AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Radiosensitization effect of Selenium on the ''Warburg effect'', Metabolism of hypoxic Oral Squamous cell Carcinoma

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ABSTRACT

One of the hallmarks of oral cancer is its inherent insensitivity to Radiotherapy. We have analysed the tumor metabolites and quantization of biochemical and bioenergetic metabolites by NMR spectroscopic analysis. In this study, trace element selenium exhibit dual nature in oxic and hypoxic conditions as an antioxidant and anticarcinogen respectively, indexing its radiosensitising effect, denoting a remarkable decrease in tumor mass with the gradual disappearance of tumor characteristics and oxidative stress in oral cancer cases undergoing radiotherapy. Dual role of selenium as a radio sensitizer and radio-protector could warrant a significant progress in the cancer patients, as an adjuvant to the conventional therapies in oral cancer management.

Key words: Selenium, Free radicals, Oral cancer, Radiosensitisation, Radiotherapy.

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Received 30 September 2016, Accepted 07 October 2016

Please cite this article as: Elango S *et al*., Radiosensitization effect of Selenium on the "Warburg effect", Metabolism of hypoxic Oral Squamous cell Carcinoma. American Journal of PharmTech Research 2016.

INTRODUCTION

Tumor hypoxia has been considered to be a potential therapeutic problem since it renders solid tumors more resistant to radiation therapy [1]. Detecting hypoxic tumor cells at the start of therapy would provide an opportunity to include treatment strategies designed to improve tumor oxygenation or kill hypoxic tumor cells. Therefore, an agent which involves an additional mechanism to radiosensitise the hypoxic cells resistant to the irradiation is needed. Selenium, a natural antioxidant mineral exhibits dual nature in oxic and hypoxic conditions as antioxidant and anticarcinogen respectively. Selenium deficiency is associated with increased occurrence of malignancies like breast, gastrointestinal chronic lymphatic leukemias, oral, etc. [2,3] and also the lower concentration of selenium is associated with distinct metastasis, multiple primaries, recurrences and decreased survival time.

Oral cancer usually develops from hyperplasia through dysplasia to carcinoma in the manner of 'field cancerization' due to carcinogen exposure mostly of tobacco origin, with highly altered metabolic profile inside the tumor [4]. Tumor cells are more efficient in utilizing energy substrates for growth, amino acids for gluconeogenesis and protein synthesis from the host increasing the metabolic burden. Reactive oxygen species (ROS) are the mutagenic compounds known to favor cell transformation and to mediate the effect of ionizing radiation. The ionizing radiation producing free radicals from the radiolysis of cellular water includes hydroxyl radicals, hydrated electrons and hydrogen peroxide[5,6]. Thus the radiation mediated oxidative stress aggravates the already existing oxidative stress in the cancer condition. In the present study, trace element selenium was supplemented to oral cancer patients undergoing radiotherapy to examine its capacity of eliminating tumor hypoxia and thus radiosensitising the cancer cells, through observations on the alterations in the tumor characteristics, and the degree of intracellular perturbation. The putative role in reducing the radiation mediated oxidative stress were also analyzed for its radioprotectant activity, in the concept of combating free radical level exclusively after the cessation of irradiation.

MATERIALS AND METHOD

Patients

Oral cancer patients attending the out-patient clinic and the In-patients were recruited in the study as per the process and approval by the hospital ethical review board. Patients under the study was divided into two groups. Group I comprised of control (Normal healthy volunteers; n=10), Group II comprised of oral cancer patients untreated; n = 20 (At the initial, 24 untreated oral cancer patients recruited for the study. Later, there was 2 dropouts and 2 casualty death). Group II was

bifurcated into Group IIa and IIb. Group IIa comprised of oral cancer patients who underwent radiotherapy alone (n = 10) and Group IIb constituted of oral cancer patients who underwent radiotherapy along with supplementation of selenium (n = 10). Both Group IIa and IIb were followed up for 6 months and all the patients completed the long time therapy. The study protocol was followed strictly and the patients consented prior to this study. Eligibility criteria include patients with age within 40-65; patients having no systemic diseases such as hyper tension, diabetes mellitus or any infections (since these might interfere with the results of the study);patients not undergoing any other treatment (surgery, chemotherapy or any other drug supplementation).Table 1 A,B details the anatomic sites and clinico pathological features of the Patients .

