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RESEARCH ARTICLES

Deranged Antioxidant Status and Oxidative Stress in Patients with Cervical Cancer Receiving Radiotherapy

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ABSTRACT | Cervical cancer is the seventh overall, but the second most common cancer in women worldwide. It is the second most common cancer in women of all ages in Ethiopia. Radiotherapy is one of the treatment strategies, but its effect on production of oxygen free radicals that leads to oxidative stress requires proper attention and investigation. In this study oxidative stress in 43 patients with cervical carcinoma before and in the course of radiotherapy was assessed versus 20 normal subjects. Sera were collected at 4 time points from patients, i.e., before radiotherapy, and after 2, 4, and 8 weeks of radiotherapy, but only once from normal controls. Reduced total antioxidant capacity (TAC) was observed in patients than normal subjects before and after radiotherapy. Significantly elevated total peroxide concentration (TPC) was seen in the course of radiotherapy. TPC values at the end of radiotherapy were significantly higher compared to before and after radiotherapy. Oxidative stress index in patients was significantly elevated compared to controls during and at the end of therapy. Levels of lipid peroxides in patients were markedly elevated compared to controls. Our findings showed that cervical cancer induces increased production of oxygen free radicals and radiotherapy has a synergetic effect on the production of oxygen free radicals leading to oxidative stress.

KEYWORDS | Cervical cancer; Free radicals; Oxidative stress; Radiotherapy

ABBREVIATIONS | ABTS, 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]; BHT, butylated hydroxytoluene; HPV, human papillomavirus; MDA, malondialdehyde (MDA); OSI, oxidative stress index; ROS, reactive oxygen species; TAC, total antioxidant capacity; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive species; TPC, total peroxide concentration

CONTENTS

1. INTRODUCTION

Cancer of the cervix uteri is the third most common cancer among women worldwide, with an estimated 83,195 new cases and 35,673 deaths in 2012. Worldwide, mortality rates of cervical cancer are substantially lower than incidence with ratio of mortality to incidence reduced to 50.3% [1] The highest incidence rates are observed in sub-Saharan Africa, Melanesia, Latin America, the Caribbean, and Southeast Asia. Incidence rates are now generally low in developed countries, with age standardized rates less than 14.5 per 100,000 women [2]. In Ethiopia 7,095 new cervical cancer cases are diagnosed annually. Moreover, cervical cancer is the second most common female cancer in women aged 15 to 44 years in Ethiopia [3]. Current experimental and epidemiological information undoubtedly point to the human papillomavirus (HPV) as the primary causal agent in the development of cervical cancer [4]. According to the World Health Organization (WHO)'s International Agency for Research on Cancer (IARC), at least 200 types of HPV were identified and classified into 16 groups. HPVs are members of the *Papovaviridae* family, which also includes polyomavirus and simian vacuolating virus. Although HPV is a necessary cause of cervical cancer, other factors are responsible for its progression, which include environmental cofactors (e.g., hormonal contraceptives, tobacco smoking, parity, nutrition and sexually transmitted diseases), viral cofactors (e.g., co-infection with other HPV types and HSV-2, HPV variants), and host cofactors (e.g., endogenous hormones, genetic factors, and factors related to the immune response) [5]. Deaths from cervical cancer can be prevented by cytological screening, which can detect pre-neoplastic and subclinical lesions. The long interval between the appearance of intraepithelial lesions and the development of invasive disease provides opportunities for preventive interventions against malignant transformation [6]. Treatment of invasive cervical cancer is affected by the stage of the disease. Surgery and radiotherapy have been used as a primary treatment for patients with early stage cervical cancer. The currently used International Federation of Gynecology and Obstetrics system of staging is based on anatomical extent and clinical evaluation of the disease [7].

