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A comparative evaluation of the different ferroxidases activities in sera and saliva of Iraqi patients with alcoholic and non-alcoholic liver disease

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Abstract:

The present study focused on multicopper oxidase enzymes status in serum and saliva of patients with non- alcoholic fatty liver disease (NAFLD), and alcohol-related liver disease (ALD). Four groups of Iraqi males, 18 patients with NAFLD, 18 patients with ALD, 30 non-smokers and non-alcoholic control (control 1), and 20 smokers with non-alcoholic control (control 2) were the participants of this study. The changes in the activity of three different ferroxidases: Total, non- ceruloplasmin (non-Cp), and ceruloplasmin (Cp) ferroxidase, were investigated in the serum and saliva of these participants, as well as the measurement of total protein concentration, and copper concentration. The results reflected the presence of a highly significant decrease ($p<0.001$) in serum total ferroxidase activity and specific activity as well as in Cp ferroxidase activity for both patient's groups and in non-Cp ferroxidase activity in ALD patients, with a highly significant increase in Cp ferroxidase specific activity in ALD patients group only. Meanwhile, in the saliva of all studied groups, different ferroxidases activities and specific activities except in ALD showed non- significant differences ($p>0.05$), except in that of Cp ferroxidase specific activity in the ALD patients they showed a significant increase ($p<0.001$) compared to its control group. The copper level was found to be highly significantly decreased ($P<0.001$) in serum of the patients with NAFLD and highly significantly increased in saliva of the ALD group. The results of the correlation between the changes in the studied parameters that occurred in serum and saliva indicated that there was a significant correlation in non-Cp ferroxidase activities ($p<0.01$) in the case of control 1, a highly significant correlation in total and non-Cp ferroxidase activities ($p<0.01$), as well as a significant correlation ($p<0.05$) in non-Cp specific activity only in NAFLD patients. Meanwhile a highly significant correlation was found in non-Cp activity ($p<0.01$) of the control 2 group, and a highly significant correlation ($p<0.01$) was clear inCp ferroxidase activity in the ALD patients group. This study's results indicated the activity of the different ferroxidases and copper concentration contribute in a different manner to the oxidative stress and iron overload reported previously in our laboratory in serum and saliva of ALD and NAFLD patients. Furthermore the results of Pearson correlations indicated the possibility of using saliva instead of serum to measure a limited number of parameters.

Keywords:total ferroxidase, ceruloplasmin ferroxidase, non-cerulopasmin ferroxidase, Iraqi male patients.

1. Introduction

The term cirrhosis (scirrhous in Greece) was firstly introduced by Laennec in 1826 to describe the orange color surface of the liver seen at autopsy (1). The most serious diseases of the liver are viral hepatitis, alcohol-related liver disease (ALD), and the non-alcoholic fatty liver disease (NAFLD) which affects all the liver and may cause liver cirrhosis, that in some cases developed into hepatocellular carcinoma which means the life cannot repair itself (2). Oxidative stress is an imbalance between oxidants and antioxidants in the body (3). Where the antioxidants are any substance that inhibits, or prevents cell and tissue damage via ability to neutralize free radicals by donating them one of their own electrons, these antioxidants are a range of enzymatic and non-enzymatic mechanisms (4-7). Among the enzymatic antioxidants is a family of many enzymes called multicopper oxidases (MCOs) that serves as antioxidants because of their ferroxidase activity. This important physiological

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function is involved in the conversion of highly toxic ferrous iron to its non-toxic ferric form(8), and is directly or indirectly mediated by their binding to copper, and its activities to decrease copper deficiency (9)**.** Such deficiency can lead to increase ROS and oxidative damage of DNA, lipids, and proteins and contribute to several diseases(10). In the human body, three MCOs have been identified to date: ceruloplasmin (ferroxidase I), and non-ceruloplasmin (ferroxidase II) which included hephaestin, and zyklopen both of these activities are involved in what is known as total ferroxidase activity (11).