Table 1A: Anatomic sites of patients with Squamous Cell Carcinoma of Oral Cavity

Site	No. of Cases
Tongue	4
Floor of the Mouth	6
Palate	2
Buccal Mucosa	8

Treatment

Radiotherapy was delivered with a tele-cobalt beam using anterior and lateral wedge pair or lateral parallel portals (Theratron-780-^{60}Co; phoenix-^{60}Co; Gammatron-^{60}Co) at a dosage of 6000 cGy (200 cGy/day) in five fractions per week for a period of six weeks. Radiotherapy employs a daily tumor dose of 180-225 rads.

Selenium supply

Selenium capsule containing 400 µg of selenium (sodium selenite) was administered in patients for period of 6 months, right from the day one after the cessation of the Radiotherapy. Selenium capsules procured from Cassel research laboratories, Chennai, India. Dosage of selenium was fixed with reference [7].

Sample collection

Oral cancer tissue samples were collected before initiating radiotherapy and after 6 months in both Group IIa and (Group IIb), and following analysis were investigated: i. Analysis of tumor metabolites by NMR (Nuclear Magnetic Resonance): Quantization of biochemical and bioenergetic metabolites by NMR spectroscopic analysis were done by the method [8]. ii. Degree of perturbation by Spin echo analysis: Spin echo analysis by NMR spectroscopy was done by the method [9]. iii. Electron Spin Resonance (ESR) detection of free radicals: Free radicals were detected by ESR - Spectrometer according to the method [10]. iv. Quantization of tumor necrosis

factor (TNF-α): The level of TNF-α in cancer tissue was measured by ELISA according to the method [11]. Mouse monoclonal antibody against human TNF-α (1:5,000 dilution) were used. TNF-α was expressed as pg/ml. v. Expression of Caspase-3: Activity of Caspase-3 in Cancer tissues were analyzed by Western Blot method according to the method [12]. Mouse monoclonal antibody against human Caspase-3 (1:1000 dilution) were employed (Transduction Laboratories, Lexington, KT, USA). Blots were photographed and visualized on Biomax MR film (Kodak, Rochester, NY).

Statistical analysis

Statistical significance of the differences between all the groups were determined by one way analysis of variance (ANOVA) using Statistical package for Social Sciences (SPSS) version 17 software package for Windows, USA. Values of $p < 0.05$ were considered to be significant and are expressed as mean ± Standard deviation.

RESULTS AND DISCUSSION

Biochemical and Bio energetic metabolites

Hypoxic tumor cells exhibit severely altered metabolite profile. The proton spectra of oral tissues from normal, untreated oral cancer patients, radiation treated and the selenium supplemented oral cancer patients, were shown in Figure 1 and 2.

