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The Variations in Saliva and Serum Total Peroxidases System's Activity in Patients with Different Oral Tumors

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Abstract

Salivary peroxidases have biological functions of particular importance to oral health. The aim of this paper is to shed the light on saliva and serum total peroxidases activity as well as the activity of each of salivary peroxidase (SPO) and myeloperoxidase (MPO) in patients with oral tumors. The studied participants were divided into two groups: the first group included 18 oral squamous cell carcinoma patients and 20 age and gender-matched healthy controls while the second group consisted of 20 oral ossifying fibroma patients and 23 age and gender-matched healthy controls. Total peroxidases activity was determined, and its specific activity was calculated in serum and whole mixed saliva as well as in the supernatant and pellet fractions of saliva. Furthermore, the activities of SPO and MPO were determined in each of saliva's supernatants and saliva's pellet fractions, and the thiocyanate (SCN^-) concentration was measured in the supernatants fraction only. The results indicated the presence of a significant increase in the activity of both total peroxidase and MPO ($p = 0.0001$) in the salivary supernatants of oral squamous cell carcinoma patients relative to the control group. A significant increase ($p = 0.0001$) in total peroxidase activity in patients with oral ossifying fibroma was also found in serum compared with healthy individuals. In this study, we have shown that the measurement of total peroxidase and MPO activities in saliva may be used as an adjuvant tool for monitoring patients with oral malignancies.

Keywords: Oral squamous cell carcinoma, Ossifying Fibroma, Salivary peroxidase, Myeloperoxidase, Thiocyanate.

1. Introduction

Oral cancer is one of the most common cancers in the world, with approximately 274300 new cases and 127500 deaths occurring each year. Over 90% of total oral cancers reported being squamous cell carcinomas (OSCC) (Scully, 2013). OSCC occurs in all sites of the oral cavity, but the majority of cases involve the tongue, oropharynx, and floor of mouth (Chi *et al.,* 2015). Ossifying fibroma (OF) is a true neoplasm that is composed of fibrous tissue containing a variable mixture of bony trabeculae, cementum-like spherules, or both) with significant growth potential. It has been suggested that the origin of these tumors is either odontogenic or from periodontal ligament**.** Ossifying fibroma occurs over a wide age range with the greatest number of cases encountered during the third and fourth decades of life (Neville *et al.,* 2009.).

Peroxidase enzymes have been reported to play a key role in many human diseases where the activity of these species can be both beneficial and detrimental. Generally, SPO is known to be the major peroxidase present in human saliva, while MPO is released from oral leukocytes in amounts proportional to the degree of gingival inflammation (Koss *et al.,* 2016; Moriguchi *et al.,* 2017). The two types of peroxidases enzymes are distributed unevenly between the pellet and the supernatant fraction of saliva (Thomas *et al.,* 1994).

Due to the very limited studies on SPO and MPO activities in serum and saliva of patients with different oral tumors, we previously studied this enzymatic system using electrophoresis as a separating tool (Hasan and Aburahma, 2014.). The electrophoretic profile of the total peroxidase system showed the appearance of a new band that was stained for peroxidase activity in the saliva of Iraqi patients with OSCC. Therefore, the present study aims at highlighting the variations in the activity of this system in saliva and serum of the two most common types of oral tumors in Iraq, OSCC and OF.

2. Materials and Methods

2.1. Studied groups

A total of 38 non-alcoholic and non-smoking patients with primary oral tumors, who were attending the Department of Oral and Maxillofacial Surgery (Al- Wasity Hospital and Hospital of Specialized Surgeries, Medical city**,** Baghdad, Iraq) were included in the current study. The samples were collected from these patients during the period from May to November of 2016. Patients who were presented with distant metastases, patients who underwent radiotherapy and those whose saliva samples were mixed with blood were excluded from the study. Moreover, according to the type of tumor, the patient's groups were divided into two groups: The first group included 18 patients with OSCC while the second group included 20 patients with oral OF.

For comparison purpose, 43 non-alcoholic and non-smoking healthy, age and gender matched, volunteers were

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used as a control, where 20 of them were used as a control for the first group of the patients and 23 individuals for the second group. The protocol of the present study was approved by the Ethics Committee of the College of Science, University of Baghdad, Baghdad, Iraq.

