Evaluation of Glutathione S-Transferase Activity in Venous and Finger-Prick Blood in Healthy Smoking and Non-Smoking Men in Kuwait

Abeer M Almutairi[1], Yazan Akkam[2], Mohammad F Alajmi[3]

[1] Department of sciences, faculty of fundamentals, The Public Authority for Applied Education and Training, Kuwait.
[2] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Yarmouk Universty, Irbid, Jordan
[3.] Department of Mathematics and Natural Sciences, College of Arts and Sciences, Gulf University for Science and Technology.

ABSTRACT

BACKGROUND: Smoking has long been hypothesized to be an inducer of oxidative stress; hence antioxidant enzymes such as glutathione S-transferases (GSTs) are an important defense line against prospective oxidative cell damage. OBJECTIVES: This work investigated whole blood GST activity with respect to tobacco smoking. Also, it evaluated the possibility of using capillary blood (finger-prick) as a route of sampling for GST activity in comparison to the conventional venous blood sampling. METHODS: Whole blood GST activities were measured from healthy volunteers, as well as from distinct categories of smokers classified according to type of tobacco and number of cigarettes. RESULTS: No statistically significant difference was detected in the GST level finger-prick vs. venous blood. The GST activity was 5.249 ± 0.2 U/g/Hb for control group, 9.6 ± 1.71 U/g/Hb for one pack smokers, 7.2 ± 1.6 U/g/Hb for more than one pack smokers, and 3.7 ± 0.24 U/g/Hb for Hookah smokers. CONCLUSIONS: This study provides evidence that finger brick sampling can replace venous blood for measuring GST activity. Also proofs that tobacco smoking (especially Hookah) has an extreme influence on GST activity, which may lead to neutralizing the GST mechanism, and subsequently rendering an insufficient self-defense against oxidative stress.

Keywords: Oxidative Stress, Glutathione S-transferases, Reduced Glutathione, 1-Chloro-2,4-Dinitrobenzene, Electrophilic, Antioxidant.

1. INTRODUCTION

Smoking can cause serious effects on almost every organ of the body; it increases the risk of lung cancer and other cancers, cardiovascular disease and chronic pulmonary disease[(1)]. Furthermore, WHO estimated approximately six million deaths every year worldwide caused by smoking[(2,3)].

The prevalence of smoking rates varies considerably from one region to another. In 2015, more than 1.1 billion people smoked tobacco worldwide. For instance, in Kuwait, the most recent results of the male smoker survey ion 2016 showed that the rates of cigarette smokers accounted for 31.8% and 35.4% of tobacco smokers[(4,5,6)].

Smoking has long been hypothesized to be an inducer of oxidative stress; hence antioxidant enzymes such as glutathione S-transferases (GSTs) are an important defense line against oxidative cell damage[(7)]. Glutathione S-transferases catalyzes the conjugation of the reduced thiol-containing tripeptide glutathione (GSH) with electrophilic substrates. Moreover, GSTs can prevent damage to important cellular components caused by reactive oxygen species[(8)]. Furthermore, GSTs can serve as a reliable defensive response biomarker that develops in an organism in the case of environmental/chemical pollutants[(9)].

Naturally, the blood cells and plasma contain large amounts of GSTs, therefore the sampling procedures for blood or plasma are exceptionally critical. The most

*.am.almutairi@paaet.edu.kw
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widely used methods are finger-prick and venous blood sampling, each method has its advantages and disadvantages. In general, finger-prick blood collection is much preferred by both patients and laboratory technicians because it is relatively painless, allows faster results and simple as the patients can be taught to do capillary blood collection at home. Finger-prick blood collection can be used to measure blood clotting, anemia, lipids, basic chemistry plates and many more[10, 11].

This study was conducted to assess the blood glutathione s-transferase activity as a biomarker in healthy smokers and non-smokers males in Kuwait, along with determining the ability of finger-prick technology to identify GSTs activity as an oxidative stress biological marker.

2. Patients, Materials and Methods

2.1. Study design and participants:

Samples from 85 healthy volunteers (males) living in two areas of Kuwait; Ali Sabah Al-Salem residential and Al-Qairawan, were collected. Females were excluded due to cultural and traditional barriers. All the volunteers were men and had been reached by passing through doors or located in cafe shops. Twenty-two healthy non-smoker volunteers were assigned as a "reference", while 63 healthy-smoker subjects were tagged as "tested". The volunteers were divided into four groups, as assigned in table (1).

