Journal of Medical Genetics and Clinical Biology
Homepage : https://journal.antispublisher.id/index.php/JMGCB
Email : admin@antispublisher.com

e-ISSN : 3032-1085
JMGCB, Vol. 3, No. 6, June 2026
Page 52-65

Impact of Smoking and Athletic Activity on Salivary Malondialdehyde, Copper, and Zinc Levels in Male Student Athletes in Babylon Province, Iraq

Rajaa Abdulrazzaq Al Anbagi[1], Baqir Habib Abis[2], Zainab Hassan Abdullah[3], Fakhir R. H. Alshuwaili[4]
[1,2,3]Department of Medical Biotechnology, Al-Qasim Green University, Iraq
[4]Department of Applied Biotechnology, Al-Qasim Green University, Iraq

DOI : https://doi.org/10.61796/jmgcb.v3i6.1778

Sections Info

Article history:
Submitted: March 15, 2026
Final Revised: April 05, 2026
Accepted: April 20, 2026
Published: May 07, 2026

Keywords:
Iraq
Malondialdehyde
Oxidative stress
Physical exercise
Smoking
Trace elements

ABSTRACT

Objective: *The smoking habits and physical exercise are two important factors associated with human health. The current study was designed to investigate the influence of cigarette smoking habits and athletic activity on salivary factors linked to oxidative stress and trace elements in male smokers and athlete have been studied by investigating effects of smoking and regular exercise on the flow rate and malondialdehyde (MDA) levels as a biomarker for quantifying oxidative stress in the salivary samples.* ***Method****: A total of forty university students were selected for the study. The sample was classified equally into two groups smokers and non-smokers and each group were divided into another two groups based on the physical exercise. The levels of some trace metals including saliva zinc (Zn), copper (Cu), Cu/Zn ratio and MDA levels were measured in Iraqi students as an important parameter involved in several physiological process including antioxidant system.* ***Results****: Findings of this effort had pointed the investigated salivary parameters were affected by physical stress and cigarette smoking. It was found significant observed variations between groups of smoking habits and exercises when being taken independently and their interactions. The highest salivary MDA levels were in the smokers/ non-athletes (1.469 µmol/L) and non-smokers/athletes (1.349 µmol/L) and differed significantly ($p < 0.05$) from non-smokers/non-athletes (0.71403 µmol/L). The concentrations of Cu were substantially higher in salivary of non-smokers/non-athletes compared to other three groups. The Cu/Zn ratio is driven by a rise in Cu levels and decrease in Zn levels among the fourth groups.* ***Novelty****: Although some study investigated effects of MDA and some trace metals such as Zn and Cu, there is an obvious lack of comprehensive studies in Iraq that evaluate salivary MDA, Zn and Cu levels among athletes, specifically in relation to smoking status. Thus, this study provides important data to fill this gap and better understand these interactions related to oxidative stress and antioxidant defense systems.*

INTRODUCTION

Reactive oxygen species (ROS) are naturally occurring in living organisms from endogenous sources such as mitochondria, endoplasmic reticulum and plasma membrane due to cellular metabolisms. These reactive molecules are the main effectors of oxidative stress in human cells and play important roles in cellular homeostasis (1, 2]. The generated radical molecules are continuously removed by a host of antioxidant system. Evidences have accumulated that excessive production of ROS and damage by free radicals may lead to some clinical conditions such as diabetes, irritable bowel disease and ageing [1].

Other exogenous factors such as some human behaviors and UV exposure, also, increase the production of ROS. Physical excises, for example, are increasingly

performed and habitually recommended for their beneficial effects on human physiques. However, exercises usually lead to an increase in the productions of reactive oxygen and nitrogen species [3, 4]. During exercises, radical species are generated continuously due to contracting skeletal muscles [1,5]. The excessive productions of free radicals may trigger damage to DNA, proteins and lipids in tissues [1]. This leads to contractile dysfunction due to decrease in the capacity of the antioxidant system related to exceeding levels of ROS. On the other hand, the regular exercise also plays important roles through enhancement in the activity of DNA repair enzymes involved in ROS-mediated damage [3]. Many studies have proposed there is a close relation between oxidative, in combination with chronic inflammatory conditions and development of metabolic diseases such as diabetes mellitus type 2 and cardiovascular diseases and oral disease such as oral submucous fibrosis [6, 7].

