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Research Article

Comparison of different blood biomarkers of cervical cancer patients with control subjects

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ABSTRACT

Cervical cancer shows a high prevalence among Bangladeshi women. Reports indicated that oxidant-antioxidant balance plays a role in cervical cancer pathogenesis. Therefore, this study explores the change of different blood-derived enzymes, antioxidants, and biomarkers to understand the oxidative stress status of Bangladeshi cervical cancer patients. A clinician recruited the case and control participants for the study based on histopathologic reports. No significant difference was observed in the random blood glucose level, blood pressure, total cholesterol, triglycerides (TGs), HDL cholesterol, LDL cholesterol, and total protein levels in case participants compared to age and sex-matched control subjects. However, serum albumin, vitamins like alpha-tocopherol and ascorbic acid, antioxidant enzymes like SOD, and catalase levels significantly decreased in patients compared to controls. Other parameters, like, ALT, AST, ALP, phospholipid hydroperoxide, TBARS, protein carbonyl content, serum nitric oxide, cholesterol/phospholipid ratio (C/P) of erythrocyte and osmotic fragility were significantly increased in the case subjects compared to control. All of these reflect an oxidant-antioxidant imbalance in this setting. Thus, this study provides a detailed profile of the oxidant-antioxidant levels of the Bangladeshi cervical cancer patients, which revealed that the compromised antioxidant system in cervical cancer patients is associated with damage to various biomolecules and ultimately impairs cellular functions.

Introduction

Cervical cancer is the fourth most common carcinoma among women worldwide. Less-developed countries experience a very high prevalence of cancer, where 87% of the world's cervical cancer occurs (Ferlay et al., 2015). Over 2.7 million people died were between the ages of 25 and 64 worldwide, of which 2.4 million deaths reported in developing areas and 0.3 million in developed countries (Yang et al., 2004; Castle and Pierz, 2019). In Bangladesh, the rate is also very high. It is the second most common cancer amongst Bangladeshi women, with an estimated 11,956 incidents and 6582 deaths in 2012 (Islam et al., 2018). Current evidence indicates that the leading cause of cervical cancer is cervical infection with certain types of Human Papilloma Virus (HPV), transmitted sexually (Kjaer et al., 2001). Besides, many data have recently been published supporting the fact that oxidative stress plays a significant role in aggravating the pathogenesis of cervical cancer (Ebrahimi et al., 2019). Moreover, a severe imbalance was observed during the oxidative stress between the production of reactive species and antioxidant defenses, leading to potential tissue damage and carcinogenesis

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(Rani et al., 2016). Oxidative stress is persistent during aerobic metabolism when highly reactive free radicals-ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) such as superoxide, hydroxyl radical, nitric oxide, and hydrogen peroxide are continuously produced and cause oxidative damage to DNA, carbohydrates, proteins, and lipids. This leads to fragmentation and cross-linking cellular macromolecules (Rani et al., 2016; Kruk and Aboul-Enein, 2017). It also modulates the function of cancer cells, such as cell proliferation, alterations in cellular sensitivity to anticancer agents, invasion, and metastasis by increasing mutations, and genetic instability (Zelenka et al., 2018; Wang et al., 2018; Saha et al., 2017).

