A Biochemical Study of α-Amylase Activity in Saliva of Some Libyan Cigarette Smokers

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Abstract: Tobacco consumption alters many biological parameters, including α-amylase activity. This study reports the effect of cigarette smoking on salivary α-amylase activities of some male heavy smokers (100) in apparent good health compared with (40) male non-smokers as control. The salivary α-amylase activity was assayed using the standard colorimetric method. The α-amylase enzyme was characterized from the saliva samples collected from healthy adult smokers and non-smokers (control) to determine the effects of temperature, pH, and substrate concentration on the kinetic parameters of the enzyme. The analysis of the saliva samples showed that there were significant increases (P<0.05) in the α-amylase activity values in the saliva of cigarette smokers when compared with non-smokers at optimum conditions (2.74±0.37 nmol/min/mg-protein and 2.01±0.16 nmol/min/mg-protein, respectively). The Michaelis constant (K_m) values of 0.5±0.02 mM and 1.12±0.08 mM observed for non-smokers and smokers were obtained from the double reciprocal plot of initial velocity (*1/V*) and substrate concentration (*1/[S]*). The maximum activities obtained at an optimum temperature of 37ºC, and an apparent pH of 7.0 for both subjects were 31.25±8.24 nmol/min/mg protein and 18.10±4.06 nmol/min/mg protein for non-smokers and smokers respectively. Measurements of salivary α-amylase activity could be useful in the early detection and diagnosis of parotitis. Subsequent studies are required to specify the mechanisms responsible for such a risk.

Keywords: Cigarette Smokers; Salivary α-Amylase; Kinetic Parameters; Optimum Conditions.

INTRODUCTION

Tobacco dependence is still the leading cause of avoidable mortality in the world (WHO, 2017; Da Costa & Sylva, 2005). The World Health Organization estimated in 2017 that tobacco use killed 5 million people at a global level, half of which were in developing countries. According to forecasts for 2025- 2030, the death toll is expected to rise to 10 million people, including 7 million in developing countries (WHO, 2017). The works of many chemists and biologists over the past 50 years have shown the harmful effects of numerous tobacco components (Mohammed et al., 2016). Nico-

tine addicts in Libya get their daily dose of this toxicant by different smoking habits, including smoking cigarettes, water pipes (narghile, hookah, shisha), and inhaling snuff, known locally as naffa. In addition to nicotine, it has been reported that tobacco products contain other toxic substances, including some heavy metals (Abd El-Samad & Hanafi, 2017; Caruso et al, 2014). Cigarette smoke (CS) contains over 4000 different chemicals, 400 of which are proven carcinogens. These carcinogens include aromatic amines, nitrosamines, oxidants such as oxygen free radicals, and also high concentrations of toxic volatile aldehydes, such as acrolein, crotonaldehyde (α,β-unsaturated

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aldehydes), and acetaldehyde (saturated aldehyde), all of which presumably are major causes of damage to various molecules exposed to CS (Nagler et al., 2000). Saliva is an exceptional fluid, as it is the first biological fluid to encounter the inhaled CS gaseous and water-soluble extracts. Salivary biomarkers have gained popularity in stress research, as it has proven superior to testing biomarkers in blood. (Yi & Moochhala, 2013). This is based on the fact that saliva samples have many advantages such as wide range of constituents, being non-invasive and easy to perform, and do not need trained staff. It also does not cause additional stress in sample collection unlike blood or urine collection (Heberd et al., 2015).

Alpha-amylase (EC 3.2.1.1) in animals is a major digestive enzyme. It is secreted by the salivary gland and pancreas, and therefore, present in saliva and serum. It hydrolyzes the α-1, 4 linkages of starch to glucose and maltose (Gomina et al., 2013). The noxious effects of tobacco dependence on human health are known. In fact, tobacco consumption is the cause of cardiovascular pathologies, pleuro-pneumonias, and cancers (Callegari & Lami, 1984). In addition, tobacco consumption modifies several biological parameters, including α-amylase activity (Weiner et al., 2009).

