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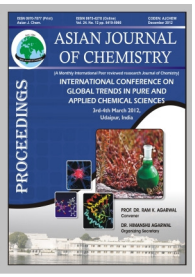

Study of Peroxidase Enzyme and Related Trace Elements in Serum and Saliva of Patients with Oral Epithelial Tumors†

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The present study was designed to investigate the changes in the activity and specific activity of peroxidase and related to iron concentration in serum and saliva samples of patients with oral epithelial tumors (benign and malignant). Anodic and cathodic conventional PAGE (7.5 %) were used to illustrate the changes of the enzyme due to the presence of the tumor. Thirty three patients with oral tumor (benign and malignant) aged 15-75 years attending the specialized surgery hospital/Baghdad medical city were include in this study. Thirty two healthy individuals of matched age and gender were utilized as control. The results indicated the presence of highly increase ($p < 0.001$) of both serum and salivary peroxidase activity (U/L) in both benign and malignant groups in comparison to that of control. The specific activity of peroxidase showed a significant increase ($p < 0.05$) in saliva sample of benign group. Serum iron concentration results showed a highly significant decrease ($p = 0.01$), ($p < 0.01$) in benign and malignant groups respectively in comparison to the control, while the salivary iron concentration showed a highly significant and significant increase ($p = 0.001$), ($p = 0.05$) in benign and malignant groups respectively. The ratio of iron concentration/peroxidase activity showed a significant difference ($p < 0.01$) in serum samples of benign and malignant groups in comparison to the control.

Key Words: Antioxidant enzymes, Oral cancer, Saliva, Peroxidase.

INTRODUCTION

Oral cancer is the 6th most common cancer in the world, with a high morbidity rate and a 5-years mortality rate of about 50 %[1]. Saliva has many diagnostic uses, like large scale screening and epidemiologic studies[2,3], whole saliva however, is most frequently used for diagnosis of systemic diseases, since it is readily collected and contains serum constituents. These constituents are derived from the local vasculature of the salivary glands and reach the oral cavity *via* the flow of gingival fluid[4]. It is well known now that oxidative stress is one of the etiological factors of many pathological cases[5] including cancer; it has been implicated in the cancer process, either by increasing the formation of free radicals or by a decrease in the antioxidant defense[6]. Reactive oxygen species such as superoxide radicals ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are postulated to be involved in neoplastic transformation. The antioxidant defense system limits cell injury induced by reactive oxygen species[7]. One of the important antioxidant enzymes is Peroxidase (donor- H_2O_2 oxidoreductase, EC. 1.11.1.7); it is a hemoprotein catalyzing the oxidation by hydrogen peroxide of number of substrates. Peroxidase, having a wide range of biological functions and present in most cell organelles and take part in either antibacterial actions or cellular defense against oxidative damage by reactive oxygen species[8]. Salivary peroxidase enzymes (often called SPO system); this system is composed of the enzyme salivary peroxidase (SPO), the thiocyanate ion (SCN^-) and hydrogen peroxide (H_2O_2). Salivary peroxidase secreted from the major salivary glands, mainly the parotid gland contributes 80 % of oral peroxidase activity and myeloperoxidase produced by leukocytes in inflammatory regions of the oral cavity contributes the remaining 20 % of the oral peroxidase activity[9]. Salivary peroxidase system (SPO system) has a dual function in the oral cavity, generating the antibacterial agent hypothiocyanite anion ($OSCN^-$) and detoxifying H_2O_2, which secreted by bacteria and leukocytes present in the oral cavity.

The aim of this study is to investigate the effect of oral tumors on the activity and specific activity of total peroxidases enzyme in serum and saliva samples and distinguish the variation of its isoenzymes on poly acrylamide gel electrophoresis. Since peroxidase is a metalloenzyme (haemoprotein), so it is

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worthy to estimate the iron concentration which also plays a role in oxidant/antioxidant status.

EXPERIMENTAL

Sixty five individuals were included in the present study. Thirty three of them were clinically and histologically diagnosed as patients with oral epithelial tumors (benign and malignant). The samples were collected from patients attending the hospital of Specialized Surgery in Baghdad Medical City. Patients were evaluated by full medical history to exclude any existing of systemic disease or taking any drug that may affect the parameters to be examined. The results were compared with those obtained from a control group of 32 ages and gender matched healthy individuals.