Table (1)
Demographics of the volunteers

Group	# volunteers	Age (± standard deviation)
The non-smoker (reference)	22	33.4 ± 9.2
Smoke less than one pack (20 cigarettes in a pack)	21	33.1 ± 9.5
Smoke a full pack or more	21	32.2 ± 7.6
Smoke one pack with hookah (sometimes also called shisha).	21	37.6 ± 7.5

The data was acquired through a questionnaire enclosed questions about volunteer's medical history, and specific questions for smokers and non-smokers.

Volunteers with a history of uncontrolled systemic hypertension, hyperlipidemia, cardiac failure, hepatic failure, kidney diseases, diabetes, cerebrovascular diseases, peripheral vascular diseases, or coagulopathy were excluded.

Smoking volunteers confirmed the regularity of smoking, the number of cigarettes consumed per day, and the history of smoking. All smoker volunteers were regular smokers and have been practicing smoking (cigarette or hookah) for at least 5 years. This study defined hookah smoker as regular cigarette smoker who smoked hookah at least three times weekly besides smoking 1 pack of cigarettes.

The non-smokers were asked if they were previous smokers or they have been subjected to other people's smoke, and the exposure time was then estimated (hours/day). In this study, all previous smokers have been excluded.

All volunteers gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by Scientific Research Committee- Public Authority for Applied Education and Training- Kuwait.

2.2. Blood Samples: Blood from the tip of the left ring finger was collected using a blood sampling needle (Ruiqi Technology Ltd, Chengdu, Sichuan, China), and the

venous blood was collected from the left median cubital vein. For the finger- stick sampling, one skin puncture was made on the tip of the left ring finger using a blood sampling needle (Accu-Chek Safe-T-Pro Plus Lancets, Roche Diagnostics USA). The first and second drops of blood produced after gentle squeezing were discarded, and then an aliquot (250 µL) of blood was taken in a K_2 EDTA 0.5 ml micro-container tube, using an Innovac Quick-Draw device (Innovative Med Tech USA), the larger volume collected decreases finger-stick drop-to-drop variability. For the venous blood, 4 ml of blood was taken from each person using a vacuum tube with EDTA-K_2 (Ruiqi Technology Ltd) for anticoagulation. Aliquot of both blood routes were immediately frozen in dry ice and stored in a -80 C freezer.

2.3. GSTs Activity: The activity of GSTs in total blood was determined spectrophotometrically with the microplate reader (Bio-Tek ELx800, USA). Essentially as described in the literature[12]. To get total blood GST activity, RBCs should be lysis first which was achieved by mixing RBCs with distilled water. Briefly, 0.01 ml of blood was diluted in 50 volumes (0.5 ml) of ice-cold bi-distilled water, and then they were mixed using a vortex. After 2 min, 0.02 aliquots of hemolyzed samples were incubated with 0.5m M GSH and 1 mM of 1-chloro-2,4-dinitrobenzene in 0.197 ml of 98.5 mM potassium-phosphate buffer, pH 6.5. The enzymatic activity assay was performed (37 C) at 340 nm in a Greiner Bio-one -96-well plate (Catalog Number 655101), where the enzymatic product, the S-glutathionyl-2,4-dinitrobenzene, absorbs (ε_{340}nm = 5.3 mM^{-1}). Each spectrophotometric determination was subtracted by the spontaneous reaction of GSH with CDNB. The absorbance at 340 nm was measured after a 1-minute lag time, every minute for 10 minutes. Each assay was done in duplicate, and the results were repeated at least four times.

The concentration of hemoglobin was measured for each lysate sample using a Hemocue HB 201^+ device (HemoCue America, USA) to normalize the activity of the enzyme for inter-sample comparison. GST activity was expressed as enzyme units (U) per gram of hemoglobin (Hb)[15]. One unit represents the amount of enzyme that catalyzes the conjugation of 1 µmol of GSH to CDNB in 1 min at 25ºC. Importantly, the activity of e-GST is linearly related to its expression[16]. Thus, the increase of e-GST activity can be considered as equivalent to the hyper-expression of e-GST.

