Antioxidant status in advanced cervical cancer patients undergoing neoadjuvant chemoradiation

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ABSTRACT

Cervical cancer is the most common cancer in Indian women. The aim of this study is to assess the alterations in the circulating lipid peroxide, antioxidant components and activities of defence enzymes in advanced cervical cancer patients, and to monitor the variations in their levels before and after neoadjuvant chemoradiation. Sixty patients with advanced cancer of the cervix (FIGO IIIa–IVb) are included in the study, along with 60 healthy controls. Blood samples are collected before the start of therapy (S_1), two weeks after the second course of chemotherapy (S_2) and two weeks after completion of tele/brachyradiation (S_3). Single blood samples are taken from controls. Lipid peroxides, conjugated dienes, reduced glutathione (GSH), catalase (CAT) and glutathione-S-transferase (GST) are estimated using standard methods. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) are assayed using commercially available kits. The pretreatment levels of plasma lipid peroxide were significantly elevated in cancer patients, while significantly lowered levels of GSH, GPx, GST, SOD and CAT were observed when compared to controls. After chemotherapy, the levels of lipid peroxidation showed a significant decline ($P<0.05$), which became highly significant after chemoradiation ($P<0.01$). Levels of GSH, GPx, SOD, GST and CAT showed a mild increase after chemotherapy. After chemoradiation, levels reverted to normal or near normal ($P<0.01$). Low levels of antioxidants in the circulation of patients with cervical cancer may be due to their increased utilisation to scavenge lipid peroxidation as well as their sequestration by tumour cells. The observed increase in antioxidant concentration after therapy might be due to the death of tumour cells or the arrest of tumour growth by chemotherapeutic agents. The normalisation of these parameters may provide information about the efficacy of neoadjuvant chemoradiation. A larger patient cohort with a longer follow-up period for therapeutic response studies may yield more significant data.

KEY WORDS: Antioxidants. Catalase. Cervical neoplasms. Drug therapy. Glutathione. Lipid peroxides. Neoadjuvant therapy. Superoxide dismutase

Introduction

Cervical cancer is the second most common cancer among women worldwide, with an estimated 493,000 new cases and 274,000 deaths in the year 2002. It is much more common in developing countries, where 83% of cases occur. Cervical cancer accounts for 15% of female cancers, with the risk before age 65 of 1.5%.[1]

In India, about 100,000 women are affected by cervical cancer every year. The majority present with a locally advanced stage of the disease, due to low socio-economic status, illiteracy and a lack of screening procedures in India.

Development of cervical cancer is due mainly to human papillomavirus (HPV) infection,[2] but may also be associated with other factors such as oral contraceptive use,[3] poor genital hygiene and low socio-economic status,[4] malnutrition,[5] smoking,[6] age at first coitus[7] and a large number of sexual partners.[4] However, studies show that human cervical cancer due to HPV infection is the most common form of the disease,[8–10] as infection is accompanied by damage to DNA and other constituents of the cell.[11]

Oxidative stress is potentially harmful to cells, and reactive oxygen species (ROS) are involved in multistage carcinogenesis, in initiation and promotion.[12] Thus, ROS can damage cellular components such as lipids, proteins and DNA, affecting enzyme activity and membrane function.[13]

Free radicals are highly reactive compounds that activate pro-carcinogens and alter the cellular antioxidant defence system. This includes enzymic and non-enzymic antioxidants such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), ascorbic acid (vitamin C) and α-tocopherol (vitamin E). Under conditions of excessive oxidative stress, however, cellular antioxidants are depleted.[14]

Enzymes such as SOD, CAT and GPx are considered to be the primary antioxidant enzymes, as they are involved in the direct elimination of active oxygen species. Secondary antioxidant enzymes (e.g., GST and GR) help in the detoxification of ROS by decreasing peroxide levels (GST) or by maintaining a steady supply of metabolic intermediates (GR) for the primary antioxidant enzymes. Antioxidants have been shown to inhibit initiation and promotion in carcinogenesis, and counteract cell immortalisation and transformation.[15]

Moreover, the extent of ROS-induced oxidative damage can be exacerbated by decreased efficiency of antioxidant defence mechanisms.[16,17] However, very few studies have

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addressed the effect of treatment on antioxidant levels in patients with advanced cervical cancer.[18,19]

Although radiotherapy is the main form of treatment for cervical cancer, this alone has not shown consistent improvement in cure rates over the past three decades.[20] Thus, combined approaches have been used in an attempt to increase therapeutic response. Chemotherapy and radiation have a synergistic effect and interact by spatial cooperation, with radiation focusing on local disease and chemotherapy on systemic subclinical boundaries.