Previous studies carried on tobacco dependence's impact on the activity of salivary alpha-amylase resulted in divergent viewpoints. For Nater et al, (2007) smoking tobacco increases the value of alpha-amylase activity in saliva. On the contrary, for other authors, smoking tobacco does not affect the value of serum and salivary alpha-amylase activity (Nagaya & Okuno, 1993; Kivela et al., 1997; Aysun et al., 2009). Nevertheless, investigations on the influence of smokeless tobacco consumption on alpha-amylase activity are very few. Likewise, reports on alpha-amylase activities among cigarette smokers have remained scarce in the Libyan society. The present study, therefore, attempts to report the activities of α–amylase in the saliva of some Libyan cigarette smokers.

MATERIALS AND METHODS

All chemicals used throughout this study were obtained from Sigma-Aldrich. Absorbance measurements were made with Beckman Coulter Model DU 800spectrophotometer. One hundred male cigarette smokers in apparent good health were chosen as test subjects from the smoking population and were randomly selected from Al-Bayda city, Libya. The volunteers comprise 100 heavy smokers (>11 sticks/day) (Enemchukwu et al., 2011). Also, 40 individuals who do not smoke or drink alcohol were included as the control subjects.

Sample collection: Saliva samples were collected from the volunteers (100 smokers and 40 non-smokers) in apparent good health. For the collection, which was performed at least 1 h after eating; volunteers were asked to generate saliva in their mouths and to spit it into a wide test tube for 10-15 min. Following collection, saliva was immediately centrifuged (1000xg for 3 min) to remove squamous cells and cell debris. The fresh supernatant was then transferred to the plastic containers and stored at 4°C until analysis. The cigarettes used in this study were popular commercial cigarettes (Milano, Oris, and Super Grand) containing 5, 3, and 1mg of tar and 0.5, 0.3, and 0.1mg of nicotine per cigarette, respectively. About 1000 μl of saliva was collected from each subject using specimen sample bottles and diluted to 1:100 with physiological saline (0.85% NaCl) using the technique proposed by Enemchukwu et al., (2011).

Measurement of salivary α-amylase activity: The activity of the enzyme was determined using starch as substrate. An appropriate amount (100μl) of the enzyme solution (diluted saliva) was incubated with 800 μl of a substrate (starch) in an incubator at 37°C for 30 min. The reducing sugars resulting from the starch hydrolysis were assayed by the 3, 5-dinitrosalicylic acid (DNS) method (Enemchukwu et al., 2011). The aldehyde group of reducing sugars converts 3, 5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid, which is

the reduced form of DNS. The formation of 3-amino-5-nitrosalicylic acid results in a change in the amount of light absorbed at 540 nm. The absorbance measured using a spectrophotometer is directly proportional to the amount of reducing sugar (Miller, 1959). The α-amylase activity in each sample was determined by using the following formula:

α-amylase activity in each assay tube =

$$\frac{\frac{\Delta ABS}{30.0} min * Final\ volume}{1.0 cm}$$

Where, ΔABS = absorbance change

Protein concentration was estimated according to the method described by Bradford depending on the bovine serum albumin standard curve (Bradford, 1976). The specific activity was determined by using the following equation:

$$Specific\ activity\ (U/mg) = \frac{Enzyme\ activity\ \frac{U}{ml}}{Protein\ concentration\ \frac{mg}{ml}}$$

Effect of pH and temperature on salivary α-amylase activity: In order to determine the effects of temperature (25-60°C) and pH 3-13 (50 mM acetate buffer and 50 mM phosphate buffer) at 37°C on salivary α-amylase activity among cigarette smokers as well as non-smokers, routine analysis of the saliva samples was carried out with the buffered starch substrate using the enzymatic method for the α-amylase assay for human biological fluids (Enemchukwu et al., 2013). The salivary α-amylase was assayed by the 3, 5-dinitrosalicylic acid method (Enemchukwu et al., 2011).