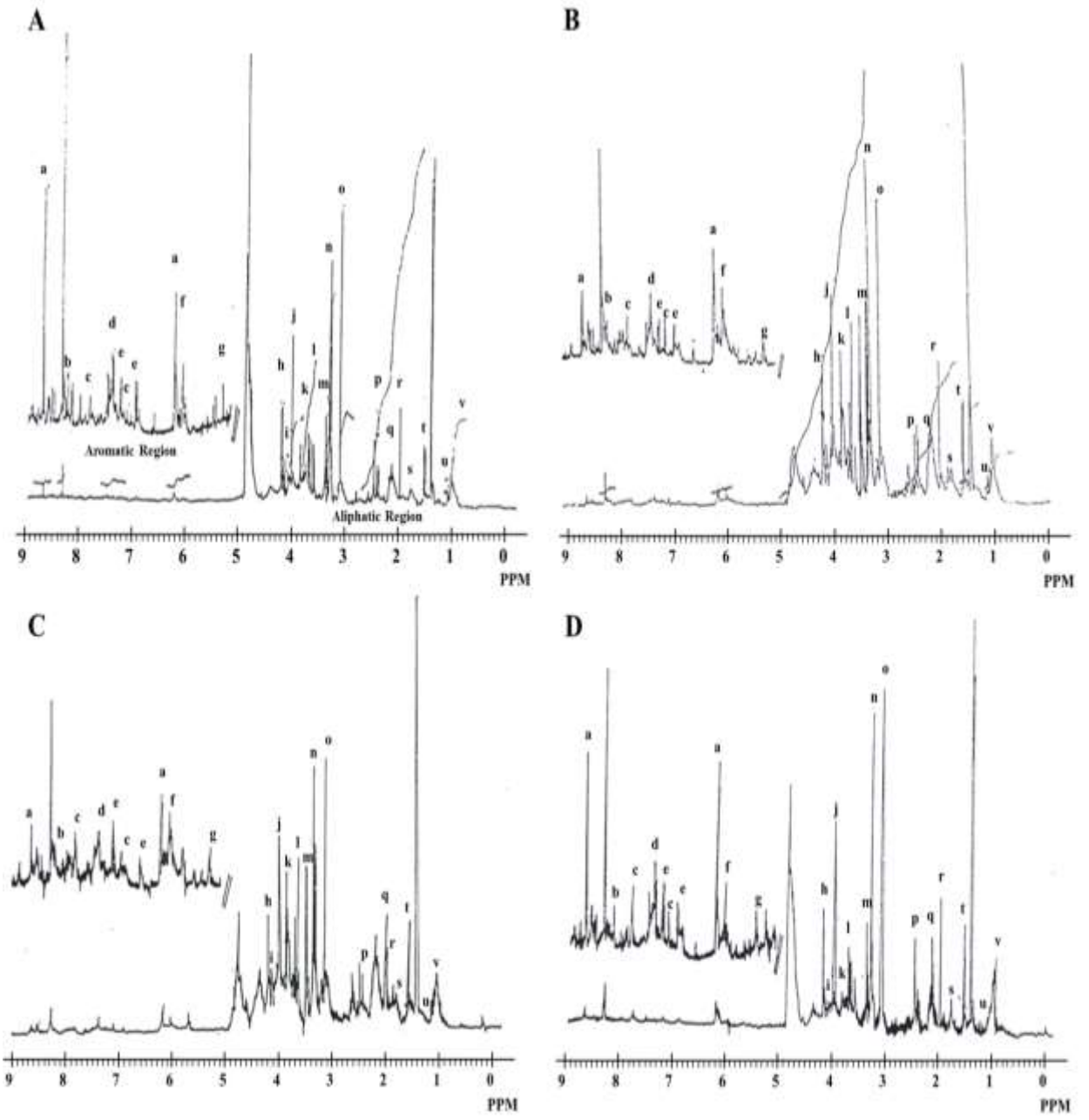

Figure 1: Water suppressed 1H (proton) Nuclear Magnetic resonance spectrum of normal and cancer tissues of all study groups (400 MHz). Aromatic region: a-Adenine; b-guanine; c-Histidine; d-phenylalanine; e-tyrosine; f-uracil+cytosine+guanine, g-glycine.Aliphatic region: h-lactate; i-Phosphoryl ethanolamine; j-phosphocreatine; k-creatine; l-Glycine+glutamine; m-taurine; n-phosphoryl choline; o-phosphocreatine; p-proline+glutamine; q-proline+glutamine+glycine,r-acetate, s-leucine; t-alanine; u-valine, v-leucine.

