بسم الله الرحمن الرحيم

Sudan University for Science and Technology

Collage of Graduate Studies

Assessment of Salivary Urate, Superoxide Dismutase, and Related Trace Elements [Copper and Zinc] in Sudanese Tobacco Users in Khartoum State

تقويم حامض اليوريك و السوبراوكسايد دسميوتيزوالعناصر ذات الصلة (النحاس و الزنك) الموجودة في لعاب السودانيين مستخدمي التمباك في ولاية الخرطوم

A dissertation submitted in partial fulfillment for the Master Degree in Medical Laboratory Science (Clinical Chemistry)

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2016

بسم الله الرحمن الرحيم

قُل لَو كانَ البَحرُ مِدادًا لِكَلِماتِ رَبّي لَنَفِدَ البَحرُ قَبلَ أَن تَنفَدَ كَلِماتُ رَبّي وَلَو جِئنا بِمِثلِهِ مَدَدًا

صدق الله العظيم

سورة الكهف، الآية(109)

الإهداء

إلى:

هادياً و راعياً لفكري و روحي... إليك يا من استلهمت حكمتك و زادني شموخاً صدى صوتك يملأ مساحة الكون...أبي

إليك:

إلى ذلك النجم الذي أضاء ظلمة حياتي و سطع شمساً فبددت دياجير جهلي و تربع على عرش قلبي... أمي

إليك:

أنت يا رفيق دربي و نبراس حياتي، إلى من سعى معي في نجاح مسيرة العلم ...

زوجي

إليكم:

جواهر انتظمت و تفردت وكانت محوظات همم بجمالها الوجود فزادني بريقاً و ألقاً ...

إخوتي

إلى:كل من ساندني ووقف بجانبي في حياتي

Acknowledgment

First of all a great thanks to Allah for giving me the power and willing to complete this study.

Great appreciation to my friends for their supporting and to Dr. Noon Babiker Mohammed for her guidance to finish this work.

Finally, Thanks to the staff of clinical chemistry Department and Research Laboratory

Abstract

Tobacco using is known as one of the most important risk factor for the oral diseases such as oral cancer and keratoses. Antioxidants prevent diseases such as cancer and coronary heart disease, one such class of protective enzymes is the superoxide dismutase.Uric acid is by far the highest concentration antioxidant in human blood. Zinc and copper is trace element work as cofactor for nearly 300 enzymes in different species.

This study was done to assess the salivary urate, superoxide dismutase, and related trace elements (copper and zinc) in Sudanese Tobacco users by compare them with control group. Also to correlate the salivary superoxide dismutase, uric acid, copper, and zinc level with number of using Toombak per day and duration of using Toombak.

Material and Methods:

This is case control study. The study was conducted from February to May 2016, fifty samples from saliva of non-tobacco users (Toombak) chosen randomly as control and fifty samples from saliva of tobacco users as test group.

All samples were tested for superoxide dismutase activity using Fortress diagnostic reagent by spectrophotometer analyzer, uric acid level using biosystem diagnostic reagent by spectrophotometer analyzer, and copper, zinc level using atomic absorption spectroscopy, and results were analyzed using statistical of package social science [SPSS]

Results:

The study result showed that the level of superoxide dismutase activity was significantly decreased in Toombak users compared with non-Toombak users control group [mean ± SD: 2.16 ± 0.76 versus 4.96 ± 2.21, P = 0.02], uric acid level and copper showed insignificant result, uric acid level[mean ± SD: 1.44 ± 0.89 versus 2.36 ± 1.33, P = 0.15], copper level [mean ± SD: 1.07 ± 1.02 versus 1.47 ± 0.97, P = 0.1] and zinc level showed significant

increase in Toombak users compared with non-Toombak users [mean ± SD: 0.15 ± 0.04 versus 0.23 ± 0.15, P = 0.00]

In Toombak users groups, the study showed that the superoxide dismutase

[r = -0.40, P = 0.00], and uric acid[r = 0.02, P = -0.33] have a negative weak correlation with number of Toombak using per day and no correlation between the level of copper [r = -0.17, P = 0.25] and zinc [r = -0.03, P = 0.82] with a number of Toombak using per day.

Also the study showed that the superoxide dismutase has a negative weak correlation with the duration of Toombak using per years [r = -0.35, P = 0.01].

Uric acid [r = -0.14, P = 0.33], copper [r = 0.13, P = 0.38], and zinc[r = -0.03, P = 0.82] have no correlation with duration of Toombak using per years.

Conclusion:

From the result of this study, it is concluded that superoxide dismutase was decreased and zinc increased in Toombak users group. There are a weak correlation between superoxide dismutase and uric acid with a number of using Toombak per day. Also the superoxide dismutase has a correlation with duration of using Toombak per years.

المستخلص

استخدام التمباك يعتبر من أهم العوامل الخطيرة في الإصابة بأمراض الفم مثل سرطان الفم وتصبغ الفم،وتعمل مضادات الأكسدة على منع العديد من الأمراض مثل السرطانات و أمراض القلب، ويعتبر أحد الإنزيمات المضادة للأكسدة إنزيم السوبر أوكساید دسميوتيز.

كذالك يعدحامض اليوريك من أكثر المواد المضادة للأكسدة تركيزاً في الدم،ويعتبر كل من النحاس والزنك من العناصر النادرة التي تدخل كعوامل مساعدة لاكثر من ٣٠٠إنزيم من أنواعٍ مختلفة .

أجريت هذه الدراسة لتقييم حامض اليوريك والسوبراوكساید دسميوتيز وبعض العناصرذات الصلة (النحاس و الزنك) الموجودة في لعاب السودانيين مستخدمي التمباك و مقارنتهم مع مجموعة التحكم، و كذلك لايجاد علاقة بين نشاط إنزيم السوبرأوكساید دسميوتيزومستويات كلٌ من حامض اليوريك و النحاس والزنك مع عدد مرات استخدام التمباك في اليوم و إدمانه بالسنين.

المتطلبات العملية وطرق البحث:

أجريت الدراسة لمقارنة نشاط إنزيم السوبر أوكساید باستخدام كتشف التشخيص، وتم قياس الإنزيم بواسطة جهاز مطياف الضوء ومستوى حامض اليوريك بواسطة جهاز مطياف الضوء وقياس مستويات النحاس و الزنك باستخدام مطيافية الامتصاص الذري في مستخدمي التمباك في الفترة من فبراير إلى مايو ٢٠١٦.

٥٠ عينة من غير مستخدمي التمباك تم اختيارهم عشوائياً، و ٥٠ عينة من مستخدمي التمباك كمجموعة اختبار، وتم تحليل النتائج باستخدام الإحصاء من حزمة العلوم الإجتماعية.

النتائج:

أظهرت نتائج الدراسة أن مستوى نشاط إنزيم سوبرأوكساید دسميوتيزيتناقص بشكلٍ ملحوظ لدى مستخدمي التمباك وكان الاحتمال الإحصائي للمقارنة ٠,٠٢ و كانت النتيجة كالآتي: (المتوسط ± الانحراف المعياري عند مجموعة التحكم مقارنةً بمستخدمي التمباك :- (٢,١٦±٠,٧٦مقارنةً ٤,٩٦±٢,٢١)

بينما أظهر حامض اليوريك (١,٤٤±٠,٨٩مقارنةً ٢,٣٦±١,٣٣) والنحاس (١,٠٧±١,٠٢ مقارنةً ١,٤٧±٠,٩٧) عدم وجود تغير في القراءات بين المجموعتين، و أوضح الزنك (٠,١٥±٠,٠٤مقارنةً ٠,٢٣±٠,١٥) تزايد ملحوظ لدى مجموعة مستخدمي التمباك.

في مجموعة مستخدمي التمباك أوضحت الدراسة أن إنزيم السوبر أوكسايد دسميوتيز بدلالة إحصائية معنوية (٠,٠٠)، و حامض اليوريك بدلالة إحصائية معنوية (٠,٠٢) لديهما علاقة سالبة ضعيفة مع عدد مرات استخدام التمباك في اليوم الواحد و أنه لا توجد علاقة بين النحاس و الزنك مع عدد مرات استخدام التمباك.

كذلك أظهرت الدراسة أن هناك علاقة سالبة ضعيفة في نشاط إنزيم السوبر أوكسايد دسميوتيز بدلالة إحصائية معنوية (٠,٠١) مع فترة استخدام التمباك بالسنين و أنه لا توجد علاقة بين حامض اليوريك و النحاس و الزنك مع مدة استخدام التمباك.

الخاتمة:

من نتائج الدراسة، توصلنا إلى أن مستوى إنزيم السوبر أوكسايد دسميوتيز يتناقص لدى مستخدمي التمباك و يتزايد الزنك لدى مجموعة مستخدمي التمباك وأن هناك علاقة بين عدد مرات استخدام التمباك و تركيز كل من إنزيم السوبر أوكسايد دسميوتيز و حامض اليوريك. كذلك بينت الدراسة وجود علاقة بين إنزيم السوبر أوكسايد دسميوتيز و فترة استخدام التمباك بالسنين.

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Abbreviations

Abb.	Full term
CAT	Catalase
DNA	Deoxyribose nucleic acid
FDA	Food and drug administration
GSH	Growth stimulating hormone
INT	Iodophyenyle nitrofenol
PCM	Protein calorie malnutrition
RNA	Ribose nucleic acid
RNS	Reactive nitrogen specious
ROS	Reactive oxygen specious
SIADH	Syndrome inappropriate antidiuretic hormone
SOD	Superoxide dismutase
SPSS	Statistical of package social science
TSNAs	Tobacco specific nitrosamine

Chapter One

1. Introduction

1.1 Introduction:

Saliva is a biological diluted solution of water, ions and proteins such as mucine, lysozyme and immunoglobulins, its secretion is under autonomic control and can be triggered by multiple stimuli such as the sight, smell, and touch and even thought of food, it serves multiple purposes; water and mucus soften and lubricate food to make it easier to swallow, saliva begins chemical digestion with the secretion of salivary amylase and small amount of lipase **[Silverthorn.2004]**

Antioxidants is a molecule that inhibit the oxidation of other molecules, its consist of vitamin C, A, E, catalase and superoxide dismutase, its function to prevent diseases such as cancer and coronary heart disease **[Fischbach.1999]**

Superoxide dismutase is the antioxidant enzyme that catalyzed the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. Peroxide can be destroyed by CAT and GPX reaction **[Zheng,et al.2001]**

The function of superoxide dismutase is protecting aerobic organisms against the potential deleterious effect of superoxide. The enzyme occurs in different compartment of the cells **[Monry.1999]**

Zinc [Zn] is a bluish white lustrous metal that is stable in dry air and becomes covered with a white coating when exposed to moisture. Zinc is an essential trace element and deficiency is common throughout life, especially in individuals that do not ingest meat **[Bishop.2013]**

Copper [Cu] is a relatively soft yet tough metal with excellent electrical and heat conducting properties, its widely distributed in nature both in elemental form

and in compounds. Copper is an essential element found in four oxidation states, Cu[0], Cu[$^{2+}$], Cu[$^{2+}$] and Cu[$^{3+}$], with Cu[$^{2+}$] the most stable of all oxidation states. Copper is an important cofactor for several metaloenzymes and is critical for the reduction of iron in heam synthesis **[Bishop.2013]**

Uric acid is the product of catabolism of the purine nucleic acid, such as guanine from the breakdown of ingested nucleic acids or from tissue destruction. Uric acid is transported in the plasma from the liver to the kidney. It is measured to confirm diagnosis and monitor treatment of gout **[Bishop.2013]**

Toombak using is one of the most important risk factors for the oral cancer, because of containing carcinogenic substances which affect the salivary antioxidant **[Idris.1998]**

1.2 Rationale:

Abuse of tobacco is a major problem in the society, its famous because of it is cheap, available and simple in manufacturing.