Determination of kinetic parameters for salivary α-amylase: The kinetic parameters [Michaelis-Menton constant (K_m) and maximum velocity (V_{max})] of invertase activity of α-amylase activity were obtained individually from Lineweaver Burk (LB) plot following the determination of salivary α-amylase activity at the optimal assay conditions 37°C, pH 7.0 at 30 min in the presence of various starch concentrations: 0.25, 0.5, 1, 2, 5, 10 and 20 mM (Hadrich *et al.*, 2015). The intercept of the LB plot on the y-axis corresponds to *1/V$_{max}$*, while the slope corresponds to K_m/V_{max} (Hadrich et al., 2015).

Statistical analysis: Results were expressed as means ± standard division of the mean (n = 6). Statistical significance was set at P<0.05 by using a t-test. Microsoft Excel has calculated kinetic parameters for α-amylase activity.

RESULTS

The results presented in Table 1 are the demographic data that were observed in both groups. The data indicated the selectivity in studied groups that were included in the present study.

Table (1). Demographic data of the two studied groups (Mean ± SD)

Characteristic	Smokers [n=100]	Non – smokers [n=40]
Gender	Male	Male
Age (year)	47.70 ± 6.12	43.25 ± 4.19
Method of Smoking	Cigarette	-
Type of cigarettes	Milano, Oris and Super grand containing 5, 3 and 1mg of tar and 0.5, 0.3 and 0.1 mg of nicotine per cigarette, respectively.	-
Period of Smoking (year)	13.48 ± 3.10	-
Number of smoking (times per day)	(>11 sticks/day)	-
Workplaces	Non polluted	Non polluted
Medical History	-	-

Present data showed that there were significant increases (P<0.05) in the α-amylase activity values in the saliva of cigarette smokers when compared with non-smokers at optimum conditions (2.74±0.37 nmol/min/mg-protein and 2.01±0.16 nmol/min/mg-protein, respectively). In the present work, the effects of pH on salivary α-amylase activity were assessed for cigarette smokers as well as non-smoker subjects (control). The effect of pH on the activity of

salivary α-amylase was determined in the pH range of 3.0-13.0 for both subjects. The assay result for optimum pH determination for smokers and non-smokers showed an apparent pH optimum of 7.0 for both subjects corresponding to specific activities of 2.54 nmol/min/mg-protein and 2.01 nmol/min/mg-protein respectively, as could be observed from the graphs (Figure 1 and Figure 2). The specific activities of salivary α-amylase increased to 89.39% from pH 6.0 to pH 7.0 for smokers and 73.45% from pH 6.0 to pH 7.0 for non-smokers.
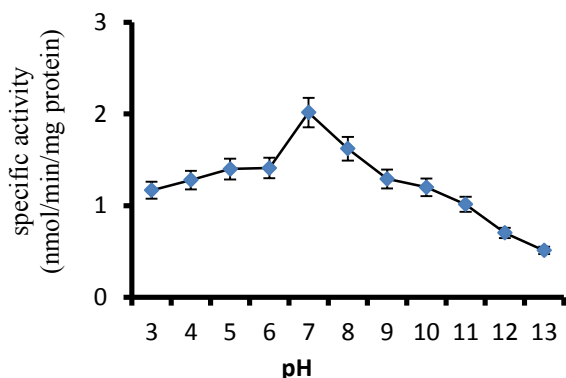

Figure (1). Effect of pH on salivary α-amylase of some Libyan cigarette smokers
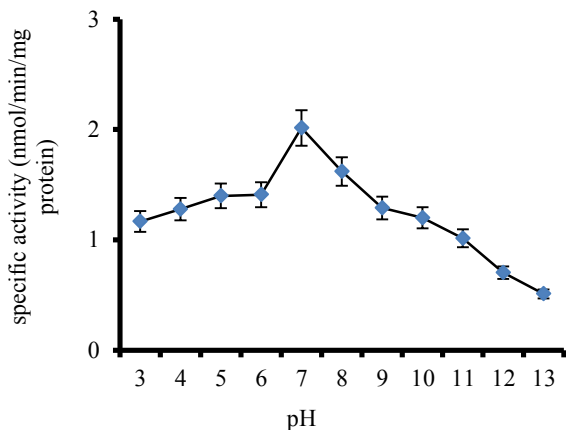