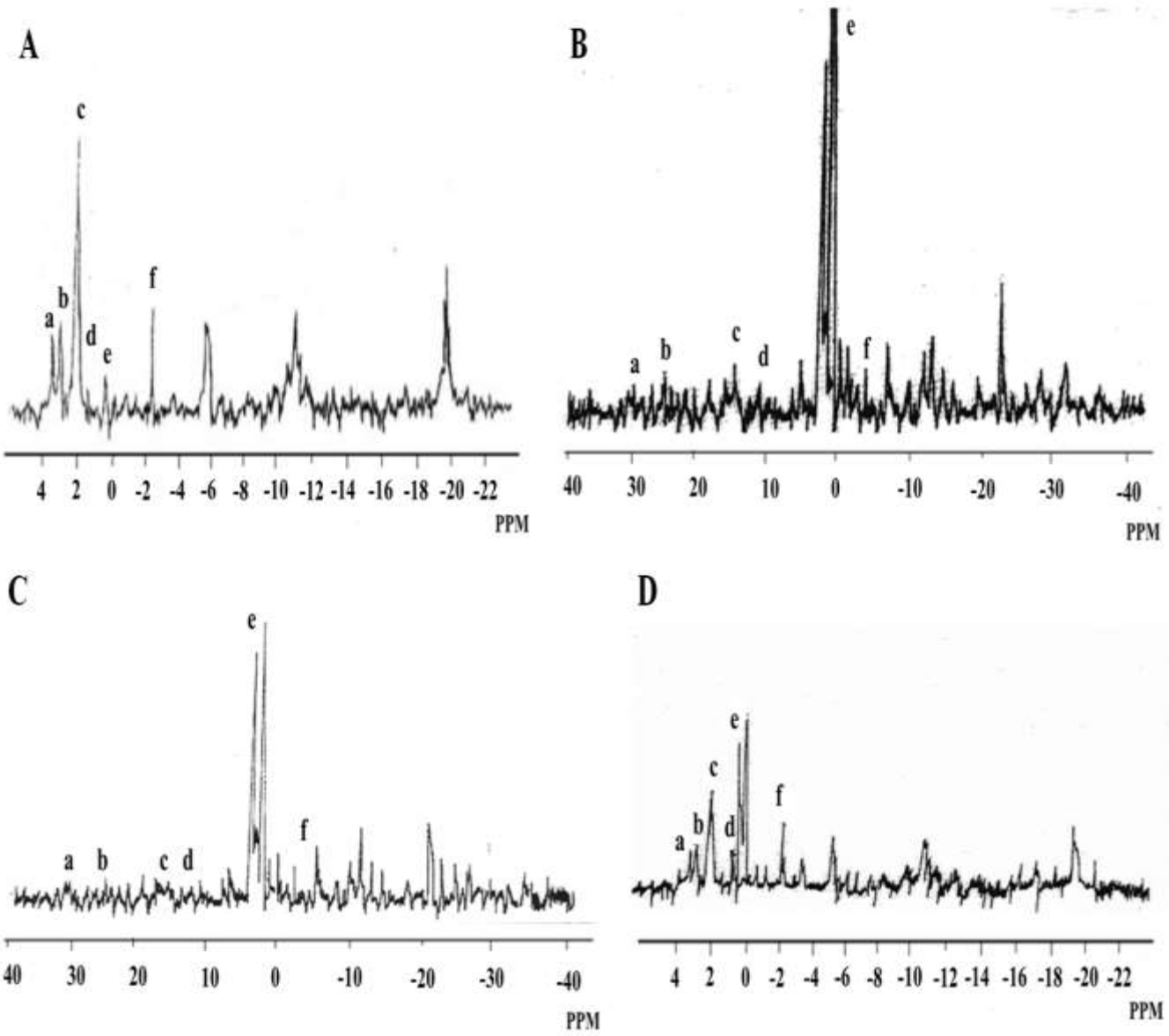

Figure 2: ^{31}p (Phosphorous-31) nuclear magnetic resonance spectrum of normal and cancer tissues of all study groups (400 MHz). a-phosphoryl choline; b-phosphoryl ethanol amine; c-inorganic phosphate; d-Glycerophosphoryl ethanolamine; e-glycerophosphorylcholine; f-phosphocreatine

Well resolved resonances of various aminoacids, nucleotides, creatine, phosphocreatine, phosphoryl ethanolamine, choline and glycerol phosphoryl choline and lactate were clearly distinguishable (Figure 1) in all the four groups in NMR spectrum. Resonance assignments were done using methods, including analysis of chemical shifts, pH dependence, two-dimensional J resolved and cosy spectroscopy (Figure 3).