One such approach is anterior/neoadjuvant chemotherapy; a regime involving the use of chemotherapy prior to definitive radiation therapy. This induces shrinkage of macroscopic disease, controls micrometastases early in the course of the disease, and reduces the local tumour mass, all of which is reported to lead to longer survival.[21]

The effects of radiation and anticancer drugs are mediated by production of free radicals, which act on DNA to produce lethal cell damage.[22] Hence, the aim of the study is to assess alterations in circulating lipid peroxidation, antioxidant components and the activities of defence enzymes in advanced cervical cancer patients, and to monitor variations before and after neoadjuvant chemoradiation.

Materials and methods

Subjects

Sixty patients with advanced cervical cancer (histologically proven cases of squamous cell carcinoma, FIGO stage IIIa–IVb) referred to the Department of Radiotherapy, Maulana Azad Medical College, New Delhi, were enrolled in the study. The mean age of the patients was 48.2±5.6 years. Clinical staging was assessed by a senior gynaecologist and confirmed by an oncologist in a jointly run gynaecological cancer clinic and classified according to FIGO staging.[23] At the same time, a punch biopsy was taken and sent for histopathological examination. An experienced pathologist reviewed all histological samples for dysplasia and invasive carcinoma.

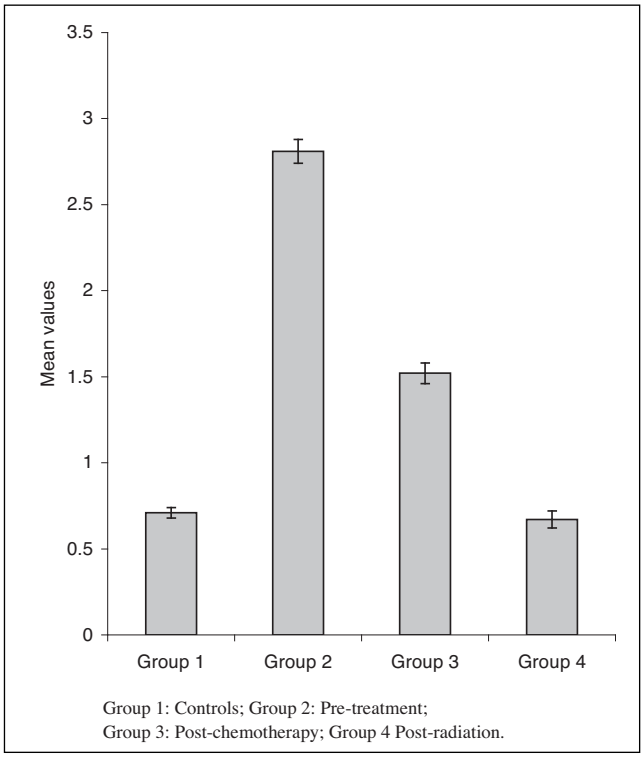

Fig. 1. Levels of conjugated dienes (µmol/mL) in each of the study groups.