Smoking habit is another exogenous factor increase the ROS production. The habitual smoking can lead to continuous changes in brain cells, resulting in changes in mental functions, memory and intelligence and to numerous disorders including cardiovascular diseases and various types of cancer [8, 9]. Cigarette puffs contain oxidant molecules that can alter endogenous macromolecules and lipid phases into peroxynitrite which forms superoxide radicals producing persistent oxidative stress. These oxidative stress cause cell oxidative damage after entering cells up to nucleuses [10, 8]. Furthermore, various metal ions are found in cigarette smoke. These are generated from tobacco leaves and other components of cigarettes such as printing ink and wrap papers. Metal ions including Cadmium, Chromium, Chlorine, Copper, Iron, Mercury, Manganese, and Zinc are commonly detected in different cigarette products [11]. These metal ions react with cysteine, thereby depleting the antioxidant thiol pool. After few years of consistent smoking, it reduces the lung capacity and aerobic performance and increases the threat of cardiovascular diseases [9, 12].

Saliva is the first line of the body fluid that encounters some human lifestyles such as training and cigarette smoke and attempts to maintain a redox homeostasis [13, 14]. Saliva has been used as a biological sample for medical research refracting oral health. It constitutes from more than 99% of water and the remaining composed of organic and inorganic components including trace elements, vitamin C, malondialdehyde (MDA), amylase, and proteomes [15, 16]. The physiochemical features and amount of saliva are affected by an individual activity and a variety of diseases [14, 17, 18]. Studies showed that some blood immunological and parameters in patients who recovered from COVID-19 and or vaccinated, for example, have changed compared with other groups and showed salivary disorders and Zn dyshomeostasis [19].

Because of an easy sampled and non-invasive method, saliva sampling usually prefers for some researchers that does not require immediately sample preparation enabling multiple collections [14].

Malondialdehyde originates from systematic sources and one of the final decomposition products of lipid peroxidation (LPO) by ROS. It has been reported as a biomarker of oxidative stress reflecting changes in LPO levels and assessing cellular

damage [20, 21]. MAD have been used as an indicator of the effect of exercise on individuals [20, 22]. Significant increase in MDA levels has been reported in different exercise levels, owing to the aggravated LPO [23]. Similarly, it has been applied as a biomarker of smoking impact on the oral health in smokers [21].

Trace minerals are found in human saliva and most human cells and involved in any physiological processes. Copper (Cu) and Zinc (Zn) are essential trace minerals and contribute to various metabolic processes [4, 23]. Several oral diseases are linked to change in salivary composition including trace elements [24, 2]. Both Cu and Zn levels in human saliva and plasma, for example, alter during different conditions providing information on the immune response and inflammation as well as oxidative stress in a body [24, 25]. Both are affected by physiological stress and might influence on oral health [24]. Moreover, Cu and Zn act as cofactors of antioxidant enzymes that involve in antioxidant mechanisms, protecting human cells from oxygen reactive radicals. Thus, their concentrations in the human body significantly influence on protection against oxidative stress [26]. With biotechnic development, detections of trace element concentrations in human body became easy, highly efficient and less sample required with ability to analyses a large number of samples in a short time [23].

The Cu/Zn ratio has been used as biomarker for antioxidant status, oxidative stress and inflammation in oral cavity [27,25]. Both Cu and Zn are a part of several antioxidant systems including the copper-zinc superoxide dismutase (CuZnSOD) and metallothionein that act as a bulk scavenger of superoxide radicals [28]. If the balance of Cu/Zn ratio is interrupted, the functions of antioxidant enzymes are disrupted, and the oxidative stress is developed [29]. Thus, the evaluation of its ratio may provide knowledge on its association with a case study and with the antioxidant system. Taken together, evaluations of the biomarkers for oxidative stress and the antioxidant system are essential to explain the effects of inhaled smoke, regular exercise training and their interactions. The association between trace elements in saliva athletes and smokers have been investigated frequently [4].

In Iraq, there have been no study investigated the association between physical exercise and smoking on salivary factors linked to oxidative stress and trace elements in male smokers and athletes. Thus, the current study aimed to investigate the effect of smoking and regular exercise on the values of slivery flow rate and Cu/Zn ratio. The levels of MDA, Zn, and Cu were also measured and compared between Iraqi students in Babylon province.

RESAERCH METHODS

Study groups

A total of forty university students were selected for the study on the basis of voluntary participation. The study group included males only with an average age 25.1 years. The body mass index (BMI) of participants was 25.76 kg/m^2 [30]. The sample was classified equally into two groups smokers and non-smokers. Later, each group were divided into another two groups based on the physical exercise: cigarette smokers who

exercised 4 days in a weak for a duration 1-2 h (smokers/ athletes), cigarette smokers who did not exercise, smokers/ non-athletes, nonsmokers/ athletes and nonsmokers/ non-athletes.