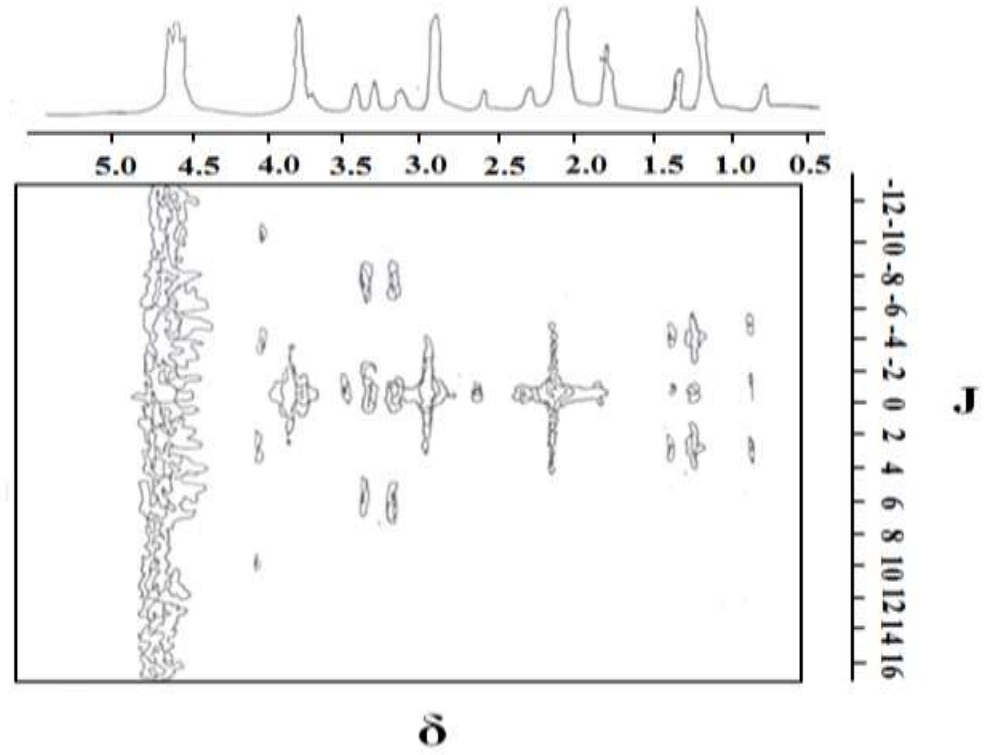

Figure 3: Contour plot depicting 400 MHz, IH homonuclear shift-correlated two dimensional nuclear magnetic resonance spectrums.

The NMR spectrum of tumor tissue Figure 1B (Group II) showed certain resonance peaks (aliphatic region) compared to the normal tissue Figure 1A (Group I). In addition certain resonance signals absent in normal tissue were seen clearly in untreated tumor which corresponds to the methyl protons of alanine at 1.47 ppm, the protons of glutamine, proline and glutamic acid near 2.1 ppm. Methyl methylene protons of leucine at 0.98 and 1.7 ppm, methyl resonance from valine 1.21 ppm resonances of choline, glycerol phosphoryl choline and S-CH2 of taurine (triplet) near 3.43 ppm were clearly identifiable.

Figure 1C and 1D show the proton spectrum of tissue from Group IIa and IIb, respectively. In the aliphatic region, in contrast to the Group II and IIa , the Group IIb (compare Group II b with Group II a) showed less resonances, unseen in normal tissue. The disappearance of certain resonance bands in Group IIb , indicates the favorable modulations in the tumor characteristics.

The level of aromatic metabolites is indicated in Figure 1 (aromatic region). In the aromatic region, tumor tissue of Group II (Figure 1B) relatively showed a low concentration of metabolites compared to Group I (Figure. 1A). Group II a (Figure 1C) showed some similarity with Group I, yet in aromatic region most of metabolites were absent except that of adenine derivatives, which appears to resemble the metabolites of untreated tumour. In contrary, the resonances of Group II b (Figure 1D) showed increased concentration of metabolites compared to Group IIa .This appeared

to be similar to Group I. These results show that the tumor characteristics still present in Group IIa , was found to be decreased with Group IIb. Concentration of metabolites like lactate, creatine/phospho creatine, choline/phosphocholine, alanine/proline/glutamine/glycine, valine/leucine of Group II showed marked increase in Group I (Table 2). This showed significant decrease at six months in Group II a, while much more decrease was observed in Group II b ($p<0.05$).