Radiotherapy, which uses controlled high-energy rays of ionizing radiation to destroy malignant tumor cells, is one of the therapeutic modalities used to treat early and locally advanced stages of cervical cancer. Cancer cells are more sensitive to radiation because of their rapid division rate. The sound repair system for DNA damage in normal cells makes them less prone [8]. Inside cells, reactive oxygen species (ROS) like hydroxyl radical, superoxide, nitric oxide, and peroxynitrite are produced via intrinsic and extrinsic mechanisms. Mitochondria of cells during aerobic metabolism produce a variety of ROS. However, cells can neutralize their effects using enzymes, antioxidants like glutathione, and vitamins (A, C, and E). When there is imbalance between ROS production and antioxidant capacity, it leads to oxidative stress [9]. Photons generated by clinical radiotherapy machines induce a range of biochemical lesions in genomic DNA, which leads to cell death via DNA double strand break. This can be induced by direct ionization of DNA or indirectly via the generation of free radicals [10]. Alternatively, radiation has an indirect action by interacting with other atoms or molecules such as water to produce a free radical [11]. When the defense mechanism fails, membranes, DNA, and proteins may be damaged causing cell death. The aim of the present study was to assess the nature of oxidative stress and lipid peroxidation in patients with cervical carcinoma before and during the course of radiotherapy as compared to healthy control subjects. Such a study is the first of its kind in this country.

2. MATERIALS AND METHODS

The study proposal was reviewed and approved by the Ethical Review Committee of the Medical Faculty of Addis Ababa University, Faculty Research and Publication Committee (FRPC). Permission to conduct the study was obtained from the Department of Internal Medicine. Informed consent was given and signed by every participant of the study before sample collection. This prospective longitudinal study was conducted on 43 patients with cervical carcinoma (stages IIB–IVA and postoperative cases) attending the radiotherapy center of Tikur Anbasa Specialized hospital (TASH), Addis Ababa, Ethiopia. As the treatment protocol followed by Radiotherapy unit of TASH all the patients were treated by external beam radiotherapy using a cobalt 60 machine (dose 45–60 Gy, but given in fractions). Blood samples (5 ml) were obtained from 43 patients by antecubital arm vein puncture at four time points: before therapy, and after 2, 4, and 8 weeks of

radiotherapy. Blood samples were also obtained (once) from 20 socioeconomic and age matched healthy female control subjects. The whole blood samples were left to stand in ice bath for 30 min to clot. The clot was separated from the wall and specimens were centrifuged at 1500 *g* for 15 min using a Beckman centrifuge at 4°C. The supernatant hemolysis free sera were separated and stored at –70°C in aliquot till analysis.

Analytical grade reagents chemicals and kits were utilized during analysis. ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ammonium ferrous sulphate, butylated hydroxytoluene (BHT), xylenol orange, thiobarbituric acid (TBA), and 1,1,3,3-tetramethoxypropane were from Sigma-Aldrich (Gillingham, UK); CH_3COONa was from MAY and BAKER LTD (Dagenhan, England); glacial acetic acid, trichloroacetic acid, and 35% HCl were from Fischer Scientific (Leicestershine, UK); H_2O_2 was from El-Nasr Pharmaceutical Chemical Co. (Cairo, Egypt); K_2HPO_4 and KH_2PO_4 were from Carloerba (Peypin, France); NaCl was from E. Merk (Darmstadt, Germany); HPLC-grade methanol was from Labort Fine Chemicals Private Ltd (Surat, India); and H_2SO_4 was from BDH Chemical Company (Poole, UK). The water used was deionized water.

Total antioxidant capacity (TAC) was assayed by the ABTS radical cation decolorization assay [12]. Total peroxide concentration (TPC) was determined using the ferric–xylenol orange "FOX" method with minor modifications according to Harma et al. [13]. The oxidative stress index (OSI) is an indicator for oxidative stress. It is the percent ratio of the TPC to the TAC. It was calculated for serum according to the following equation after Harma et al. [13].

$$OSI = [(TPC, \mu M) \div (TAC, \mu M \text{ Trolox equivalent})] \times 100$$

Lipid peroxide levels were measured indirectly through malondialdehyde (MDA) measurement as one of the thiobarbituric acid reactive species (TBARS) according to Buege and Aust [14] modified by adding BHT to prevent further peroxidation during boiling. The extracted purple color developed due to TBA reaction was monitored spectrophotometrically using a UV-VIS spectrophotometer. Information obtained from questionnaire and laboratory investigations were analyzed using the computer statistics Prism 3.0 package (GraphPad Software, San Diego, CA, USA) and Microsoft Excel (for constructing the standard curves and calculating concentrations). The minimum level of statistical significance was set at $p < 0.05$. The data were expressed as mean ± SEM. Results were analyzed statistically using column statistics; t-test for comparison of unpaired two-tailed variables. Group differences were determined by ANOVA with post hoc testing using the Newman–Keuls method. Correlation among the investigated parameters in each group was tested by the non-parametric Spearman's analysis.