Over the past several decades, numerous studies have examined the relationship between oxidative stress status with cervical neoplasia and cancer risk (Castle and Giuliano, 2003; Tong et al., 2015; Cruz-Gregorio et al., 2018). ROS are essential contributors linking environmental toxicity to the multistage carcinogenic process. These oxidants can be generated in response to both endogenous and exogenous stimuli. To counter balance ROS-mediated injury, an endogenous antioxidant defense system exists. The antioxidant system consists of enzymatic and non-enzymatic components (Rani et al., 2016). Superoxide dismutase (SOD) and catalase are the antioxidant enzymes among those components. SOD protects by catalyzing the dismutation of superoxide radicals to H_2O_2. Increased lipid peroxidation occurs due to decreased SOD levels, resulting in deformation and rigidness of cells (Islam et al., 2019; Bairova et al., 2014). Another enzyme, catalase, prevents the cell membrane from being damaged by detoxifying H_2O_2 to water and molecular oxygen. It is one of the ways by which catalase protects erythrocytes from oxidative stress (He et al., 2017). The decreased level of catalase is linked with the low detoxification of oxidants in cancer. However, when oxidation exceeds the control mechanisms, oxidative stress begins. Chronic and cumulative oxidative stress induces deleterious modifications to various macromolecular components, such as DNA, lipids, and proteins (Glorieux and Calderon, 2017). Recent studies showed decreased antioxidant concentrations and increased oxidative stress in cervical cancer (Cruz-Gregorio et al., 2018). Moreover, the cervical cancer cell line shows that ascorbic acid has potential in the treatment of cervical cancer by inhibiting critical steps in cancer development and spread (Mane et al., 2016; Blaszczak et al., 2019). Protective effects were also observed for vitamins A, C, and E and beta-carotene (Jain et al., 2017). Therefore, the purpose of the present study is to evaluate the antioxidant status in cervical cancer patients by estimating different serum biomarkers in addition to the level of nitrite, antioxidant enzymes, and structural integrity of erythrocytes, since oxidative stress is considered to play a major role along with HPV in the progression of cervical cancer.

Materials and Methods

This case-control study was carried out in the Department of Biochemistry and Molecular Biology of Dhaka University, involving 60 Bangladeshi participants. Participants were distributed equally among the control- and patient-group based on the inclusion and exclusion criteria of the study. The study was explained to all the participants to get written consent. An expert clinician confirmed the patients using the acetic acid test of the cervix (VIA) in the clinic of Gynecological Oncology, Department of National Institute of Cancer Research and Hospital (NIRCH), Mohakhali, Dhaka. Moreover, participants were informed about their rights to withdraw their involvement from the study at any time. Study subjects completed a structured questionnaire about their age, age at the time of marriage, no. of children, medical, residential, occupational, and family history of chronic diseases, along with other details. At the same time, 30 healthy female subjects from the same

socioeconomic status, having no history of smoking, alcoholism, or carcinoma, were recruited as controls. Ethical Review Committee of the Department of Biochemistry and Molecular Biology, University of Dhaka, approved the study and all the participants provided written informed consent before participation.

Blood samples from the cervical cancer patients and the control subjects were collected under protocols approved by the Department of Biochemistry and Molecular Biology, University of Dhaka. About 5 mL of venous blood was drawn from each individual using a disposable syringe with the help of a trained person, following all aseptic precautions. Blood was maintained with EDTA for erythrocyte membrane preparation and without EDTA for serum preparation and osmotic fragility test. The drawn blood was transported to the laboratory using an icebox and then centrifuged for 10 minutes at 3,000 rpm. The serum was collected from the centrifuged tube carefully with a Pasteur pipette. The samples were either analyzed on the same collection day or stored at -20° C until further use.