2.2. Saliva samples

Unstimulated 5 to 10 mL whole, mixed-saliva samples were collected on ice, under resting conditions in a quiet room between 8.0-9.0 A.M. The Patients and healthy individuals were asked to rinse their mouth with normal saline, then to generate saliva in their mouth and to spit into a plastic container for 10 minutes. After collection, the saliva was immediately divided into two test tubes, the first one was labeled as "total saliva" and stored frozen, while the remainder saliva sample (3 mL) was centrifuged at (2000 ×g) for 10 minutes at 4℃ to obtain the supernatant and the pellet fractions. The obtained fractions were stored frozen at –20℃ in polyethylene tubes until used for the different assays. Just before analysis, the peroxidases were extracted from the pellet fractions using phosphate-buffered saline 0.2M; pH 7.0 containing 0.1% cetyl-tri-methyl ammonium bromide (CTAB) as reported by Thomas *et al.* (Thomas *et al.,* 1994). The pellet was washed with cold phosphate-buffered saline and centrifuged at (2000 ×g) for 5 minutes at 4℃. Then the supernatant fraction was discarded and the pellet was suspended to the original volume of cold phosphate-buffered saline 0.2M; pH 7 containing 0.1% CTAB in an ice bath and sonicated at 10 µm Amplitude for 1 minute at intervals of 15 second using MSE SONIPREP 150 and incubated for one hour at 4℃ to complete the extraction. Finally, the sample was centrifuged for 10 minutes at (3000 ×g) and the supernatant was used for protein and enzyme activity measurements on the same day.

2.3. Serum Samples

Ten milliliters of venous blood samples were collected using plastic disposable syringes from overnight fasting patients (before surgery) and the control groups, in plain polyethylene tube. The blood samples were allowed to clot for ten minutes at 37℃ in a water bath, then they were centrifuged at (3000 ×g) for 10 minutes. The obtained clear serum supernatant was stored frozen at -20℃ until being assayed for the different parameters.

2.4. Determination of total peroxidases

Total peroxidases activity was measured in saliva and serum, as well as its distribution between the saliva's supernatant and pellet fractions by colorimetric method using phenol, 4- aminoantipyrine and H_2O_2 as the dye-generating compounds (Song *et al.,* 2005). The activity was expressed as the increase in absorbance at $\lambda = 510$ nm resulting from the decomposition of hydrogen peroxide per time of incubation (ΔA/min) using a molar extinction coefficient of 7100 $M^{-1}cm^{-1}$. The specific activity is expressed for all studied enzymes as a unit of enzymatic activity/mg of protein concentration, where the protein concentration was estimated as previously described using a modified Lowry method (Hasan and Abdelwahab, 2014).

2.5. Determination of salivary peroxidase and myeloperoxidase

In order to be able to determine each of SPO and MPO separately, the saliva samples were desalted using PD10 column to remove the SCN^- and chloride ions (Cl^-) from the saliva samples (Helmerhorst and Stokes, 1980). Then, SPO and MPO activities were determined using the method described in Mansson-Rahemtulla *et al.,*1986. Briefly, this method was based on the oxidation of the intensely yellow 5-thio-2-nitrobenzoic acid (TNB) to the colorless 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) by $OSCN^-$ ions generated during the oxidation of SCN^- . The presence of Cl^- only in the assay mixture makes the method suitable for the determination of MPO activity, since Cl^- is oxidized to OCL^- by this type of peroxidase, but not by the SPO type (Kaczmarek, 2005). One unit of enzyme activity was expressed as the level of enzyme activity needed to cleave 1 µmol of TNB/min at 22ºC, using a molar extinction coefficient of 12.800 $M^{-1}cm^{-1}$ (Goi *et al.,* 2007).

The activity of SPO = Total peroxidases activity – MPO activity

2.6. Determination of salivary thiocyanate (SCN-) concentration

Salivary thiocyanate concentration was measured spectrophotometrically as described by Aune and Thomas (Aune and Thomas, 1977). This concentration (expressed in mM) in saliva's supernatant fractions was calculated using the linear equation derived from the standard curve which was constructed by plotting the absorbance at 450 nm of the produced $FeSCN^{2+}$ in presence of different concentrations of KSCN (ranging between 0.2-2.4 mM) treated as above.