Saliva collection

Unstimulated whole saliva specimen from the selected students was collected by the splitting method between 10 AM and 12 AM. Each participant was asked not to smoke, eat, or drink 1 h before collection and then spits saliva in 10 ml sterile Falcon tubes for 5 minutes. The saliva pH was determined immediately after collection saliva using pH indicator stripes. The flow rate of unstimulated saliva was detected by dividing the volume (mL) of saliva secreted five minutes. Later, the collected saliva samples were centrifuged at 1200 g for 5 min at the cold centrifugation This process provides saliva samples free of large debris and of reduced viscosity, allowing more accurate and reproducible analysis. The salivary supernatant was stored at (-20 ^{o}C) in polyethylene tubes for subsequent chemical analysis.

Determination of malondialdehyde

The content of malondialdehyde (MDA, µmol/L) in the saliva samples was determined colorimetrically using thiobarbituric acid reactive substances (TBARS) method with 1,3,3,3-tetraethoxypropane. The measurement was performed based on the reaction of MDA with thiobarbituric acid (TBA) at 90-100°C in acidic conditions. That reaction leads to making the pink colored trimethine complex. 2 ml of the reaction mixture constituted from 1:1:1 ratio TBA– trichloroacetate –HCl reagent were added to 100 µl of saliva in the test tube. The reactive tube was placed in a heated water bath for 15 min, and then allowed to cool. The sample was then centrifuged for 5 min at 5000 rpm. Absorbance of the supernatant was measured spectrophotometrically at 532 nm against reagent blind samples containing the distal water instead of saliva [31].

Determination of copper and zinc in saliva samples

The quantifications of copper (Cu) and zinc (Zn) concentrations (µg/dL) in saliva samples were performed using the colorimetric process with Dibromo-PAESA and with 5-Bromo-PAPS. respectively. After thawing of the saliva samples, the Salivary copper was quantitated based on the principle that released copper in saliva reacts with 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-sulphopropylaniline to form a colored complex. That colored matrix can be measured due to its increased absorption and that absorption is directly proportional to the concentration of total copper in the sample. The analysis was standardized at the wavelength/filter (580 nm (Hg 578 nm), room temperature and Light path (1 cm) before starting the examination. Then, the supernatant saliva, distilled water, standard solutions were pipetted into three clean dry test tubes labeled as test (T), blank (B), and standard (S), respectively. Then, the above samples were mixed and incubated at room temperature for 10 min. Later, the absorbance of the standard (Abs.S) and test samples (Abs.T) against the blank was measured within 30 min. Salivary copper levels (µg/dL) obtained from samples were estimated based [6]. Similarly, the quantification of zinc (µg/dL) in salvia is performed based on the assay principle that included the ability of zinc to form a red chelate complex when reacting with 2-(5-

Bromo-2-pyridylazo)-5-(N-propyl-Nsulfopropylamino)-phenol. Similar to the coper analysis, the analysis was standardized at the same conditions except the wavelength which was 546 nm. Finally, the values of Cu/Zn ratio were derived from the above estimated levels.

Statical analysis

The Shapiro-Wilk analysis test was applied for the normality distribution of the investigated parameters prior to any analysis, which showed normal distribution ($p > 0.05$). Data were firstly analyzed using Student t-test. One-way analysis of variance (one-way ANOVA) was performed to test for significant changes in salivary flow rate, MDR, Cu, Zn, Cu/Zn ratio. Turkey post hoc tests were then applied to determine the variations among groups. Two-way univariate analysis of variance (two way-ANOVA) analyses were also applied to test for the significant variations in previous parameters between four groups. Data were expressed as mean ± standard deviation and $p \leq 0.05$ were considered statistically significant.

RESULTS AND DUSCUSSION

The results of the present study have pointed the salivary parameters that were affected by physical stress and cigarette smoking. These showed significant differences between these two variables and their interactions on some investigated parameters of man salivary samples. The salivary profile of investigated groups showed the salivary pH levels (6.1) were not affected by cigarette smoking and physical exercise independently or their interactions which are comparable with the previous findings [32, 33]. The results also signified the unstimulated salivary flow rate were 0.465 ml/min in smokers and 0.685 ml/min in non-smokers (Figure 1A). These results differ significantly (p=0.0003) and are consistent with previous studies [34, 17]. The current differences may be related to functional and structural changes in saliva during exposure to cigarette smoke, resulting in formations of numerous toxic substances. That causes alterations in the structure and function in saliva ([35], resulting in reducing saliva [36]. However, the current reported findings the flow rates of unstimulated saliva are still within the range values (0.3-0.65 ml/min) mentioned in the previous results [34,17].