All the methods were standardized first, and standard graphs were obtained. Random Blood glucose was determined by the glucose oxidase (GOD) method according to the protocol provided by the reagent kit of Linear chemicals, Spain. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and ALP (Alkaline Phosphatase) were determined by the reagent kit of Randox, UK. Serum total protein was determined by a modification of the Lowry technique (Okutucu et al., 2007). In the modified method, the protein binds to copper in an alkaline medium and produces Cu^{++}. This catalyzes the oxidation of aromatic amino acids by reducing phosphomolybdotungstate to heteropolymolybdanum blue, which predominantly depends upon the tyrosine and tryptophan protein content. In another protocol, albumin reacts with the anionic Bromocresol green (BCG) and produces a green color, which is measured at 546 nm (Duly et al., 2003). Furthermore, serum carbonyl groups were measured using a reaction with dinitrophenylhydrazine (DNPH), forming of stable hydrazone products and measuring the absorbance at 370 nm (Colombo et al., 2016). Total cholesterol and triglyceride were determined according to the protocol provided by Linear Chemicals Limited, Spain. HDL- and LDL- cholesterol were determined according to the protocol supplied by the Erba diagnostics Mannheim GmbH, Germany, and Friedword's formula, respectively. The thiobarbiturate reactive substances (TBARS) were determined by the method of Yagi (1998). Serum lipid peroxide was measured by precipitating lipoproteins with trichloroacetic acid and boiled with thiobarbituric acid, which reacted with malondialdehyde to develop pink color, which was quantified at 535 nm (Dasgupta and Klein, 2014). Phospholipid hydroperoxide was determined by a colorimetric method based on the oxidation of ferrous to ferric ions in the presence of xylenol orange. Serum vitamin A and vitamin E were measured simultaneously by the high-pressure liquid chromatography (HPLC) method. Acetonitrile and methanol (88:12) were used as mobile phase, Nucleosil C18; 5µ, 4.6 X 250 mm column, and UV detector (absorbance at 292 nm for both retinol and tocopherol) were used. 50 µL sample was injected, the flow rate was 1.0 mL/min, temperature was ambient, and sensitivity was 0.0005. Serum ascorbic acid was measured by reacting with dinitrophenylhydrazine. The level of serum nitrite (NO_2^-) was determined by the reduction of nitrate by copper-cadmium alloy, followed by color development with Griess reagent (sulfanilamide and N-naphthylethyl enediamine) in an acidic medium according to the method of Sastry et al. (2002). The activity of serum SOD was determined by using of either ferricytochrome c, an electron-carrying protein containing one heme group found in mitochondria of all aerobic organisms, or nitro blue tetrazolium according to the method described by Beyer and Fridovich (1987). The serum catalase level was determined by the breakdown of hydrogen peroxide by the action of catalase and the measurement of the ultraviolet absorption of peroxide according to the method of Beers and Sizer (1952). Erythrocyte membrane cholesterol was determined by the method

of Allain et al. (1974). According to this method, cholesterol esters were hydrolyzed to free cholesterol by cholesterol ester hydrolase. The free cholesterol produced was oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-amino antipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm. Erythrocyte membrane phospholipid was determined by the method of Bartlett, where phosphorus reaction mixture was heated in the presence of strong sulfuric acid, and absorption was taken at 830 nm (Bartlett, 1959). Erythrocyte membrane protein was determined by Folin phenol reagent after alkaline copper treatment according to the method of Lowry (Nayak et al., 2008). Osmotic fragility was determined using whole blood by the method of Parpart et al. (1947) and Layton and Roper (2016). The degree of resistance of red cells to a decrease in salt content has long been used as a measure of their viability and clinically as diagnostic characteristics. When any red cell reaches the hemolytic volume, the hemoglobin of that cell diffuses to equilibrium, usually without rupturing the plasma membrane of the cell (Toepfner et al., 2018).

Data obtained from the experiments were analyzed using the GraphPad Prism version 6.0 and Microsoft Excel 2007. Data are expressed as mean ± SEM. The student's t-test was used for statistical analysis. p-value <0.05 was considered a statistically significant.

Results

In this case-control study, 30 female subjects were enrolled as the patient (case) who was diagnosed as cervical cancer positive with pap smear tests (Shlay et al., 1998), and 30 age-matched control subjects with no history of cervical cancer. Interestingly, when we analyzed the average age at which they got married, we have found that the patient group married at a significantly earlier age (case vs. control, 17 vs. 23 years, $p<0.05$) compared to the control subjects, which indicates marriage age could be a potential risk factor for cervical cancer (Table 1).

Table 1. Characteristics of the study subjects

Parameters		Control (n=25)	Patient (n=25)
Age (GM in years)		49	52
Marriage Age (in years)		23	17 *
Married life (in years)		27	34 *
No. of Children		2	4*
Systolic BP (mmHg)		110	104
Diastolic BP (mmHg)		70	65
Random blood glucose (mg/dL)		132.07	113.14
Educational Level	Illiterate	6	12
	Primary	9	7
	Secondary	7	4
	Above secondary	3	2

GM means Geometric Mean, ns means non-significant, *p<0.05.