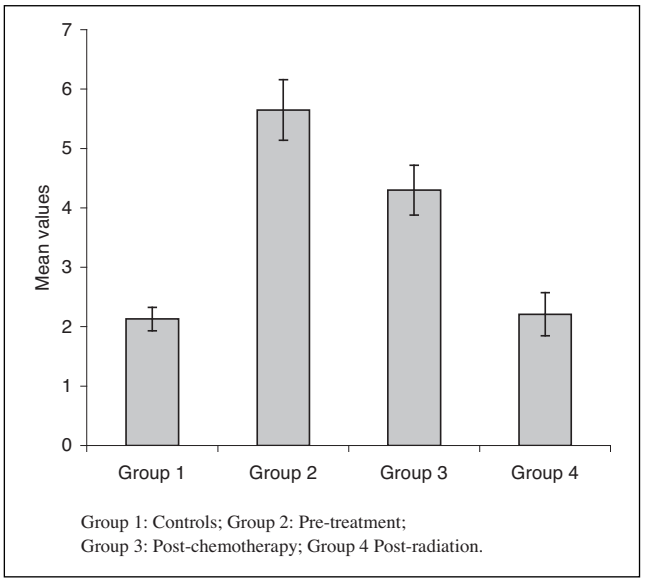

Fig. 2. TBARS level (nmol MDA formed/mL plasma) in each of the study groups.

Sixty healthy control subjects (no history or laboratory evidence of malignancy, inflammation or prior gynaecological disease, with normal pelvic examination and negative Papanicolaou smear) were also enlisted. Mean age of the control group was 47.3±5.7 years. All study participants were non-smokers, and none had diabetes mellitus, liver disease or rheumatoid arthritis. The study protocol was approved by the hospital ethics committee and patients and controls gave informed consent

All patients received two courses of chemotherapy, with a 21-day gap between the two courses. Each course was given by sequential infusion of 5-fluorouracil, followed by cisplatinum and bleomycin for five days. All patients received ondansetron tablets (4 mg, twice daily) to avoid cisplatinum-induced nausea.

Two weeks after the second course of chemotherapy, whole pelvic irradiation was given using 50 Gray telecobalt therapy (25 fractions in five weeks at the rate of 200 cGy/day in each fraction). Two weeks after the end of teleradiation, intracavitary cesium brachyradiation (30 Gray) was given over 18 hours in a single application.

Sample collection

In the patient group, samples were collected before the start of therapy (S_1), two weeks after the completion of the second course of chemotherapy (S_2) and two weeks after completion of tele/brachyradiation (S_3). Single blood samples were taken from control subjects.

Half of each sample was used for GPx and SOD estimations. Plasma from the other half was separated by centrifugation at 1000 x*g* for 15 min. After removing the buffy coat and plasma, the packed erythrocytes were washed (x3) with physiological saline.

To determine the activity of RBC antioxidant enzymes, the

haemolysate was prepared by lysing a known volume of erythrocytes in cold hypotonic phosphate buffer (pH 7.4). The haemolysate was centrifuged at 2500 x*g* for 15 min at 4°C. Biochemical estimations were carried out immediately.[24]

Table 1. Antioxidant enzyme activities in each of the study groups.

	GPx (U/g Hb)		SOD (U/mL)	
	Mean±SD	*P* value	Mean±SD	*P* value
Controls (*n*=60)	31.36±5.43		199.94±28.48	
Cancer patients (*n*=60)				
Pre-treatment	13.44±4.50	<0.001*	50.53±18.11	<0.001*
Post-chemotherapy	19.06±2.31	<0.05†	108.63±20.16	<0.03†
Post-radiation	31.13±4.26	<0.02†	198.68±23.46	<0.01†

*versus controls.

†versus pre-treatment levels.

Biochemical estimations

Lipid peroxidation was estimated by measurement of thiobarbituric acid reacting substance (TBARS) in plasma by the method of Yagi.[25] The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde (MDA), a secondary product of lipid peroxidation, was estimated at 532 nm.

Conjugated dienes were estimated in plasma by the method of Rao and Recknagel,[26] based on the arrangement of the double bonds in polyunsaturated fatty acids (PUFA) to form conjugated dienes with an absorbance maximum at 233 nm.

Reduced glutathione was assayed in plasma by the method of Ellman.[27] Estimation is based on the development of yellow colour when 5, 5′dithio 2-nitrobenzoic acid (DTNB) is added to compounds containing sulphydryl groups. Whole blood GPx levels were measured using a commercially available kit (Ransel, Randox Laboratories, UK) using the method of Paglia and Valentine,[28] where GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione is converted immediately to its reduced form, with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was expressed as units/g haemoglobin.