Table 1B: Clinic Pathological Features of Patients

Characteristics	No. of Patients
T. Classification	
T1	0
T2	1
T3	3
T4	16
Node	
Negative	17
Positive	3
Metastasis	-
Tumor Thickness	
<= 10mm	2
>10mm	18
Histological Grading	
(G1) Well Differentiated	1
(G2) Moderately Differentiated	3
(G3) Poorly Differentiated	16

Table 2: ^{1}H NMR: Concentration of biochemical metabolites in normal and oral carcinomatous tissues of all study groups creatine/phospho creatine (Cr/Pcr), phosphocholine (PC), alanine/proline/glutamine/glycine (Ala/Pro/Glu/Gly)

Groups	N	AIa/Pro/Glu/Gly				Valine/Leucine			
		Mean (SD)	SE	F st	p value	Mean (SD)	SE	F st	p value
Groups I (Normal)	10	2.423 (0.059)	0.019	982.51	< 0.0001	0.304 (0.052)	0.016	78.47	< 0.0001
Groups II (B T)	20	3.909 (0.066)	0.015			0.791 (0.045)	0.01		
Groups II a (RT)	10	3.506 (0.066)	0.021			0.710 (0.12)	0.038		
Groups II b (RT + selenium)	10	3.095 (0.101)	0.032			0.610 (0.12)	0.038		

Groups	N	Choline/PC			
		Mean (SD)	SE	F st	p value
Groups I (Normal)	10	2.205 (0.057)	0.018	1817.5	< 0.0001
Groups II (B T)	20	4.901 (0.091)	0.02		
Groups II a (RT)	10	4.404 (0.154)	0.049		
Groups II b (RT + selenium)	10	3.417 (0.074)	0.024		

B T - before treatment; RT – radiotherapy

Figure 2F, 2G, 2H and 2I show the phosphorus–31 NMR analysis of all Groups. Concentration of phosphorus metabolites phosphoryl choline, phosphoethanol amine, phosphocreatine , glycerophosphoryl choline , glycerophosphoryl ethanolamine showed marked increase in Group II ($p<0.05$) compared to Group I (Table 3). In Group IIa except GPC, other parameters showed a moderate decrease at 6 months ($p <0.05$). Yet Group IIb decreased the level of these metabolites to a greater extent ($p<0.05$).

Table 3 31P **NMR: Concentration of bioenergetic metabolites in normal and oral carcinomateous tissues**

Groups	N	PC				PEth			
		Mean (SD)	SE	F st	p value	Mean (SD)	SE	F st	p value
Groups I (Normal)	10	19.19 (0.44)	0.14	80.42	< 0.0001	16.10 (0.49)	0.155	406.09	< 0.0001
Groups II (B T)	20	23.23 (0.42)	0.094			22.39 (0.58)	0.13		
Groups II a (RT)	10	2.600 (0.41)	0.129			19.84 (0.46)	0.146		
Groups II b (RT + selenium)	10	2.002 (1.48)	0.467			17.96 (0.34)	0.108		

Groups	N	PCr				GPC			
		Mean (SD)	SE	F st	p value	Mean (SD)	SE	F st	p value
Groups I (Normal)	10	7.2 (0.14)	0.045	721.47	< 0.0001	4.0 (0.27)	0.086	444.74	< 0.0001
Groups II (B T)	20	12.89 (0.23)	0.051			7.11 (0.23)	0.051		
Groups II a (RT)	10	12.11 (0.45)	0.143			6.93 (0.16)	0.052		
Groups II b (RT + selenium)	10	11.11 (0.44)	0.14			5.67 (0.27)	0.084		

	N	GPE			
		Mean (SD)	SE	F st	p value
Groups I (Normal)	10	3.10 (0.24)	0.075	146.48	< 0.0001
Groups II (B T)	20	5.12 (0.24)	0.053		
Groups II a (RT)	10	4.09 (0.25)	0.08		
Groups II b (RT + selenium)	10	3.97 (0.32)	0.101		

B T - before treatment; RT – radiotherapy

Spin echo resonance measurements

The spin echo resonance measurements of intracellular water of malignant and normal tissues are listed in the form of Table 4 A. The contrast between the spin-lattice (T1) and spin-spin (T2) relaxation rates of Group II(T1 $p < 0.05$; T2 $p < 0.05$) showed an marked increase compared to Group I.This illustrates high degree of perturbation in the endosolvent structure that accompany malignant transformation and status. A considerable decrease in relaxation times (T1 $p<0.05$; T2 $p<0.05$) was observed in Group II a, while apparent decrease in Group II b(T1 $p<0.05$; T2 $p<0.05$) suggests a notable change in the degree of ordering of intracellular water in the malignant tissue ,by selenium.

