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Oxidative stress status in sera and saliva relevance to alcoholic and non-alcoholic fatty liver disease in Iraqi patients.

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ABSTRACT

Oxidative stress is an imbalance due to an excess of oxidants and a decrease in the antioxidant resulting in cells damage. The aim of this project is to look for the differences in the oxidative stress status in patients with non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) compared to that of control and to assess whether this status can be measured in saliva as an alternative to that of serum. The present study included four groups of Iraqi male, (18 patients with NAFLD), (18 patients with ALD), (30 nonsmokers control), and (20 smokers control). The measured parameters included serum and salivary total oxidant status (TOS), total antioxidant capacity (TAC), and oxidative stress index (OSI). The results in comparsion with that of the control group's showed :-

1). No significant differences in serum (TOS)&(OSI), salivary (TAC) of NAFLD group, as well as in salivary (TAC)&(OSI) of ALD group.
2). A significant differences in TOS in saliva of both patients groups, (TAC) in serum of NAFLD group.
3). A highly significant differences in serum (TOS), (TAC)& (OSI) of ALD group & in salivary (OSI) of NAFLD group.

The results showed no strong correlation in the measured parameters between serum and saliva of both studied patients groups. There is oxidative stress in both saliva &serum samples of (ALD) patients, whereas this situation of oxidative stress was present in only saliva sample of (NAFLD) patients.

Keywords: Alcoholic & nonalcoholic liver disease, Serum &saliva, Total oxidant status, Total antioxidant capacity ,Oxidative stress index.

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INTRODUCTION

The liver, which is the largest organ in the human body, plays a key role in many intermediary metabolic processes [1]. The changes in the morphology of its diseases are generally associated with biochemical alteration as well [2]. All over the world, one of the most increasing recognized as a morbidity significant cause, is a non-alcoholic fatty liver disease (NAFLD) [3]. In this disease, the earliest and most prevalent stage is accumulation of fat in the liver (fatty liver) , followed as next stage by steatosis (macrovascular steatosis), then steato-hepatic and severe inflammatory cirrhosis, which ultimately leads to liver failure and hepatocarcinoma in some cases [4]. This disease is of multifactorial nature that involves mitochondrial dysfunction, imbalance of amino acids, hyperglycemia, and imbalance in ketogenic and antiketogenic hormones in the portal blood [5]. On the other hand, the other type of chronic liver disease is an alcoholic liver disease (ALD) which arises from chronic alcoholic consumption. This type of liver disease as well covers a range of stages starting from fatty liver to alcoholic hepatitis and cirrhosis, which may eventually lead to the development of hepatocellular carcinoma [6,7]. Thus the the liver disease pathogenesis is based on the disrupted uptake, synthesis, and oxidative export of fatty acids, such imbalance leads to excessive accumulation of fat in the liver [8].

Normally, an efficient antioxidant system is present in the liver to maintain the redox hemostasis [9]. Many studies like those cited in [10] have suggested the intermediates of oxygen reduction, as one of the biochemical parameters associated with the development and progression of liver disease.

Several studies measured the serum, or plasma concentration of antioxidants individually in NAFLD patients. Such as a study carried in Chile which determined the activities of catalase (CAT) , and glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), as well as the concentration of total glutathione (GSH) in obese patients with NAFLD [11]. In addition, there are many studies carried out in Turkey, such as the study by Horoz, et al which measured total antioxidant response and the concentration of total peroxide [12] in obese children with NAFLD. Another study in Turkey estimated total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI) in obese children with NAFLD [13]. Meanwhile Baskol, et al, studied the advanced oxidation protein products (AOPP), total thiol (T-SH) levels and total oxidant/antioxidant status in Turkish obese NASH adolescents [14].