Sera samples: Six mL of venous blood taken from each individual, allowed to stand at room temperature for 0.5 h, centrifuged at (2000xg) for 10 min, the obtained sera transferred immediately to another test tubes. These samples were estimated directly for enzymes activities or frozen at - 20 ºC for subsequent analysis. Hemolyzed samples were discarded.

Saliva samples: About 10 mL unstipulated (resting) whole saliva were collected after the diagnosis, under resting conditions between 8.0-10.0 A.M. Patients were asked to rinse their mouth with water and to generate saliva in their mouth and to spit into a wide test tube[10]. The collection period was 20 min. Following the collection, the saliva was centrifuged at (2000 xg) for 10 min. The resulting supernatant was stored at - 20 ºC in polyethylene tubes until assayed.

Determination of protein concentration: Total protein concentration of all samples (serum and saliva) was determined using a modified Lowry method by Hartree[11] bovine serum albumin (BSA) was used as a standard.

Determination of total peroxidase activity: Peroxidase activity was determined colourimetrically. In this study an improved assay was adopted using 4- amino antipyrene as hydrogen donor[12]. The activity was determined by measuring the increase in absorbance at λ = 510 nm resulting from the decomposition of hydrogen peroxide per time of incubation (ΔA/min). One unit of the enzyme activity represents the decomposition of one µmole of hydrogen peroxide per min at 25 ºC and pH = 7 under the specified conditions.

The test tube contained 1.4 mL of phenol (0.17 M) in 4-aminoantipyrine solution (0.0025 M) and 1.5 mL of (0.0017 M) hydrogen peroxide in (0.2 M) phosphate buffer. The test tube was incubated at 25 ºC for 3-4 min to achieve temperature equilibration. The reaction was initiated by the addition of (0.1 mL) of the sample (serum, saliva), with mixing. The increase in the absorbance at λ = 510 nm, was recorded for 5 min, to obtain ΔA/min.

(ΔA/min) was calculated, since ΔA is the difference in absorbance between zero time and 5 min.

$$\text{Peroxidase activity U/L} = \frac{\Delta A/\text{min}}{\varepsilon} \times \frac{Vt}{Vs} \times 10^6$$

where, Vt = total volume; Vs = sample volume; ΔA/min = (Abs. at 5 min - Abs. at the zero time)/ incubation time (5min.)

Determination of the related metal ion concentration: Iron concentration was determined using atomic absorption spectrophotometer (GBC 933 plus) at wavelength 248 nm[13].

Peroxidase electrophoretic profile: Peroxidase enzyme was reported to consist of acidic and basic isoenzymes, so anodic and cathodic conventional PAGE (7.5 %) were used to illustrate the differences in separation profile of the sera and saliva peroxidase isoenzymes of the studied groups. A conventional polyacrylamide gel electrophoresis (7.5 %) was carried out according to LKB 2117 note, using *tris*-glycine buffer, stock solution (0.15 M) pH 8.9 and a volume of (10 µL) of the samples were applied into the wells in the gel and concentrated for 5-10 min with a current of 20 mA, electrophoresis was continued, using 40 mA, until the bromophenol blue dye reached the gel margin. Cathodic polyacrylamide gel electrophorysis (7.5 %) carried out according to Reisfel *et al.*[14] using β-alanin buffer (pH 4.5), the samples(10 µL) were applied to the gel, electrophorysis was continued using 30 mA until the dye methylene green reached the gel margin. The presence of peroxidase activity was detected by using two different staining methods:

Tetramethylbenzidine was prepared stain according to Thomas *et al.*[15]. The tetramethylbenzidine solution (6.3 mM) was freshly prepared in methanol.

Staining mixture: This solution was prepared immediately before use: 3 parts of the tetramethylbenzidine solution were mixed with 7 parts of sodium acetate buffer 0.25 M (pH 5).

H_2O_2 solution with final concentration of 30 mM: After electrophoresis, the gels were immersed in staining mixture for 1-2 h, at room temperature in the dark with occasional mixing (every 10-15 min). H_2O_2 solution was added to a final concentration of 30 mM. The staining was visible within 3 min and increased in intensity over the next 30 min. Photographs should be taken after bands appearance.

Ortho-dianisidine stain was prepared according to Andrews and Krinsky[16]. After electrophoresis, the gel was incubation at room temperature in a solution of 1 mM of *ortho*-dianisidine for 5 min and then followed by incubation for 5 min in 10 mM H_2O_2.

Statistical analysis: The findings were expressed as the mean ± standard deviation. The data were analyzed with student's independent *t* test. All statistical analyses were performed with the program statistical package for the social science (SPSS) for windows, version 10.0. A (P value) of < 0.05 was accepted as statistically significant.