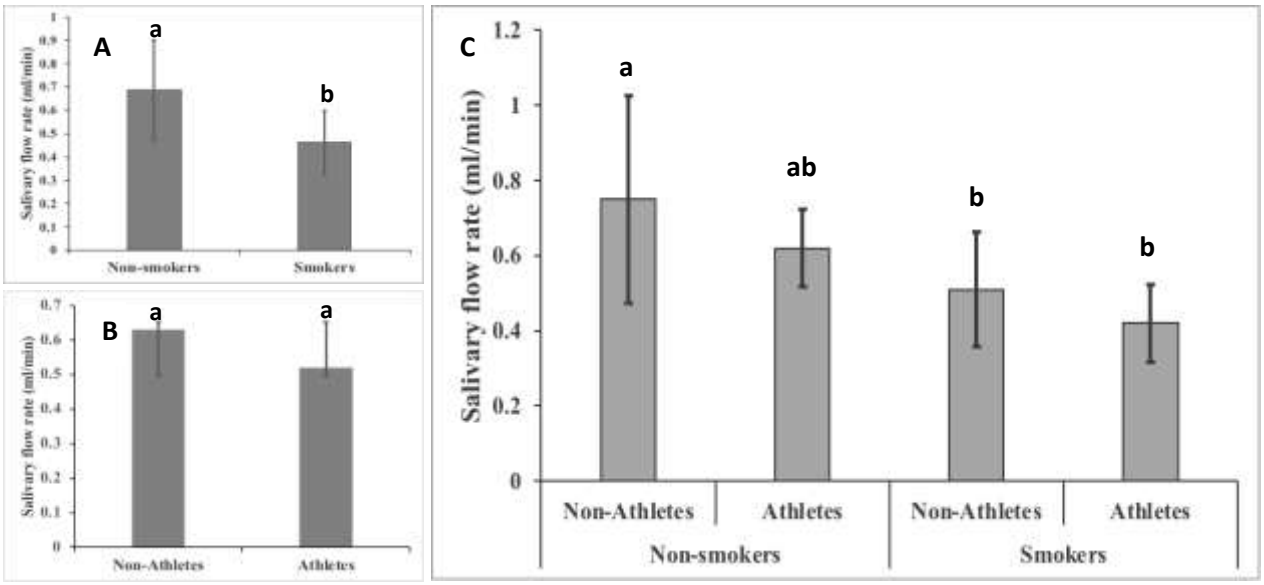

Figure 1. The values of salivary flow rate determined by cigarette smoking in both oral cavities of athletes and non-athletes. The values with same letters are not significantly different.

The physical exercises did not affect significantly (p=0.0528) on the values of salivary flow rate in the present study (Figure 1B) in agreement with previous studies [33,17]. However, the interactions between both physical stress and cigarette smoking showed the mean values of the salivary flow rate (0.75 ml/min±0.077) in non-smokers/non-athletes were significantly higher (p=0.001) than in smokers/ non-athletes (0.51 ml/min±0.077) and smokers/ athletes (0.42 ml/min±0.077). However, no significant differences between non-athletes and athletes who were not cigarette smoking (Figure 1C). Studies pointed the smokers and athletes are at a higher risk of sever xerostomia (dry mouth) and microbial infections [34, 33]. Altercations in the whole salivary flow rate have an important role in the oral pathogenesis. Also, these disrupts may influence on the concentrations of saliva substances including oxidants, antioxidants, and their trace minerals [17].

The results of MDA levels revealed no significant variations were reported when taken in account the smoking and regular exercise independently (Figure 2 A&B). However, substantial variations were observed in their concentrations between the four investigated groups (Figure 2C). The highest salivary MDA levels were in the smokers/ non-athletes (1.469 µmol/L) and non-smokers/athletes (1.349 µmol/L) and differed significantly ($p < 0.05$) from non-smokers/ non-athletes (0.71403 µmol/L). Although the MDA levels of the smokers/ athletes were 1.109 µmol/L and did not vary considerably from the previous three groups. MDA derived from lipid peroxidation, have been applied to evaluate cellular damage and to indicate of health and physiological adaptation in athletes [22] and in cigarette smokers [21]. In this study, insignificant differences in the levels of man salivary MDA between the two groups (smokers and non-smokers) and other two groups (athletes and non-athletes) independently (Figure 2A&B) were in agreement with studies by Sari-Sarraf et al. [37] and Oda et al. [18].