As far as our knowledge, no study has been reported in the literature that deals with the measurement of oxidative stress parameters in the saliva of NAFLD patients ,neither with measurement of (TAC), (TOS), and (OSI) as a collective term in ALD patients. But there are several studies spot light on oxidative stress in serum by studying antioxidants and oxidants parameters in a single form. Some of these studies were done in India such as one study focused on serum concentration of malondialdehyde (MDA), vitamin C, and vitamin E [15]. In the same year and country, another study conducted to estimate the levels of (GSH), (T-SH), and (MDA) in the serum of twenty alcoholic male patients [16]. Another study by Deshpande, et al in India, in which the concentration of MDA, the activity of SOD, and (GSH-Px) were measured [17]. Meantime, a study by Balu, et al measured serum (MDA and vitamin C) concentration [18]. Meanwhile, Bhardwaj, et al estimated serum (SOD) activity, and thiobarbituric acid reactive substances (TBARS) as lipid peroxidation products, ferric reducing ability of plasma (FRAP) and vitamin C concentration in both ALD and NAFLD patients [19]. In Taiwan, a study was carried out by Chen, et al measured the activities of glutathione peroxidase (GPx), glutathione reductase (GRD), superoxide dismutase (SOD), and catalase [20].

Generally, the oxidative stress can be measured in vivo in multiple sample types including cells, solid tissues, blood, saliva, and urine [21]. Several investigations have explained the corresponding modality of oxidative stress between serum and saliva, suggesting that saliva contains oxidation biomarkers approaching to those in blood [22]. A lack of consequence on the possible association between liver disease and the alternation of some biochemical parameters related to oxidative stress in saliva and serum motivated us to conduct the present work, which intended to explore the differences in the oxidative stress status in these two fluids of ALD and NAFLD patients groups in comparison to that of control and to assess whether such difference can be measured in the saliva to reflect what is happening in their serum.

PATIENTS AND METHODS

PATIENTS AND PARTICIPANTS

Two groups of male patients were included in the present project. Patients with nonalcoholic fatty liver disease (NAFLD, n =18) who were diabetic and non-smokers, and alcoholic liver disease patients (ALD, n = 18) who were alcoholic and smokers for at least 20 years. For comparison purpose, two groups of age-matched healthy controls were also included in the study. The first control group was 30 non-smokers to be used as a control for NAFLD patients, while the second control group was 20 smokers, healthy individuals to be used as a control for ALD patients. The patients were attending to the center of Gastroenterology and Hepatology in Baghdad city. They were referred from different hospitals in Baghdad and other governorates of Iraq during the period of November 2015 to July 2016. Their ages ranged from 35-60 years. The ethics committee of Baghdad University/ College of Science approved the study project. The participants were informed about the nature of the study. With their consent, a questionnaire linked to their study along with their personal full details history including medical history, laboratory evaluation, a radiographic study including computed tomography (CT), magnetic resonance imaging (MRI), as well as, the results of liver biopsy examination, and the results of biochemical tests which were carried out at the hospital laboratory, that included the following tests: body mass index (BMI), prothrombin time (PT), partial thromboplastin time (PTT), the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Level of total serum bilirubin (TSB) and fasting blood sugar (FBS).

INCLUSIVE CRITERIA OF THE PARTICIPANTS

According to the hospital laboratory tests, the participants were chosen, so they had no significant variation in BMI, PT, PTT, TSB, and ALP ($p>0.05$). The NAFLD patients were diabetic with a mean value of blood sugar concentration (274.88±66.27mg/dl). While the ALD patients were non-diabetic. Age-matched healthy control who fulfilled inclusion criteria were also included in the present study.

EXCLUSIVE CRITERIA

For both ALD, NAFLD the excluded criteria were: non-alcoholic cirrhosis due to hepatitis B, C, and Wilson disease, α1-antitrypsin deficiency Haemochromatosis, obese person, and vitamin supplements usage. A diabetic ALD patients, as well as alcoholic NAFLD patients, were excluded from the present study.

SAMPLES

Blood and unstimulated saliva samples were taken from the individuals of all groups after overnight fasting and the participants were asked to rinse their mouth with saline before collecting the saliva samples. The saliva samples were centrifuged for 10 minutes, at (2400×g), then the supernatant was kept frozen to be used for the desired measurements. At the same time, 10 mL of blood were withdrawn from the same individual in serum tube then centrifuged at (3000 x g) for 5 minutes. The sera samples were collected and kept frozen to be used for the determinations of the different studied parameters.