RESULTS AND DISCUSSION

Total peroxidase activity (U/L) and specific activity (U/mg) of sera samples are presented in Table-1 and reveal a highly significant increase ($P < 0.001$) and significant increase ($P < 0.01$) in benign and malignant groups respectively in comparison to that of the control. On other hand, a non significant difference ($P > 0.05$) in the specific activity was observed in benign and malignant groups, when compared with the control group. The results (Table-2) present the mean values of the activity (U/L) and specific activity (U/mg) of salivary peroxidase of control and patient groups. The results indicate the presence of highly specific increase ($P < 0.001$) in malignant groups in comparison to the control. While the specific activity results show a significant increase ($P < 0.05$) in malignant group in comparison to that of the control group.

TABLE-1
MEAN VALUES OF SERA TOTAL POD ACTIVITY AND SPECIFIC ACTIVITY IN CONTROL AND PATIENT GROUP

Group	Sample number	Age (year) Mean ± SD	Activity (U/L) Mean ± SD	Specific activity × 10^{-3} (U/mg) Mean ± SD
Control	32	35.66 ± 11.64	13.39 ± 9.20	0.24 ± 0.23
Benign	14	35.78 ± 15.63	28.83± 13.49**	0.33 ± 0.18
Malignant	19	46.57 ± 15.21	25.61 ± 20.26*	0.27 ± 0.24

**Highly significant difference in comparison to control at (P < 0.001)
*Significant difference in comparison to control at (P < 0. 05)

TABLE-2
MEAN VALUES OF SALIVARY TOTAL POD ACTIVITY AND SPECIFIC ACTIVITY IN CONTROL AND PATIENT GROUP

Group	Sample number	Age (year) Mean ± SD	Activity (U/L)	Specific activity × 10^{-2} (U/mg)
Control	32	35.66 ± 11.64	24.24 ± 18.85	1.71 ± 1.38
Benign	14	35.78 ± 15.63	29.89 ± 23.7	1.74 ± 1.53
Malignant	19	46.57 ± 15.21	79.26 ±73.26**	4.01 ± 5.56*

**Highly significant difference in comparison to control at (P < 0.001)
*Significant difference in comparison to control at (P < 0. 05)

Iron level in sera and saliva samples of control and oral tumor patient groups are represented in Figs. 1 and 2 respectively and show a highly significant decrease (P = 0.01), (P < 0.01) in sera samples of benign and malignant groups respectively, in comparison to that of control group. Whereas Salivary iron show a highly significant increase (P = 0.001) in benign group. Meanwhile, in malignant group, the increase was significant (P = 0.05) in comparison to that of the control.

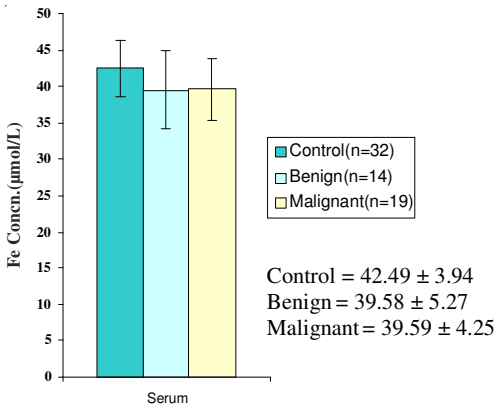

Fig. 1. Mean values of serum Fe level (µmol/L) in control and oral tumor patient groups

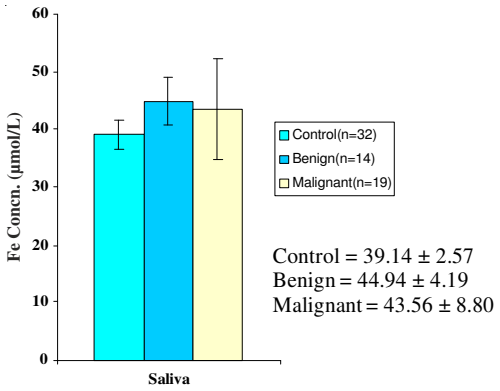

Fig. 2. Mean values of saliva Fe level (µmol/L) in control and oral tumor patient group

Table-3 shows the mean value of [Fe]/peroxidase activity in sera samples, which reflects a significant decrease, (P < 0.05), (P < 0.01) in malignant and benign groups respectively, in comparison to that of the control. Meanwhile the results in Table-4 show the [Fe]/peroxidase activities in saliva samples and reveal a non significant increase (P > 0.05) in both benign and malignant groups in comparison to the control group.