3. RESULTS

The average age group for the patient and control groups was 46.21 ± 1.63 and 41.85 ± 1.66 years, respectively, with no significant difference. However, parity for the patient group was significantly higher (5.33 ± 0.492) than that for the control group (2.70 ± 0.317) with p value < 0.01. While parity is one of the cofactors that may contribute to the transformation of HPV infections to invasive cervical cancer (ICC), non-significant difference in oxidative stress was seen. Statistical analysis showed non-significant differences among the patients with surgery and with different cancer stages considering all parameters investigated at all time points. Hence, patients were presented as one population versus controls. The TAC of patients was found to be significantly lower than that in controls ($p < 0.001$) as indicated in **Table 1**. However, the difference between varied time point measurements of the TAC for patients receiving radiotherapy was non-significant with a weak trend of progressive decline.

Figure 1 presents the changes in TPC in patients group before and during radiotherapy as compared to healthy controls. The TPC level in controls subjects and pre-radiotherapy patients was 21.66 ± 0.54 μM and 22.25 ± 0.47 μM, respectively, with no significant difference, but a progressive significant increase ($p < 0.001$) in TPC was observed during radiotherapy. The values obtained for the 2, 4, and 8 weeks radiation treated patients were 25.24 ± 0.51 μM, 25.30 ± 0.37 μM, and 27.32 ± 0.33 μM, respectively. There was no significant difference in the TPC levels between 2 weeks and 4 weeks radiotherapy, but the difference was significant when comparing 2 weeks versus 8 weeks, and 4 weeks versus 8 weeks radiotherapy patients with $p < 0.01$ and $p < 0.001$, respectively.

TABLE 1. Time-dependent change in the total antioxidant capacity (TAC) in cervical carcinoma patients before and during radiotherapy versus control

	Controls	PreRT	2 wks on RT	4 wks on RT	8 wks on RT
N	20	43	43	43	43
Range (mM)	1.28–1.92	1.00–1.78	0.83–1.76	0.94–1.67	0.65–1.62
Mean ± SEM (mM)	1.62 ± 0.042	1.32 ± 0.029	1.31 ± 0.032	1.28 ± 0.024	1.26 ± 0.03
p < vs. Controls		0.001	0.001	0.001	0.001
p < vs. PreRT			ns	ns	ns
p < vs. 2 wks RT				ns	ns
p < vs. 4 wks RT					ns

Note: Shown are group number (N), range mean ± SEM, and results of ANOVA statistical analysis (p value). PreRT, pre-radiotherapy; wks, weeks; RT, radiotherapy; ns, non-significant.

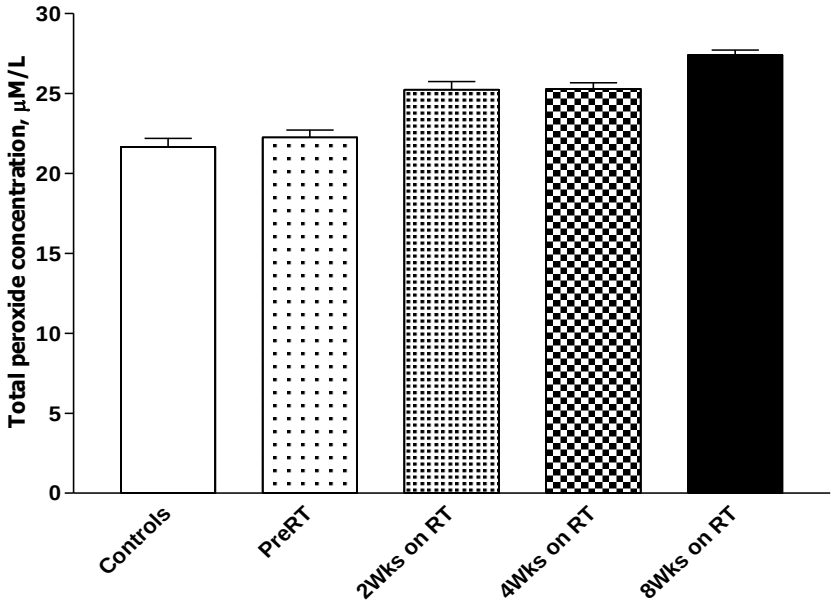

FIGURE 1. The control and time-dependent change in the total peroxide concentration (TPC) in cervical carcinoma patients before and in the course of radiotherapy. Data shown are mean ± SEM. RT, radiotherapy; wks, weeks.