Ranganathan, *et al*. studied serum ceruloplasmin concentration and it's ferroxidase activity in ALD French patients (12). While in NAFLD patients, Nobili, *et al*. measured ceruloplasmin concentration and its ferroxidase activity in 100 Italian children with NAFLD (13). While a study by Aigner, *et al.* in Austria, reported a potential association between the copper status and the disturbances of iron homeostasis measured in NAFLD (14).

In saliva, in addition to the enzymatic system, Low molecular antioxidants glutathione, ascorbic acid, and uric acid, as well as melatonin were reported to be present. Their function is to protect oral cavity against the negative effects of endogenous and exogenous ROS/RNS (15). It is worth to mention that so far no study had been found in the literature that deals with multicopper oxidase activity in saliva of patients with liver disease. Therefore, the present study focused on investigation the activities of multicopper oxidase enzymes status in serum and saliva of NAFLD, and ALD patients. The measured parameters included the activity of each of total ferroxidase, non-ceruloplasmin (non-Cp) ferroxidase, and ceruloplasmin (Cp) ferroxidase, total protein concentration and copper concentration.

2. Materials and methods:

2.1 Patients

A total of 86 individuals were the participants in this study, the patients were of two types of liver disease: the first group were 18 nonsmoker's patients with nonalcoholic fatty liver disease (NAFLD), and were diabetic with type 2 diabetes mellitus, while the second group were 18 patients with alcoholic liver disease (ALD) and were smokers. All patients were attending Baghdad Center of Gastroenterology and Hepatology during the period from November 2018 to July 2019, they were referred from different hospitals in Baghdad and other governorates in Iraq. For comparison purposes, two groups of age-matched healthy controls were also included in the study. The first control group consisted of 30 nonsmokers and non- alcoholics to be used as a control for NAFLD, while the second control group included 20 smokers and nonalcoholic, healthy individuals to be used as a control for ALD patients. The mean age of these participants was (50±12) years.The baseline examination included a personal health interview, a health questionnaire linked to their study along with their personal full details history including computed tomography (CT), magnetic resonance imaging (MRI), as well as, the results of liver biopsy examination. The ethics Committee of Baghdad University/ College of Science has approved the study project.

*2.*2 Inclusive criteria of the participants*:*

The NAFLD patients were diabetic with a mean value of blood suger concentration (274.88±66.27mg/dl). While the ALD patients were non-diabetic. The control groups included in the present study were age-matched individuals who fulfilled inclusion criteria.

2.3 Exclusion criteria*:*

The excluded criteria for both ALD and NAFLD groups were: patients with, nonalcoholic cirrhosis due to Wilson disease, hepatitis B, C, α1-antitrypsin deficiency, haemochromatosis, obese person, and vitamin supplements usage. An alcoholic smokers NAFLD patients as well as nonsmokers and diabetic ALD patients were also excluded from the present study.

2.4 Samples:

After overnight fasting, 10ml of blood were withdrawn in plain tubes, left for ten minutes at RT, then were centrifuged at (3000xg) for 5 minutes. At the same time, from the same individual, unstimulated saliva samples were taken, and the participants were asked to rinse their mouths with saline solution before donating the saliva samples. The saliva samples were centrifuged for 10 minutes at (2400×g).The collected sera and saliva samples were kept frozen to be used for the determinations of the different studied parameters.

2.5 Chemicals:

All chemicals used in this study were of the highly analytical grade and were from BDH, Fluka, Sigma, and Randox companies.

Methods:

2.6 Determination of total ferroxidase activity

Total ferroxidase activity in the collected saliva and serum samples was estimated by a method described by Erel(16). The decrease in the concentration of the substrate (ferrous ion) upon its

incubation with the enzyme gives the total ferroxidase activity in sera and concentrated saliva.

2.7 Determination of ferroxidase II activity (non-Ceruloplasmin ferroxidase activity)

In order to measure ferroxidase II activity, Topham method was followed (17). In this method, ceruloplasmin ferroxidase activity was completely inhibited by the inclusion of sodium azide (1 mM) in the enzymatic mixture.