Moreover, the duration of marriage life also a significantly impacts cancer prognosis. Table 1 showed that the patient group has a significantly longer marriage life compared to the control (case vs. control, 34 vs. 27 years, $p<0.05$). Additionally, the patient group experienced a significantly higher number of childbirths than the control group (case vs. control, 4 vs. 2 children, $p<0.05$). However, no differences were found in the blood pressure level, random blood glucose level, or educational status with the disease outcome between the two groups (Table 1). When fasting blood lipid profiles were analyzed, it was found that both the case and control groups have similar lipid profiles, where total cholesterol levels were (135.5±15.56 vs. 122.4±27.29 mg/dL, $p=0.69$), triglycerides levels were (113.9±15.29 vs. 102.6±29.76 mg/dL, $p=0.74$), HDL cholesterol (34.60±1.50 vs. 31.6±3.17 mg/dL, $p=0.42$), and LDL cholesterol levels were (78.2±11.77 vs. 49.61±3.93 mg/dL, $p=0.051$) case vs. control, respectively (Table 2). The blood levels of enzymes (ALP, ALT, and AST) were significantly ($p<0.05$) higher in the cervical cancer subjects compared to the control subjects (1361.0±15.4 vs. 895.3±7.6 U/L, 2646.0±4.3 vs. 1723.0±7.1 U/L, and 1340.0±21.9 vs. 910.9±6.5 U/L, respectively) (Table 2).

Table 2. Lipid profiles and serum enzymes in control and cervical cancer subjects (n=5).

Parameters	Control	Patient
Cholesterol mg/dL	135.5±15.56	122.4±27.29
Triglycerides mg/dL	113.9±15.29	102.6±29.76
HDL Cholesterol mg/dL	34.60±1.50	31.6±3.17
LDL Cholesterol mg/dL	78.2±11.77	49.61±3.93
Alkaline phosphatase U/L	1361.0±15.4	895.3±7.6*
Alanine transaminase U/L	2646.0±4.3	1723.0±7.1*
Aspartate transaminase U/L	1340.0±21.9	910.9±6.5*

The data are expressed as mean±SEM. *Differences were considered significant at p<0.05.

In similar experiments, different redox parameters of the blood were analyzed, where the case group differs significantly from the control. The case group had phospholipid hydroxide at a level of 4.1±0.08 vs. 2.3±0.07 nmol/ml in the control group, which was significantly different, p<0.05. The levels of TBARS were also significantly different in case vs. control (2.70±0.29 vs. 1.1±0.13 nmol/ml, respectively, p<0.05). Similarly, protein carbonyl contents were significantly higher (0.23±0.02 nmol/mg of protein) in case subjects vs. (0.14±0.01 nmol/mg of protein) control subjects (Fig. 1).

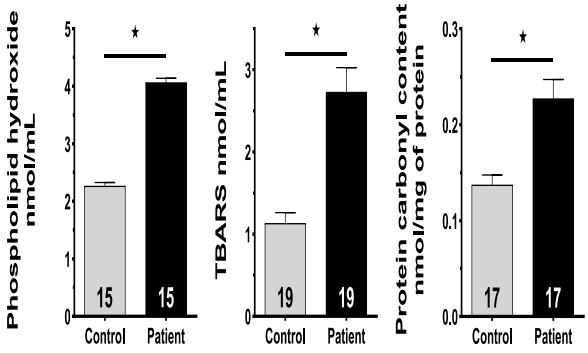

Fig. 1. Redox status in control and cervical cancer subjects. The number inside the bars indicates the number of participants. *indicates statistically significant differences at p<0.05.

Total protein content, albumin, and globulin levels were also analyzed on the blood samples and found that the total protein level (42.2±2.45 vs. 42.6±1.91 g/L, p=0.90) and globulin levels (29.3±3.39 vs. 24.9± 1.80 g/L, p=0.28) were comparable between the case and control groups, whereas the blood albumin level was significantly lower in the case group compared to the control group (18.9±1.20 vs. 24.5±1.52 g/L, p<0.05) (case vs. control, respectively; Fig. 2).