Figure 2 shows the changes in the OSI in patients group before and during radiotherapy as compared to healthy controls. The healthy control OSI (1.36 ± 0.061) was significantly lower than the values for pre-radiotherapy patients (1.72 ± 0.048, $p < 0.01$) and for 2, 4, and 8 weeks in the course of radiotherapy [(2.00 ± 0.073, $p < 0.001$), (2.00 ± 0.043, $p < 0.001$), and (2.23 ± 0.069, $p < 0.001$)], respectively. Pre-radiotherapy OSI was significantly lower than the 2, 4 and 8 weeks post-radiotherapy ($p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively). The two and four weeks OSI levels were non-significantly different, but each was significantly lower than the 8 weeks level ($p < 0.05$ and $p < 0.01$, respectively).

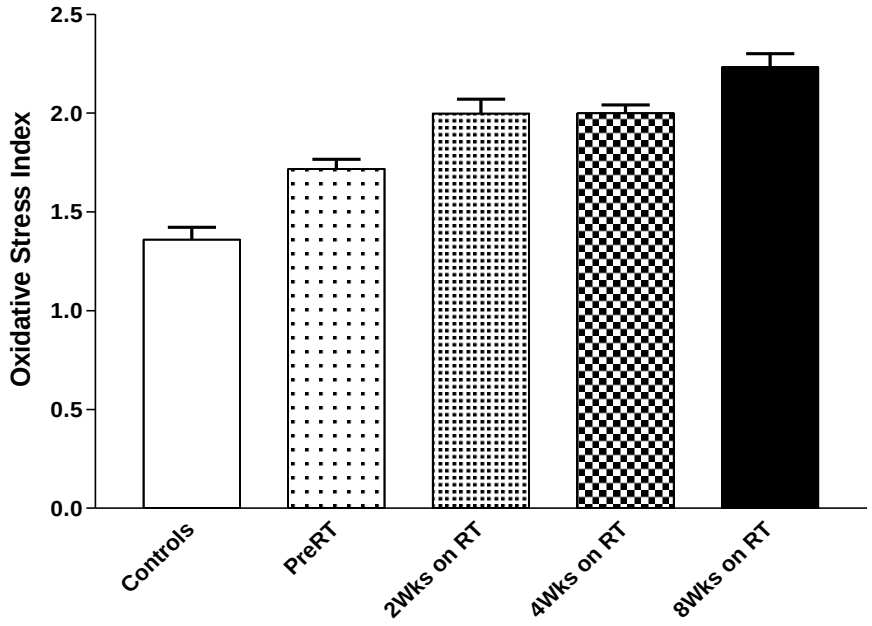

FIGURE 2. The control and time-dependent change in the oxidative stress index (OSI) in cervical carcinoma patients before and in the course of radiotherapy. Data shown are mean ± SEM. RT, radiotherapy; wks, weeks.

A significantly higher level of lipid peroxide was observed in pretreated patients as compared to control subjects (**Table 2**). However, the level declined through the treatment period among patients. The MDA levels among patients and controls were 1.59 ± 0.109 μM and 0.57 ± 0.031 μM ($p < 0.001$), respectively. There was no significant difference between values obtained for patients during the various treatment periods, i.e., 2, 4, and 8 weeks of radiotherapy (**Table 2**).

Correlation analysis of parameters for control group, patients, and between patients and controls was done, and the following results were obtained. Correlation analysis between TAC, TPC, OSI, and MDA in the control group showed that TAC correlated negatively and significantly with each of TPC and OSI, but TPC correlated positively and significantly with OSI. Other relationships were non-significant as indicated in **Figure 3**.

Figure 4 presents results of the correlation analysis between results of parameters for patients before radiotherapy. Pretreatment TAC significantly correlated negatively with OSI and positively with MDA. The MDA values correlated negatively and significantly with OSI. TPC correlated positively and significantly with OSI. Other relationships were non-significant.

