells at 37^{0}C for 10 minutes in a shaking water bath. Vitamin C [21] and superoxide dismutase activity [22] were measured after freeze thawing three times followed by centrifugation. Neutrophils were lysed with 0.2% Triton X-100 and then utilized for the assay of catalase [23] .

STATISTICAL ANALYSIS.

All quantitative estimations were made on 30 patients in each group . The values were expressed as mean ± SD. Statistical analysis was done by students "t" test and "p" value was arrived at to assess the statistical significance of changes observed. P values less that 0.02 were considered significant.

RESULTS

Figure 1 shows the level of MDA and conjugated dienes in neutrophils of normal subjects, cervical cancer patients and in ^{60} Co treated subjects. The levels of MDA and conjugated dienes increased significantly (P< 0.001) in neutrophils of cervical cancer patients when compared with healthy controls. Upon cobalt – 60 radiotherapy, these levels showed a significant decrease (P < 0.001) in neutrophils when compared to that of cervical cancer patients.

Fig.1
LEVELS OF OXIDATION PRODUCTS PRESENT IN NEUTROPHILS OF NORMAL SUBJECTS, CERVICAL CANCER PATIENTS AND ^{60}CO TREATED SUBJECTS

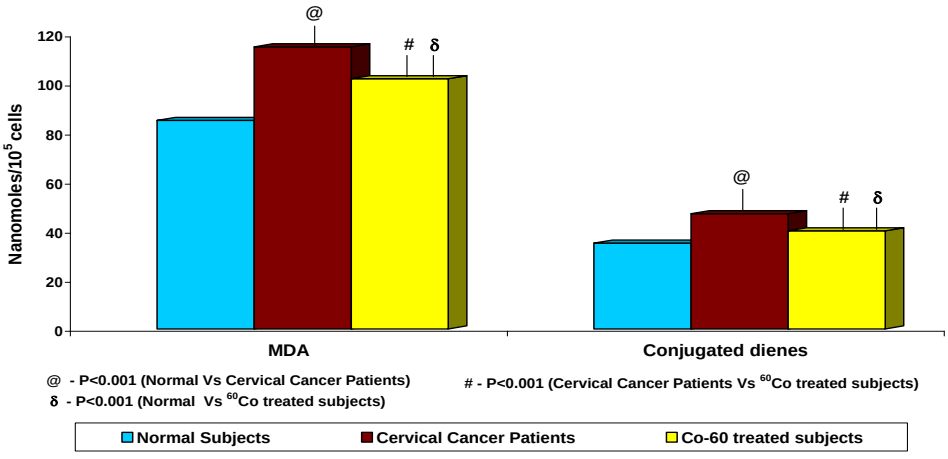

Figure 2 shows the level of lipid hydroperoxides present in neutrophils of normal subjects, cervical cancer patients and in ^{60} Co – treated subjects. The level of hydroperoxides was found to be significantly increased (P < 0.001) in neutrophils of cervical cancer patients when compared with healthy controls. Upon radiation treatment, hydroperoxides level was decreased significantly (P < 0.001) in neutrophils as compared with that of cervical cancer patients.

Fig.2
LEVEL OF LIPID HYDROPEROXIDES PRESENT IN NEUTROPHILS OF NORMAL SUBJECTS, CERVICAL CANCER PATIENTS AND ^{60}Co TREATED SUBJECTS