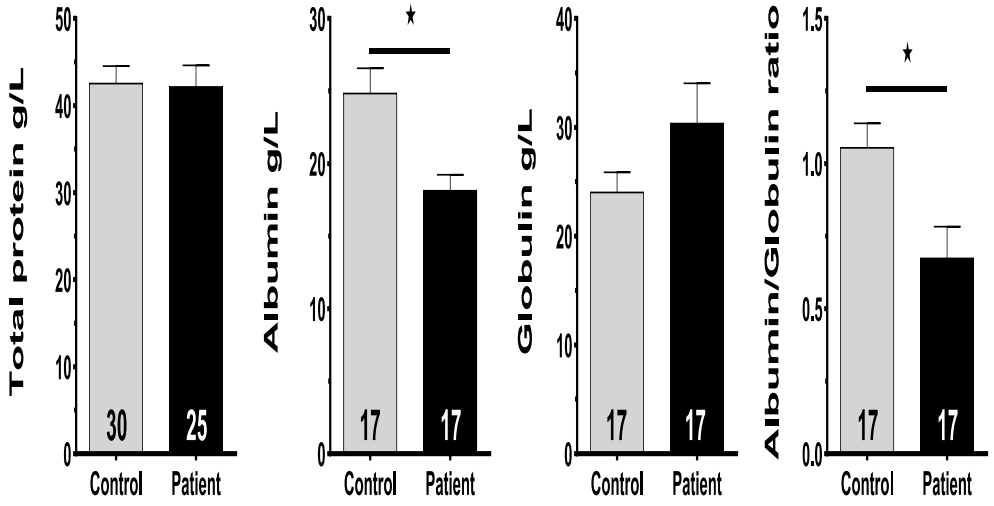

Fig. 2. Blood levels of proteins in control and cervical cancer subjects. The number inside the bars indicates the number of participants. *indicates statistically significant differences at p<0.05.

When levels of different vitamins were analyzed in the blood, it was found that the case group had significantly lower levels of ascorbic acid (0.78±0.12 vs. 1.50±0.12 mg/dL, p<0.05), retinol (38.0±5.04 vs. 62.2±2.76 g/mL, p<0.05) and alpha-tocopherol (6.3±0.85 vs. 14.1±1.90 g/mL, p<0.05) (case vs. control, respectively; Fig. 3).

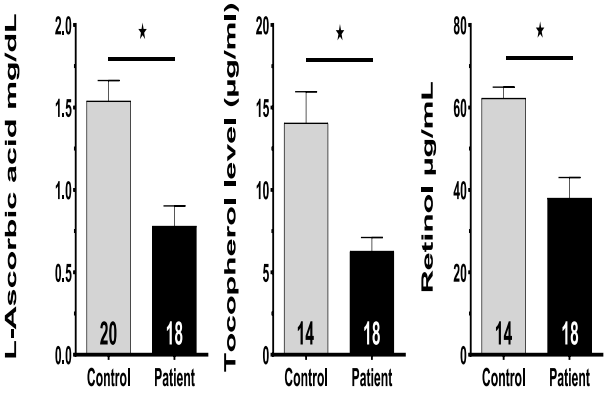

Fig. 3. Vitamin levels in control and cervical cancer subjects. The number inside the bars indicates the number of participants. *indicates statistically significant differences p<0.05.

The blood maintains a different antioxidant system to control the redox status of the body. When serum nitrate, superoxide dismutase (SOD) and catalase levels were analyzed, it was found that serum nitrate was 5.6±0.09 vs. 3.0±0.05 µmol/L, p<0.05, SOD was 4.1±0.09 vs. 10.30±0.74 units/mL, p<0.05 and catalase was 0.93±0.04 vs. 10.3±0.49 units/mL, p<0.05 (case vs. control, respectively; Table 3). Significantly increased cholesterol and decreased phospholipid in the erythrocyte membrane of the case subjects were observed compared to the control (Table 4). Besides, a significant increase in the C/P ratio was observed in the case subjects compared to the control subjects (Table 4).

Table 3. Level of nitrite and antioxidant enzymes in the blood of control and case participants. (n=30).

Parameters	Control	Patient
Nitrite (µmol/L)	3.0± 0.05	5.6 ± 0.09*
SOD (units/mL)	10.30±0.74	4.1±0.09*
Catalase (units/mL)	10.30±0.49	0.93±0.04*

The data are expressed as mean±SEM. *Differences were considered significant at p< 0.05.

Table 4. Cholesterol, phospholipid content, and C/P ratio of erythrocyte membrane in control and cervical cancer samples (n=30).

Parameters	Control	Patient
Cholesterol (µg/mg protein)	212.2±11.0	277.8±8.18*
Phospholipid (µg/mg protein)	593.5±30.42	221.9±12.10*
C/P ratio	0.35±0.01	0.54±0.01*

The data are expressed as mean±SEM. *Differences were considered significant with p< 0.05.