The same type of analysis was done for patients after four weeks of radiotherapy and results obtained are shown in **Figure 5**. TAC positively but non-significantly correlated with TPC and it negatively and significantly correlated with OSI. TPC correlated positively and significantly with OSI and insignificantly with MDA. Oxidative stress index negatively, but non-significantly, correlated with MDA.

4. DISCUSSION

For maintenance of redox balance against oxidant conditions, the blood plays a central role because it transports and redistributes antioxidants to every part of the body. Synergistic interactions among different antioxidants in cells provide greater protection against attack by ROS than single antioxidant alone. Moreover, plasma antioxidant status is the result of the interaction of many different compounds and systemic

TABLE 2. Time-dependent changes in malondialdehyde (MDA) among cervical carcinoma patients before and in the course of radiotherapy versus controls

	Controls	PreRT	2 wks RT	4 wks RT	8 wks RT
N	20	43	43	43	43
Range (µM)	0.29–0.96	0.46–3.92	0.13–3.67	0.23–3.66	0.29–2.56
Mean ± SEM (µM)	0.57 ± 0.031	1.59 ± 0.109	1.26 ± 0.109	1.21 ± 0.103	1.17 ± 0.067
p < vs. Controls		0.001	0.001	0.001	0.001
p < vs. PreRT			0.05	0.05	0.01
p < vs. 2 wks RT				ns	ns
p < vs. 4 wks RT					ns

Note: Shown are group number (N), range mean ± SEM, and results of ANOVA statistical analysis (p value). PreRT, pre-radiotherapy; wks, weeks; RT, radiotherapy; ns, non-significant.

	TAC	TPC	OSI	MDA
TAC		–0.474 (0.05)	–0.879 (0.001)	–0.001 (ns)
TPC			0.784 (0.001)	0.063 (ns)
OSI				0.147 (ns)

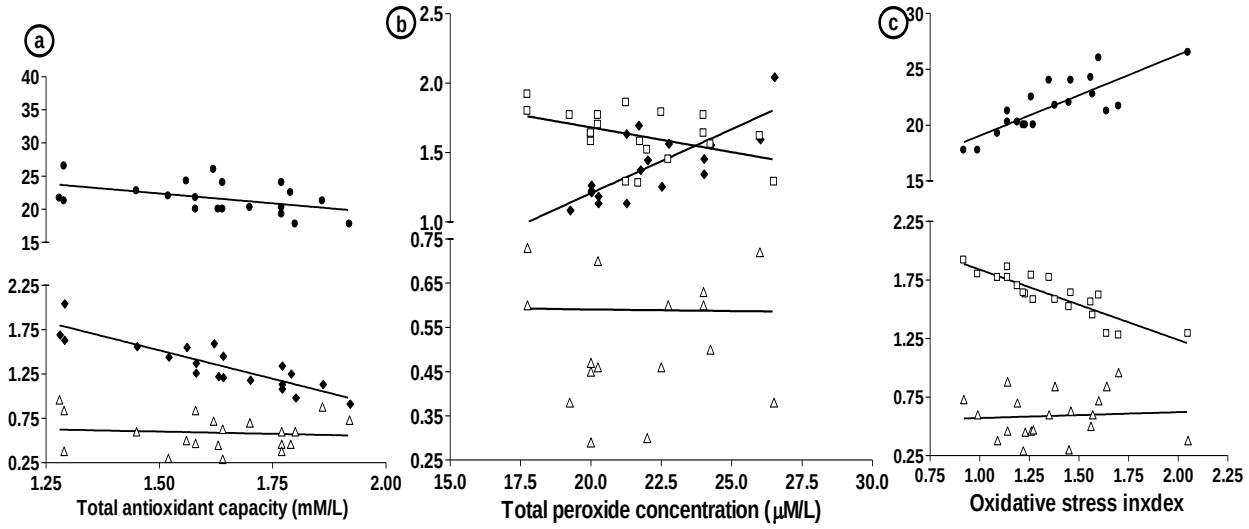

FIGURE 3. Correlation among the investigated parameters in the control group with a table showing p values and Spearman's r values. (a) Correlation between TAC and each of TPC, OSI and MDA. (b) Correlation between TPC and each of TAC, OSI, and MDA. (c) Correlation between OSI and each of TAC, TPC, and MDA. Values on x-axis are TAC in mM (□), TPC in µM (●), oxidative stress (■) and MDA µM (△) vs. the other three parameters presented on y-axis. The p values in parenthesis and spearman's r values are shown in the table attached (top panel).

metabolic interactions. Thus, TAC, measured in patients with cervical carcinoma in the present study, may give more biologically relevant information than measuring concentrations of individual antioxidants.