Figure (2). Effect of pH on salivary α-amylase of some Libyan cigarette non-smokers

The result from the analysis of the temperature effects on salivary α-amylase activity showed that the enzyme had maximum specific activities of 2.39 and 2.11 nmol/min/mg protein at 37ºC for smokers and non-smokers respectively as shown in Figure 3 and Figure 4.
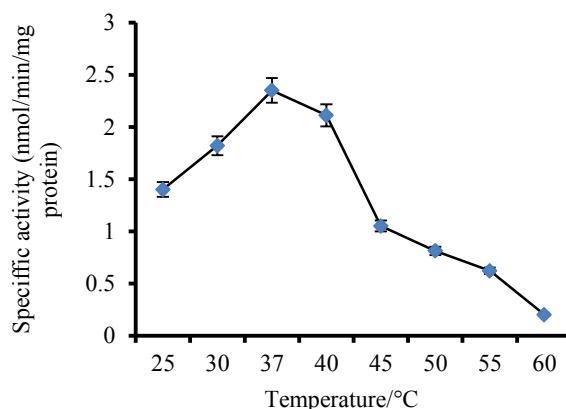

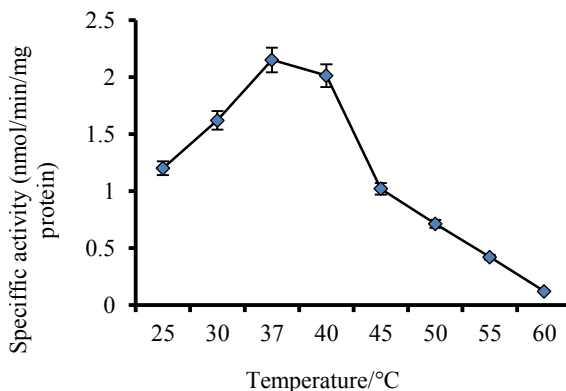**Figure (3).** Effect of temperature on salivary α-amylase of some Libyan cigarette non-smokers

Figure (4). Effect of temperature on salivary α-amylase of some Libyan cigarette smokers

The kinetic constants (V_{max}, K_m, $Kcat$, Ks (V_{max}/K_m) and $Kcat/K_m$) for salivary α-amylase from non-smokers and smokers were determined by incubating a fixed amount of enzyme with varied concentrations of soluble starch as a substrate (0.25 to 20 mM). The enzyme followed the Michaelis-Menten kinetics of catalysis. The K_m and V_{max} values of different enzymes are difficult to compare as they depend on the substrate used and the reaction conditions.

In the present research, the reciprocal velocity ($1/V$) at optimum temperature 37ºC was plotted

against reciprocal substrate concentration ($1/[S]$) in Figures 5 and 6, for non-smokers and smokers subjects respectively. Extrapolation of the line gave the K_m values for non-smokers and smokers, which are found to be 0.50±0.02 mM and 1.12 ± 0.08 mM respectively. The enzyme turn-over number for non-smokers and smokers (*Kcat*) and specificity constant (*Kcat/K_m*) for salivary α-amylase were determined (Table 2).

Table (2). The catalytic properties of salivary α-amylase from cigarette smokers and non-smokers

Parameters	Non-smokers	Smokers
K_m (mM)	0.5 ± 0.02	1.12 ± 0.08
V_{max} (nmol/min/mg protein)	31.25 ± 8.24	18.10±4.06
[a]*Kcat* (min^{-1})	2.66 ± 0.07	1.80 ± 0.05
Kcat/K_m (mM^{-1} min^{-1})	5.33 ± 1.03	1.61 ± 0.04
Ks (V_{max}/K_m) (ml/min/mg protein)	62.50± 15.21	16.50±6.41