3. Statistical analysis

Data are expressed as mean ± SD. Comparison between venous and finger-prick blood was made using unpaired *t*-test; GSTs activity between two types of blood samplings was performed using one-way ANOVA (analysis of variance). The 95% confidence intervals (CIs) were calculated. Bland- Altman plots were constructed to determine the agreement between the two methods of blood testing. Differences were considered significant at $p < 0.05$.

4. Results

GST activity: The linearity of GST assay over a 10 min period was confirmed for each sample using the coefficient of determination (R^2) as seen in the example (Figure-1). Any results with R^2 less than 0.95 was rejected. The GST assay was

Repeated for each sample 3 times. Numerically, the R^2 values were 0.998, 0.998, 0.999, 0.987, for non-smokers, smokers (<20 cigarettes), smokers (>20 cigarettes), and hookah smoker, respectively.

4.1. Comparison between Venous and Finger-Prick Blood in control group

To investigate the possibility of using finger-prick blood samples for GST activity determination, samples from the control groups (non-smokers) were used. The activity of GST from venous samples was compared to figure-prick samples. The mean age of the 22 participants with analyzable samples was 33.4 years (range 19 to 54).

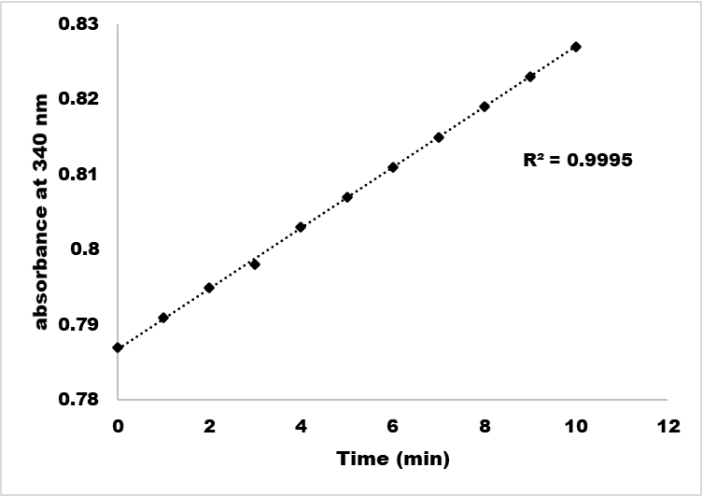

Figure (1): Linear regression for GST assay. The coefficient of determination (R^2) was measured for each GST assay. The graph represents an example from non-smokers group

As shown in Figure (2) (A), the mean activity of GST obtained from finger-prick blood and venous blood were 5.249 ± 0.2334 U/g/Hb and 5.235 ± 0.2262 U/g/Hb respectively. There were no statistically significant differences in the two blood collection methods and the 95% confidence interval (CI) was -0.6704 to 0.6415.

To determine whether a different statistical conclusion would be reached, the GSTs levels were compared between the two types of blood samplings based on one-way ANOVA. The difference between two means was (-0.01445 ± 0.3250; $p< 0.05$).

Moreover, Bland-Altman plot was further used to emphasize the similarity, where the differences between the two techniques were plotted against the averages of the two techniques. As demonstrated in Figure 2(B), all 22 values were within the limit of agreement of 95% (± 2SDs). Likewise, a good agreement in the Bland-Altman plot between fingertips and venous blood testing was instituted. There was a good correlation between the two methods of assessment for GSTs activity ($r = 4.705e^{-005}$, $p< 0.0001$).

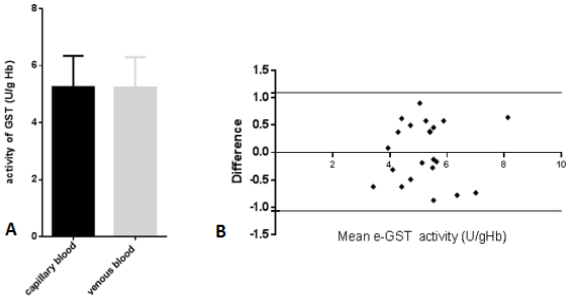

Figure (2-A): Comparison between GSTs Level in Venous and Finger-Prick Blood.B) Bland-Altman plot for comparing finger-prick and venous blood measures for e-GST (n=22)