However, some results showed a significant increase in the MDA levels in cigarette smokers [21]. Free radicles formed in exposed organs through cigarette smoke or found with many oxidants in cigarette constituents are able to cause oxidative stress leading to increase MDA concentrations [38] The smoking habit also activates some endogenous mechanisms that increased the oxidants when inhalation of smoke into lung [28]. The significant increase in the MDA of non-smokers/ athletes compared with non-smokers/non- athletes in this study disagreed with findings by Susanto et al. [22]. They found the MDA levels were decreased significantly during exercise and the training levels and nutritional interventions both affects the MDA concentrations [22,23]. Although the differences did not significant between non- smokers/ non- athletes and smokers and athletes, smoking can potentially impact on health and exercise performance [39]. The results also indicate the physical exercise may alter the harmful effect of cigarette or engage in healthier lifestyle outside of training. It seems that the age and smoking duration of participants in the current study may have not accumulate sufficient long-term damage. However, there is disagreement regarding the exact cause of these results between the studies and more studies are recommended [40, 41, 39]. Several factors may affect the different results including participant age, health and lifestyle and the methods of measuring MDA [18, 39].

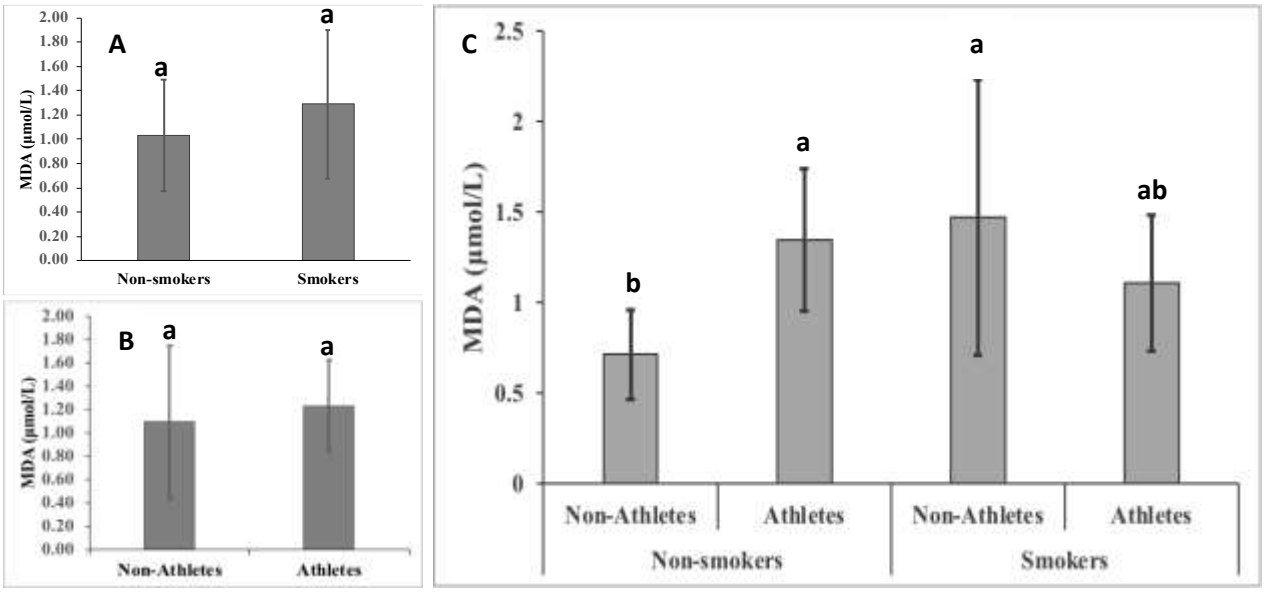

Figure 2. The levels of salivary MDA determined by cigarette smoking in both oral cavity athletes and non-athletes. The values with same letters are not significantly different.

In the present data, the concentration of salivary copper (Cu) in the non-smokers (1.723 µg/dL) was significantly (p<0.001) higher than in the smokers (1.566 µg/dL, Figure 3A). There were no significant differences (p >0.05) between non-athletes (1.639 µg/dL) and athletes (1.651 µg/dL, Figure 3B). However, the interaction effects of the physical training and the smoking habits on the salivary copper levels revealed significant variances were recorded (Figure 3C). The levels of copper were substantially

higher in salivary of non-smokers/ non-athletes (1.781) compared to smokers/ non-athletes (1.497 µg/dL) and in those non-smokers (1.666 µg/dL) and smokers (1.637 µg/dL)/ athletes. Theses trace elements play essential roles in redox reactions protecting the body from oxygen free radicals (Osredkar et al., 2011; Yadav et al., 2025). Both Cu and Zn are an integral component of the antioxidant enzyme, superoxide dismutase (SOD) degrading the superoxide anion dismutation into the hydrogen peroxide and oxygen [26].