It indicates that the fluidity of the RBC was significantly decreased in case subjects. Moreover, when fragility was considered, it was found that the erythrocytes of cervical cancer patients were more fragile than the control subjects. When the osmotic pressure-induced RBC fragility was tested, a rightward shift of the fragility curve, meaning more hemolysis, in cervical cancer patients was observed compared to the control subjects (Fig.4).

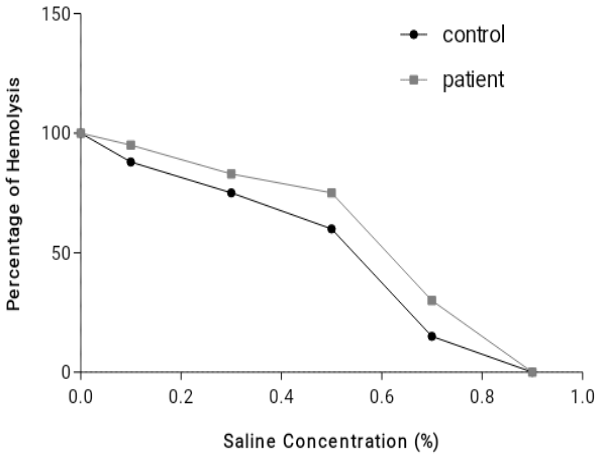

Fig. 4. Red blood cell fragility in control and cervical cancer subjects. Each dot indicates the mean value of at least 5 samples.

Discussion

It is now a well-established concept that oxidative stress has a pathogenic effect and plays a crucial role in developing many diseased conditions, including cancer, neurological diseases, and cardiovascular diseases (Halliwell and Gutteridge, 2015). The pathogens are

initiated due to inadequate performance of the antioxidant defense mechanism and cause constant damage to the cellular macromolecules, which ultimately leads to alteration of cellular physiology (Cruz-Gregorio et al., 2018; Devi et al., 2000).

Altered biochemical and nutritional parameters levels were found in cervical cancer patients (Orr et al., 1985). When we compared blood glucose or blood pressure, it was found that both the study groups had similar patterns. Although scientists believe that hyperglycemia is strongly associated with a poor prognosis of cervical cancer, the high prevalence of diabetes and hypertension in this country may be the reason for similar blood glucose and blood pressure patterns in these study groups. Interestingly, significantly higher levels of triglycerides and lower total cholesterol levels were observed in previous cervical cancer patients (Preetha et al., 2016). Ray et al. showed, on the other hand, lower levels of all lipid fractions (Ray et al., 1997). However, in our study, both the cervical cancer patients and control subjects had comparable lipid profiles (Table 2). Moreover, different serum enzymes like aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) activity were reported to be higher in the circulation of cervical cancer patients (Manju et al., 2002). Similarly, in our study, ALT, AST, and ALP levels of the cervical cancer patient group were significantly increased compared to the control group. This may be because carcinogenic agents alter cellular growth, leading to various biochemical changes in the blood (Chougule et al., 2008).

Lipid peroxidation appears to be a significant cause of human's endogenous DNA damage that may contribute significantly to cancer (Gentile et al., 2017). Among the peroxidation end products of membranous polyunsaturated fatty acids, Malonaldehyde (MDA) was reported as mutagenic in bacterial and mammalian cells and carcinogenic in rats (Niedernhofer et al., 2003). MDA reacts with thiobarbituric acid (TBA) to form a fluorescent red adduct and can be used as an indicator of oxidative stress (Marnett, 2000; Balas et al., 2011). Several studies showed thiobarbituric acid reactive substance (TBARS) levels were significantly higher in the cervical cancer patient group than in control (Kolanjiappan et al., 2002; Chiou and Hu, 1999; Manju et al., 2002; Kim et al.; 2003; Jelić et al., 2018; Balasubramaniyan et al., 1994). Moreover, myeloperoxidases and lipid peroxide-modified proteins were also increased in gynecological malignancies (Song and Santanam, 2001). In the present study, we reported similar findings where cervical cancer patients have significantly higher levels of phospholipid hydroperoxide, and TBARS compared to control subjects.