Calculation:-

The corresponding total ferroxidase and non-Cp ferroxidase activities were calculated from the equation bellow with the consideration to sample volume.

Enzyme activity (U/L) = [C_1-C_2] / t × [Vt / Vs]

Where: C_1 = The substrate concentration at the beginning of the enzymatic reaction (60 µmol/L).
C_2= The substrate concentration at the end of the enzymatic reaction (calculated from the standard curve). V t = Total volume 1374µL.
Vs = Sample volume (9 µL for serum, 25 µL for concentrated saliva). , t = the incubation time (4 minutes).

To calculate theCeruloplasmin ferroxidase(Ferroxidase I) activity,the following equation was used:

Ferroxidase I activity (U/L) = Total ferroxidase activity – Ferroxidase II activity

2.8 Enzyme specific Activity:

The specific enzyme activity was expressed as mUnit of activity divided by mg of protein.

2.9 Procedure used to concentrate saliva:

In order to concentrate the saliva sample, Saul and Don's method was used (18).

2.10 Determination of total protein concentration

The total protein concentration of all samples (sera and saliva) was determined using modified Lowery method by Hartree(19). Total protien concentration of serum and saliva were expressed in g/100ml.

2.11 Determination of copper concentration

Flame atomic absorption spectrophotometer is the preferred technique for the determination of copper concentration in serum and saliva (20), which proved a rapid and sensitive method for the detection of numerous elements. Copper was measured at a wavelength of 324.8 nm using a hallow cathode lamp. The results were expressed in (µg/mL).

2.12 Statistical Analysis

The data were analyzed using SPSS by Licensed materials Version 25 Computer software. Data in this study were present as mean± standard deviation (Mean± SD) using independent-samples T-Test to compare the result mean. A ($p \leq 0.05$) value was approved as statistically significant, while a ($p < 0.001$) value was approved as a highly significant.

3. Result and desiccation

The results of the different ferroxidase activities that were measured in sera and saliva samples of all the participants of this study were presented in Table 1 and Table 2 respectively. In comparison to their corresponding control groups, the above results illustrated the presence of a highly significant decrease ($p<0.001$) in serum total ferroxidase activity for the two patients groups, non-Cp ferroxidase activity for ALD group, Cp ferroxidase activity for both patients groups, with a none significant difference ($p>0.05$) in non-Cp ferroxidase activity in serum of NAFLD group. These results of NAFLD group in serum agreed with many studies that measured the activity of serum total ferroxidase, such as a study by Nobili, *et al* (13), Aigner*et al* (14), Meanwhile the results of this activity in ALD patients, agreed with the result of a study by Uhlikova, *et al* (20), and disagreed with that obtained by Lan, *et al* (21). Meanwhile, no significant differences in all parameters measured in the two patients groups compared to that of their values in their corresponding controls groups ($p>0.05$). It is worth to mention that there is no study in the literature that deals with the different ferroxidase activities in saliva of liver cirrhosis patients. The total ferroxidase activity is contributed from Cp ferroxidase (ferroxidase I) and none Cp ferroxidase (Ferroxidase II) that is due to other proteins named: hephaestin and zyklopen both are multi copper oxidase which through their ferroxidase activity contribute in the conversion of the highly toxic ferrous iron that contributes in free radical production, to a less toxic ferric form (11, 21), and as illustrated in the bellowed equations (22, 23):

$$4Fe^{2+} + 4Cu^{2+} - MCO \rightleftarrows 4Fe^{3+} + 4Cu^{3+} - MCO$$

$$4Cu^{+} - MCO + 4H^{+} + O_2 \rightleftarrows 4Cu^{2+} - MCO + 2H_2O$$

$$4Fe^{2+} + 4H^{+} + O_2 \rightleftarrows 4Fe^{3+} + 2H_2O$$

. **Table (1): Theactivityof total ferroxidase, non- Cp, and Cp ferroxidase in sera ofthe groups under investigation.**