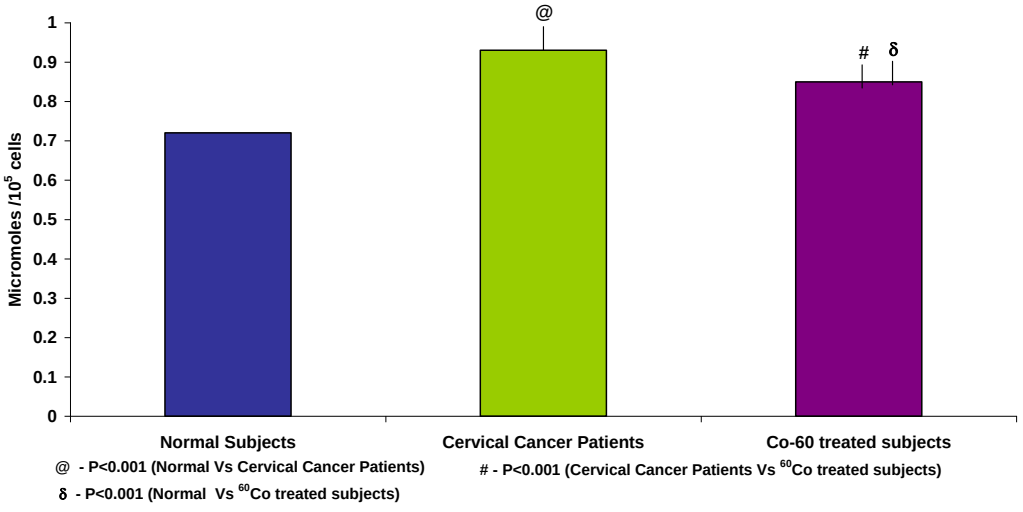

Figure 3 summarizes the level of nitrite in neutrophils of normal subjects, cervical cancer patients and ^{60} Co – treated subjects. The level of nitrite in neutrophils of cervical cancer patients increased significantly (P < 0.001) when compared with healthy controls. Upon ^{60} Co radiotherapy, its level was found to be decreased significantly (P < 0.001) when compared with neutrophils of cervical cancer patients.

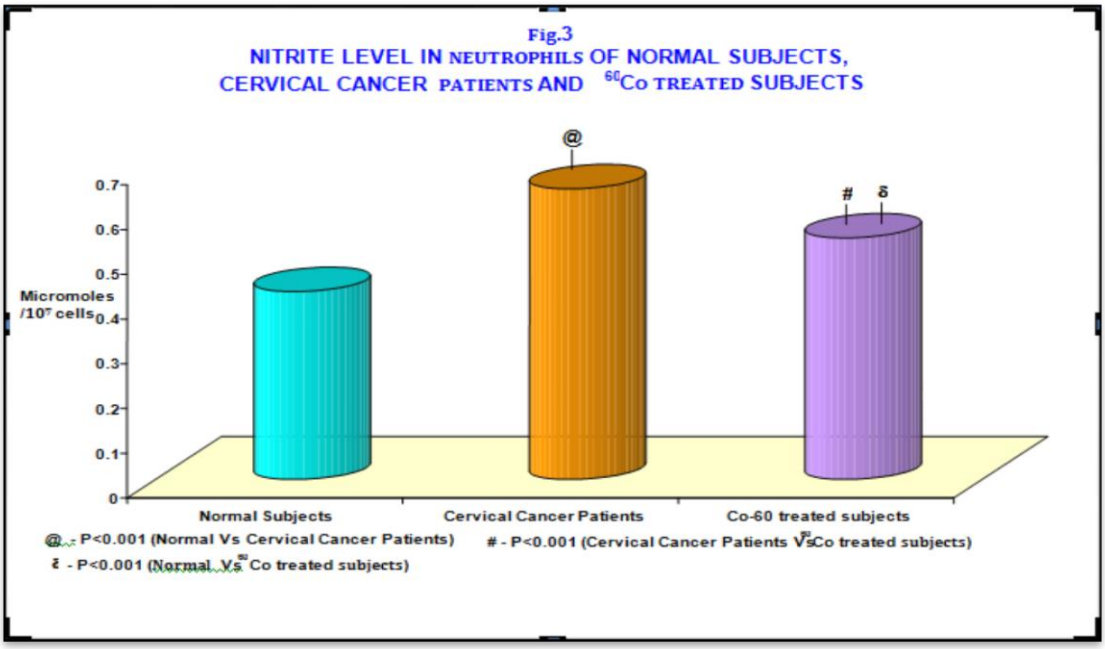

Table 2 depicts the level of non-enzymatic antioxidants like GSH and Vitamin C in neutrophils of normal subjects, cervical cancer patients and ^{60} Co – treated subjects. The level of reduced glutathione and vitamin C were decreased in cervical cancer patients. On treatment with cobalt – 60 external radiation, their levels were significantly increased(P < 0.001) and brought near normal.

Table 2: levels of non-enzymatic antioxidants present in neutrophils of normal subjects, cervical cancer patients and ^{60}co treated subjects.

The values are expressed as **mean ± SD**

Parameter	Normal Subjects	Cervical cancer patients	^{60}Co treated subjects
GSH-reduced n.moles /10^7 cells	15.2 ± 0.7	a)* 10 ± 0.9	b) * c) * 13 ± 0.5
VITAMIN C µmOLES/10^7 cells	1.21 ± 0.08	a) * 0.9 ± 0.05	b) * c) * 1.0 ± 0.09

***P < 0.001**
a) Comparison between normal subjects and cervical cancer patients
b) Comparison between cervical cancer patients and ^{60}Co treated subjects.
c) Comparison between normal and ^{60}Co treated subjects

Table 3 shows the activities of enzymatic antioxidants in neutrophils of normal subjects, cervical cancer patients and ^{60} Co – treated subjects. The activities of SOD, Catalase and GSH – PX were decreased (P < 0.001) in cervical cancer patients when compared with normal subjects. SOD and GSH- PX activities were increased significantly (P < 0.001) upon radiotherapy with cobalt – 60. But their levels were low when compared to normal subjects.