	TAC	TPC	OSI	MDA
TAC		–0.027 (ns)	–0.738 (0.001)	0.559 (0.001)
TPC			0.570 (0.001)	–0.046 (ns)
OSI				–0.466 (0.001)

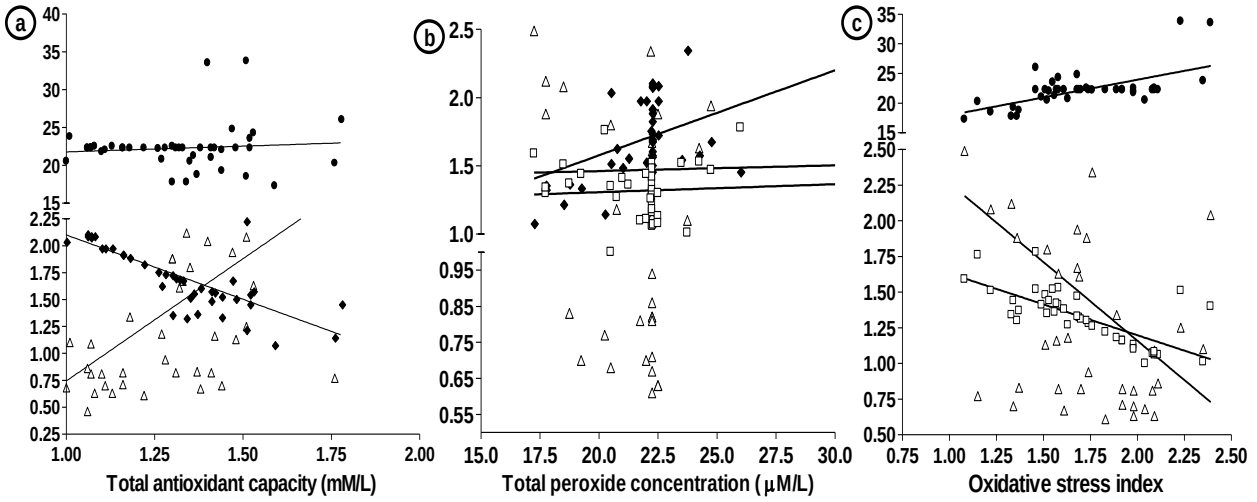

FIGURE 4. Correlation among the investigated parameters in cervical carcinoma patients before radiotherapy with a table showing p values and Spearman's r values. (a) Correlation between TAC and each of TPC, OSI, and MDA. (b) Correlation between TPC and each of TAC, OSI, and MDA. (c) Correlation between OSI and each of TAC, TPC, and MDA. Values on x-axis are TAC in mM (□), TPC in µM (●), oxidative stress (■) and MDA µM (△) vs. the other three parameters presented on y-axis. The p values in parenthesis and Spearman's r values shown in small table attached (top panel).

Extensive work has been carried out on the relationship between free radical activity, antioxidants scavenging of free radicals, and different types of cancer, including cancer of the uterine cervix [15, 16]. Impaired antioxidant defense system observed in cancer patients at multiple sites reflects the excessive free radical production. In the present study, significantly lowered levels of TAC in patients with cervical carcinoma both at pretreatment and at the three different time points in the course of radiotherapy were observed compared to healthy controls. While there are a number of previous studies that investigated plasma concentration of individual enzymatic and non-enzymatic components of the antioxidant defense in cervical carcinoma, reports on TAC are scarce. Levels of MDA in the pretreatment and at the three different time points during radiotherapy of patients with cervical carcinoma were significantly higher than those in the control group. This is a clear indication of increased ROS production and lowered antioxidant capacity leading to increased lipid peroxide levels that result in increased oxidative stress in patients with cervical carcinoma. Similar results of increased MDA level were obtained for stage IV cervical carcinoma patients in other studies [6, 17, 18]. In addition, TPC in the pretreatment patients was non-significantly higher than that in the control groups. However, radiotherapy for 8 weeks significantly increased the TPC, which was even higher than that in the pretreated patients. This adds an additional clue to the increased ROS production (beyond the natural antioxidant capacity of cells) due to radiotherapy [19].