The substrate efficiency is denoted by $Ks = V_{max}/K_m$; the turnover number (*Kcat*) = $V_{max}/[e]$, where [e] = Molar concentration of enzyme; [a]calculated estimating a molecular mass of 62 kDa for the active enzyme; K_m and V_{max} are the kinetic constants, while *Kcat/K_m* is the specificity constant.
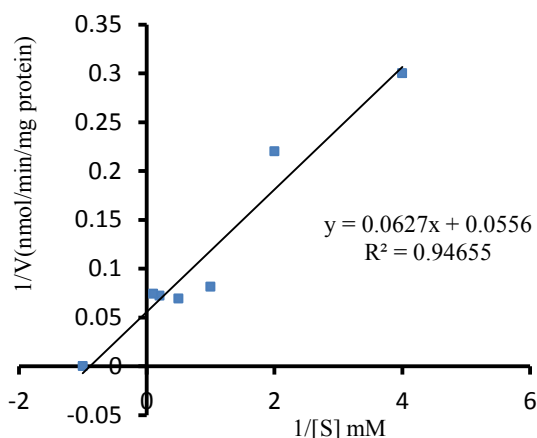
Figure (5). Lineweaver-Burk plot for determination of K_m and V_{max} of salivary α-amylase for cigarettes smokers. (Lineweaver Burk plot; pH 7, 37°C, substrate concentration ranging from 0.25-5 mM; Mean and standard deviation were determined from three replicates). The intercept on the y-axis corresponding to $1/V_{max}$ = 0.0556, slope corresponding to K_m/V_{max} = 0.0627
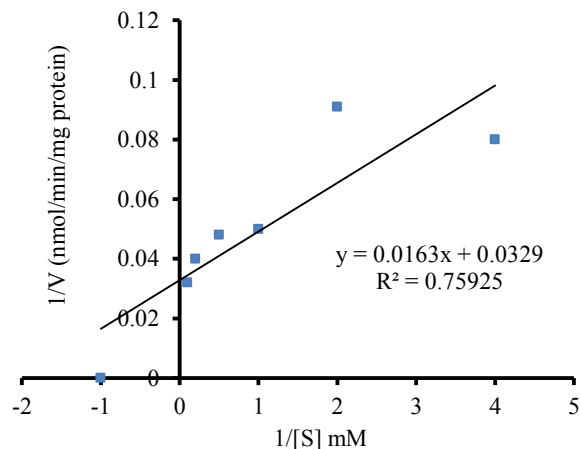
Figure (6). Lineweaver Burk plot for determination of K_m and V_{max} of salivary α-amylase for non-smokers. (Lineweaver Burk plot; pH 7, 37°C, substrate concentration ranging from 0.25-5 mM; Mean and standard deviation were determined from three replicates). The intercept on the y-axis corresponding to $1/V_{max}$ = 0.0329, slope corresponding to K_m/V_{max} = 0.0163

DISCUSSION

Many studies reveal that cigarette smoking causes an increase in saliva α-amylase activity values (Nater et al., 2007; Onyesom et al., 2012). These demonstrated increases have been contradicted by reports of other researchers. Some researchers report that smoking cigarettes do not affect the α–amylase activity value in saliva. Nagaya and Okuno, (1993) studied the effects of smoking habits on salivary amylase and reported that cigarette smoking does not have any significant influence on salivary amylase activity in the subjects. Also, Kivela et al. (1997) stated that the degree of amylase activity in the saliva of people who smoke cigarettes was not significantly different from that present in the saliva of non-smokers.

Several studies suggest that nicotine (which is a major component of cigarettes) accounts for the increase in α-amylase activity in people who smoke cigarettes. Maier et al. (1991) report that after the intravenous infusion of nicotine into some volunteers, an increase in salivary amylase activity was observed. Acute administration of nicotine to non-smokers was as-

sociated with increased salivary amylase activity (Maier et al., 1991). Thus, the increase in salivary amylase could be a result of decreased metabolic clearance of amylase, pancreatitis, or parotitis (Onyesom et al., 2012). Parotitis is a salivary disease that is associated with an increase in S-type isoamylase. Parotitis is usually caused by trauma, stress, or surgery to the salivary gland, radiation to the neck area involving the parotid gland, and subsequently causing duct obstruction, or calculi of the salivary duct. Evidence shows a measure of asymptomatic pancreatic, salivary and parotid glands' dysfunction among cigarette smokers.