Tobacco using is known as one of the most important risk factor for the oral diseases such as oral cancer and keratoses. Tobacco consumption has a direct correlation with DNA damage. When a cell with DNA damage divides, metabolism and duplication of cells become deranged and mutations can arise, which is important factor in carcinogenesis. Reactive oxygen species, free radicals and reactive nitrogen species in snuff have been suggested to induce a gradual evolving process, because of direct contact with tissue; localized absorption. Toombak contains oxidants and pro oxidant agents leading to dysplastic lesions which then transformed into carcinoma lesions. This evidence emphasizes the role of Toombak on salivary antioxidants in the pathogenesis of oral cancers.

Free radical formation is naturally controlled by antioxidants. Antioxidants are capable of deactivating or stabilizing free radicals before they injure cells.

Using of saliva sample rather than blood sample because of direct presence of Toombak in the oral tissue and its local absorption.

This is why this study is done to evaluate this hypothesis.

1.3 Objectives:

1.3.1 General Objective:

To assess the salivary urate, superoxide dismutase, and related trace elements [copper and zinc] in Sudanese Tobacco users.

1.3.2 Specific objectives:

- To compare the salivary superoxide dismutase activity, uric acid, copper, and zinc level in control and cases.
- To correlate the superoxide dismutase activity, uric acid, copper, and zinc level with number of using Toombak per day and duration of using Toombak per years.

Chapter Two

2. Literature review

2.1. Saliva:

The composition of whole saliva consist of secretion of the salivary gland, microorganisms, their enzymes and metabolic products desquamated epithelial cells, leucocytes, and tissue enzymes, secretions of mucosa, gingival fluids and probably food remainants. Salivary mucines have been found to coat bacteria and to protect the organism against phagocytosis. **[Nolte.1982]**

The composition of saliva are electrolytes [Na^+, K^+, Cl^-, HCO_3^-, Ca^{2+}, Mg^{2+}, HPO_4^{2-}, SCn^- and F^-], secretory proteins[amylase, proline-rich proteins, mucines, histatin, cystatin, peroxidase, lysozyme, and lactoferrine], immunoglobulins [secretory immunoglobulins A, IgG and IgM], small organic molecules [Glucose, amino acids, urea, uric acid and lipids], other components EGF, insulin, cyclic adenosine monophosphate binding proteins and serum albumin. **[Nanci.1998]**

2.1.1. Salivary flow rate:

The effectiveness of the saliva's flow rate and cleansing action is influenced by the location of the salivary gland and their ducts. The quantity of saliva secreted between meals is reported to be somewhat less than at meal time. Minimum salivary flow is reported during sleep. The flow of saliva from the large salivary duct demonstrate a protective mechanism in that it restrains the movement of microorganism into the duct. The Corkscrew characteristic of stensen's duct retard the reflux of saliva from the mouth to the parotid gland. Gravity also influences the drainage of the parotid secretion downward from the buccinators muscle which is surrounding the duct. Salivary flow of the sublingual and submaxillary glands is not enhanced by gravity, and these glands are more liable to infection than is the

parotid gland. The slowing down of salivary flow, noted to occur in the state of shock and dehydration, appears to favor infection of the glands. **[Nolte. 1982]**

2.1.2. Salivary pH:

Normally mixed saliva has a pH range of 5.6-7.0, averaging 6.7. The pH of buffering capacity of saliva increase with enhancement of flow brought about by the chewing process and to some extent by the appearance or aroma of certain food. The pH of saliva was vary during the day, during sleep the pH falls. **[Nanci. 1998]**

2.1.3. Functions of saliva:

A- Protection (by provides a washing action that flushes away non adherent bacteria and other debris also mucin and other glycoproteins provide lubrication, preventing the oral tissues from adhering to one another, allowing them to slide easily over one another and form a barrier against noxious stimuli, microbial toxins, and minor trauma)

B- Buffering (by the bicarbonate, some extent, phosphate, ions, and some basic proteins which provide a buffering action that helps to protect the teeth from demineralization caused by bacterial acids produced during sugar metabolism)

C- Pellicle formation (by proteins and calcium bind to the surfaces of the teeth and oral mucosa, forming a thin film, which help to protect the tooth surface)

D- Maintains of tooth integrity (by calcium and phosphate ions. The solubility of these ions is maintained by several calcium binding proteins, specially the acidic proline-rich proteins and satherin, at the tooth surface, the high concentration of calcium and phosphate results in a post eruptive maturation of the enamel, increasing surface hardness and resistance to demineralization. Remineralization of

initial caries lesions also can occur, these is enhanced by the presence of fluoride ions in saliva)

E- Antimicrobial actions

F- Tissue repair (by many of the growth factors, other biological active peptide and proteins the tissue growth, differentiation, wound healing and other beneficial effect)

G- Digestion (by the solublization of food substances and the action of enzymes such as amylase and lipase, begin the digestive process)

H- Taste (by solublilizing food substances located in taste buds as taste receptors)

[Nansi.1998]

2.2. Antioxidants:

Oxygen is the highly reactive molecule that damages living organisms by producing reactive oxygen species. Consequently organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids. In general, antioxidant systems either prevent these reactive species from being formed or remove them before they can damage vital components of the cell. However, reactive oxygen species also have useful cellular functions such as redox signaling. Thus, the function of the antioxidant system is not to remove oxidant entirely, but instead to keep them at an optimum level. **[Percival.1998]**

2.2.1. Metabolites of antioxidants:

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water [hydrophilic] or in lipids [lipophilic].In general, water soluble antioxidants react either oxidants in the cell cytosol and blood plasma,

while lipid soluble antioxidants protect cell membrane from lipid peroxidation. These compounds may be synthesized in the body or obtained from the diet. The different antioxidant are present at a wide range of concentration of body fluids and tissues with some such as glutathione or upiquinone mostly present within cells, while others such as uric acid are more evenly distributed .In food, antioxidant are highly regulated to extremely small percentages in most countries-down to the law fractions of one percent composition of the substrate, processing conditions, impurities, and desired shelf life are among the most important factors in selection the best antioxidant system for a given food product. **[Considine, et al.2005]**

The desirable features of the antioxidants may be summarized as:

1- Effectiveness at law concentration

2- Compatibility with the substrate

3- Nontoxic to consumers

4- Stability in terms of conditions encountered in processing and storage including, temperature, radiation, pH, etc

5- Non volatility and non-extractability under the conditions of use

6- Ease and safety in handling

7- Freedom from off-flavors, off-odors and off-colors that might be imparted to the food product

8- Cost effectiveness. **[Considine, et al. 2005]**

2.2.2. Mechanism of oxidative degradation:

It could appear that in as much as oxidative degradation occurs in a variety of organic materials that are dissimilar in appearance and have entirely different applications and different properties, with degradation producing different effects, the oxidation mechanism itself might be different. Current knowledge indicate, however, that the mechanism of oxidative degradation is the same for the all organic substrate. They appear to degrade by the same free radical mechanism. **[Considine, et al. 2005]**

2.2.3. Functions of antioxidants:

Antioxidants functions are improve health; prevention of diseases such as cancer or coronary heart disease, general promotion of health, and industrial function as preservatives in food, cosmetics and to prevent the degradation of rubber and gasoline. The antioxidant involves a variety of components, both endogenous (e.g. billirubin, uric acid, and enzymes) and exogenous (e.g. vitamin A&E, and metal binding proteins) in origin. **[Kala Chandra.2015]**

2.2.4. Enzyme systems:

As with the chemical antioxidants, cells are protected against oxidative stress by an interacting network of antioxidant enzymes. Here, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutase catalyzing the first step and then catalases and various peroxidases removing hydrogen peroxide. As with antioxidant metabolites, the contributions of these enzymes to antioxidant defenses can be hard to separate from one another, but the generation

of transgenic mice lacking just one antioxidant enzyme can be informative. **[Fukai, et al.2011]**

2.3. Toombak:

In Sweden, snuff [locally known as snus], was introduced since the year 1937. Presently, Sweden has the highest per capita consumption and sale figures of snuff in the world, and the habit is coming increasingly popular. Snus is manufacturing into a dry form used in the nasal cavity and the moist form used in the oral cavity. Snus manufactured for oral use is moist ground tobacco of dark kientucky or virginia species mixed with an aqueous solution of water and other blending ingredients. This form of snuff is found in two types:
1- Loose and 2- Portion-bag-packet. These are the most widely used. The loose moist form [1-2g a quid] is the most popular type consumed by 73% of the males, followed by the portion-bag-packet form [0.5-1 g a quid], consumed by13% of the males, while 14 % of the males are mixed users. The majority of the snuff users place the quid in the vestibular area of the upper lip, and the prevalence among persons 15 years of age or older in 15.9% among males and 0.2 % among females. The pH of snuff has declined from a previous range of 8-9 to a range of 7.8-8.5, moisture content ranges 35-60% and nicotine content is in the order of 5-11mg/g dry wt tobacco-specific N-nitrosamines [TSNAs] in micrograms **[Idris.1998]**

The toxic components of tobacco include: tar, nicotine and carbon monoxide. Tar is a by-product of burning tobacco. Its composition is a dark, sticky substance that can be condensed from cigarette smoke. Tar contains many potent carcinogens and chemicals that irritate tissue in the lungs and promote chronic bronchitis and emphysema. These substances paralyze and destroy the cilia that line the bronchi, causing [smoker’s cough]. Long term exposure of extremely toxic tar to lung tissue can lead to the development of cancer.