Glutathione-S-transferase activity in the haemolysate was determined by the method of Habig *et al.*,[29] by following the increase in absorbance at 340 nm using 1-chloro, 2-4-dinitrobenzene (CDNB) as substrate.

Whole blood SOD levels were measured using a commercially available kit (Ransod, Randox Laboratories). Xanthine and xanthine oxidase were used to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl- tetrazolium chloride (INT) to form a red formazan dye. Superoxide dismutase activity was measured by the degree of inhibition of this reaction. One unit of SOD causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay.[30]

Catalase activity was determined by the method of Sinha,[31] based on the utilisation of hydrogen peroxide by the enzyme. The colour developed was read at 620 nm. Haemoglobin in the haemolysate was measured by the method of Drabkin and Austin.[32] Blood was diluted in an alkaline medium containing potassium cyanide and potassium ferricyanide. Haemoglobin oxidised to methaemoglobin combines with cyanide to form cyanmethaemoglobin, which was measured at 540 nm.

Statistical analysis

Biochemical data are expressed as mean ± SD for patients and controls separately. Comparison of data in all groups was performed by non-parametric Mann-Whitney U test and significance was calculated by the Kruskal-Wallis test.[33] Comparisons were made between the control group and the cancer group, and within the cancer group comparing results before treatment with those achieved after each stage of treatment. $P<0.05$ was considered to be significant. Statistical analysis was performed using SPSS version 12.0.

Results

Figures 1 and 2 show the levels of circulating conjugated dienes and TBARS. Lipid peroxidation, indicated by circulating TBARS, and levels of plasma conjugated dienes were significantly higher ($P<0.001$) in the patients with cervical cancer.

Tables 1 and 2 show the levels of circulating antioxidants The non-enzymic antioxidant GSH and enzymic antioxidants GST, GPx, SOD and CAT were significantly lower in the cancer patient group.

Table 2. Circulating antioxidant levels in each of the study groups.

	Plasma GSH (mg/dL)		GST (U/mg Hb)		Catalase (U/g Hb)	
	Mean±SD	*P* value	Mean±SD	*P* value	mean±SD	*P* value
Controls (*n*=60)	39.23±3.52		2.51±0.28		5.96±0.61	
Cancer patients (*n*=60)						
Pre-treatment	18.06±2.47	<0.001*	1.34±0.3	<0.001*	3.2±0.41	<0.001*
Post-chemotherapy	26.31±3.16	<0.05†	1.82±0.41	<0.05†	4.5±0.29	<0.03†
Post-radiation	37.30±2.67	<0.02†	2.43±0.16	<0.01†	5.87±0.43	<0.01†

*versus controls.

†versus pre-treatment levels.

After chemotherapy, levels of lipid peroxidation and conjugated dienes showed a significant decline ($P<0.05$) and the antioxidant parameters (GSH, GPx, SOD, GST and CAT) showed a slight rise ($P<0.05$). After completion of therapy, the decrease in lipid peroxidation and the level of conjugated dienes became highly significant ($P<0.01$). On completion of neoadjuvant chemoradiotherapy, the antioxidant parameters (GSH, GST, GPx, SOD and CAT) showed significant elevation ($P<0.01$), but then returned to normal or near-normal levels.

Discussion

Enhanced lipid peroxidation observed in the circulation of cervical cancer patients in the present study can be attributed to a deficiency in antioxidant defences.[34] Antioxidant depletion in the circulation may be due to the scavenging of lipid peroxides, as well as sequestration by tumour cells. Epidemiological studies reveal that low levels of antioxidants are associated with an increased risk of cancer.[35,36] Significantly increased levels of lipid peroxidation, with a concomitant decrease in antioxidant levels, in cancer cervix patients was observed by Manoharan *et al*.[37] and Mila-Kierzenkowska *et al*.[38] Similar changes in other cancers[39–43] have also been reported.