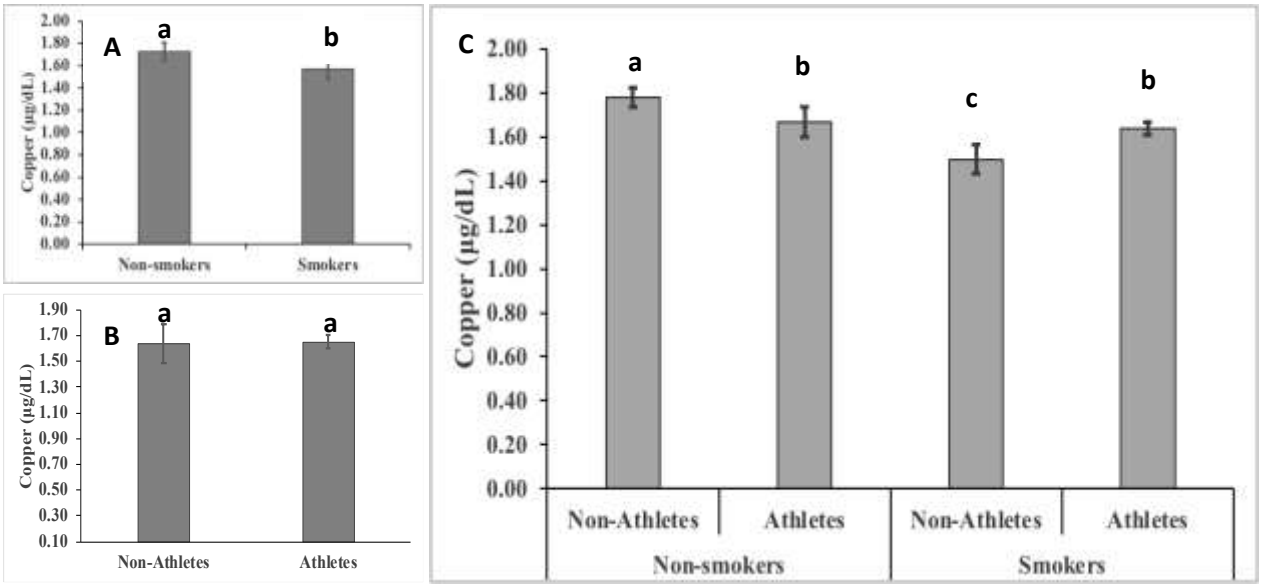

Figure 3. The levels of salivary Cu determined by cigarette smoking in both oral cavity athletes and non-athletes. The values with same letters are not significantly different.

The salivary Cu levels were significantly higher among non-smokers than the smokers in the current findings may be relate to redox reactions. It has been found that cigarette smoke alters the salivary antioxidant activity [42]. The salivary Cu levels didn't substantially differ between non-athletes and athletes. That results were harmonized with Zeffa et al., [14] and Xue et al. [23] who reported that Cu concentrations in the professional athlete group were significantly lower than those in the sedentary group. However, Wang and colleagues [43] observed high levels of the plasma Cu levels in athletes after intense exercise with gradually returned to early levels after resume training. That suggest the exercise efforts influenced the Cu secretion [14]. When being taken the interaction between the exercise and smoking habits, smoking behavior still impacts on the compared pervious fourth groups. The higher concentrations of Cu in the salivary of non-smokers and non-athletes compared to other three groups may be because there wasn't oxidative stress status that usually originated due to exercise training and/ or cigarette smoking and lower metabolic consumption of antioxidants. Evidenced showed that individuals in relaxed state had higher salivary Cu than those under social stress or training [24,23]. In the present data, the Cu levels were lowest in smokers/ non-athletes. The possible reason is redistribution or increased demand of Cu due to oxidative or metabolic stress. The Cu

is an important component of antioxidant enzymes including SOD that reduces the damage of human cells due to generation free radicals [27, 16]. The Cu is frequently found in cigarette products may involve in depleting the antioxidant thiol pool through its reaction with cysteine [11, 9].

In this study, no significant differences in concentrations of saliva Zn were recorded between both regularly smoking and exercise independently as well as four investigated groups when taking each habit (Figure 4). These results contradict previous studies. It was reported significant decreases in different exercise levels when comparing their levels to the sedentary group [23]. The routine exercise can reduce the levels of serum Zn in adolescent gymnasts [44]. It was found significant correlations between the training levels and the trace elements [4, 14]. The results of influence the smoking status and athletic activity on zinc contain in the human saliva revealed that **non-smokers/athletes** exhibit the highest mean value (1.39 µg/dL), while smokers**/athletes** present the lowest mean values (1.33 µg/dL). The variations between these groups were statically noteworthy. However, the zinc concentrations in saliva of other two groups smokers/non-smokers and non-athletes showed intermediate overlapping results without significant variances**.**

