IMPACT OF RADIOThERAPY ON OXIDATIVE STRESS IN NEUTROPHILS OF CERVICAL CANCER PATIENTS
GAYATHRI GUNALAN AND VIJAYALAKSHMI KRISHNAMURTHY*
Department of Biochemistry, Bharathi women's College, Chennai-108, TamilNadu, India, Email: vij42research@yahoo.co.in
Received: 9 November 2011, Revised and Accepted: 3 March 2012
ABSTRACT
Cervical carcinoma is one of the second 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/IVF 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 60Co 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 60Co 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 superoxide (O$_2^-$) anion. By the reduction of O$_2^-$, H$_2$O$_2$ 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$_2$O$_2$. 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 /chromide system 7. HOCI is the most potent bactericidal oxidant known to be produced by the neutrophils 8 and MPO is the only enzyme that produces HOCI 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 ability 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 (HOCI) 9.
Neutrophils contains large amount of GSH predominantly in the reduced form 10 as well as GSH-Px and GSGG-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 Rajivpattnah 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 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), conjugated dienes and hydroperoxides.

**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.

**Assay of Myeloperoxidase.**

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

**Antioxidants analysis.**

Glutathione and Glutathione peroxidase activity was measured after pre incubating the cells at 37°C for 10 minutes in a shaking water bath. Vitamin C and superoxide dismutase activity 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.

**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 60Co 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 as compared with that of cervical cancer patients.

Figure 2 shows the level of lipid hydroperoxides present in neutrophils of normal subjects, cervical cancer patients and in 60Co 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.
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

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

*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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Subjects</th>
<th>Cervical cancer patients</th>
<th>$^{60}$Co treated subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD units/10$^7$ cells</td>
<td>0.65 ± 0.02</td>
<td>0.42 ± 0.03</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>Catalase μmole/10$^7$ cells</td>
<td>470 ± 20</td>
<td>420 ± 31</td>
<td>443 ± 35</td>
</tr>
<tr>
<td>GSH – Px</td>
<td></td>
<td>a) *</td>
<td>b) *</td>
</tr>
<tr>
<td>μmOLES/10$^7$ CELLS</td>
<td>6.5 ± 0.3</td>
<td>5.1 ± 0.2</td>
<td>5.7 ± 0.4</td>
</tr>
</tbody>
</table>

* 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 ⁶⁰Co - 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>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Subjects</th>
<th>Cervical cancer patients</th>
<th>⁶⁰Co treated subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase</td>
<td>11.0 ± 0.9</td>
<td>9.3 ± 0.7</td>
<td>10.1 ± 0.8</td>
</tr>
</tbody>
</table>

* P < 0.001
a) Comparison between normal subjects and cervical cancer patients
b) Comparison between cervical cancer patients and ⁶⁰Co treated subjects.
c) Comparison between normal and ⁶⁰Co 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. Higher levels of lipid peroxides were found in cervical tumor tissues that in normal tissue samples.

Lipid hydroperoxides being more hydrophobic, they perturb the plasma membranes of cancer cells. 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. NO also promotes tumor growth and metastasis. Macrophages, neutrophils appear to produce reactive nitrogen species. NO is a gaseous signaling molecule involved in host defence and immune response. Neutrophils produce NO in response to extra cellular stimuli. Dormant neutrophils incubated at 37°C produce NO continuously but activation arrests this pathway in favour of oxidative burst. Nitric oxide may contribute to the microbidal activity of neutrophils by reacting with ROS to form secondary cytotoxic species such as peroxynitrite. 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. NO can also stimulate H₂O₂ 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. Upon radiotherapy, the level of nitrite was decreased as the tumor size was reduced.

**Antioxidants analysis**

Neutrophils contain large amounts of GSH predominantly in the reduced form. GSH redox system is of vital importance for the protection of neutrophils against their own oxidative bacterial products. GSH is an important protectant of microtubule synthesis in neutrophils. It was observed from our study that there is depletion in GSH content in neutrophils of cervical cancer patients which may lead to malfunction 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 O₂⁻, H₂O₂, 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 antioxidative defense system and upon treatment with ⁶⁰Co 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. 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 immune surveillance. It also preserves neutrophil integrity and protects the host tissue by inactivating free radicals and oxidants. Intracellular ascorbate was utilized for the protection against permanent oxidants made by activated neutrophils. 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. SOD is a 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 H₂O₂ mediated lipid peroxidation. Catalase activity has been reported to decrease in cervical cancer patients. Catalase utilizing the reducing equivalents of GSH to reduce H₂O₂ 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. 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. Since the levels of oxidants were...
decreased after radiotherapy, the antioxidants levels were increased significantly.

**Assay of Myeloperoxidase**