The percentage ratio of the TPC to TAC, also known as OSI, reflects the redox balance between oxidation and anti-oxidation. In this study, OSI values of pretreatment and the three radiotherapy time points

	TAC	TPC	OSI	MDA
TAC		0. 071 (ns)	–0.711 (0.001)	0.142 (ns)
TPC			0.553 (0.001)	–0.081 (ns)
OSI				–0.008 (ns)

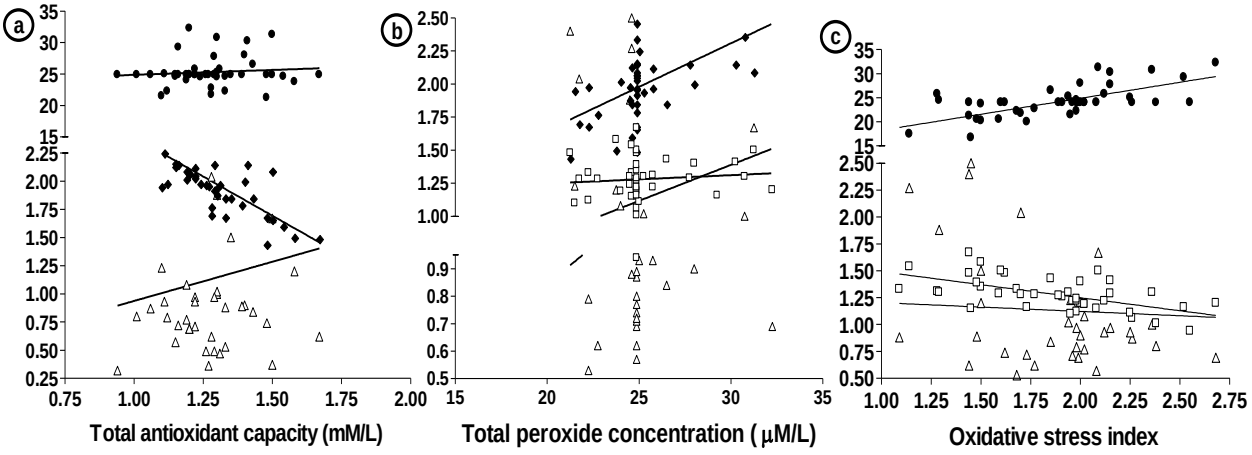

FIGURE 5. Correlation among the investigated parameters in cervical carcinoma patients at 4 weeks on radiotherapy with a table showing p values and Spearman's r values. (a) Correlation between TAC and each of TPC, OSI and MDA. (b) Correlation between TPC and each of TAC, OSI and MDA. (c) Correlation between OSI and each of TAC, TPC and MDA. Values on x-axis are TAC in mM (□), TPC in µM (●), oxidative stress (■) and MDA µM (△) vs. the other three parameters presented on y-axis. The p values and Spearman's r values are shown in small table attached (top panel).

are significantly higher than those of the healthy control group. The OSI values progressively and significantly increased in pre-radiotherapy cancer-induced state and in the course of radiotherapy time points. This shows that in addition to the disease itself, patients on radiotherapy face more oxidative stress due to the synergetic effect of the radiotherapy in the production of ROS. To the best of our knowledge, there are no reports on the association between TAC, TPC, and OSI in any cancer case (including cervical cancer) and on the effect of radiotherapy in relation to oxidative stress in Ethiopia. The disturbance in antioxidant status and the danger of increased oxidative stress in late stage cervical cancer patients and the effect of radiotherapy is verified through correlation analysis of results obtained in this research work. The negative correlation between TAC and OSI and positive correlation of TPC with OSI and MDA are clear indications of the imbalance between antioxidant status and increased production of ROS.

5. CONCLUSION

The results of this work show that cervical cancer patients are at a risk of oxidative stress that can lead to complications and worsening of the disease. This is verified through observations of increased TPC and MDA levels in the serum of cervical cancer patients enrolled in the study. Moreover, radiotherapy is likely to have a synergetic effect on the production of ROS which results in increased TPC and MDA, and a decreased TAC. Therefore, it would be wise to keep cervical cancer patients on antioxidant supplements to help them avert the effect of ROS and consequent oxidative stress. This will improve treatment outcomes

of cervical cancer patients as suggested in a previous report [20].

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