Nicotine is a colorless, oily compound that is extremely poisonous in concentrated amounts. This highly addictive drug is a major contributor to heart and respiratory diseases causing short-term increases in blood pressure, heart rate and blood flow from the heart resulting in narrowing of the arteries. A strong dependence on nicotine can occur after as little as three packs of cigarettes and it is more addictive than cocaine or heroin. Because of its addictive effects, The Food and Drug Administration [FDA] has determined nicotine should be regulated. **[Baker. M. 2003]**

At first, nicotine acts as a stimulant and then it tends to tranquilize the nervous system. The effects depend largely on how one chooses to smoke. Shallow puffs seem to increase alertness because low doses of nicotine facilitate the release of acetylcholine, which create feelings of alertness. Long, deep drags tend to relax the smoker because high doses of nicotine block the flow of acetylcholine. Ninety percent of the nicotine inhaled while smoking is absorbed into the body, while twenty to thirty percent of nicotine is absorbed if the smoke is drawn only into the mouth, not the lungs. Other side effects include inhibiting formation of urine, discoloration of the fingers, dulling the taste buds, and irritating the membranes in the mouth and throat. Because nicotine constricts blood vessels, it causes the skin to be clammy and have a pallid appearance, as well as reducing body temperature. The highly addictive nature of nicotine can cause withdrawal symptoms to occur quit suddenly. These symptoms include irritability, anxiousness, hostility, food cravings, headaches, and the inability to concentrate. Carbon Monoxide is an odorless, tasteless gas that is highly toxic. Reduces the amount of oxygen the blood can carry, causing shortness of breath. Carbon monoxide ultimately damages the inner walls of the arteries, thus encouraging a buildup of fat on the walls of the

arteries, this is called atherosclerosis. Over time, this causes the arteries to narrow and harden, which may lead to heart attack. **[Baker.2003]**

Several health and addiction hazards may be associated with the use of spit tobacco because of the ready absorption of nicotine and other molecules through the oral mucosa. A variety of local oral alteration also are found in chronic users, one of the most common local changes is a characteristic painless loss of gingival and periodontal tissues in the area of tobacco contact. This gingival “recession” frequently include destruction of the facial surface of the alveolar bone and correlate well with the quantity of daily use and the duration of smokeless tobacco habit. Dental carries also has been reported to be more prevalent in spit tobacco users, perhaps because of the high sugar content of some brand. Long-term use may lead to localized or generalized wear of occlusal and incisal surfaces, especially in person employed in dusty environment. A brown-black extrinsic tobacco stain is typically found on the enamel and cementum surfaces of the teeth adjacent to the tobacco. In addition, halitosis is a frequent finding in chronic users. **[Damm, et al.2002]**

2.3.1. Smokeless tobacco-related keratoses:

Hyperkeratosis mucosal lesions can result from smoking or use of smokeless tobacco [topical tobacco-snuff dipping and tobacco chewing]. By contrast, there is no characteristic hyperkeratosis lesion associated with the far more common habit of cigarette smoking. Tobacco chewing and snuff dipping [holding flavored tobacco powder in an oral sulcus] are popular habits in the USA and some parts of the Europe. **[Cowson, et al. 2000]**

The development of this lesion is most strongly influenced by habit duration and also by the brand of the tobacco used, early onset of spit tobacco use, total

hours of daily use, amount of tobacco consumed daily, and number of site routinely used for tobacco placement. **[Damm, et al.2002]**

2.3.2. Clinical features:

The habit of snuff dipping or tobacco chewing may be maintained for decades and give rise to keratoses in the buccal or labial sulcus, where the tobacco is held. Early changes are erythema and mild, whitish thickening. Long-term use gives rise to extensive white thickened and wrinkling of the buccal mucosa. Malignant change can follow, but only after several decades of use, a high proportion of carcinoma in snuff users are verrucous in type, but if they remain untreated invasive squamous carcinoma may develop. **[Damm, et al.2002]**

2.4. Cancer:

- Cancer cells characterized by three properties:

- Diminished or unrestrained control of growth

- Invasion of local tissues

- Spread or metastasis to other parts of the body

- Agents causing cancer fall into three broad groups:

- Radiant energy

- Chemical compounds

- Viruses

In general, these act by causing mutation or by introducing novel genes into cells. There are also a number of familial conditions that cause cancer, these are due to mutation in specific genes [e.g. tumor suppressor genes]. A wide varieties of chemical compounds are carcinogenic. Many substances are associated with

development of cancer in a human caused by environmental factors. Exposure to such compounds can occur because of a person's occupation [benzene, asbestos], diet [e.g. aflatoxine B1, which is produced by the mold, and sometimes found as contaminated food stuffs] or life style [e.g. a cigarette smoking]. **[Monry, etal.1999]**

All forms of smoking tobacco are associated with an increased risk of mouth cancer particularly if reversed smoking is practiced. **[Chestnutt, et al.2007]**

Tumor may be differentiated into benign and malignant:

-Benign tumors remain at their site of origin

-Malignant tumors: there is abnormal growth with the invasion and distant metastasis

Carcinomas are malignant tumors of epithelial tissue. The most common malignant tumor of the oral cavity is the squamous cell carcinoma. Sarcomas malignant is tumor of connective tissue, e.g. liposarcoma. . **[Chestnuttig, et al.2007]**

2.4.1. Mouth cancer:

Marked geographic variations in incidence worldwide in the UK mouth cancer accounts for only 1-2 % of all malignant tumors. Ninety to 95% of all mouth cancer is squamous cell carcinoma, which mainly seen in middle aged and elderly but as yet an explained increasing incidence among younger adults. **[Chestnuttig, et al.2007]**

2.4.1.1 Etiological factors:

1- Tobacco: all forms of smoking tobaccoare associated with an increased risk of mouth cancer. Use of snuff and chewing tobacco increasing the risk

2- Alcohol: increased risk in association with alcohol consumption

3- Diet and nutrition: poor diet increases risk

4- Ultra violet light: important risk factor of carcinoma of the mouth

5- Immunosuppression: increases risk factor of lip cancer among transplant recipient. **[Chestnuttig, et al.2007]**

2.4.1.2. Clinical features:

Clinical presentation varies considerably. Early mouth cancers are very often asymptomatic. Common patient presentation include the following:

- **Early lesion:** painless solitary ulcer, exophytic growth, white patch, erythroplakia, erythroleukoplakia, chronic crusted being on the vermillion boarder of the lip
- **Advanced lesion:** pain, exophytic masses, necrotic; bleeding of warty surface, deep; createred ulcers with indurated edges and invasion leading to possible altered sensation and pathological fracture **[Chestnuttig, et al.2007]**

2.5. Tumor marker:

Biochemical laboratory tests help in the management of patients with cancer. Many cancers are associated with the abnormal production of enzymes, proteins and hormones which can be measured. These molecules are known as tumor marker. Three measure conclusions have emerged from the study of tumor markers:

- No single marker is useful for all types of cancer

- Markers are most often detected in advanced stages of cancer rather than early stage when they would be more helpful

- Uses of marker the most successful have been the monitoring of responses to therapy and detection of early recurrences. **[Monry. 1999]**

2.6. Free radicals:

Free radical is an atom or molecule that has one or more unpaired electrons. It is consequent tendency to acquire an electron from other substances makes it highly reactive. However, not all reactive oxygen species are free radicals, e.g. singlet oxygen and H_2O_2. When oxygen is reduced to water by cytochrome oxidase four electrons are acquired. The individual molecules in univalent reduction are highly reactive and potentially damaging to tissues. They are the superoxide free radical, hydrogen peroxide and the hydroxyl free radical. **[Monry. R. K. 1999]**

Many of the compounds in the body are capable of being converted to free radicals by natural events that remove one of their electrons or by radiation. Radiation, for example dissociates water into the hydrogen atom and the hydroxyl radical:

$$H_2O \leftrightarrow H^+ + OH^-$$

[Lieberman, et al. 2007]

Cells adapted to oxidative stress by induction of antioxidant enzymes. Many of these are controlled by the antioxidant response element [ARE]. ARE-dependent enzyme include catalase [CAT] and superoxide dismutase [SOD], and other enzymes that catalyze the oxidation and conjunction of carcinogens and oxidants for excretion. One of these enzymes, hepatic glutathion S-transferase catalyzes conjunction with GSH. The conjugate are then excreted in urine as mercapturic acid which are S-substituted N- acetyl- cysteine derivatives. Reactive oxygen species [ROS] are the sparks produced by oxidative metabolism, and oxidative stress may be viewed as the price we pay for using oxygen for metabolism. ROS

and reactive nitrogen species [RNS], such as superoxide, peroxide, hydroxyl radical, and peroxy nitrate, are reactive and toxic, sometimes difficult to contain, but the production is important for regulation of metabolism, turnover of biomolecules and protection against microbial infection. ROS and RNS cause oxidative damage to classes of biomolecules: proteins, lipids and the DNA. There are a number of protective antioxidant mechanisms including sequestration of redox-active metal ions, enzymatic inactivation of major ROS, inactivation of organic radicals by small molecules, such as GSH and vitamins, and, when all else fail, repair and/or turnover. Biomarkers of oxidative stress are readily detected in tissues in inflammation, and oxidative stress is increasingly implicated in the pathogenesis of age-related, chronic disease **[Bayness, et al. 1999]**

2.6.1. Reactive Oxygen Species [ROS]:

The hydroxyl radical is probably the most potent of the ROS. It initiates chain reactions that form lipid peroxides and organic radicals and adds directly to compounds. The superoxide anion is also highly reactive, but it has limited lipid solubility and cannot diffuse far. However, it can generate the more reactive hydroxyl and hydroperoxyl radicals by reacting non enzymatically with hydrogen peroxide [H_2O_2].H_2O_2, although not actually a radical, is a weak oxidizing agent that is classified as an ROS because it can generate the hydroxyl radical [OH^-]. Transition metals, such as Fe^{2+} or Cu^+, catalyze formation of the hydroxyl radical from H_2O_2 in non enzymatic reactions. Because H_2O_2 is lipid soluble, it can diffused through membranes and generate OH^- at localized Fe^{2+}-or Cu^+-containing sites, such as the components of the electron transport chain within the mitochondria. **[Devtin, et al .2011]**

2.6.1.1. Damage caused by reactive oxygen species:

Reactive Oxygen Species cause damage to all major classes of macromolecules in cells. The phospholipids of plasma and organelles membranes are subject to lipid peroxidation, a free radical chain reaction initiated by removal of hydrogen from a polyunsaturated fatty acid by hydroxyl radical. The resulting lipid radical then react with O_2 to form lipid peroxy radicals and lipid peroxide along with malondialdehyde, which is water soluble and can be detected in blood. The effect of lipid peroxidation in human is exemplified by the brown spots commonly observed on hands of the elderly. One significant consequence of lipid peroxidation is increased membrane permeability leading to an influx of Ca^{2+} and other ions with subsequent swelling of the cell. Similar increases in permeability of organelle membranes may result in maldistribution of ions and cause intracellular damage for example, accumulation of excessive amount of Ca^{2+} in mitochondria may trigger apoptosis. The most important consequences of oxygen radicals is damage to mitochondrial and nuclear DNA resulting in mutation. The nonspecific binding of ferrous ion [Fe^{2+}] to DNA may result in localized formation of hydroxyl radicals that attack individual bases and cause strands breaks. Mitochondrial DNA is more susceptible to damage. Since the electron transport chain is a major source of toxic oxygen radicals. In addition, nuclear DNA is protected from permanent damage by a protective coat of histones as well as by active and efficient mechanisms for DNA repair. **[Devtin, et al. 2011]**