METHODS

CHEMICALS: All chemicals used in this study were of the highly analytical grade.

MEASURMENT OF TOTAL OXIDANT STATUS [TOS]

The total oxidant status of saliva and serum were measured using a colorimetric method that developed by Erel [23]. The results were expressed as (µmol H_2O_2 Eq. /L).

DETERMINATION OF TOTAL ANTIOXIDANT CAPACITY [TAC]

The total antioxidant capacity of saliva and serum were determined using Erel method [24]. The results were expressed as (mmol uric acid Eq./L).

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CALCULATION OF OXIDATIVE STRESS INDEX (OSI)

Oxidative stress index value calculated from the following formula [25]:
OSI =TOS (µmol H_2O_2 Eq./L) / TAC (µmol uric acid Eq./L)

STATISTICAL ANALYSIS

The data were analyzed using SPSS by Licensed Materials version 22 computer software. Data in this study was presented as Mean± Standard deviation (Mean± SD) using Independent-samples T-Test to compare the mean. A value of ($p<0.05$) &($p<0.001$), were considered as statistically significant, & highly significant respectively.

RESULTS AND DISSCUSION

Many studies have suggested that the ratio of AST/ALT can be used to differentiate between patients with ALD and NAFLD; if it is >2 this indicates that the patient has ALD, while if it is <2, it indicates that the patient has NAFLD [26-29]. Meanwhile, Obika, et al suggested that NAFLD patient with a ratio of less than 1 has either no, or minimal fibrosis, while a ratio of value >1 indicates the development of the disease to the cirrhosis stage [30]. When this ratio was calculated for both ALD and NAFLD patients included in the present study, it was found that all ALD patients had a ratio of >2. And when the NAFLD patients group were screened for this ratio, it was found that 28% (n=5) of them had a ratio of AST/ALT less than 1 i.e they had no or minimal fibrosis. While 72% (n=13) of them had a ratio more than 1 i.e that they had cirrhosis. Therefore, in order to check if the stage of the disease has any effect on the measured parameters in serum and saliva of NAFLD patients, a comparison of the results of (TOS, TAC, and OSI) was done between these patients. No significant variations in the measured (TOS, TAC, and OSI) were observed in saliva, neither in serum between those patients of the NAFLD with cirrhosis and that with no, or minimal fibrosis (the results not shown). Based on these results, data for both stages in NAFLD patients were combined for further analysis.

Table 1: Personal correlation between serum & saliva oxidative stress profile all study groups.

Parameters	Control 1 n=30	NAFLD n=18	Control 2 n=20	ALD n=18
TOS	0.112	-0.042	0.144	0.026
TAC	-0.227	-0.006	0.149	0.066
OSI	-0.198	0.180	0.216	-0.193

When a comparison of (TOS) between ALD group and NAFLD group with that of their corresponding control group were done. The results presented in **(Figure 1a)** show that there is no significant increase ($p>0.05$), in (TOS) of NAFLD patients group compared to that of its control group, while a highly significant increase ($p<0.001$) of this parameter in serum of ALD patients in comparison to its control group. Meanwhile in saliva, a significant increase ($p<0.05$) is found in that of both patients groups compared to their corresponding controls groups **(Figure 1b)**.

The result of NAFLD in serum agrees with many studies in different countries measured individual oxidative stress parameters, which showed that there was an oxidative stress in sera of their patient's groups. Such as a study by Horoz, et al which reported an increase in total peroxide levels [12], and study by Pirgon, et al who measured an increase in total oxidant status in their studied NAFLD patients [13]. As well as, a study by Baskol et al in Turkey which showed an increase in the advanced oxidation protein products (AOPP) and (TOS) [14]. Meanwhile, there are no studies reported in the literature which deals with the measurement of salivary oxidative stress in NAFLD patients.