TABLE-3
MEAN VALUES OF FE CONCENTRATION/POD ACTIVITY IN SERA OF CONTROL AND PATIENT GROUPS

Group	Sample number	Age (year) Mean ± SD	Fe conc./POD activity (µ mol/U) Mean ± SD
Control	32	35.66 ± 11.64	7.10 ± 5.08
Benign	14	35.78 ± 15.63	1.69 ± 0.91**
Malignant	19	46.57 ± 15.21	3.04 ± 3.21*

** Significant difference in comparison to control at (P < 0.01)
*Significant difference in comparison to control at (P < 0.05)

TABLE-4
MEAN VALUE OF FE CONCENTRATION/POD ACTIVITY IN SALIVA OF CONTROL AND PATIENT GROUPS

Group	Sample number	Age (year) Mean ± SD	Fe conc./POD activity (µ mol/U) Mean ± SD
Control	32	35.66 ± 11.64	2.36 ± 2.3
Benign	14	35.78 ± 15.63	3.45 ± 4.7
Malignant	19	46.57 ± 15.21	3.70 ± 4.9

Peroxidase electrophoretic profile: The electrozymogram of the two different staining methods indicate that both methods are suitable for the detection of the peroxidase activity zone, since they gave clear distinct bands. Meanwhile it was noticed that when tetramethylbenzidine stain was used, the resolution of the bands were better, but these bands disappeared quickly. On contrast, with *o*-dinasidine stain, was produced less resolution, but the bands were stable by time. Upon the comparison of the localized activity bands in the zymograms of the studied groups, Fig. 3A and B represents the conventional electrophoresis, where Fig. 4A and B represents the catodic electrophoresis using the both staining methods. It is clear that band no.1 is present in the sera of all the studied groups (Lane 1, 2, 3). In malignant group (Lane 3) more aggregated bands, which migrate faster in to the gel than in the control and benign groups (lane 1, 2). The salivary peroxidase isoenzymes seems to migrate faster (low molecular weight) than the isoenzymes in the sera samples, especially in the benign and malignant groups (Lane 4, 5 and Lane 6), respectively.

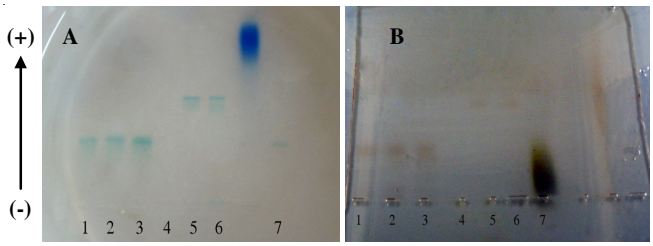

Fig. 3. Conventional poly acrylamide gel electrophoresis 7.5 %, using *Tris*-glycin buffer, pH 8.0 as electrode buffer. Electrophoresis was carried out for 3 h at 10 ºC. The gel was stained for peroxidase using two types of substrate: A) tetramethylbenzidine B) *o*-dianisidine

The samples used were: 1) Crude pooled serum (control); 2) Crude pooled serum (benign); 3 crude pooled serum (malignant); 4 Crude pooled saliva (control); 5) Crude pooled saliva (benign); 6) crude pooled saliva (malignant).

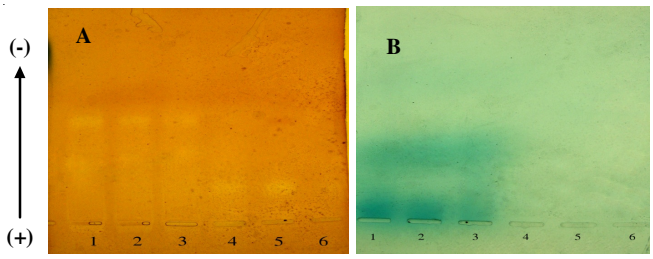

Fig. 4. Cathodic poly acrylamide gel electrophoresis (PAGE) 7.5 %, using β-alanine/glacial acetic acid pH 4.5 as electrode buffer. Electrophoresis was carried out for 3 h at 4 ºC by using a constant current of 30 mA. The gel was stained for peroxidase activity using two types of substrates: A) *ortho*-dianisidine; B) tetramethylbenzidine