2.7. Statistical Analysis

Statistical analysis was carried out using the program Statistical Package for the Social Science (SPSS for Windows, version 21 software packages). The differences between groups were tested by the Student t-test, and the *p*-value was considered significant if it was < 0.05.

3. Results

The baseline characteristic of the studied groups is illustrated in Table 1. The study population consisted of a total of 38 patients including 18 with OSCC and 20 with OF: the age ranges were (59.2±11.6 and 23.4±16.0) respectively, with two groups of healthy individuals serving as control corresponding to each patients group who were within about the same age range.

Table 1. Characteristics of the studied population.

Group	Sub-Group	Age (year) (Mean±SD)	Gender	Site of tumors	Histopathological Type	Clinical staging of patients	Histopathological grading
First group	Control (n=20)	56.5±10.2	10 males 10 females	-	-	-	-
	Malignant (OSCC) (n=18)	59.2±11.6	10 males 8 females	Tongue	Squamous cell carcinoma	Stage II	Grade II - Moderately –well differentiated
Second Group	Control (n=23)	26.0±12.2	10 males 13 females	-	-	-	-
	Benign (OF) (n=20)	23.4±16.0	6 males 14 females	jaw	Ossifying fibroma	-	-

3.1. Total salivary peroxidase activity and specific activity

The results of total peroxidase activity's measurement in the saliva samples of all studied groups and the calculation of the specific activity are presented in Table 2.

Table 2. Total peroxidase activity and specific activity in the salivary samples of patients with different oral tumors OSCC and OF: first and second groups, respectively.

Group	Sub-Group	Activity (U/L) *P*-value			Specific Activity (U/g) *P*-value		
		Total saliva	Supernatant of Saliva	Pellet of saliva	Total saliva	Supernatant of saliva	Pellet of saliva
First group	Control (n=20)	365.8±118.5	125.5±53.3	147.6±59.5	142.2±31.4	59.6±20.0	599.7±107.4
	Malignant (OSCC) (n=18)	485.7±186.1*	240.7±99.7*	141.3±59.4	110.8±26.0*	101.2±37.0*	329.9±183.4*
		0.023	0.0001	0.750	0.002	0.0001	0.0001
Second group	Control (n=23)	347.4±97.6	112.3±40.7	148.2±59.9	116.1±43.1	55.4±17.3	417.7±131.6
	Benign (OF) (n=20)	411.7±199.3	199.9±87.6*	107.9±48.3*	104.5±42.7	88.4±30.9*	288.0±71.5*
		0.179	0.0001	0.021	0.380	0.0001	0.001

* Significant difference in comparison to control at ($p < 0.05$).

From the results presented in the table 2 above, a significant increase ($p < 0.05$) in salivary total peroxidase activity and specific activity of malignant tumors (OSCC**)** patients in comparison to that of healthy control was observed. In the benign tumors (OF) patients' group, however, the increase in total saliva peroxidase activity in comparison to that of healthy controls was non-significant ($p > 0.05$).

Upon calculating the percentage of peroxidase activity present in the salivary supernatant and pellets in relation to the activity of peroxidase in total saliva, the percentage of distribution of this activity appeared to be as follows: 49.8% and 30.5% in the supernatant and pellet fractions respectively in malignant tumors patients' group (OSCC**)** and 50.5% and 27.7% respectively in benign tumors patients' group (OF).

3.2. Serum peroxidase activity and specific activity.

The activity of total peroxidase was measured in the serum samples as described in the methods section and its specific activity was calculated. The results in Table 3 reveal the presence of a significant increase ($p < 0.05$) in serum peroxidase activity of the (OF) group in comparison with that of their corresponding control. On other hand, the increase in this activity was observed to be non-significant ($p > 0.05$) in sera samples of (OSCC**)** patients in comparison with that of the corresponding control group.

Table 3. Total peroxidase activity and specific activity in the serum samples of patients with different types of oral tumors; OSCC and OF.