4.2. Comparison between Venous and Finger-Prick Blood among All Groups

A comparison of GSTs finger-prick and GSTs venous blood was evaluated between all tested samples using the Bland-Altman plot to stress the similarity. Figure 3 shows the difference against the average results between GSTs in a finger-prick blood sample and venous blood sample. Bland-Altman plot shows a tendency toward decreasing scatters with increasing the number of smoked cigarettes. When the scores were standardized for the Bland-Altman plot most scores fell within ± 2SDs, with only two outliers for GSTs one above the + 2SDs and the other sit on the -2SD line. A good correlation between the two methods of assessment for GSTs activity (r = 0.938, $p< 0.0001$) was inaugurated. Similarly, unpaired *t*-test shows no significant difference in means between two measures ($p< 0.05$, $p = 0.9648$).

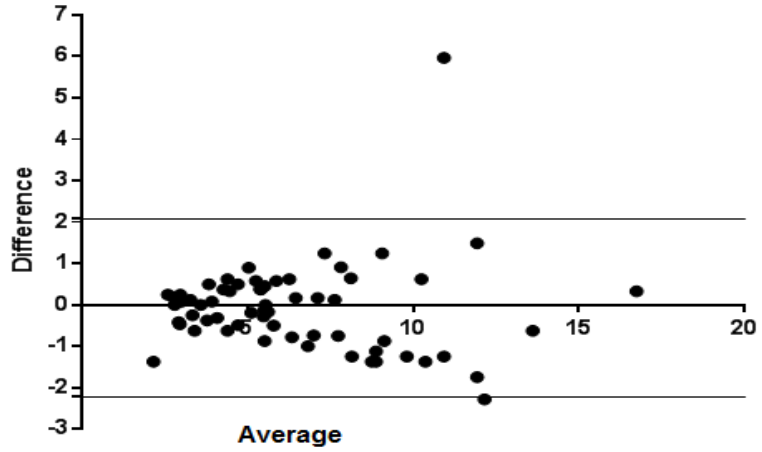

Figure (3): Bland and Altman plot for comparing finger-prick and venous measures for GSTs (n=85)

4.3. Comparison between Groups in GST Activity

The effect of smoking on GST activity was determined in term of whole blood GST. As shown in Figure (4), GST activity was elevated in the one pack smokers group reaching 9.6 ± 1.71 U/g/Hb. The GSTs activity was then decreased gradually upon increasing the number of cigarettes reaching 7.2 ± 1.6 U/g/Hb (Fig.4). Further reduction in the GST activity reaching 3.7 ± 0.24 U/g/Hb was noticed after consuming tobacco as Hookah. GSTs activities were expressed as means ± SE; $p < 0.0001$ according to *one-way ANOVA* comparing values obtained from studied groups.

5. Discussion

Pollution has been associated with oxidative stress, consequently assigned as initiator of several human's chronic disease[13]. Oxidative damage caused by several types of reactive oxygen species is normally minimized by a combination of biological antioxidant systems including enzymatic and non-enzymatic reactions[14]. Increased exposure to pollutants has created a critical need for biological markers to detect oxidative stress. Smoking-as pollutant- may enhance oxidative stress not only through the production of reactive oxygen species, but also through weakening of the antioxidant defense mechanisms[15,16]. It has been argued that the increased production of reactive oxygen species associated with smoking may exceed the

capacity of the oxidant defense system[16].

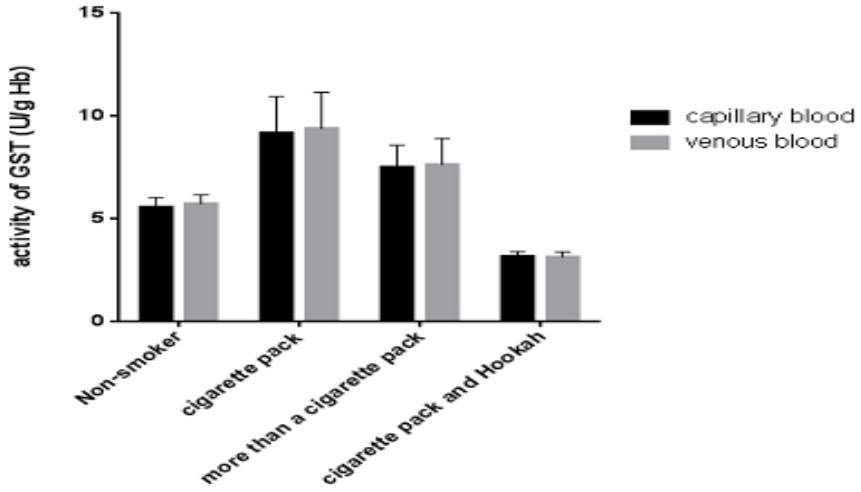

Figure (4): GSTs activity changes in finger-prick and venous blood between studied groups. Bars represent the standard errors of the means and n=85; p < 0.01