2.6.1.2 Cellular defenses against reactive oxygen species:

Cells that live in an aerobic environment have developed multiple ways to remove reactive oxygen species and thus protect themselves against their deleterious effects. Protection against reactive oxygen species may also be gained by ingestion of oxygen scavengers such as; vitamins C, E, B and carotene, recent evidence has indicated that substances present in green tea, beans and red wine may also protect against oxidative damage by ROS. **[Devtin, et al. 2011]**

2.7. Superoxide dismutase:

In human both iron-containing hemoglobin and zinc-contain carbonic anhydrase play pivotal roles in binding oxygen and delivering it to the cells. Moreover, enzymes developed to protect cells from high levels of oxygen also contain metals. One such class of protective enzymes is known as the superoxide dismutase [SODS]. Mammals have three different isoenzymes of superoxide dismutase that catalyze conversion of superoxide to peroxide. The cytosolic form of superoxide dismutase contains Cu/Zn at its active site, as does the extracellular enzymes; however, the mitochondrial enzyme contains Mn at its active site. Hydrogen peroxide is removed by catalase, a heme-containing enzyme present in highest concentration in peroxisomes and to lesser extent in mitochondria of cytosol. **[Considine, et al.2005]**

The superoxide radical is more toxic than previously believed. These radical is produced as a by-product of many oxidative reactions, but most of it probably arises as an abbreviation of the mitochondrial electron transfer element. It has been estimated that as much as 5% of the O_2 consumed in respiration might be converted to O_2^-in young rat heart mitochondria, these increases with the animal's

age. Fortunately all aerobic cells contain enzymes, superoxide dismutase, that scavenge and detoxify O_2^- by catalyzing the dismutation reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

These enzymes are formed in species ranging from bacteria to mammals. They are found in mitochondria, the cytosol of liver, in erythrocytes and in other tissues. Superoxide dismutases are metalloenzymes, but the metal requirement depends on the enzyme source. **[Montgomer, et al .1990]**

2.8. Trace elements:

A number of trace elements in the body are essential to the life process, many are involved in the activation or deactivation of enzymes. Others are involved in vitamins, hormones, skeleton and other controls. Some evidence has been obtained indicating that trace metals may be associated with RNA. Some elements are essential in trace quantities but become toxic in large amounts. **[Christian. G. D. 1986]**

Trace elements are considered essential when deficient intake produces an impairment of function and when restoration of physiological amounts of only that elements prevents or alleviates the impairment. In absolute deficiency, death result in limited intake, the organism may survive, but biological functions are impaired. The nutrient is given in excess, marginal toxic response is attained followed by mortal toxic response. Both defined biochemical functions of signs of deficiency in humans are only known for iron, zinc, copper, cobalt, iodine, molybdenum and selenium **[Burits, et al. 1996]**

2.8.1. Characteristics of trace element functions:

Essential trace elements are specific for their in vivo functions; they cannot be effectively replaced by chemically similar elements.The essential trace metal or element interact with electron donor atoms such as nitrogen sulfur and oxygen; the types of interaction depend on configurational preferences and bond type. Certain trace elements are stable in more than one valance state [e.g. Fe, Cu, Mo], which allows for biological oxidation-reduction function, whereas others are stable in only single state [e.g. Zn^{+2}], which allows for more of conformation or substrate-binding rout. Transition metals with partially filled d-electron orbital [e.g. Fe, Cu, Co] tend to coordinate with a larger number of electron donor metals than metals with filled d-orbitals [Zn^{+2}]. **[Burits, et al. 1996]**

Metal ions are required as active components of several proteins. The most obvious of these is iron and its function as part of the proteins involved in the transfer of molecular-oxygen. Other metals have been found to be essential for normal biological functions. These include metals previously thought to be toxic; indeed environmental excesses of these result in toxicity such elements include chromium, selenium, manganese, copper and zinc, and are now termed essential trace elements. **[Devtin.2011]**

2.9. Zinc:

Zinc [Zn] is second only to iron in importance as an essential trace metal and is present in abundance in the body. **[Burits, et al. 1996]**

The 2.3g of zinc in the human body are widely distributed in every tissue and tissue fluid about 90 wt % is in muscle and bone; unusually high concentrations are in the choroid of the eye and in the prostate gland. Almost all of the zinc in a blood is associated with carbonic anhydrase in the erythrocytes. Zinc

is concentrated in nucleic acid and found in the nuclear mitochondrial, and supernatant fractions of all cells. **[Considine, et al. 2005]**

Zinc is an integral component of nearly 300 enzymes in different species. Important zinc containing metaloenzymes in humans include carbon anhydrase, alkaline phosphatase, ribonucleic acid [RNA] and deoxyribonucleic acid [DNA], polymerase, thymidine kinase, carboxy peptidase, and alcohol dehydrogenase. The zinc atoms are an integral, firmly bound part of the metaloprotein molecule and are often involved in the active site. Loss of zinc metaloenzyme activity with zinc depletion varies with the different zinc enzymes. Activity loss is dependent on the tissues, enzymes turnover rate, and the affinity if the enzymes for zinc. Zinc is an important element in wound healing, several studies have implicated zinc as a necessary factor in the biosynthesis and integrity of connective tissue, for these reason, adequate zinc nutrition is especially important for the post-surgical patient **[Burits, et al. 1996]**

2.9.1. Metabolism of zinc:

Approximately 20 % to 30% of ingested dietary zinc is absorbed. Absorption of zinc from the diet is an active process and shares gut transport mechanism with cupper and iron. On absorption, zinc is found bound to the protein metal-lothioneine, a cysteine-rich protein, which is also associated with the binding of other divalent metal ions, e.g. cupper. Its synthesis is dependent on the amount of zinc in the diet and may delay a role in reducing toxicity, and it is probably the least toxic of the trace metals by increased oral doses of zinc interfere with cupper absorption leading to the deficiency of the latter. **[Iczak. 1999]**

The major role of zinc excretion is via the feces. Pancreatic excretion, biliary losses are small, urinary losses, and sweat losses. **[Burits, et al. 1996]**

2.9.2. Clinical significance:

Nutritional zinc deficiency in humans is fairly prevalent throughout the world. Clinical features include mild zinc deficiency, weight loss, hyper ammonemia and lowered ethanol tolerance. Moderate zinc deficiency is characterized by growth retardation, hypogonadism in male, mild dermatitis, delay wound healing and impaired immune responses. Manifestations of sever cases of zinc deficiency include bullous-pusular dermatitis, diarrhea, recurrent infection and ultimately, if no treatment is given death. **[Burits, et al. 1996]**

The size of the human fetus is correlated with zinc concentration in the amniotic fluid and habitual low zinc intake in the pregnant female. **[Considine, et al. 2005]**

2.10. Copper:

All human tissue contain copper. The highest amount found in the liver, brain, heart and kidney. In a blood, plasma, and erythrocyte contain almost equal amount of copper, i.e., ca 110 and 115 mg/100ml respectively. In plasma, ca 90 wt% of copper is in the metaloprotein ceruloplasmin, also known as a_2-globuline, mol wt 151000, which contains 8 atoms of copper per molecule. Creuloplasmin has been identified as a ferroxidase [I] which catalyses the oxidation of aromatic amines and of Fe^{+2} to Fe^{+3}. The ferric ion is then incorporated into transferrin which is necessary for the transport of iron in tissues involved in the synthesis of iron-containing compounds, e.g. hemoglobin. Lowered level of ceruloplasmin interferes with hemoglobin synthesis. **[Considine, et al. 2005]**

Copper deficiency is characterized by poorly formed collagen which leads to bone fragility and spontaneous bone fractions in animals, and also results in cardiac hypertrophy. Abnormal electrocardiographs have been noted when low

copper diet were fed to humans. Anemia, neutropenia and bone disease have been reported in children having protein calorie malnutrition [PCM] and accompanying hypo copraemia. At least two genetic diseases involving copper are known: Wilson's disease, an autosomal recessive disease, usually detected in adulthood and Menke's Kinky hair syndrome. Excessive copper has been reported to be fatal for oral dose levels of copper sulfate of 200mg/kg body weight for a child and 50mg/kg for adults. **[Considine, et al. 2005]**

The major function of copper metaloproteins involve oxidation, reduction reactions; most known copper-containing enzymes binding and react directly with molecular oxygen. Copper is an integral component of many metaloenzymes including cereuloplasmin, cytochrome C, oxidase, superoxide dismutase, dopamine-B-hydroxylase, ascorbate oxidase, lysyloxidase and tyrocinase. Cereuloplasmin, the major copper-containing protein in plasma. **[Burits, et al. 1996]**

Copper is also required for appropriate cross linking of collagen, being an essential component of lysyl oxidase. **[Bayness, et al. 1999]**

2.10.1. Metabolism of copper:

Copper absorption is maximal in the duodenum and may be absorbed from the stomach. Within the intestinal mucosal cells, copper can react with metalothionine, a sulfhydryl group-rich protein that's binds copper through the formation of mercap-tide bonds. Other metal ions, particularly zinc and cadmium, compete with copper for sulfhydryl binding sites which explain the antagonism of these metals toward copper absorption. Absorbed copper is rapidly transported to the liver where it is stored, mostly as metalothionin-like cuproprotein. Copper is released from the liver, mainly as ceruloplasmin and transported to cells for

incorporation into copper-containing enzymes by several identified transport mechanisms. These includes ceruloplasmin, transcuprein, copper-albumin, and copper-aminoacid complexes. The movement of copper within the cell and its incorporation into cuproproteins may be regulated by both glutathione and metalothionein. **[Burits, et al. 1996]**

2.10.2. Clinical significant:

Elevated serum copper concentrations are seen in portal cirrhosis, biliary tract disease, rheumatoid arthritis and hepatitis. Hypocopraemia has been observed in hemolytic jaundice, hemochromatosis and some type of hepatic cirrhosis. Signs of copper deficiency include: neuroneutropenia and hypochromic anemia, osteoporosis, decrease pigmentation of the skin, and the later stages, possible neurological abnormalities.The copper deficiency diseases are Menke's syndrome and Wilson diseases. **[Burits, et al. 1996]**

2.11. Uric acid metabolism:

Purine nucleutides are essential components of nucleic acid; they are intimately involved in energy transformation and phosphorylation reaction and act as intracellular messengers. There are three sources of purine in human; the diet, degradation of endogenous nucleotide and de novo synthesis. Since purines are metabolized to uric acid, the body urate pool and hence plasma concentration depends on the relative rate of both urate formation from these sources and urate excretion. **[Marshall. W. J. 2000]**

Uric acid is by far the highest concentration antioxidant in human blood. Studies of high altitude acclimatization support the hypothesis that urate act as an antioxidant by mitigating the oxidative stress caused by high altitude hypoxia. In animal studies that investigate diseases facilitated by oxidative stress, introduction

of uric acid both prevents the disease or reduces it, leading researcher to propose this is due to uric acid antioxidant properties. **[Ames, et al. 1981]**