Table 4A: Spin echo proton signals of intracellular water of normal and oral malignant tissues

Groups	N	T1				T2			
		Mean (SD)	SE	F st	p value	Mean (SD)	SE	F st	p value
Groups I (Normal)	10	0.46 (0.143)	0.045	87.47	< 0.0001	0.058 (0.003)	0.001	18.62	< 0.0001
Groups II (B T)	20	0.786 (0.025)	0.006			0.119 (0.025)	0.005		
Groups II a (RT)	10	0.707 (0.025)	0.008			0.105 (0.029)	0.009		
Groups II b (RT + selenium)	10	0.434 (0.039)	0.012			0.077 (0.025)	0.008		

T1 – Spin Lattice relaxation times; T2 – Spin Spin relaxation times; B T - before treatment; RT – radiotherapy

Table 4B: Free radical levels in normal and oral carcinomatous tissues

Groups	N	Free radicals			
		Mean (SD)	SE	F st	p value
Groups I (Normal)	10	5.89 (0.17)	0.053	239.6	< 0.0001
Groups II (B T)	20	15.85 (1.14)	0.254		
Groups II a (RT)	10	15.29 (1.38)	0.437		
Groups II b (RT + selenium)	10	9.32 (1.14)	0.361		

B T - before treatment; RT – radiotherapy

Table 4C: Concentration of TNF-alpha in normal and oral carcinomatous tissues

Groups	N	TNF - alpha			
		Mean (SD)	SE	F st	p value
Groups I (Normal)	10	5.45 (0.34)	0.108	3917.74	< 0.0001
Groups II (B T)	20	3.50 (0.27)	0.06		
Groups II a (RT)	10	13.89 (0.17)	0.053		
Groups II b (RT + selenium)	10	9.81 (0.25)	0.08		

B T - before treatment; RT – radiotherapy

Free radical estimation

Table 4 B, demonstrates the levels of free radical in oral tissues of normal and all study groups. Free radical levels are evidenced with increased spin concentration confirming the over production of reactive oxygen species during carcinogen metabolism in untreated oral cancer patients. These are hydroxyl radicals corroborating the oxidative stress and hence destroying the cancerous cells. Several fold increase in free radical levels observed in radiation treated oral cancer patients (Group II a) at initial months decreased at sixth month. Yet selenium highly reduced the free radical content at 6^{th} month ($p<0.05$).

Level of TNF-α

Table 4 C, represents the level of tumor necrosis factor-α (TNF-α) in normal and all study groups. Untreated oral cancer tissue showed decrease in the level of TNF-α. While increased TNF-α level noticed in radiation treated group (Group II a) . Selenium supplemented group showed suppressed level of the TNF-α expression ($p<0.05$).

Caspase-3 expression

The Caspase-3 level in cancer tissues was measured by western blot (Fig. 4). In group I, normal level of Caspase-3 was observed. There was an obvious decrease in Caspase-3 level in cancer tissues. However, radiation treatment resulted with increase in Caspase-3 protein levels, which was maintained until six months. Cancer patients supplemented with selenium showed remarkable increase in Caspase-3 level (Figure. 1) at initial months and gradually suppressed at the end of six months.

Figure 4: Western blot analysis of Caspase-3 expression in oral tissues of normal and all study groups.

Lane: 1. Protein marker; 2. Group I; 3. Group II ; 4. Group IIa ; 5. Group II b

DISCUSSION

Cancer cells are characterized by the alterations in their cell metabolism which include increased glucose consumption and aerobic glycolysis, commonly known as the"Warburg effect" [13]. In our study, the observed resonances peaks in the NMR analysis are assigned to specific metabolites and

its alterations, reflect the presence and status of tumor burden. The relative levels of lactate, creatine, phosphocreatine, choline and phosphor glyceryl choline were found to be higher in untreated tumor tissue, as compared to normal. The acidosis resulting from accumulation of lactic acids in hypoxic tumor cells may be the reason for increased level of lactate , which have been already documented as increased lactate production in tumor bearing patients [14]. This increase is consistent with increased glycolytic flux. A high rate of glycolytic activity and lactate production is commonly seen in cancer tissues [15]. The host, recycles the lactate produced by the tumor during anaerobic glycolysis.