Glutathione S transferase (GST) main function is conjugating reactive oxygen species (ROS) with glutathione to produce less reactive water-soluble compounds that can be more easily excreted[17].

Among GSTs classes, blood GSTs have been the main emphasis because it is thought to be subject to a high internal rate of ROS produced from Hb auto-oxidation[18,19]. Blood is the first compartment to be exposed to oxidative stress, including red blood cells. Furthermore, erythrocytes are subjected to high risks of oxidative damages due to the exposure of high oxygen tension, and lacking damage repair mechanisms[20,21]. Consequently, erythrocytes are completely relying on the antioxidant defensive components throughout their life span[22]. So, the effect of pollution on induction of oxidative stress may be monitored or measured via studying the blood GST which includes serum GST and erythrocyte GST.

To measure the activity of blood GST, two technical aspects of sampling have been tested; finger-prick and venous blood. Vein carries deoxygenated blood, while finger-prick is a mixture of deoxygenated and oxygenated blood. Moreover, finger-prick is likely to be contaminated with interstitial fluid during the blood sampling but has a better patient compliance. The disadvantages of venous sampling can be summarized by the following: it requires a rained phlebotomist or nurse, it can cause significant discomfort to the patient, and it is difficult to obtain blood from the elderly, infants and young children, or very sick patients[23]. Differences in biochemical indicators and hematological parameters between the methods have been documented[24].

In term of studying blood GST activities, the result suggests the possibility of using figure-prick sampling as *t*-test and one-way ANOVA showed there was no statistical differences between the mean of the two methods among all tested subjects with $p<0.0001$ (Figure 2). Furthermore, Bland-Altman analysis also confirmed that there was no overall bias between GST activity using finger-prick and venous blood (Figure-3).

Smoking tobacco-in general- can cause a variety of harmful complications in the body and may lead to death,

along with psychological, physical and economic damage[25]. Tobacco may entitle under different names and forms, and the most common are cigarette, cigar, pipe, and hookah. Despite the title variation, all tobacco forms carry the same health risks[26].

Substantial evidence showing that free radicals and peroxides produced via smoking enhance oxidative stress. Most studies showed an immediate increase in oxidative stress after smoke inhalation[27].

Tobacco smoke contains about 4700 hazardous chemical compounds, including a high concentration of free radicals and other oxidants[28] such as H_2O_2 and HOCl. Thereby, these compounds enhance the development of oxidative damage, consequently the body needs higher activity of antioxidant mechanisms including GST activity[29], and that is what has been observed (Figure 4). After smoking 1 pack of cigarettes, a one-fold increase in the GST activity was observed in comparison to the non-smokers. This finding is expected as tobacco smoking may cause imbalance between production and detoxification of ROS.

With increasing the number of cigarettes (more than 1 pack); the GST activity was still higher than the control group, but surprisingly less than the activity observed in 1 pack. This might be explained by the fact that heavy cigarettes smokers are exposed to higher concentrations of oxidizing toxins to a limit beyond the capacity of the oxidant defense system. Hence, oxidative damage may encounter proteins, lipids and DNA, and subsequently altere the expression of some xenobiotic metabolizing enzymes and antioxidant proteins[31]. This finding is suited well with what has been published[16].

Hookah (also referred to as Shisha, waterpipe, narghile or hubble-bubble) is a popular, and a traditional method of smoking in the Middle East. The composition of tobacco used in hookah smoking is variable and not well standardized as in cigarettes. The contents of nicotine have been reported for hookah almost double the amount for cigarettes. The concentrations of carbon monoxide in the hookah smoke were much higher than cigarette smoke[32,33]. Hookah's smoke contained 2.94 mg nicotine, 802 mg tar, 145 mg CO and relative to a smoke of single cigarette, greater quantities of chrysene, phenanthrene and flouranthrene[34]. Several researches have dissected the effect of hookah on oxidative stress in general. However, the majority of the research have been conducted on either mice or rat models, not human[35-39].