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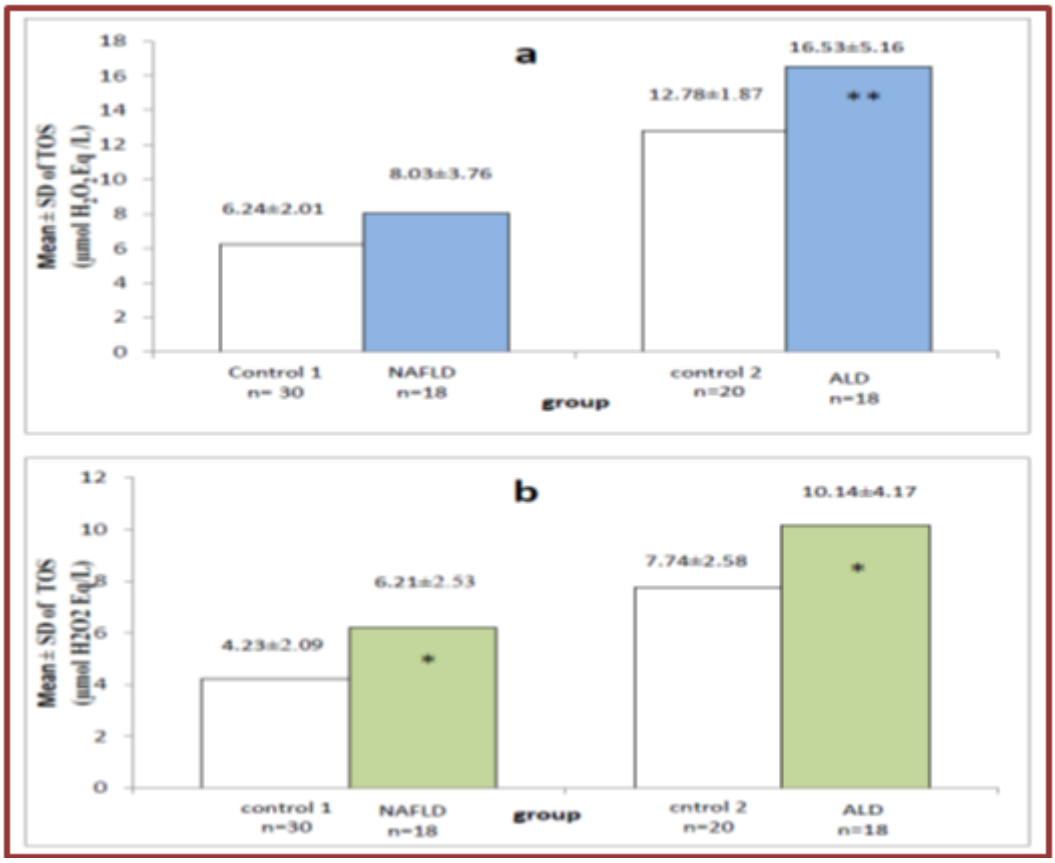

Figure 1: Mean value ±SD of (TOS) in (a) serum and (b) saliva of all studied groups. **The difference is highly significant at the 0.001 level. *The difference is significant at the 0.05 level.

The observed increase in the level of TOS in serum and saliva of NAFLD patients compared to their control group may be due to insulin resistance (IR). Insulin, in the liver, exerts two predominant actions: increases the synthesis of fatty acids and triglycerides (lipogenesis), and reduces glucose production (gluconeogenesis). In the case of insulin-resistant state, one of these actions is blocked. Insulin retains its ability to enhance lipogenesis but loses its ability to reduce gluconeogenesis [31]. In adipocytes, IR increases the release of free fatty acids (FFAs) from stored triglyceride, and increased oxidation of FFAs in aortic endothelial cells due to lack of insulin stimulation of malonyl-CoA production, this causes an increased superoxide production by the mitochondrial electron transport chain [32].