Parameters	Group	number	Mean± SD	P-value
Total Cp ferroxidase (U/L)	NAFLD	18	621.28±161.9	0.001**
	Control 1	30	869.40±109.57	
	ALD	18	581.28118.558	0.001**
	Control 2	20	877.95±146.45	
Non-Cp ferroxidase (U/L)	NAFLD	18	424.56±123.6	NS
	Control 1	30	463.30±75.82	
	ALD	18	398.33±75.35	0.001**
	Control 2	20	479.70±49.55	
Cp ferroxidase (U/L)	NAFLD	18	194.15±69.39	0.001**
	Control 1	30	406.10±127.28	
	ALD	18	193.89±86.26	0.001**
	Control 2	20	398.25±146.71	

**The difference is a highly significant at the $p<0.001$ level. NS: non-significant difference $p>0.05$.

Table (2): The activityof total, non- Cp, and Cp ferroxidase in the saliva of all study groups.

Parameters	Group	number	Mean± SD	P-value
Total ferroxidase (U/L)	NAFLD	18	233.88±27.25	NS
	Control 1	30	226.35±29.24	
	ALD	18	227.10±32.97	NS
	Control 2	20	222.44±19.13	
Non-Cp ferroxidase (U/L)	NAFLD	18	179.00±22.35	NS
	Control 1	30	185.22±37.65	
	ALD	18	190.10±23.33	0.061
	Control 2	20	206.44±7.57	
Cp ferroxidase (U/L)	NAFLD	18	54.87±26.9	NS
	Control 1	30	41.13±30.46	
	ALD	18	37.00±27.98	0.058
	Control 2	20	16.00±14.06	

NS: non-significant difference $p>0.05$

Table (3): The concentrations oftotal protein in serum and saliva of all studied groups.

Parameters	Group	number	Mean± SD	P-value
Total protein concentration in serum (g/100mL)	NAFLD	18	7.14±0.80	NS
	Control 1	30	7.37±0.53	
	ALD	18	6.12±0.89	0.001**
	Control 2	20	6.91±0.27	
Total protein concentration in saliva (g/100mL)	NAFLD	18	0.199±0.07	NS
	Control 1	30	0.190±0.04	
	ALD	18	0.157±0.06	NS
	Control 2	20	0.168±0.02	

****The difference is a highly significant at the 0.001 level.NS: non-significant difference p>0.05.**

Table (4): Mean value± SD of total, nonCp, and Cp ferroxidase activities and specific activities in sera and saliva of all study groups.

Parameters	Group	n	Serum $*10^{-2}$	p	Saliva	P-value
Total specific activity (mU/mg)	NAFLD	18	9.91± 1.41	0.001**	0.11±0.03	NS
	Control 1	30	11.74±1.79		0.09±0.02	
	ALD	18	9.78±2.15	<0.001**	0.17±0.10	NS
	Control 2	20	12.72±2.11		0.13±0.02	
Non-Cp specific activity (mU/mg)	NAFLD	18	6.95±1.04	NS	0.08±0.02	NS
	Control 1	30	6.26± 1.24		0.08±0.02	
	ALD	18	6.65± 1.7	NS	0.14±0.09	NS
	Control 2	20	6.97±0.85		0.12±0.02	
Cp specific activity (mU/mg)	NAFLD	18	3.14±0.82	NS	0.02±0.01	NS
	Control 1	30	5.48±1.76		0.02±0.01	
	ALD	18	3.13±1.22	<0.001**	0.03±0.02	0.036*
	Control 2	20	5.75±2.06		0.01±0.01	

**The difference is a highly significant at the 0.001 level. *The difference is significant at the 0.05 level.NS: non- significant difference > 0.05.

Table (5): The concentrations of copper in serum and saliva of all study groups.