The samples used were: 1) Pooled crude serum (control); 2) Pooled crude serum (benign); 3) Pooled crude serum (malignant); 4) pooled crude saliva (control); 5) Pooled crude saliva (benign); 6) Pooled crude saliva (malignant)

Reactive oxygen species are constantly generated and eliminated in the biological system and play important role in a variety of normal biochemical functions and abnormal pathological processes[8]. In the current study, total peroxidase activity in sera samples show an increased level, this result seem to agree with those obtained by other investigators on oral squamous cell carcinoma patients[17]. As well as in patients with advanced laryngeal carcinoma[18] and breast cancer[19]. The elevation of sera total peroxidase, which was observed in this study might be due to the enhanced free radical activity, which causes endothelial damage. So, in order to combat this oxidative stress, the body raises the level of its antioxidants. The synthesis of some enzymes of the oxidative system was reported to be induced by free radicals which being increased at oxidative stresses[20]. In other words, over production of hydrogen peroxide (as a result of both dismutation of superoxide radicals by SOD and immune cells) leading to increase peroxidase activity. During current study, salivary peroxidase show a highly significant increase in malignant group and this is in agreement with many studies on OSCC patients[21]. In the reaction catalyzed by salivary peroxidase:

$$SCN^- + H_2O_2 + H^+ \rightarrow HOSCN + H_2O$$

Thiocyanate is the electron-donating component (similar to glutathione in other biological system) and the reaction is catalyzed by peroxidase[20], two potent antibacterial oxidizing products evolve out of this reaction: hypothiocyanous acid (HOSCN) and its conjugated hypothiocyanite anion (OSCN). The acculamated antibacterial activity of the combination of peroxidase, hydrogen peroxide and thiocyanate is much more potent than that of hydrogen peroxide alone[22]. Salivary peroxidase and myeloperoxidase, catalyze the reaction involved in the inhibition of bacterial growth and metabolism and the prevention of hydrogen peroxide accumulation, thus protecting proteins from the action of oxygen and reactive oxygen species[23]. However, the elevated level of peroxidase activity may be attributed to that: in the inflamed tissue the elevated activity of the peroxidase contributed by myeloperoxidase activity[24]. The gingival cervicular fluid (GCF) is constantly mixed with saliva and its flow rate increase with gingival inflammation, this increased in gingival cervicular fluid flow relates to increased polymophonuclear nutrophil (PMN) levels which is rich with myeloperoxidase and as a result, the overall peroxidase activity increased contributing to the entrance of myeloperoxidase to the saliva[25]. Higher myeloperoxidase levels was suggested to be present in low flow rate whole saliva supernants of subject with sever gingival inflammation probably owing to the enhanced number of polymophonuclear nutrophil, which enter the oral cavity[25]. It is worth to mention that almost all the patients included in the current study had a low flow rate of saliva. Serum iron levels are considered as biochemical indicators for nutritional assessment. In most cases clinical anemia may be a contributing factor. Iron deficiency is known to be occurred in oral cancer[26]. In the current study, there was a highly significant decrease in Iron level in sera samples of oral tumor patients and this is in accordant with many studies on oral cancer[27,28] and also different malignancies such as gastric cancer[29] and larynx cancer[30]. Inadequate intake of food due to burning sensation and vesiculation in the oral cavity might also be an important factor. Reduction in the serum iron level may be due to malnutrition caused by the tumor burden in cancer patients[31]. Salivary iron show an increased level and this is disagree[28,32] studies on OSCC patients, where they explained the decrease in salivary Fe level to the malnutrition of cancer patients. Excess iron as with excess copper can cause free radicals production and more cell damage[33]. Iron is regarded as co-factor of peroxidase enzyme, which its concentration was observed to increase in oral cavity fluid in oral tumor patients as described previously. In order to indicate the relationship between iron concentration and peroxidase activity, the ratio of this element concentration to peroxidase activity ([Fe]/peroxidase activity) was calculated (Table-3). This ratio represent the non- peroxidase metal fraction and show a significant decrease in sera samples of benign and malignant patients, this finding confirm the depletion of iron level in the sera of oral cancer patients (Fig. 1) and as discussed in this part of the study. On the contrary, the ratio [Fe]/peroxidase activity in saliva (Table-4) show a non significant increase, which mean increased non-peroxidase iron, such increase and in the presence of overproduction of H_2O_2 in lesion area lead to more free radical production through Fenton reaction and Haber-weiss reaction since Fe is one of the pro-oxidant elements, excess iron as with excess copper can cause free radical production and cell damage[34,35].

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