The other source of free radicals is glucose oxidation. The enediol form of glucose is oxidized to an enediol radical anion in a transition-metal dependent reaction. This Enediol radical anion is then converted into reactive ketoaldehydes and to superoxide anion radicals [33]. The superoxide anion radicals are converted to hydrogen peroxide, which in the presence of transition metals, can lead to the production of extremely reactive hydroxyl radicals or react with nitric oxide to form reactive peroxynitrite radicals [34,35].By a superoxide-dependent pathway, hyperglycemia was found to promote lipid peroxidation of low-density lipoprotein (LDL) resulting in the generation of free radicals [36]. Another important source of free radicals in diabetes is the interaction of glucose with proteins, leading to the formation of an Amadori product and then advanced glycation endproducts (AGEs) [37]. The AGEs, via their receptors (RAGEs), inactivate some enzymes and alter their structures and functions, quench and block antiproliferative effects of nitric oxide, and promote free radical formation [38]. The results in this Figure, also show an increase in (TOS) in ALD in serum and saliva compared to their corresponding control group. The results in serum are in line with the results presented by many researchers who worked on individual oxidative stress markers in serum such as measurement of the activity of (SOD), (CAT), and (GSH-Px), as well as, the concentration of vitamin C, vitamin E, and MDA. Among these studies,many studies in India which showed an increase in MDA levels in serum of ALD patients [16,18-20]. While in saliva again, no studies are found in the literature deals with oxidative stress in the saliva of ALD. The presence of this oxidants situation may arise from two sources. The first source is through the oxidative

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metabolism of alcohol, while the other one is due to smoking effect. The major pathway of oxidative metabolism of ethanol in the liver involves the production of acetaldehyde, by alcohol dehydrogenase (ADH) , a highly reactive and toxic byproduct that may contribute in tissue damage and, possibly, the addictive process [39]. This compound has the capacity to bind to proteins such as enzymes, microsomal proteins, and microtubules [40]. Acetaldehyde, produced by alcohol oxidation is rapidly metabolized by acetaldehyde dehydrogenase (ALDH2) to acetate and NADH [40]. The acetate increases blood flow into the liver and depresses the central nervous system, as well as affects various metabolic processes [39]. In the mitochondria of peripheral and brain tissues acetate is metabolized to acetyl-CoA, which is involved in lipid and cholesterol biosynthesis [41]. Ethanol metabolism tends to increase the hepatocytes' oxygen uptake from the blood. When the hepatocytes which are located close to the artery supplying oxygen-rich blood to the liver take up more than their normal demand of oxygen, not enough oxygen may be left in the blood to adequately supply other liver regions with oxygen [42]. Ethanol indirectly increases the cells' oxygen use by activating Kupffer cells in the liver, in addition, to directly increasing hepatocytes' oxygen use as described above [39]. The oxidation processes of the acetaldehyde by ALDH2 involves an intermediate electron carrier, nicotinamide adenine dinucleotide (NAD+) that will be converted into NADH by accepting electrons. This results in a significant increase in the hepatic NADH/NAD+ ratio in both the cytosol and mitochondria thus increase the possibility of producing more free radicals [43,44]. The other source of free radicals in ALD patients is smoking. Cigarette smoke contains a lot of reactive oxygen species since this smoke contains many chemicals, that cause the formation of free radicals such as phenols, aldehydes, hydrocarbons and quinone radicals [45-47]. The observed results in (Figure 1) of control 2 (who were smokers) compared with that of control 1 (who were nonsmokers) confirm such effect of smoking.