Table 3: Activities of enzymatic antioxidants present in neutrophils of normal, cervical cancer patients and ^{60}co treated subjects. The values are expressed as mean ± SD

Parameter	Normal Subjects	Cervical cancer patients	^{60}Co treated subjects
SOD units/10^7 cells	0.65 ± 0.02	a) * 0.42 ± 0.03	b) * c) * 0.46 ± 0.01
Catalase µ moles/10^7 cells	470 ± 20	a) * 420 ± 31	b) # c) ** 443 ± 35
GSH – Px NMOLES/10^7 CELLS	6.5 ± 0.3	a) * 5.1 ± 0.2	b) * c) * 5.7 ± 0.4

*** P < 0.001, ** P<0.002, # P <0.02**
a) Comparison between normal subjects and cervical cancer patients
b) Comparison between cervical cancer patients and ^{60}Co treated subjects.
c) Comparison between normal and ^{60}Co treated subjects

Table 4 depicts the activity of myeloperoxidase in neutrophils of normal subjects, cervical cancer patients and ^{60} Co – treated subjects. The activity of MPO was found to be decreased significantly (P < 0.001) when compared with normal subjects. Its activity was increased significantly (P < 0.001) upon radiotherapy.

Table 4: Activity of myeloperoxidase in neutrophils of normal subjects, cervical cancer patients and ^{60}co treated subjects.

The values are expressed as **mean ± SD**

Parameter	Normal Subjects	Cervical cancer patients	^{60}Co treated subjects
			b) *
Myeloperoxidase		a)*	c) *
μ moles/10^7 cells	11.0 ± 0.9	9.3 ± 0.7	10.1 ± 0.8

*** P < 0.001**

a) Comparison between normal subjects and cervical cancer patients

b) Comparison between cervical cancer patients and ^{60}Co treated subjects.

c) Comparison between normal and ^{60}Co treated subjects

DISCUSSION

Lipid peroxidation and its end products analysis

Lipid peroxidation is a chain reaction that involves the oxidation of lipids and generates cytotoxic compounds capable of damaging nucleic acids, proteins and general cellular organization. Measurement of MDA- modified DNA adducts would be useful for studies investigating the role of exogenous and endogenous agents in oxidative stress and carcinogenesis. Many authors have reported an increased level of lipid peroxides in cervical cancer patients [24,25]. Higher levels of lipid peroxides were found in cervical tumor tissues that in normal tissue samples [26].

Lipid hydroperoxides being more hydrophobic, they perturb membrane structure or function and can be deleterious to the cells. Once formed, these lipid hydroperoxides may undergo reductive degradation which either diminishes or enhances cytotoxic potential, depending on a variety of circumstances [27].

In the present study, an increased level of MDA, conjugated dienes and lipid hydroperoxides in neutrophils of cervical cancer patients were observed. This suggests that there is an excess production of oxidants in the neutrophils of cervical cancer patients. This may lead to oxidation of nucleic acid, proteins and lipids in neutrophils leading to mutations in DNA, oxidation of various proteins particularly glutathione, a cytoprotective antioxidant and may also alters the membrane permeability. As radiotherapy reduces the tumor size, the levels of oxidation products were decreased significantly when compared to cervical cancer subjects.

Nitrite analysis

Nitric oxide (NO) has many physiological functions ranging from regulation of vascular tone to neurotransmission and modulation of inflammatory process. Nitric oxide participates in apoptosis mediated cell death and in a large number of patho physiological conditions [28]. NO also promotes tumor growth and metastasis [29]. Like macrophages, neutrophils appear to produce reactive nitrogen species. NO is a gaseous signaling molecule involved in host defence and immune response [30].PMNs produce NO in response to extra cellular stimuli [31]. Dormant neutrophils incubated at 37^{o}C produce NO continuously but activation arrests this pathway in favour of oxidative burst. Nitric oxide may contribute to the microbicidal activity of neutrophils by reacting with ROS to form secondary cytotoxity species such as peroxynitrite [32]. Phagocytes may employ MPO generated reactive nitrogen intermediates as a physiological pathway for initiating lipid peroxidation and forming biologically active lipid and sterol oxidation products *in vivo* [33]. NO can also stimulate H_2O_2 induced lipid peroxidation. Since NO is a short lived radical, its measurement was done in terms of nitrite, nitrate or L-citrulline accumulation.