In the final group, the group who smoked hookah along with one and more pack of cigarettes, an extreme deficiency in GST activity was observed, 2 folds less than the normal. The lowest GSTs level in smokers with hookah can be illustrated by the fact that the coal used to heat tobacco in hookah can raise health risks by producing high levels of carbon monoxide, toxic metals and carcinogenic chemical. The injury caused by hookah may exceed the capacity of the oxidant defense system, resulting in oxidative damage to proteins, lipids and DNA, subsequently change the expression of antioxidant enzyme[40].

Two major mechanisms may have played a role in the depletion of the GSTs activity. The first mechanism is due to higher exposure to free radicals and H_2O_2 imposed by hookah caused consumption of antioxidant during the breakdown of these radicals[41], while the second involves the direct inhibition of GSH synthesis and activities of Superoxide dismutase (SOD) and GST[42,43]. Subsequently, the hookah smoker individuals suffered from a lack of cellular protection provided by the GSTs[44]. Corresponding our study, it has been reported that adult smokers have significantly lower erythrocyte Se-dependent glutathione peroxidase (SE-GSH-PX) activities than do non-smokers, and the suggested explanation was increased oxidative stress[45].

There is a perception that hookah is less harmful than smoking cigarettes. Hookah smokers believe that the nicotine content in hookah tobacco is lower than in cigarettes and that the smoke inhaled is believed to be more sedative and less harmful to the throat and respiratory

tract than cigarette smoking[45,46]. It has been published that water in the hookah may only filter a small fraction of harmful substances. Hookah smoker may absorb higher concentrations of nicotine and heavy metals due to high concentrations in the smoke itself or because of the mode of smoking, including frequency of puffing, inhalation depth and length of the smoking session[47]. A review on the toxicological of hookah has been published[40].

In Kuwait, the prevalence of cigarette smoking among students was 46%; 10% were hookah smokers, and 13% were cigarette smokers[48,49]. Due to the high prevalence-which is higher than Global prevalence (33.7%-2016), the Ministry of Health in Kuwait has launched a national program to combat smoking in public places.

In summary, whole blood GSTs activity was investigated among men who have a habit of smoking cigarettes and hookah. The normal blood GSTs activity in control group ranged from 43.7- 53.66 µmol/min/g Hb. The highest GSTs activity was found in the one pack smokers, while the GSTs activity was 2-folds less than the normal limit in hookah smokers. Although mild (one pack smokers) and moderate smokers (more than one pack smokers) did not differ significantly in GSTs activity, the correlation between GSTs activity level and the number of smoked cigarettes has been noted.

6. Conclusions

This study has shown no statistically relevant differences between whole-blood GST activity in all volunteers obtained from venous or finger-prick blood samples. Thus, finger-prick blood sampling can be considered as a reliable alternative method for evaluating the blood GSTs level and considered a useful practical field method. Hence, better patient compliance with significant comfort will be achieved with no need for trained phlebotomist.

The results shows that smoking a certain amount of tobacco (one-pack) increases whole blood GST activity to a limit, after which any additional types of tobacco (esp. Hookah) will reduce the GST activity significantly. Moreover, the results also indicate that smoking hookah reduces the effectiveness of self-defense mechanisms against the oxidizing agents via increasing the oxidative damage, subsequently decreasing in GSTs activity.

Recommendations

Smoking is harmful in all its forms, it can increase the risk of a variety of problems over several years as well as long-term effects on body systems. Smoking-mediated low GSTs level is a major cause of atherosclerosis, childhood asthma, lung cancer, chronic obstructive pulmonary disease and other more[40,42,50,51,52]. Despite substantial knowledge about the health consequences of Hookah and cigarette smoking, in Kuwait, the rate of smoking is still high. Many of the smoking effects can be reversed by quitting smoking. Therefore, it is the responsibility of educators, health planners and social scientists who should work together to initiate community interventions to reduce the prevalence of smoking in this country.