Parameters	Group	number	Mean± SD	P-value
Cu concentration in serum (µg/mL)	NAFLD	18	29.53±9.14	<0.001**
	Control 1	30	62.50±19.09	
	ALD	18	26.6.17±6.17	NS
	Control 2	20	32.29±9.50	
Cu concentration in saliva (µg/mL)	NAFLD	18	18.14±17.69	NS
	Control 1	30	18.33±14.71	
	ALD	18	36.00±12.39	0.021*
	Control 2	20	13.00±2.00	

**The difference is a highly significant at the 0.001 level. *The difference is significant at the 0.05 level. NS: non- significant difference > 0.05.

Table (6): Pearson correlation between serum and saliva parameters in all studies groups.

Serum / Saliva	Control1	NAFLD	Control 2	ALD
Tp concentration	-0.082	-0.450	0.162	0.09
Total Cp ferroxidase	-0.147	0.740**	-0.077	0.026
Total Cp specific activity	-0.162	-0.042	0.108	-0.552
Non-Cp ferroxidase	0.483**	0.855**	0.569**	-0.068
Non-Cp specific activity	0.041	-0.562*	-0.200	-0.31
Cp ferroxidase	-0.159	-0.165	-0.172	-0.223
Cp specific activity	-0.172	-0.048	-0.123	-0.655**
Cu concentration	-0.524	0.582	-0.721	-0.535

**Correlation is a highly significant at the 0.01 level.*Correlation is significant at the 0.05 level

This activity plays assists in iron transport in the plasma in association with the iron transport protein, transferrin, which can only carry Fe in its ferric state. Alcohol and smoking induces an alteration in body Fe levels, and induce an elevation of in the Fe levels in the body(24). This has been suggested to due to the elevation of iron absorption from food in the digestive tract. Such elevation leads to increase of iron deposition in the liver. The regular consumption of alcohol leads to is down regulation of hepcidin expression, this in turn results in an increase in divalent metal transport and feroportin in the duodenum causing the increase in Fe absorption. On the other hand, the cigarette smoking which cause inadequate oxygen in the blood circulation (hypoxia), with ultimate results of increasing the number of the destroyed red blood cells and subsequently increasing Fe overload which has been reported to occur in blood ALD patients.

In order to look at the level of the variations in the ferroxidases activity, the specific activity different ferroxidases were determined usingmodified Lowry method (19) for the protein concentration measurement. This method was used since the protein concentration in the saliva is very low and the modified method is more commonly used in research applications since it is ten times more sensitive than the biuret reaction (25). Therefore, when the total protein concentration was measured in serum and saliva samples according to this method, the results presented in Table (3) showed the measured mean value± SD of total protein concentration in serum and saliva samples of all participants of the present study. These results showed that there was no significant differences in protein concentration in both serum and saliva among all groups ($p>0.05$), except that in the serum of ALD compared with its control group where a highly

significant difference was measured (p=0.001). The specific activity of any enzyme depends on both the enzyme activity and the total protein concentration and reflects if the variation in the enzyme activity is because of the alteration in its synthesis (regulation at gene expression), or in the enzyme activity (regulation at activity level). It clearfrom the results presented in the above Table (3) serum, and saliva total protein concentration did not differ in NAFLD patients from that of its corresponding control group. These results agree with the results of a study by Angulo, *et al*. which reported that no significant differences were present in total protein of their NAFLD patients (26). The observed no change in protein concentration is due to an existance of a balance between the increase in the concentration of the positive acute phase proteins such as C-reactive protein (CRP), amyloid protein A, alpha-1antichymotrypsin, fibrinogen, haptoglobin, ferritin, alpha-1 antitrypsin, alpha-1 acid glycoprotein, and ceruloplasmin (27, 28)and the decrease in the concentration of the negative acute phase proteins such as albumin (27- 30).