In the present study, an increased level of nitrite in neutrophils of cervical cancer patients was observed. This may cause nitrosylation of various thiol containing compounds and proteins particularly glutathione. Since there is an increase in lipid peroxidation noticed in cervical cancer patients, nitrite level may also be increased as it is required for the initiation of lipid peroxidation .This finding was in agreement with that of Beevi SS *et al* [34]. Upon radiotherapy, the level of nitrite was decreased as the tumor size was reduced.

Antioxidants analysis

Neutrophils contain large amounts of GSH [35] predominantly in the reduced form [11]. GSH redox system is of vital importance for the protection of neutrophils against their own oxidative bactericidal products .GSH is an important protectant of microtubule synthesis in neutrophils [36]. It was observed from our study that there is depletion in GSH content in neutrophils of cervical cancer patients which may lead to malformation of microtubular system in the neutrophils. During phagocytosis, there is a decrease in the levels of GSH due to the production of large amounts of reactive oxygen species like O_2^- ,H_2O_2, HOCl, etc,. In order to compensate the increased oxidant level in neutrophils (Fig 1,2&3),GSH may be depleted as observed in our study. Hence, the level of GSH was decreased in neutrophils of cervical cancer patients suggesting a decreased antioxidant defence system and upon treatment with ^{60}Co external radiation, its levels was brought to near normal.

Antioxidative vitamins like vitamin C have a number of biological activities such as immune stimulation, inhibition of nitrosamine formation and an alternation of metabolite activation of carcinogens [37]. The non-enzymatic reaction between GSH and dehydroascorbate appears to be the major physiological important reaction. Thus glutathione deficiency can lead to alternations in the levels of ascorbic acid.

Vitamin C enhances neutrophil motility, chemotaxis and cell mediated immunity, which possibly increases the levels of immuno surveillence [38]. It also preserves neutrophil integrity and protects the host tissue by inactivating free radicals and oxidants [39]. Intracellular ascorbate was utilized for the protection against permanent oxidants made by activated neutrophils [40]. The decreased levels of ascorbate noticed in this study could be due to the increased utilization of antioxidants to scavenge the free radicals that were produced in neutrophils.

SOD, GSH-PX and catalase form the primary enzymatic defense system [41]. SOD is metalloenzyme and it catalyses the dismutation of superoxide radicals to hydrogen peroxide. The oxidants are toxic not only for other cells but also for producing cells. In this present study, the activity of SOD was decreased in neutrophils of cervical cancer patients. This decrease could be due to increased production of oxidation products in neutrophils.

Catalase protects the cells against H_2O_2 mediated lipid peroxidation. Catalase activity has been reported to decrease in cervical cancer [25, 42]. GSH-PX is a tetrameric protein and it has 4 atoms of Se bound to it. GSH –PX utilizes the reducing equivalents of GSH to reduce H_2O_2 and it may be the main mechanism for protection against the deleterious effects of hydroperoxides. For adequate antioxidant protection, the cell not only needs an active synthesis of GSH but also a high activity of the GSH redox system [43]. In the present study, the activity of GSH-PX was lowered in cervical cancer patients and this could be due to the depletion of GSH. These results were similar with that of S.Manoharan *et al* [25]. Since the levels of oxidants were

decreased after radiotherapy, the antioxidants levels were increased significantly.

Assay of Myeloperoxidase

MPO- H_2O_2 halide system may modulate the inflammatory response by impairing certain receptor-mediated recognition mechanism of phagocytic cells[44] has reported an intracellular deficiency of MPO in cervical carcinoma. Increased MPO in plasma of cervical cancer patients was observed by Song M and Santanam N [45] . In the present study, MPO activity was decreased in neutrophils of cervical cancer patients when compared to normal subjects. These results suggest that the MPO might have leached out from neutrophils due to oxidative damage to these cells. The lowered activity of MPO observed during this study could be due to the above said reason. Upon treatment with ^{60}Co external radiation, the MPO activity was increased. This may be because of increased GSH level as they play an important role in protecting the neutrophils against oxidative damage.

Thus from the present study it may be concluded that the oxidative stress was created in neutrophils during cervical cancer and they damage the neutrophils leading to decreased neutrophil function and subsequent reduction in immune status. Radiation therapy increases the level of antioxidants and protects the neutrophils from oxidative damage and thus increases the immune status of cervical cancer subjects.

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