Determination of salivary α-amylase activity could be valuable in detecting parotid/salivary gland diseases at an early stage. Overall, cigarette smokers have a considerable risk of developing such diseases as judged by their significant increases in salivary α-amylase activity values (Onyesom et al., 2012). And from the specific activities profile, activities increased to 89.39% from pH 6.0 to pH 7.0 for smokers and 73.45% from pH 6.0 to pH 7.0 for non-smokers, which is in agreement with the observation of Enemchukwu et al. (2013) who recorded increased activities of 82.1% from pH 6.0 to pH 7.0 for smokers and 76.9% from pH 6.0 to pH 7.0 for non-smokers salivary α-amylase. In the present study, α-amylase from human salivary showed the optimum pH of 7.0, which was corresponding to the other previous reports (Levitzki & Steer, 1974; Yoon & Robyt, 2003). A change in pH affects the ionization of essential active site amino acid residues that are involved in substrate binding and catalysis. The ionization of these residues may distort the active site cleft and hence may indirectly affect enzyme activity (Bodade et al., 2010).

The temperature optimum of 37ºC for α-amylase is in agreement with the report of Rudeekulthamrong and Kaulpiboon, (2012), and a little different from the optimum temperature of 40ºC obtained by Enemchukwu et al. (2013) and Tahtah & Otitoju, (2015) on a research work carried out on salivary α-amylase. The

difference may be due to the difference in population as well as the physiological conditions and other parameters under which the research was carried out. The Michaelis constant (K_m) values for cigarette non-smokers and smokers in this study were found to be 0.50±0.02 mM and 1.12 ± 0.08 mM respectively. The K_m value of the enzyme of non-smoker subjects is lower than that of the values of smoker subjects. Low values of K_m indicate a high affinity of the enzyme for the substrate (Zappacosta et al., 2002). The increase in the K_m value of the smokers' group might be either due to structural changes in the enzyme induced by CS, which could explain partially some of the protein modification caused by CS, or due to lower accessibility of the substrate to the active site of the salivary α-amylase (Kennedy et al., 1989 & Zappacosta et al., 2002).

The V_{max} of salivary α-amylase for non-smokers is 31.25±8.24 nmol/min/mg protein and for smokers 18.10±4.04 nmol/min/mg protein (Figures 5 and 6). The increase of the Km and decrease of the V_{max} value of subjects who smoke cigarettes is as expected because of less availability of the substrate to the active site of the enzyme due to chemical bonding, diffusion limitation, and confinement of enzyme molecules with polymeric support. On the other hand, results of apparent K_m and V_{max} values were reported from work carried out by (Enemchukwu et al. 2013) on urine, serum, and salivary amylases. Their results of apparent K_m and V_{max} values, which were obtained from smoker and non-smoker subjects, also revealed no statistical difference at a 95% level of confidence interval between subjects who are cigarette smokers and non-smokers.

The kinetic parameters of salivary α-amylase in Libyan cigarette non-smokers and smokers subjects' salivary α-amylase presented in (Table 2) showed the most dramatic change, with about a 69.2% reduction in $Kcat$ for smoker's salivary α-amylase. There was a big change in the Kcat/Km of salivary α-amylase. The K value of non-smokers' salivary α-amylase was about a 4-fold increase in the catalytic process.

The key advantages revealed here are the low cat/KmKm (0.5±0.02 mM), the high Vmax (31.25±8.24 nmol/min/mg protein), and the high Kcat/Km (5.33±1.03 mM^{-1} min^{-1}). This could be due to the ionized state of the non-smokers' salivary α-amylase whose charges have been reserved and that the starch binds more strongly to the active site than the transition state of the substrate substantiating (Myers et al., 1997). This indicated that the non-smokers' salivary α-amylase had a higher affinity towards binding starch to the active site than smoker's salivary α-amylase.