Human convert the major purine nucleoside adenosine and guanosine to uric acid. Adenosin is first deaminated to inosine by adenosine deaminase. Phosphorolysis of the N-glycosidic bond of inosine and gunosine catalyzed by purine nucleoside phosphorylase, release ribose 1-phosphate and purine base. Hypoexantine and guanine next form xanthine in reaction catalyzed by xanthine oxidase and guanase respectively. Xanthine is then oxidized to uric acid in second reaction catalyzed by xanthine oxidase. **[Murray, et al .1999]**

Two third of the acid produced daily are excreted by the kidneys whereas the remaining third exits by the stool. **[Burits, et al. 1996]**

Urate handling by the kidney is complex. It is filtered at the glomeruli and is almost totally absorbed in the proximal convoluted tubules; distally, both secretion and reabsorption occur. Normal urate clearance is about 10% of the filtered load. In normal subjects, urate excretion increases if the filtered load is increased. In chronic renal failure, the plasma concentration rises only when the glomerular filtration rate falls below about 20 ml/ min. Dietary purine account for about 30% of excreted urate. The introduction of a purine-free diet typically reduces plasma urate concentrations by only 10-20% **[Marshall. 2000]**

2.11.1. Clinical implications:

- Elevated uric acid level occur in the following conditions:

- Gout

- Renal disease

- Alcoholism

- Leukemia

- Starvation

- Liver disease

- Hyperlipidemia

- Hemolytic anemia

- Cirrhosis

- Decreased level of uric acid occur in the following conditions:

- Fanconi’s syndrome

- Wilson’s disease

- SIADH

- Some malignancies [e.g. Hodgking’s disease, multiple myeloma, xanthine urea [deficiency of xanthine oxidase].

[Burits, et al. 1996]

Chapter Three

3. Materials and Methods:

3.1. Study design:

Analytical case control study.

3.2. Study area:

Social base study,Khartoum state

3.3. Study periods:

The study was carried during the period from February to May 2016.

3.4. Study population:

50 healthy adult male as control compared with 50[case group]

Each groups were matched in age [25-65 years]

3.5. Selection criteria:

Inclusion criteria:

Toombak users as test group and non Toombak users as control group, both test and control group were apparently healthy individual.

Exclusion criteria:

Smoker, alcohol abuser and all Toombak users with chronic diseases, hereditary diseases, and benzene workers.

3.6. Sample size:

The study sample size was 100 (50 Toombak users as test group and 50 non Toombak users as control) were enrolled in this study.

Sample collection:

Whole saliva was collected from each subject without oral stimulus. In a sitting position, the participants were asked to a swallow saliva, then stay motionless and allow the saliva to drain passively in a sterile container.

Saliva samples where immediately centrifuged.

3.8. Ethical consideration:

Objective of this study were explained to all participating in this study.

Information were obtained from all participating in this study were kept as highly

Confidence, data and specimen result we are not be permitted.

3.9 Data collection:

Interview and questionnaire: interview with Toombak users and control were done to obtain the clinical data, and it was specifically designed to obtain information which help in either including or exclusion certain individual in or from study .See questioner sheet. **(Appendix i)**

3.10. Biochemical measurements:

3.10.1. Superoxide dismutase:

Superoxide dismutase activity was measured using spectrophotometer analyzer and reagent from fortress diagnostics. **(Appendix ii,iii)**

Principle:

Superoxide dismutase (SOD) role is to accelerate the dismutation of the toxic superoxide radical, produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen.

Fortress method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl) – 3-(4- nitrofenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye which read in 505 nm. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a50%inhibition of the rate of reduction of INT under the conditions of the assay.

3.10.2. Zn^{+2}, Cu^{+2}:

Copper and zinc were measured using atomic absorption spectroscopy. **(Appendix iv,v)**

Principle:

All modern Perkin Elmer atomic absorption instruments are capable of measuring both atom absorption and atom emission. It is important for the operator to understand the process that occur in each technique.

Every element has a specific number of electrons associated with its nucleus. The normal and most stable orbital configuration of an atom is known as the “ground state”. If energy is applied to an atom, the energy will be absorbed and outer electron will be promoted to a less stable configuration known as the “excited state”. Since this state is unstable, the atom will immediately return to the “ground state” releasing light energy.

Excitation:

Energy + Ground state atom → Excited state atom

Decay:

Excited state atom → Ground state atom + Light energy

The sample is subjected to a high thermal environment in order to produce excited-state atoms. This environment can be provided by a flame or more recently a plasma. However, since the excited state is unstable, the atoms spontaneously return to “ground state” and emit light. The emission spectrum of an elements consists of a collection of emission wave lengths called emission lines because of the discrete nature of the emitted wave lengths. The intensity of an emission line will increase as the number of the excited atoms of the element increases. **[Elmer.P, 1996]**

Atomic absorption process:

The “ground state” atom absorbs light energy of a specific wave length as it enters the excited states. As the number of atoms in the light path increases, the amount of light absorbed also increases. By measuring the amount of light absorbed, a quantitative determination of the amount of analyte can be made. The use of special light sources and careful selection of wave length allow the specific determination of individual elements. **[Elmer.P.1996]**

3.10.3. Uric acid:

Uric acid level was measured using spectrophotometer analyzer and reagent from bio system diagnostic. **(Appendix vi)**

Principle:

Uric Acid is oxidized by Uricase to allantoin and hydrogen peroxide.

The released hydrogen peroxide together with DCHPS and 4-aminoantipyrine, in the presence of peroxides, forms a red dye compound. The intensity of the red collar are produced is directly proportional to the uric acid quantity.

3.11. Data analyses:

Using for data analyses SPSS program version 16.5

3.12. Quality control:

Using quality control in our study for majoring, superoxide dismutase, Cu, Zn and uric acid in saliva.

Chapter Four

4. Results:

Antioxidant compounds [superoxide dismutase, uric acid, copper and zinc were measured in saliva of 50 Toombak users as test group and non-Toombak users as control group, during the period of February to May 2016.

Table [4-1]: showed significant decrease in superoxide dismutase activity in Toombak users compared with non-Toombak users control group [mean ± SD: 2.16 ± 0.76 versus 4.96 ± 2.21, P = 0.02], uric acid level and copper showed insignificant result, uric acid [mean ± SD: 1.44 ± 0.89 versus 2.36 ± 1.33, P = 0.15], copper level [mean ± SD: 1.07 ± 1.02 versus 1.47 ± 0.97, P = 0.1] and zinc level showed significant increase in Toombak users compared with non-Toombak users [mean ± SD: 0.15 ± 0.04 versus 0.23 ± 0.15, P = 0.01]

Figure [4-1]: A scatter plot shows negative weak correlation between the level of superoxide dismutase and a number of Toombak using per day [r = -0.40, P = 0.00]

Figure [4-2]: A scatter plot shows negative weak correlation between the level of uric acid and a number of Toombak using per day [r = 0.02, P = -0.33]

Figure [4-3]: A scatter plot shows no correlation between the level of copper and a number of Toombak using per day [r = -0.17, P = 0.25]

Figure [4-4]: A scatter plot shows no correlation between the level of zinc and a number of Toombak using per day [r = -0.03, P = 0.82]

Figure [4-5]: A scatter plot shows negative weak correlation between the level of superoxide dismutase and duration of Toombak using [r = -0.35, P = 0.01]

Figure [4-6]: A scatter plot shows no correlation between the level of uric acid and duration of Toombak using [r = -0.14, P = 0.33]

Figure [4-7]: A scatter plot shows no correlation between the level of copper and duration of Toombak using [r = 0.13, P = 0.38]

Figure [4-8]: A scatter plot shows no correlation between the level of zinc and duration of Toombak using [r = -0.03, P = 0.82]

Table (4-1) comparison of superoxide dismutase, uric acid, copper, and zinc in Toombak users and non Toombak users control group:

Variable	Toombak users N=50 Mean ± SD	Non Toombak users N=50 Mean ± SD	P-value
Superoxide dismutase activity (U/g)	2.16 ± 0.76	4.96 ± 2.21	0.02
Uric acid level (mg/dl)	1.44 ± 0.89	2.36 ± 1.33	0.15
Copper level (mg/l)	1.07 ± 1.02	1.47 ± 0.97	0.1
Zinc level (mg/l)	0.15 ± 0.04	0.23 ± 0.15	0.00

The table shows the mean ± SD, and the probability (p).

T-test was used for comparison.

P-value ≤ 0.05 is considered significant.

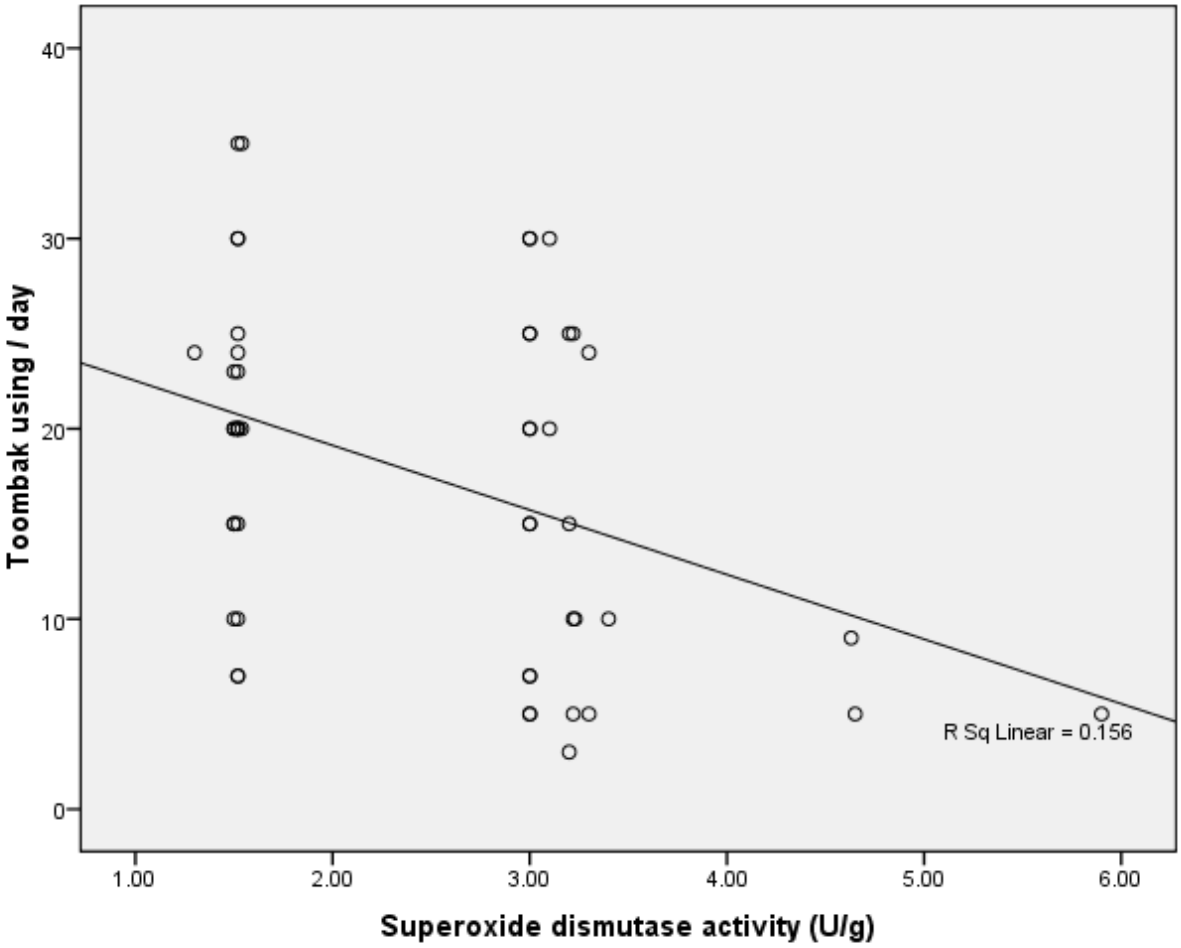

Figure (4.1) correlation between the activity of superoxide dismutase and number of Toombak using per day (r=-0.40, P=0.00).