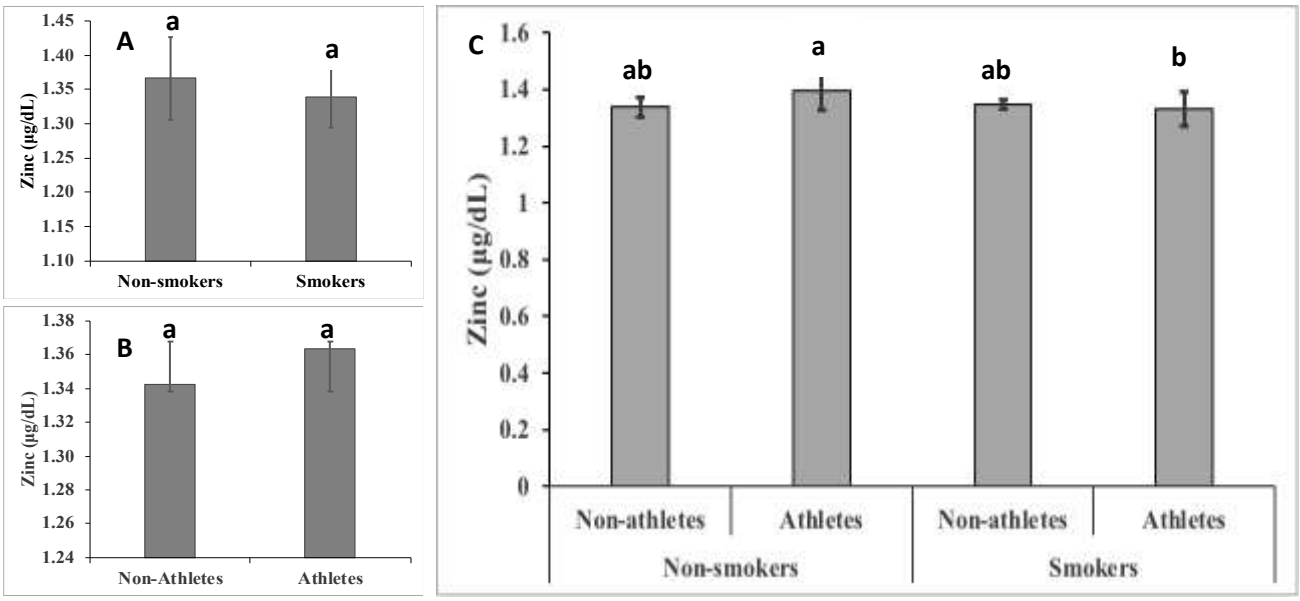

Figure 4. The levels of salivary Zn determined by cigarette smoking in both oral cavity athletes and non-athletes. The values with same letters are not significantly different.

The results highlight a potential interference consequence of smoking on athletic performance. Tobacco smoke contain many oxidants and prooxidants that rise oxidative stress in the male body due to the production of free radicals [45]. Similarly, after exercise efforts, a large number of free radicals are produced in athletes' bodies. The high concentrations of Zn can inhibit the generation of free radicals and consequently reduce the cell oxidative damage. Because of being a coenzyme of several antioxidant enzymes such as Cu/ZnSOD, it is exerting antioxidant effects [23]. Smoking

could cause oxidative damage that can disrupt in zinc metabolism and enzyme utilization [45]. Furthermore, the lowest levels of saliva Zn were found in smokers and athletes, suggesting a large amount of produced sweat and oxygen consumption in the body during exercise leading to decrease Zn levels and more Zn being transported into cells forming Cu/ZnSOD [23]. In many physiological studies, smoking is known to increase oxidative stress and impair oxygen transport (via carboxyhemoglobin), which directly counters the cardiovascular efficiency gained through athletic activity [46]. Reduction levels of Zn also linked to muscle strength, leading to reduction in physical performance. To maintain Zn levels in muscle, thus, the human body is able to prioritize and mobilize from tissue to another and Zn deficiency is commonly recorded in athletes [47].

In this study, an increase in the Cu/Zn ratio is driven by a rise in Cu levels and decrease in Zn levels among the fourth groups (Figures 3-5). It was reported a strong positive association between activities of antioxidative enzymes and their concentrations in tobacco smokers and athletes [48, 16]. Exposure oral cavity to cigarette smoke, for example, led to distinct degrease in levels of salivary antioxidants ([13] The saliva concentrations of Cu and Zn levels play essential roles as activators of antioxidant enzymes and consequently as defenders against oxidative stress [27]. Additionally, the increased MDA levels among current fourth groups, specifically in athlete groups played obvious role in raising ratios of Cu/Zn in consistence with previous results [23]. The mean values of salivary Cu/Zn ratio were significantly higher in the nonsmokers than in smokers, while no significant differences were obtained between athletes and non-athletes (Figure 5 A&B), suggesting smoking play roles in alterations of salivary compositions including trace elements [13] Both Cu and Zn are an integral component of the superoxide dismutase (SOD) degrading the superoxide anion dismutation into the hydrogen peroxide and oxygen [26].