Group	**Sub-Group**	**Total serum peroxidase**	**Activity (U/L)**	**Specific Activity (U/g)**
	Control (n=20)	Mean±SD	49.8±20.5	0.7±0.3
	Malignant (OSCC) (n=18)	Mean±SD	64.0±36.6	0.9±0.6
		P-value	0.135	0.107
	Control (n=23)	Mean±SD	30.4±9.5	0.4±0.1
	Benign (OF) (n=20)	Mean±SD	39.0±11.9*	0.5±0.1*
		P-value	0.012	0.0001

* Significant difference in comparison to control at ($p < 0.05$).

3.3. Evaluation of salivary peroxidase and myeloperoxidase activities and specific activities

The results in Tables 4A indicate the presence of a significant increase ($p < 0.05$) in MPO activity and specific activity in the salivary supernatant fractions of both studied groups in comparison to that of their corresponding healthy controls. In contrast, there is a non-significant ($p > 0.05$) slight decrease in the activity of SPO in the saliva samples of all patient's groups compared with their corresponding control groups. On the other hand, there is a significant decrease in SPO specific activity ($p < 0.05$) in patients with malignant tumors (OSCC) in comparison to that of healthy controls.

Table 4. SPO and MPO activities and specific activities in the salivary supernatant and pellet of patients with different oral tumors OSCC and OF: A: in the supernatant fraction of saliva and B: in the pellet fraction of the saliva.

A: Supernatant fraction.

Group	Sub-Group	Activity (U/L) *P*-value			Specific Activity (U/g)*P*-value		
		Total peroxidases (SPO+MPO) (U/L)	MPO (U/L)	SPO (U/L)	Total peroxidases (SPO+MPO) (U/L)	MPO (U/L)	SPO (U/L)
First group	Control (n=20)	212.8±42.7	23.5±8.9	189.3±37.3	106.4±42.5	11.9±3.9	95.2±26.2
	Malignant	218.9±70.3	58.9-21.5*	161.5±56.4	85.8±31.4	23.7±11.9*	62.8±24.9*
	(OSCC) (n=18)	0.748	0.0001	0.082	0.1	0.0001	0.0001
Second group	Control (n=23)	185.8±78.8	17.1±7.5	168.7±72.7	95.1±31.5	9.0±3.7	84.2±29.4
	Benign (OF) (n=20)	193.5±19.0	29.8±11.9*	163.7±22.3	93.9±34.3	13.5±3.9*	80.6±32.5
		0.672	0.0001	0.772	0.904	0.0001	0.705

B: Pellet fraction.

Group	Sub-Group	Activity (U/L) *P*-value			Specific Activity (U/g) *P*-value		
		Total peroxidases (SPO+MPO) (U/L)	MPO (U/L)	SPO (U/L)	Total peroxidases (SPO+MPO) (U/L)	MPO (U/L)	SPO (U/L)
First group	Control (n=20)	145.9±60.9	30.8±11.1	118.3±56.2	627.4±154.9	118.8±65.9	527.4 ±141.1
	Malignant	117.6±71.1	31.2±12.8	86.4±60.0	253.6±65.6*	79.7±36.8	188.6±67.7*
	(OSCC) (n=18)	0.202	0.915	0.104	0.0001	0.095	0.0001
Second group	Control (n=23)	190.6±84.7	48.4±23.9	142.1 ±66.3	572.9±220.9	143.5±69.4	431.6±185.3
	Benign (OF) (n=20)	224.6±98.4	96.9±48.8*	127.7±66.3	621.1±202.2	307.3±132.3*	338.1±105.2
		0.231	0.0001	0.482	0.463	0.0001	0.053

* Significant difference in comparison to control at ($p < 0.05$).

Regarding SPO in the pellet fraction of the saliva, the results from Tables 4B indicate the presence of a significant decrease in this enzyme specific activity ($p < 0.05$) in the (OSCC**)** patients upon comparison with that of its control group. Meanwhile, the activity and specific activity of (MPO) in this type of disease show a non-significant difference ($p > 0.05$) in comparison with that of its healthy controls.

In the patients with benign bone tumors group (OF), a significant increase ($p < 0.05$) in MPO activity and specific activity compared with healthy controls was recorded while for SPO activity and specific activity, a non-significant decrease ($p > 0.05$) in patients group compared with control is observed.