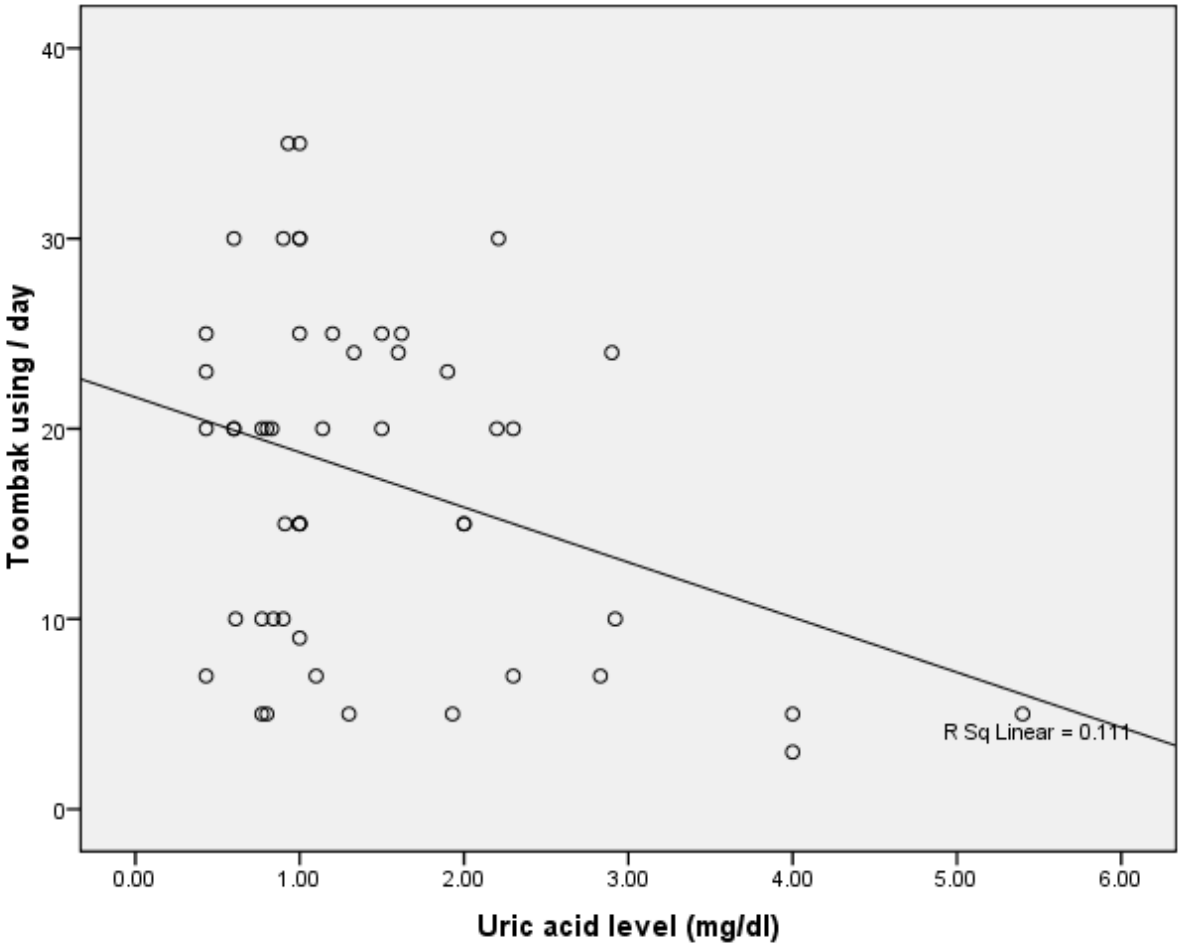

Figure (4.2) correlation between the level of uric acid and number of Toombak using per day (r =-0.33, P=0.02).

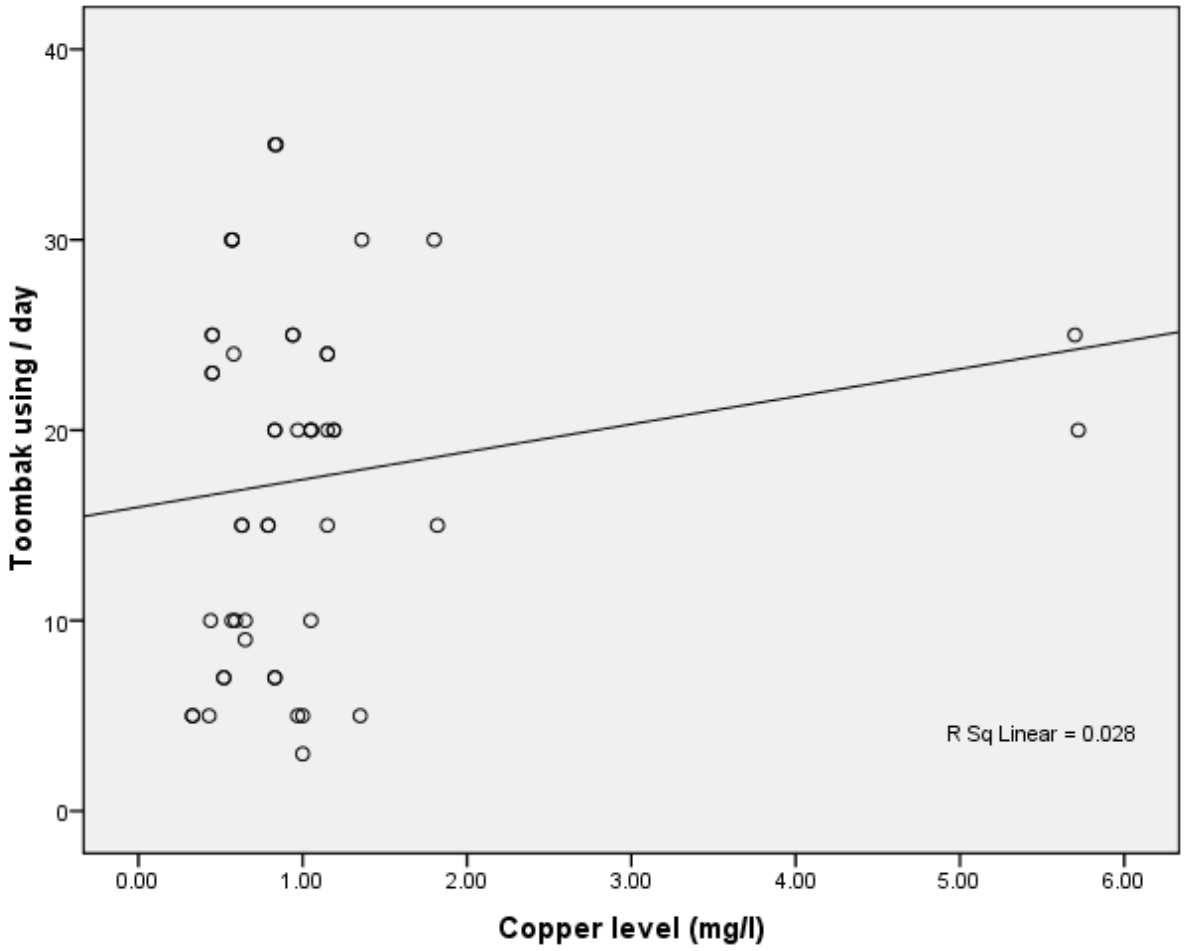

Figure (4.3) No correlation between the level of copper and number of Toombak using per day (r= 0.17, P=0.25).

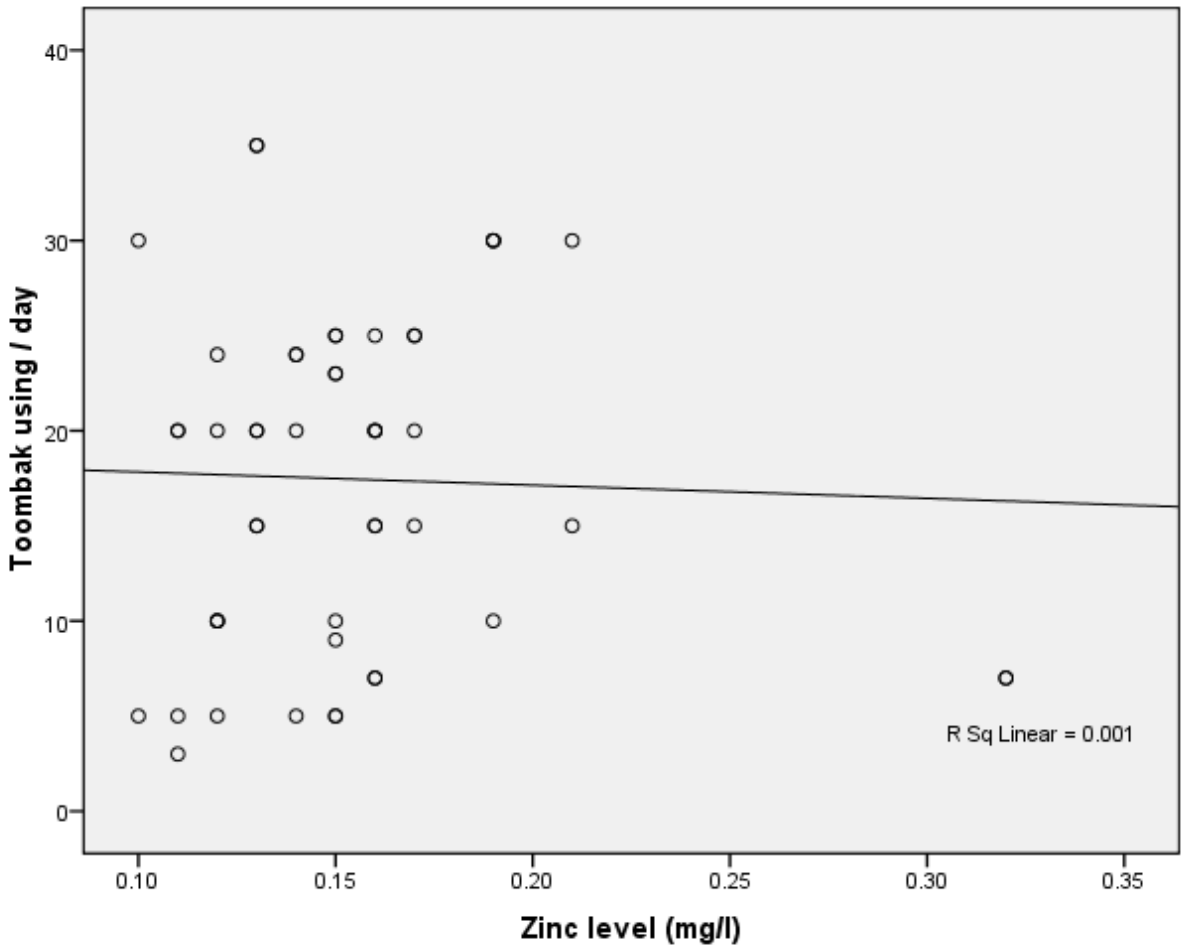

Figure (4.4) No correlation between the level of zinc and number of Toombak using per day (r=-0.03, P=0.82).