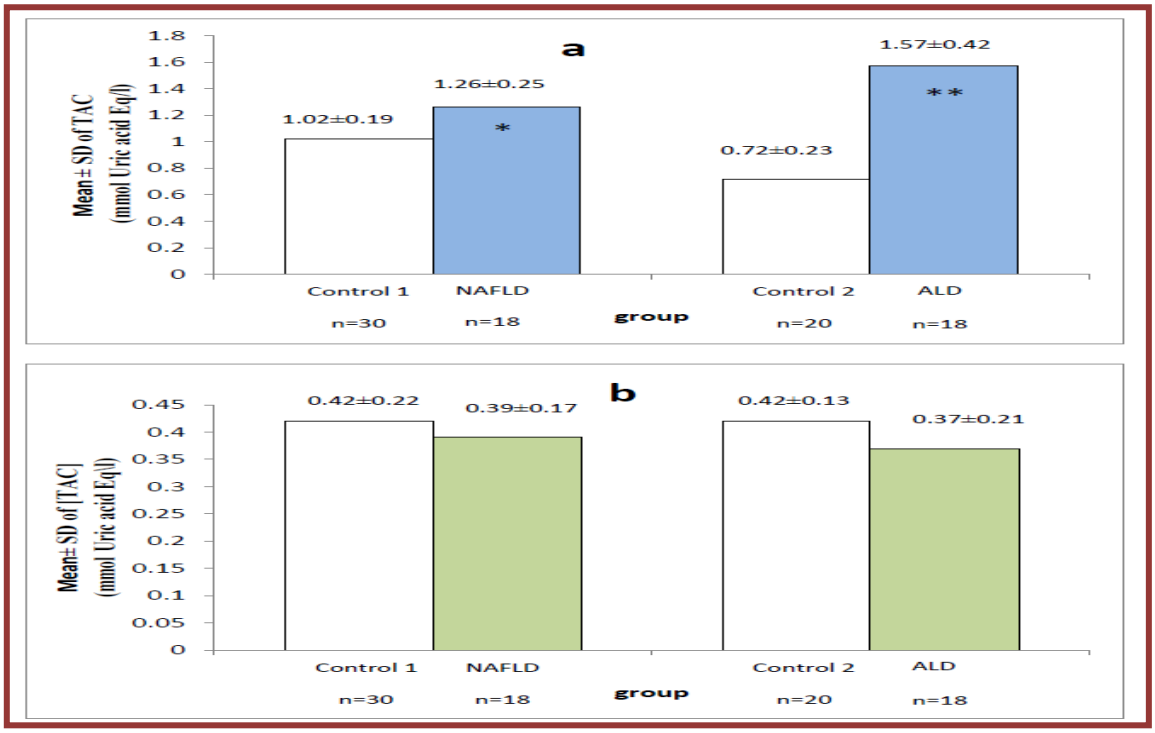

Figure 2: Mean value ±SD of (TAC) in (a) serum and in (b) saliva of all studied groups. **The difference is highly significant at the 0.001 level. *The difference is significant at the 0.05 level.

Antioxidants protect the body against free radicals. Serum and saliva contain many antioxidants such as uric acid, glutathione, and enzymes including, catalase, peroxidase, and glutathione peroxidase& superoxide dismutase [48]. In order to check antioxidant status in the different studied groups, total antioxidant capacity (TAC) was measured in serum and saliva as described in the material and method section instead of studying individual antioxidants separately. The major advantage of this test is to measure the antioxidant capacity of all antioxidants present in a biological sample and not just of a single compound [49]. **(Figure 2a)**shows that there is a significant increase in (TAC) ($p<0.05$) in the serum of NAFLD group and a highly significant increase ($p<0.001$) in that of ALD patients group in comparison with their corresponding control groups. Meanwhile, **(Figure 2b)** shows that there are no significant differences in this parameter among saliva of both patients groups ($p>0.05$).

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The result in serum disagrees with many studies in different countries that focused on measurement of individuals oxidative stress parameter, these studies showed a decrease in antioxidants parameters in their patient's groups [11-20].

When the oxidative stress index (OSI) was calculated for sera and saliva of both patients groups. The results in **(Figure 3a)** shows that for NAFLD group, there is no significant difference in serum (OSI) compared to its control group ($p>0.05$), while a highly significant decrease in that of ALD group compared to its control group ($p<0.001$). Meanwhile, in saliva, for NAFLD patients there is a highly significant increase compared to its control group ($p<0.001$), while for ALD and its control, there is no significant differences ($p>0.05$)**(Figure 3b)**. Such differences in the calculated OSI index is due to the observed variations in the measured total antioxidants capacity of the studied groups **(Figure 2)**.