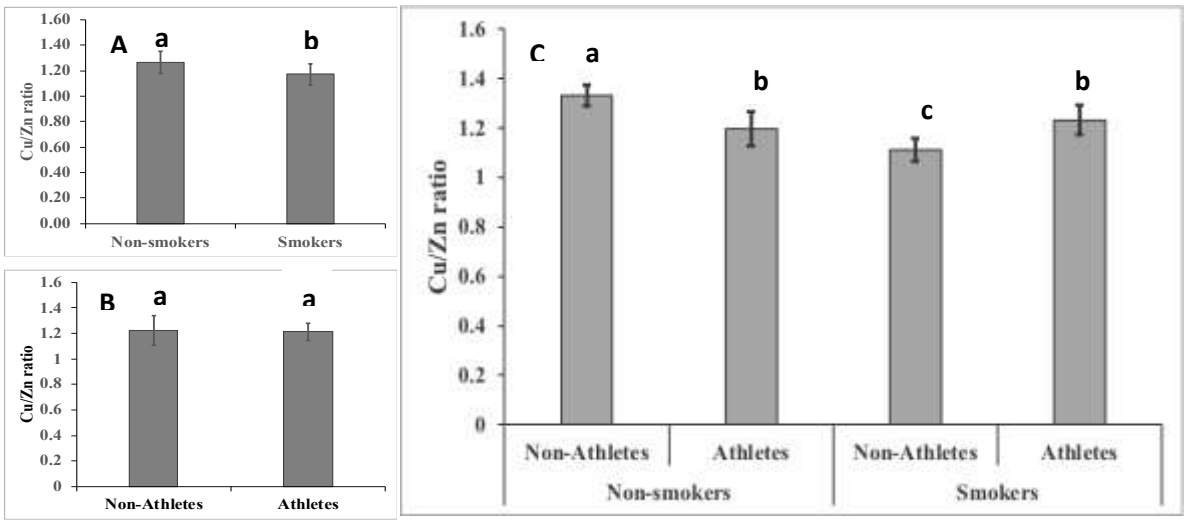

Figure 5. The values of salivary Cu/ Zn ratio determined by cigarette smoking in both oral cavity athletes and non-athletes. The values with same letters are not significantly different.

The interaction between exercise activity and smoking habit revealed noteworthy differences between these study groups. Following non-smokers/non-athletes, no statical differences were recorded between smokers/ athletes and non-smokers/athletes. The potential reason for these conditions is transfer Cu into muscle cells to produce Cu /ZnSOD to cope with the excessed free radicals [23]. It seems that the human body is capable to cope with the formation of free radicals and activate of antioxidant system under redox homeostasis. However, the lower Cu/Zn ratio in the saliva of smokers/ non-athletes in this study might consequence from the excessive production of free radicals, causing severe disruption in the salivary redox balance. Thus, Cu/Zn ratio might be used an important antioxidant, inflammatory, nutritional biomarker in human health during smoking and exercise activities.

CONCLUSION

Fundamental Finding: The current study revealed smoking habits and physical exercises influence on several saliva parameters that are related to oxidative stress and antioxidants. **Implication:** This study is among study filling a research gap in Iraq to evaluated the combined impacts of smoking and athletic activity on salivary oxidative stress marker (MDA) and important trace elements including, Zn, Cu and their ratios in student athletes as important baseline for this populace. The study also attempts to understanding interactions between smoking and exercise based on some physiochemical features of saliva. **Limitations:** The study is limited to small and homogenize sample with considering the female population and broader populations from different regions in Iraq. **Future Research:** In future study, it is important to include larger and more diverse samples. Further research is needed to comprehend the mechanisms underlying these interactions and the long-term health consequences. Other salivary biomarkers of oxidative and antioxidative should be considered.

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***Rajaa Abdulrazzaq Al Anbagi (Corresponding Author)**
Department of Medical Biotechnology, Al-Qasim Green University, Iraq
Email: ralanbagi@biotech.uoqasim.edu.iq

Baqir Habib Abis
Department of Medical Biotechnology, Al-Qasim Green University, Iraq

Zainab Hassan Abdullah
Department of Medical Biotechnology, Al-Qasim Green University, Iraq

Fakhir R. H. Alshuwaili

Department of Applied Biotechnology, Al-Qasim Green University, Iraq