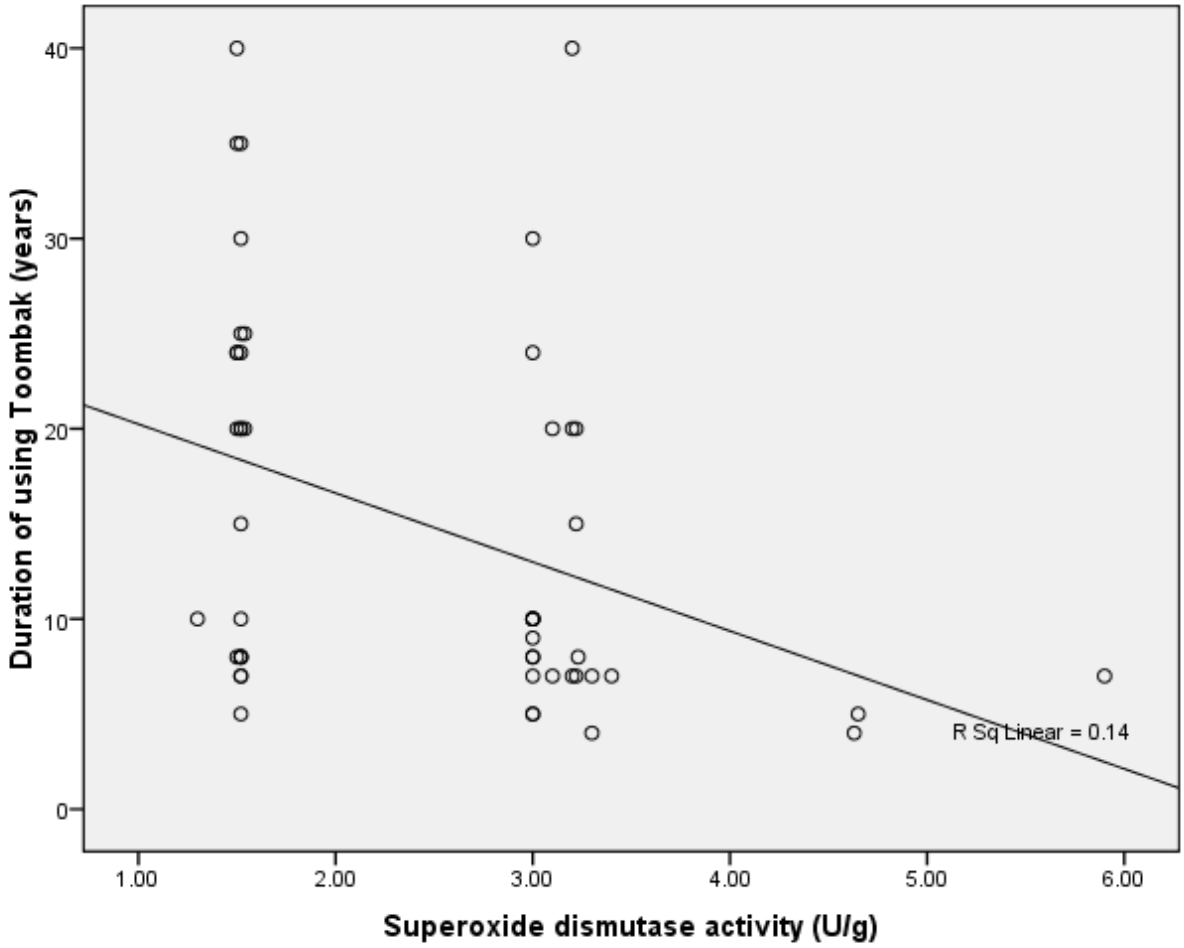

Figure (4.5) correlation between the activity of superoxide dismutase and duration of using Toombak (r=-0.35, P=0.01).

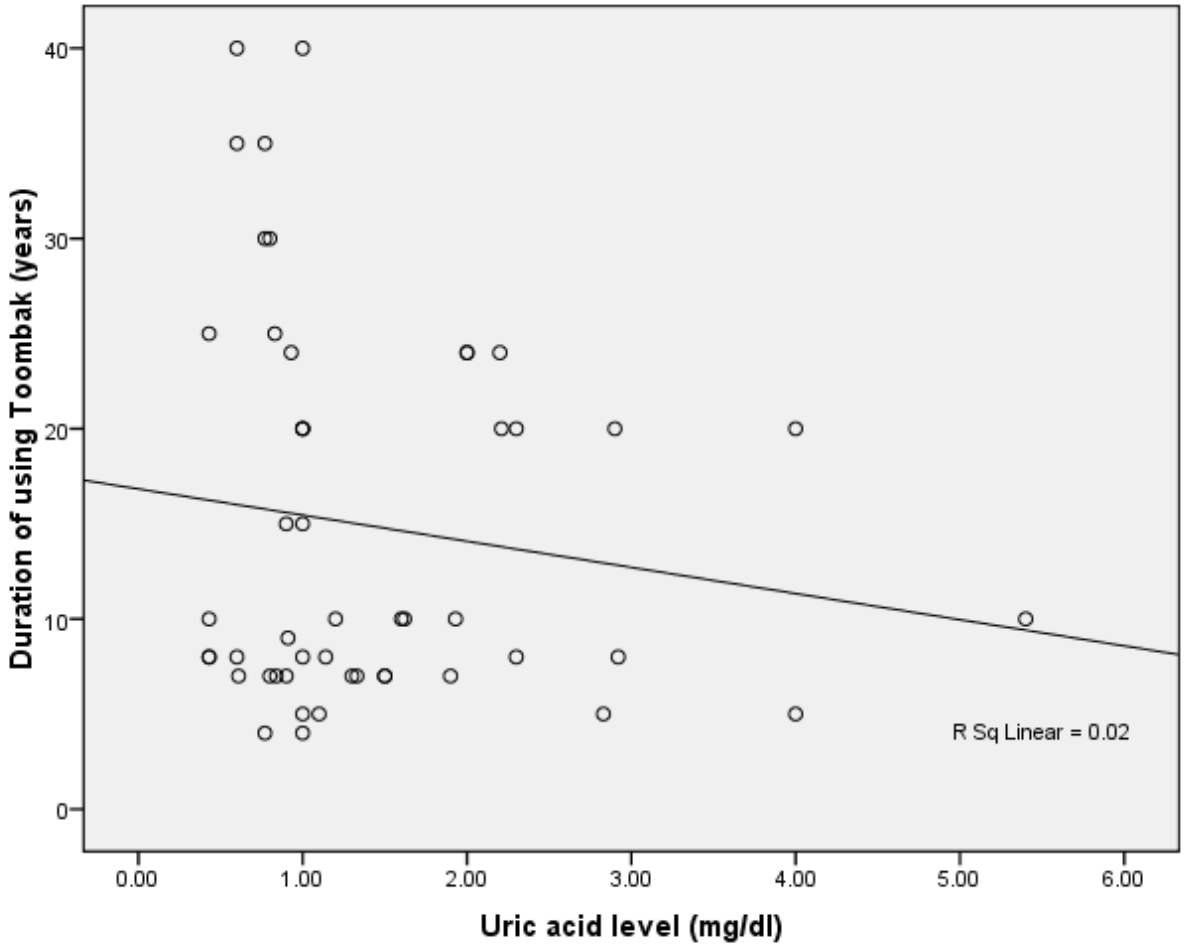

Figure (4.6) No correlation between the level of uric acid and duration of using Toombak (r=-0.14, P=0.33).