3.4. Determination of thiocyanate (SCN-) Concentration

When the SCN^- concentration in the saliva supernatant was estimated as described in the method and material section, the results reveal that the SCN^- concentration is significantly lower ($p = 0.003$) only in (OF) patients in comparison with that of the control Table 5.

Table 5. SCN^- concentration in salivary supernatant of patients with different oral tumors OSCC and OF: first and second groups, respectively.

SCN conc. (mM)	First group		Second group	
	Control (n=20)	Malignant (OSCC) (n=18)	Control (n=23)	Benign (OF) (n=20)
Mean±SD	0.6±0.2	0.4±0.2	0.6±0.3	0.3±0.3*
P-value	-	0.108	-	0.003

* Significant difference in comparison to control at ($p < 0.05$).

4. Discussion

The reported relative contributions of SPO and MPO activities in salivary total peroxidase activity were variable and sometimes contradictory, Klein *et al* and Nagler and Reznick using 2-nitrobenzoic acid-thiocyanate assay for measurement of total peroxidase and relative contribution of SPO and MPO in this total activity have concluded that the SPO is secreted from the major salivary glands, mainly from the parotid gland, and contributes approximately 80% to total peroxidase activity, whereas MPO, produced by leukocytes in inflammatory regions of the oral cavity, contributes the remaining 20% of total peroxidase activity (Klein *et al.*,2003; Nagler and Reznick, 2004). On the other hand, Thomas *et al.*, using 4-aminoantipyrine as a substrate mentioned that MPO is responsible for an average of 75% of the total peroxidase activity in the mixed saliva. The two types of peroxidases enzymes are distributed unevenly between the supernatant and the pellet fraction of saliva. SPO is found in the soluble portion and almost 80% of MPO is found in the salivary pellet (Thomas *et al.,* 1994). Unraveling this contradiction has been tested throughout the present study by measuring the relative contribution of SPO and MPO to total peroxidase activity in different salivary components, and the following results were found: in the control of the first group (Table 4) the percentage of the activities of SPO and MPO activities were 88.93% and 11% respectively in the salivary supernatant fractions. Meanwhile, approximately the same percentage is calculated in the control of the second group (90.7% and 9.2% for SPO and MPO, respectively) (Table 4 A), whereas in the salivary pellet fractions the percentages of SPO and MPO are found to be 81% and 21%, respectively, in control of the first group while they were found to be 74.5% and 25.4%, respectively in control of the second group (Table 4B). Based on these results, it can be concluded that SPO activity is the major component of total salivary peroxidase in both the supernatant and pellet of saliva. This agrees with other studies on saliva peroxidase activity of normal healthy individuals (Nickerson *et al.*, 1957; Iwamoto *et al.*, 1968; Thomas *et al.,* 1994).

The relationship between MPO and different types of tumors has not been clarified and thus needs intense research (Al-Salihi *et al.*, 2015). Furthermore, MPO has been reported to produce biochemical alterations in different antioxidative species that lead to cancer progression (Khan *et al*., 2018), and since the studies on salivary MPO in oral tumors is very limited, the aim of this study, therefore, was to investigate the alteration of MPO in serum and saliva of OSCC and OF patients.

The observed increase in MPO (Table 4) disagrees with the only study found in the literature by Ajila *et al.*, 2015 which refers to non-significant decreased MPO activity in saliva of OSCC Indian patients. On the other hand, the observed elevation of the MPO activity in saliva of present studied patients' groups agrees with some studies in different oral diseases other than tumors that have demonstrated an increase in MPO levels and/or activity in the gingival crevicular fluid (GCF) of patients with periodontitis (Karhuvaara *et al.*, 1990; Yamalik *et al*.,2000; Wei *et al.*, 2004; Borges *et al.*, 2007).

The obvious elevation in this study of MPO activity in saliva of patients with oral tumors may be related to the elevation in serum peroxidase and may give a clue that the measured serum peroxidase in our study is more or less contributing to salivary MPO activity. This type of peroxidase in the oral cavity mostly comes from a gingival crevicular fluid (GCF) (Bafort *et al.*, 2014), and gingival crevicular fluid is a serum transudate: the fluid passes from the systemic circulation through the junctional epithelium of the gingiva and into the gingival crevice/oral cavity (Davies and Finlay, 2005).