A more comprehensive study should be conducted to investigate whether there are other factors behind the decrease in GSTs activity, and more oxidative markers should be taken into consideration. Moreover, in future studies, finger-prick blood sampling should replace vein blood sampling for more volunteer's convenience and collaboration.

Study limitations

The limitations of this study are mainly due to samples collection. Kuwait has a conservative culture and -just like any other Middle Eastern country– is controlled by customs and traditions. Smoking habit for females is inadmissible in Kuwaiti culture and considered a sin. Therefore, no female will confess about smoking voluntarily. Subsequently, males were the primary subjects.

Despite the presence of a professional nurse, the fear of

getting an infection from an unsanitary needle refrained the participation. Moreover, the concept of volunteering is extremely unpopular in the community, especially (vein sampling). Also, the people are not fully aware of the importance of such research. Therefore, it took long time to collect the samples, as the team tried reaching the volunteers in their houses.

Also, blood antioxidant activity is not limited to GST, there are several other enzymes work as antioxidant. Unfortunately, GST assay sample preparation and limited sample volume confined proceeding to other markers.

Conflicts of Interest: We declare no conflict of interest. The funding agency had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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تقييم نشاط أنزيم الجلوتاثيون أس- ترانسفيريز في الدم الوريدي والوخز الاصطناعي لدى المدخنين الأصحاء وغير المدخنين في الكويت

عبير المطيري[١]، يزن العكام[٢]، محمد العجمي[٣]

[١] قسم العلوم، كلية التربية الأساسية، الهيئة العامة للتعليم التطبيقي والتدريب- الكويت.

[2] قسم العلوم الصيدلانية، كلية الصيدلة، جامعة اليرموك- اربد- الاردن.

[٣] قسم الرياضيات والعلوم الطبيعيه، كلية الآداب والعلوم، جامعة الخليج للعلوم والتكنولوجيا- الكويت.

ملخص

منذ فترة طويلة تم وضع فرضية أن التدخين محفز للأكسدة داخل جسم الإنسان. لذلك فإن الإنزيمات المضادة للأكسدة مثل الجلوتاثيون أس- ترانسفيريز خط دفاعي مهم ضد تلف الخلايا المحتمل من جراء التعرض لهذه الأنواع من المواد المؤكسدة.

الأهداف: يهدف هذا البحث الي دراسة نشاط الجلوتاثيون أس- ترانسفيريز الكلي في الدم وعلاقته بتدخين التبغ. كما تم تقييم إمكانية استخدام الدم الشعري (وخز الإصبع) لدراسة نشاط الانزيم كبديل لأخذ العينات عن طريق الدم الوريدي التقليدي.

الطريقة: تم قياس نشاط الأنزيم الكلي في الدم من متطوعين أصحاء، وكذلك من فئات مختلفة من المدخنين مصنفة حسب نوع التبغ وعدد السجائر.

النتائج: لايوجد أي فروق ذات دلالة إحصائية لنشاط الانزيم المأخوذ من وخز الإصبع مقابل الدم الوريدي. كما أن نشاط الأنزيم كان ٥,٢٤٩ ± ٠,٢ U / g / Hb للمجموعة الضابطة، ٩,٦ ± ١,٧١ U / g / Hb لمدخني علبة سجائر واحده يوميا، ٧,٢ ± ١,٦ U / g / Hb لمدخني اكثر من علبة سجائر يوميا، و ٣,٧ ± ٠,٢٤ U / g / Hb لمدخني النرجيلة.

الاستنتاجات: توفر هذه الدراسة أدلة على أن أخذ عينات الدم بواسطة وخز الإصبع يمكن أن تحل محل الدم الوريدي لقياس نشاط أنزيم الجلوتاثيون أس- ترانسفيريز. كما تثبت هذه الدراسه أن تدخين التبغ (وخاصة النرجيلة) له تأثير بالغ على نشاط الأنزيم، مما قد يؤدي إلى تحييد آلية عمل الأنزيم، وبالتالي يضعف دفاعات جسم الإنسان ضد الإجهاد التأكسدي.

الكلمات الدالة: الأكسدة، الجلوتاثيون أس- ترانسفيريز، اختزال الجلوتاثيون، ١-كلورو-٢،٤-ثنائي نتروبنزين، دم من وخز الإصبع، مضادات الأكسدة، التدخين.

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