The obtained results in ALD agreed with the result of Pujar*et al* study,which showed the presence of a significant decrease in total protein in serum of their ALD patients(31). Moreover, the measured decreased [protein] in ALD may be due to the effect of alcohol drinking, or smoking (32). Moreover, this decrease may be due to the reduction in albumin concentration, the major protein of human plasma (3.4-4.7 g/dL). This protein was reported to decrease in liver cirrhosis patients and such decrease leads to retention of fluid in the tissue spaces.

Saliva contains proteins, in a concentration of approximately equal to 3% of plasma protein concentration (33), and can be informative for disease detection and surveillance of oral health (34). The concentration of total saliva protein remains within the normal range even though the concentration of albumin decreased and [globulin] increased and the levels of many proteins like α1-antitrypsin, α1- acid glycoprotein, C- reactive protein, and ceruloplasmin, were reported to increase in case of inflammation (35). O'Connell reported that many serum-derived proteins are transferred to the saliva during inflammation (36).

The enzyme specific activity depends on both activity and total protein. Table (4) exhibited ferroxidase specific activities in sera and saliva of all current studied groups. Total ferroxidase specific activity in serum was found to decrease with highly significant differences (p< 0.001) in both patients groups and this may be due to a decrease in the synthesis of these proteins. No significant changes were detected in both non-Cp ferroxidase specific activity and Cp ferroxidase specific activity in serum NAFLD patients group. On the other hand, the disagreement in the change between non-Cp ferroxidase activity and its specific activity in ALD, and Cp ferroxidase activity and its specific activity in NAFLD patients group, despite the presence of a highly significant decrease in the activity may be due to the regulation of these proteins. In saliva, there is no significant differences in ferroxidase specific activity in all groups except in Cp ferroxidase specific activity in ALD patients which showed significantly increases compared to its control group. Because the copper-dependent ferroxidase is required for the mobilization of iron from storage sites such as the liver. The concentration of copper in the serum and saliva samples of all studied groups were measured and the results were presented in Table (5). These results showed that [Cu] decreased with a highly significant difference (p=0.000) in serum of NAFLD patients. While a significant increase in this concentration was noticed in the saliva of ALD patients. The measured Low ferroxidase activity of Cp in NAFLD patients with low copper levels may be a causative molecular mechanism underlying iron accumulation in NAFLD (37). The results of the present study in serum agreed with many studies that investigated a potential association of copper status with disturbances of iron homeostasis in NAFLD (12-14). On the other hand, the results of ALD patients agreed with the results of Uhlikova, *et al* who studied the effect of copper on ceruloplasmin concentration and oxidase activity in ALD patients(20). Meanwhile, Lan, *et al* resultsdisagreed with the results of the present study in ALD, where no decrease in the serum ceruloplasminnor in its ferroxidase activity was observed in ALD patients (21).

Pearson correlation was used to check the correlation between the ferroxidase activities profile in serum and saliva was done in order to check the possibility of using saliva as a sample of analysis instead of serum, and the results is presented in table (6).

It is clear from these results that there was a significant correlation in non-Cp ferroxidase activities (p<0.01), with no correlation (p>0.05) in the remaining parameters in the case of control 1. While in the NAFLD case there was a highly significant correlation in total, non-Cp ferroxidase activities (p<0.01), as well as a

significant correlation ($p<0.05$) in non-Cp ferroxidase specific activity, with no correlation ($p>0.05$) in the remaining parameters. Meanwhile, a highly significant correlation in non-Cp ferroxidase activity ($p<0.01$), and no correlation in other parameters ($p>0.05$) for the control 2 group. While for ALD patients group, a highly significant correlation ($p<0.01$) is clear in Cp ferroxidase specific activity, with no correlation in other studied parameters ($p>0.05$).

4 Conclusion: This study results indicated that the activity of the different ferroxidases as well as copper concentration are linked in the oxidative stress and iron overload reported previously in our laboratory in serum and saliva of ALD and NAFLD patientsin different ways (38, 39). Furthermore the results of Pearson correlations indicated the possibility of using saliva instead of serum to measure a limited number of measured parameters including in the present study.

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