The turnover number (*Kcat*) and specificity constant (*Kcat*/K_m) values of both enzymes (Table 2) showed that the activity of the non-smoker's salivary α-amylase was better compared to the smoker's salivary α-amylase. Furthermore, the value of the additional specificity constant *Ks* for non-smoker's salivary α-amylase (62.50±15.21 ml/min/mg protein) again confirmed that the non-smoker's salivary α-amylase was higher and more specific for starch as compared to smoker's salivary α-amylase (*Ks* = 16.50±6.41 ml/min/mg protein) (Table 2). It may be observed that cigarette smokers exhibit a reduction in serum type S isoamylase that correlates well with the significant reduction of salivary amylase activity. Several studies reported that tobacco smoke contains agents with cytotoxic or carcinogenic effects on the exocrine pancreas, but little is known about the effect of tobacco smoke on salivary glands (Nasrallah & Martin, 1983).

CONCLUSION

The findings of the present study indicate that salivary α-amylase activity is lower in cigarette smokers than non-smokers among Libyan subjects. From the results, it could be argued that chronic cigarette smoke may exert a toxic effect on salivary glands, thus altering the physiological salivary α-amylase activity of cigarette smokers. Therefore, further studies are needed in order to clarify the real impact or relationship between cigarette smoking and abnormalities in salivary α-amylase activity as a considerable risk of developing diseases is associated with the lifestyle.

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ETHICS

This retrospective study was conducted with the approval of the Libyan National Committee for Biosafety and Bioethics.

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دراسة بيوكيماوية لنشاط أنزيم ألفا أميليز في لعاب بعض المدخنين الليبيين

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المستخلص: تدخين التبغ يؤثر في عوامل بيولوجية عديدة، ومنها نشاط أنزيم α− الأميليز. توضح هذه الدراسة تأثير تدخين السجائر على أنشطة الأميليز اللعابي لدى بعض المدخنين الذكور الليبيين الأصحاء (100) مقارنة مع (40) من غير المدخنين كمجموعة سيطرة. تم تقييم نشاط α− الأميليز اللعابي باستخدام طريقة القياس اللونية القياسية، وتم قياس نشاط إنزيم α− الأميليز في عينات اللعاب التي تم جمعها من المدخنين البالغين الأصحاء وغير المدخنين (السيطرة) من أجل تقدير تأثير درجة الحرارة و الرقم الهيدروجيني المثالي (pH) وتركيز مادة التفاعل على النشاط الحركي للأنزيم. أظهرت نتائج تحليل عينات اللعاب وجود زيادة معنوية ($P<0.05$) في قيم نشاط α− الأميليز في لعاب مدخني السجائر عند مقارنته مع غير المدخنين في الظروف المثالية (2.74 ± 0.37 نانومول / دقيقة / ملغ من البروتين و 2.01 ± 0.16 نانومول / دقيقة / ملغ من البروتين ، على التوالي). أظهرت النتائج من خلال رسم لينويفر بيريك، وتركيز المادة الأساسية أن قيم ثابت ميكاليس (K_m) للأنزيم α− الأميليز كانت 0.5 ± 0.02 ملي مول و 1.12 ± 0.08 ملي مول لغير المدخنين والمدخنين على التوالي، وكان النشاط الأمثل للأنزيم α− الأميليز عند درجة حرارة 37 درجة مئوية، عند الرقم الهيدروجيني 7.0 لكلا المجموعتين بقيم 31.25 ± 8.24 نانومول / دقيقة / ملغ من البروتين 18.10 ± 4.06 ، نانومول / دقيقة / ملغ من البروتين للمدخنين وغير المدخنين على التوالي. يمكن أن يكون قياس نشاط الأميليز اللعابي مفيدا في الكشف المبكر عن التهاب الغدة النكفية وتشخيصها، ويلزم إجراء دراسات لاحقة لتحديد الآليات المسببة لهذا الخطر.

الكلمات المفتاحية: تدخين التبغ، α− الأميليز اللعابي، الخصائص الحركية، الشروط المثالية.

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