Protein profiling data showed no significant difference in the levels of total protein. Still, serum albumin level was significantly decreased, whereas serum globulin increased in cervical cancer patients. It has already been reported that reduced serum albumin ratio is associated with malnutrition. In contrast, an increased globulin level means the severity of the inflammatory response, which results in the cardinal features of cancer cachexia (Chi et al., 2018). The reason might be that albumin sequesters copper and other toxic transition metals to detoxify them and protects against cellular damage. Interestingly, these cancer patients had lower albumin to globulin ratio than the control. Yoshino et al. reported that low albumin to globulin ratio high globulin levels are associated with poor survival of cervical cancer patients (Yoshino et al., 2019). Moreover, a high protein carbonyl level produced due to excess ROS indicates higher oxidative stress in these cervical cancer patients. Because all the amino acid residues of a protein can be modified by $OH\cdot$ and O_2^-, which increases the formation of carbonyl groups in proteins (Shrivastava et al., 2019). In the presence of O_2^-, a high amount of peroxyl radicals and peroxides (protein peroxidation) might be formed to oxidize proteins and other targets.

In addition to increased oxidative stress, a decrease in antioxidant concentrations were also noted in cervical cancer (Palan et al., 2004; Jiang et al., 2013). For example, an increase in ascorbic acid, concentration has the potential for the treatment of cervical cancer by inhibiting cell proliferation (MTT assay), modulation of matrix metalloproteinases (MMP-2 and MMP-9) expression (gelatinase zymography), and cancer cell invasive potential (Matrigel) in cancer development and spread (Roomi et al., 2006). Similarly, in this study, it was found that cervical cancer patients have significantly decreased vitamin A, E, and C levels compared to the control group.

Nitric oxide (NO•) is an abundant radical in the biological system. It is generated during arginine to citrulline metabolism, catalyzed by nitric oxide synthases (NOSs) (Ghafourifar and Cadenas, 2005). When reacting with water, NO• also produces nitrate (NO_3-) and nitrite (NO_2-). NO•, at its lower concentration, plays an important role in the host defense system and homeostasis at a lower concentration. However, when produced at higher concentrations for a prolonged time, NO• exhibits tumorigenic effects either by the formation of carcinogenic N-nitroso compounds, direct deamination of DNA bases, or oxidation of DNA after the formation of peroxynitrite and hydroxyl radicals (Hogg et al., 1996; Liu and Hotchkiss, 1995; Sowjanya et al., 2016). Thus, an altered level of NO• can induce DNA damage when the antioxidant defense system is not functioning properly (Liu and Hotchkiss, 1995; Shrivastava et al., 2019). Antioxidant enzymes can scavenge these radicals and provide direct protection from the radical-mediated damages. However, a decreased level of SOD and catalase activity, as found in the study, cannot handle all the ROS and a high magnitude of oxidative stress that may form in cervical cancer patients.

Moreover, an increased C/P ratio in this study indicates the loss of erythrocyte fluidity in cervical cancer patients. Probably this is due to the alteration in its lipid composition (Kolanjiappan et al., 2002). A membrane's cholesterol and phospholipid content determine its fluidity (Shinitzky and Inbar, 1976; Johnson and Robinson, 1979). Increased cholesterol is a critical factor in fluidity reduction that distorts the membrane structure and might interfere with many cellular functions. In addition to the oxidative damage, this abnormal membrane structure might also be responsible, to some extent, for its increased osmotic fragility. It makes the RBC more sensitive to changing the osmotic pressure. Since it helps to determine the integrity, membrane permeability, and alterations of erythrocytes, measurement of osmotic fragility was applied previously to diagnose diseases and analytic studies (Kumar et al., 2008). All these fit together very well with the findings of this study, where excess lipid peroxidation of the erythrocyte membrane leads to decreased resistance to hemolysis.

Conclusions

From the above discussion, it is clear that the presence of cervical cancer a significantly impacts different biochemical parameters of the human body. The present study, thus, demonstrated that the antioxidant system is compromised in cervical cancer patients more than in control groups and affects cellular function and integrity. These routine biochemical parameters can be used in the early diagnosis of cervical cancer, which will be low-cost and widely available.

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