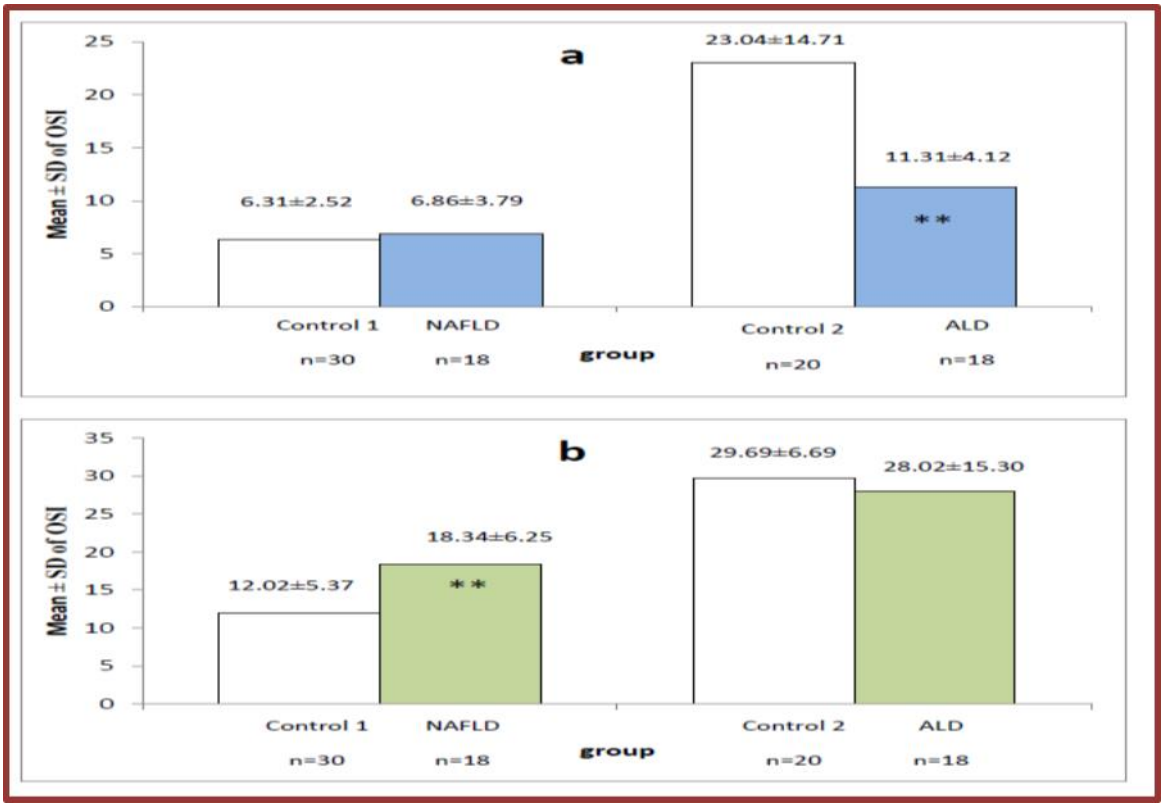

Figure 3: Mean value ±SD of OSI in (a) serum and in (b) saliva of all studied groups. **The difference is highly significant at the 0.001 level.

One sources of the increased (TAC), among other numerous sources is the activation immune system which causes an increased antioxidants activities to detoxify the effect of the produced free radicals [50-52]. The other source may be due to the reported increased levels of many antioxidants such as SOD, bilirubin [53], ferritin [54], ceruloplasmin [55], and uric acid [56,57]. The differences in the results of TAC and OSI between the present study and other studies may be due to many factors such as the difference in the gender, age, genes, and the nature of foods of the participants [58,59].

One aim of the present study was to check the possibility of using saliva as a sample of analysis instead of serum, and in order to check this possibility, a correlation between the variation in the oxidative stress profile in serum and saliva was done. As it is clear from the results presented in "Table " , no strong correlation is present between the variations in serum in respect to that of saliva in all studied groups.

CONCLUSION

According to the results of oxidative stress parameters in serum and saliva of NAFLD, ALD, in general there is oxidative stress in both saliva &serum samples of (ALD) patients, whereas this situation of oxidative

stress was present in saliva sample only of (NAFLD) patients. Such increase was observed even though an increase in total antioxidant capacity is present but it seems to be not enough to cope with the increase in the free radicals production especially in saliva of (NAFLD)patients.

Meantime the results of the correlations indicated that saliva can't be used as an alternative fluid sample to measure the variations in the oxidative stress parameters included in the present study in ALD & NAFLD patient.

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