Tumor cells are more efficient in utilizing amino acids for gluconeogenesis. Increased accumulation of amino acids like aspartic acid, serine, glutamic acid, tyrosine, glutamine inside cancer tissue (Group II) were eventually due to the dependence of tumor on amino acids. The alterations in amino acid may be the consequence of tumor growth, i.e., increased utilization of amino acid from the system for tumor growth. The decrease and disappearance of resonance peaks corresponding to the metabolites, found to be more in selenium treated tissues, indicate reduction in the tumor mass.

Neoplasia is associated with profound alterations in endosolvent structure mediated through the increase in spin relaxation times. The intracellular water (endosolvent) exists as multiple polarized layers adsorbed on to cell proteins. Altered alkali cations of biologic tissues in malignant condition indicate alterations in tissue water structure [16]. Substantially narrow line widths (the result of decreased ordering of cell water) were observed for immature muscle than for mature muscle. On this basis, the differentiated neoplastic tissue can be expected to manifest increased relaxation times (narrow line widths). Cancerous tissue has a lower degree of organization and less water structure than normal tissue. Our data showing spin – lattice, spin – spin relaxation times, confirmed with the results reported previously. There is a correlation between narrowing of the band width and relaxation time of the cell water. The increase in spin relaxation times slid down following radiation. The elimination of tumor cells following radiation after six months duration might have caused the decrease in tumor mass and therefore the decrease in spin relaxation times. Yet much decrease in spin relaxation time in the selenium supplemented cancer tissue revealed improvement in the degree of organization and water structure of the cells.

Trace element Selenium plays a critical role in anti carcinogenesis. Under the hypoxic condition, that is prevailing inside the cancer cells, selenium undergoes thiol reduction where high GSH level exists inside the cancer cells [17]. The resultant formation of anticarcinogenic metabolites (Seleno diglutathione, Selenotrisulfides, Methyl selenol) perturb the metabolism of tumor cells and induce

apoptosis. Selenodiglutathione inhibits aminoacid incorporation in a cell-free system and thus inhibits protein synthesis affecting cell proliferation. These radiosensitises the hypoxic radioresistant cells leading to the elimination of cancer cells. Our results indicate the contribution of selenium in decreasing the tumor characteristics (denoting the shrinkage of tumor mass) which is reflected through the decrease and disappearance of certain resonance peaks and also decrease in the degree of perturbation. Radiation has been shown to induce generation of reactive oxygen radicals potentiating toxicity in both animals and humans [18].The gradual decrease in the free radicals at sixth month indicates the subsidizing effects of radiation.

Yet in selenium supplemented oral cancer patients, the highly diminished levels of free radicals might be related to the free radical scavenging activity at the 'oxic' condition prevailing inside the cancer cell in consequence to the elimination of hypoxic cells and the antioxidant enhancing property of selenium. Since selenium is endowed with favorable capacity to enter into cells via diffusion, high retention of it might contribute to the elimination of free radicals [19]. Selenium compounds can act as free radical traps, scavenging the carcinogen and radiation mediated free radicals and converting them to stable products [18,20.] The significant elevation in the tissue level of TNF- α in radiation treated oral cancer patients , may be due to ROS mediated activation of nuclear factor NFκ-B, which regulates TNF-α production at transcriptional and translational levels. The active effect of Caspases, promote apoptosis by cleaving cellular substrates leading to the morphological and biochemical features of apoptosis [21].

The reduction in the level of tissue TNF-α in selenium administered for 6 months (Group IIb) in oral cancer cases may be ascribed to the inhibition of activation and translocation of NFκ-B by this antioxidant through its free radical scavenging activity. The increase in Caspase-3 level in radiation treated cells, might be due to its cytotoxicity still persisting. The significant increase of Caspase-3 level in selenium group at initial months indicates the cytotoxicity via its formation of anticarcinogenic metabolites. While, gradual reduction of Caspase-3 level shows the elimination of tumor mass through cell death.

CONCLUSION

These favorable changes following supplementation of trace element selenium project a remarkable decrease in tumor mass with gradual disappearance of tumor characteristics and oxidative stress. The dual role of selenium as a radio sensitizer and radio-protector could warrant a significant progress in the cancer patients, as an adjuvant to the conventional therapies in oral cancer management.

ACKNOWLEDGEMENTS

The author acknowledges the financial assistance rendered by the Division of Research Funds, University of Suwon, S. Korea, for to undergo this research study.

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