On the other hand, the observed decreased SPO activity in the saliva samples seems to agree with the findings of Bahar *et al*., (2007) who had found that all salivary antioxidants, including salivary peroxidase, were substantially reduced in patients with oral squamous cell carcinoma. They reported that this decrease was due to the depletion of salivary antioxidants systems as a result of the increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS). Such a state explains the oxidation of DNA and proteins and the promotion of OSCC. Cancer has been recognized as a form of chronic inflammation, involving the participation of large pool cell types (Wang *et al.*, 2020). The increased free radicals (which are highly reactive chemical compounds that has one, or more unpaired valence electrons) (Sharma, 2014) are well-known inducers of cellular and tissue pathogenesis leading to numerous disorders including tumors; such free radicals are normally scavenged by antioxidant enzymes among which salivary peroxidase is one of the most important ones present in submandibular glands. This antioxidant system acts to prevent the negative effect of these free radicals in causing the appearance of cancer (Filip *et al*., 2007). It was reported that at the time of malignant tumor setting, the reduction in SPO activity was highest and such a reduction may result from an increase in the level of oxidants (Bentur *et al*., 2006) and may be of paramount importance since SPO has a dual role. First, it controls the level of H_2O_2 excreted by bacteria and leukocytes from the salivary glands into the oral cavity (Nagler *et al*., 2002) by a mechanism that protects oral mucosa from cellular lysis induced by H_2O_2 (Filip *et al*., 2007). Meantime, H_2O_2 is considered as ROS which plays a key role in human cancer development since this compound with other free radicals can cause DNA base alterations, strand breaks, damaged tumor suppressor genes, and an enhanced expression of protooncogenes. ROS induced mutation could also result in protein damage (Bahar *et al.*, 2007). The second role of SPO is its specific antibacterial activity that inhibits the metabolism and proliferation of various bacteria in the oral cavity (Nagler *et al.*, 2002). It is known that peroxidase activity is a marker of salivary glands functionality and that ''*in vivo*'' salivary peroxidase catalyzes the oxidation of the SCN^- ion to $OSCN^-$ ion and hypothiocyanous acid (HOSCN), The latter compound inhibits the growth and metabolism of many species of pathogens. Moreover, the salivary peroxidase system maintains the thiocyanate peroxidation reactions in a state of ''*in vivo*'' dynamic equilibrium thereby minimizing the concentration of toxic H_2O_2 and maximizing the concentration of the antibacterial agent hypothiocyanite (Filip *et al.*, 2007).

The complete antimicrobial peroxidase system consists of peroxidases together with the naturally occurring SCN^- and H_2O_2 (Tonoyan *et al.*, 2017). SCN^- reduces certain tissue-damaging species [e.g., H_2O_2 and hypochlorite (OCl^-)] by subjecting itself to oxidation. In the absence of adequate SCN^-, overproduction of OCl^- by MPO during inflammation might result in severe injuries and lead to the self-destruction of white blood cells. The death of these cells would in turn cause additional destructive agents to be dumped, escalating injuries to the host. The measured decrease in the concentration of SCN^- in the current study provides inadequate protection against the overproduction of OCl^-, thus worsening inflammatory diseases and predisposing to diseases linked to MPO activity including cancer (Xu *et al.*, 2009). The development of cancer depends on the extent of DNA damage; this damage is proportional to the magnitude of oxidative and nitrative stress that reflect the net effect of both ROS and RNS, as well as the effectiveness of the antioxidant's defense system and the DNA repair system. ROS and RNS are involved in the initiation and promotion of cancer, and they are inhibited by antioxidants, however, when the equilibrium is broken DNA is oxidized and cancer evolves (Bahar *et al*., 2007).

In conclusion, although the size of the samples is limited due to the exclusion of many saliva samples of patients with OSCC because of the presence of blood mixed with the saliva, the main interesting results of the current study were the finding out of an elevation in salivary total peroxidases and myeloperoxidase activities in the patients with oral OSCC. The measurement of these activities in saliva may be proposed as an adjuvant tool for monitoring the patients for the presence of OSCC, a role which needed further study with a larger number of samples in order to be confirmed.

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