Tumour cells sequester essential antioxidants such as GSH to meet the demands of the growing tumour.[44] Reduced glutathione, an important non-protein thiol and a true scavenger of lipid peroxides, in conjunction with the glutathione-related enzymes GPx and GST, plays a pivotal role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging ROS.[17] It aids the formation of the reduced form of antioxidants (e.g., ascorbic acid) and promotes detoxification of carcinogens, free radicals and xenobiotics.

Rapid GSH synthesis in tumour cells is associated with high rates of cell proliferation, while GSH depletion can sensitise cancer cells to the cytotoxic effects of oxidative stress and make them more vulnerable to the effects of anticancer drugs or the genes that promote apoptosis.[45] The enzymes SOD and CAT catalyse cell defence reactions against the potentially harmful effects of superoxide- and hydrogen peroxide-mediated lipid peroxidation.[17,46]

The observed increase in circulating lipid peroxides in cervical cancer patients in the present study correlates with the decline in SOD and CAT activity. In tumour cells, decreased SOD and CAT may cause the accumulation of superoxide and hydrogen peroxide. The net outcome of this abnormality in the process of carcinogenesis is unknown.

A fundamental difficulty in this approach is to ascertain whether changed antioxidant enzymes are the primary metabolic disturbance that creates the cancer or they are secondary responses to neoplastic change. It seems reasonable to hypothesise that free radicals produced by carcinogens or by irradiation cause mutations and DNA damage, which may lead to neoplastic change. On the other hand, free radicals can induce antioxidant enzymes,[47] which should prevent tumour formation.

Mechanisms must exist by which cells escape the inhibitory effect of antioxidant enzymes and by which the levels of induced enzymes fall below normal, as seen in tumour cells. More work is required to illustrate the role of antioxidant enzymes in human carcinogenesis.

Moreover, the increase in circulating lipid peroxides may be related to deficiency of SOD and CAT in tumour tissue. This can result in the accumulation of superoxide anion, a highly diffusible and potent oxidising radical capable of crossing membranes, causing deleterious effects at sites far removed from the tumour.[48]

In addition to SOD and CAT, GPx and GST act as antioxidant enzymes. Kumaraguruparan *et al*.[49] have reported deficiency of these enzymes in cancer patients, and the findings of the present study are in agreement. Both enzymes, using glutathione as a substrate, play a crucial role in protecting against the deleterious effects of ROS and xenobiotics.[40]

Glutathione peroxidase is a primary antioxidant enzyme involved in the direct elimination of ROS. Glutathione-S-transferase catalyses the nucleophilic addition of the thiol of reduced glutathione to a variety of electrophiles. This enzyme has a critical role in protecting cells against ROS, due to redox cycling of exogenous and endogenous quinones.

Blood glutathione levels are believed to be predictors of morbidity and mortality.[45] Lower GSH levels and glutathione-related enzymes seen in cervical cancer patients support the hypothesis that GSH status is inversely related to malignant transformation.

Antioxidants, which scavenge free radicals, can counter the effects of radiation and anticancer drugs. If antioxidant levels are high, the tumour is radioresistant and resistant to some anticancer drugs. The complex metabolism of tumour cells produces excess free radicals and/or abnormality in antioxidant enzymes. This may be necessary for the maintenance of the malignant state, resulting in a further decrease in non-enzymatic antioxidants.

After therapy, levels of antioxidants return to normal. Mila-Kierzenkowska *et al*.[38] reported similar findings after brachytherapy, as did Bhuvarahamurthy *et al*.[19] after chemoradiotherapy. This increase in antioxidant concentration may be due to the death of tumour cells following radiation, or by the arrest of tumour growth due to the effects of chemotherapeutic agents. Lipid peroxidation levels (MDA) and conjugated dienes return to normal after therapy, indicating the curative effect of the treatment. Normalisation of enzyme activities may provide information about the efficacy of radiotherapy and combined therapy (neoadjuvant chemoradiation).

The strength of the present work lies in the fact that alterations in the levels of several oxidants and antioxidants, before and after neoadjuvant chemoradiation, are compared in a single study. However, a larger patient cohort and a longer follow-up period may yield more significant data on their utility as predictors of the chemoradiosensitivity of cervical tumours. □

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