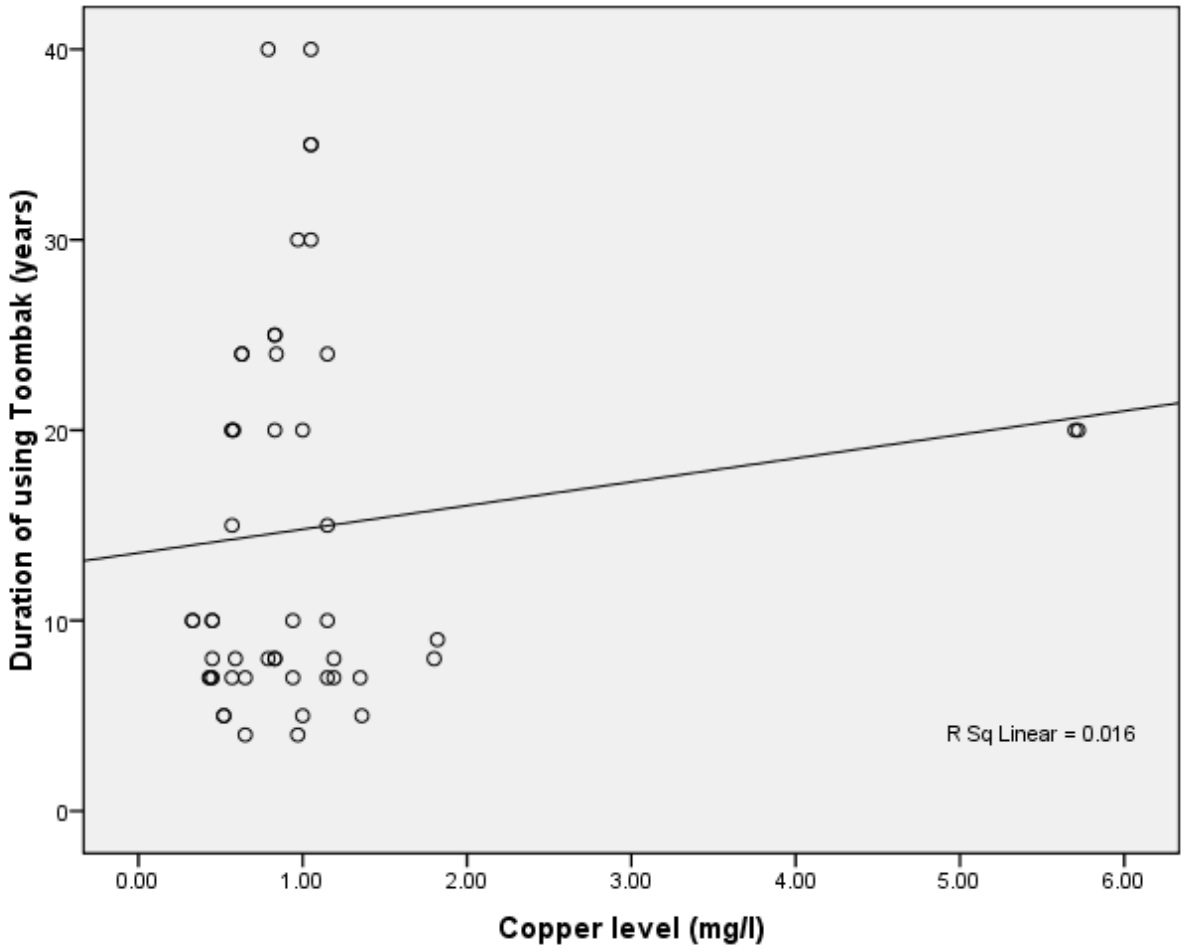

Figure (4.7) No correlation between the level of copper and duration of using Toombak (r=0.13, P=0.38).

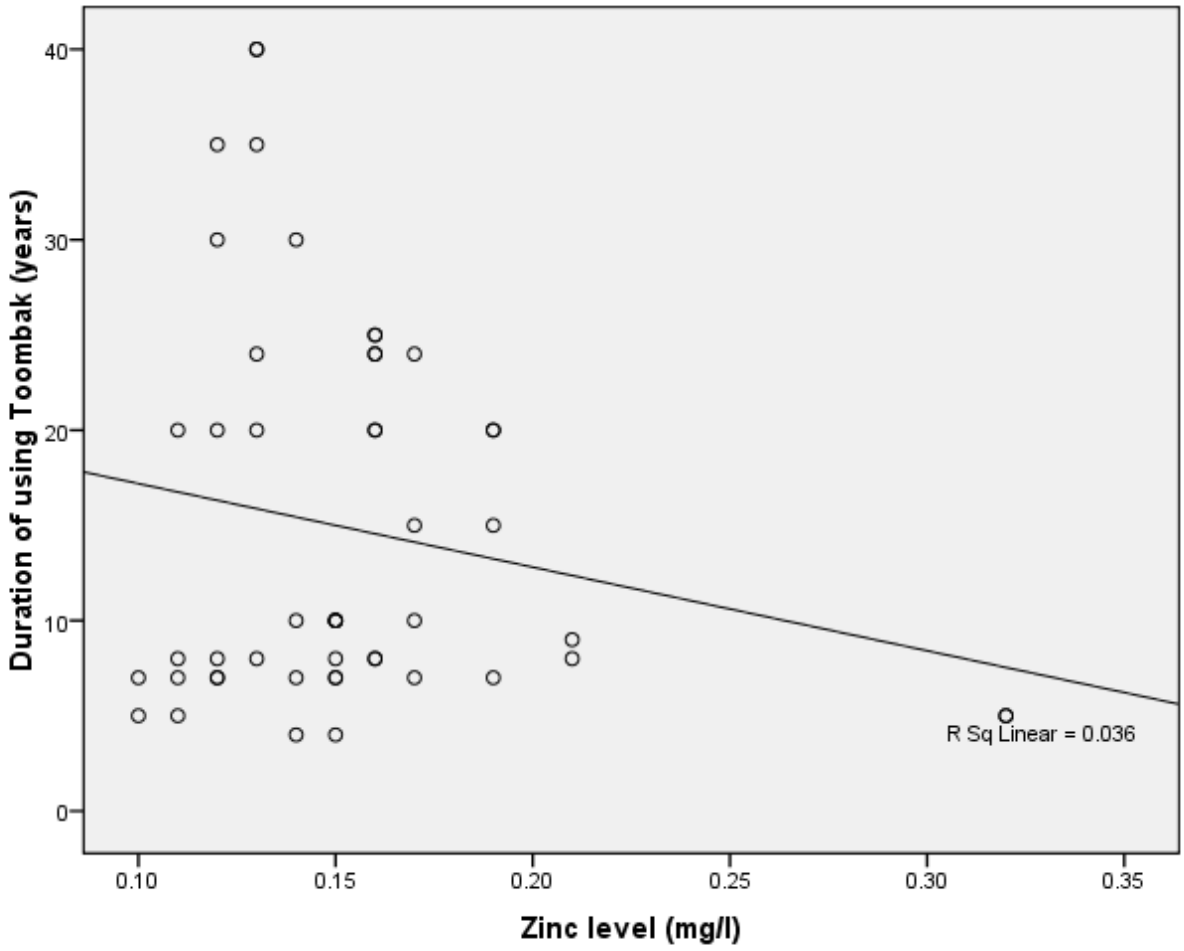

Figure (4.8) No correlation between the level of zinc and duration of using Toombak (r=-0.19, P=0.19).

Chapter Five

5. Discussion, Conclusion, Recommendation

5.1 Discussion:

Toombak using is a serious health problem and most important risk factor for oral cancer. The risk of disease increased with increased intensity and duration of Toombak using. In this study, the level of superoxide dismutase was found to be significantly decreased in Toombak users compared with non-Toombak users [2.16 ± 0.76 versus 4.96 ± 2.21] [p 0.02]. The level of uric acid and coppor were found to be insignificant result, and the level of zinc was found to be significantly increased, [0.15 ± 0.04 versus 0.23 ± 0.15] [p 0.00]. Justification of this study result is that SOD is a naturally occurring enzyme that protect the body against active oxygen free radicals by scavenging excess superoxide. Toombak contains abundant amount of oxidants and superoxide [O_2^-]. Thus damaging oral endothelium cells. Uric acid is one of the most important antioxidants and contribute approximately 70 % of the total salivary antioxidant capacity. Since zinc and copper are cofactors for SOD and they are one of the antioxidants trace elements, so the study included measuring their levels.

This finding within the same line with those obtained by [Hamid. A, et al.2011] which found decreased in SOD and uric acid activity in Toombak users compare with control [6.24 ± 2.62 versus 8.07 ± 1.30, uric acid were measured 2.8 ± 2.1 versus 3.7 ± 2.6]

In Toombak users groups, the study showed that the superoxide dismutase has a negative weak correlation [r = -0.40, P = 0.00], uric acid has a negative weak correlation [r = 0.02, P = -0.33], copper has no correlation [r = -0.17, P = 0.25], and zinc has no correlation [r = -0.03, P = 0.82], with a number of Toombak using per day.

Also, the study showed that the superoxide dismutase has a negative weak correlation [r = -0.35, P = 0.01], uric acid has no correlation [r = -0.14, P = 0.33], copper has no correlation [r = 0.13, P = 0.38] and zinc has no correlation [r = -0.03, P = 0.82], with duration of Toombak using.

5-2 Conclusion:

From this study concluded that:

1- The level of superoxide dismutase, showed significant decrease in Toombak users.
2- The level of zinc showed significant increase in Toombak users.
3- In Toombak users groups, the superoxide dismutase and uric acid has a negative weak correlation, Copper and zinc has no correlation, with a number of Toombak using per day.
4- In Toombak users groups the superoxide dismutase has negative weak correlation, uric acid, copper and zinc have no correlation, with duration of Toombak using.

5-3 Recommendations:

1- Periodic follow up of the superoxide dismutase enzyme because Toombak using is consider as risk factor.
2- Uric acid measure oxidative stress in saliva.
3-Using of SOD enzyme as a biomedical marker for oxidative stress.
4- Increase awareness to the population about the risk of using Toombak.
5- Good nutrition and healthy life style help to live healthy.

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Appendices

Sudan University for Science and Technology

Collage of Graduate Studies

Assessment of Salivary Anti-oxidant Compounds [Superoxide dismutase, Zn, Cu and uric acid] in Sudanese Tobacco Users

Questionnaire

Name:……..... **Age:**…….

Gender:…… **Occupation:**…….

Duration of use:

……

Time per day:

……

Parameters measured:

Superoxide dismutase ……....

Zn……

Cu…….

Uric acid……..

fortress diagnostics

SUPEROXIDE DISMUTASE

PRODUCT CODE: BXC0531

CE QUALITY MANAGEMENT SYSTEM CERTIFIED BY SGS TO ISO 13485 & ISO 9001 GB04/63491.00 GB04/63490.00

BXC0531A
5 x 20ml
STORE AT 2-8°C
INSTRUCTIONS FOR USE
FOR IN-VITRO DIAGNOSTIC USE ONLY

IVD For In Vitro Diagnostics Use Only
LOT Lot Number
REF Catalogue Number
Storage Temperature
Expiry Date (Year / Month)
Warning, Read Enclosed Documents
Instructions For Use
Manufactured By

SUPEROXIDE DISMUTASE

XANTHINE

Kit Contents:	BXC0531A
R1: Mixed Substrate	5 x 20 ml
R2: Buffer	1 x 100 ml
R3: Xanthine Oxidase	3 x 10 ml
R4: Standard	5 x 10 ml
R6: Sample Diluent	2 x 125 ml

Test Principle:
Superoxide dismutase (SOD) role is to accelerate the dismutation of the toxic superoxide radical (02•), produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen.
Fortress method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Summary:
This kit is used for the determination of Suoeroxide Dismutase (SOD) in whole blood samples.
Superoxide Dismutase function is to accelerate the dismutation of the toxic superoxide radical (O_2^-), produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen.
This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye.
The SOD activity is then measured by the degree of inhibition of this reaction.

$$\text{Xanthine} \xrightarrow{\text{Xanthine Oxidase}} \text{Uric Acid} + O_2^-$$

$$\text{I.N.T.} \xrightarrow{O_2^-} \text{Formazan Dye}$$

OR

$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

Reagent Concentration:

R1 Mixed Substrate	Xanthine	0.05 mmol/l
	I.N.T	0.025 mmol/l
R2 Buffer	CAPs	
	EDTA	0.94 mmol/l
R3 Xanthine Oxidase	80 U/l	
R4 Standard	Lot Specific	
R6 Sample Diluent	Phosphate Buffer pH 7.0	0.01 mol/l

Reagent Handling and Preparation:
1. Mixed Substrate (R1)
Reconstitute the contents of one vial of Mixed Substrate R1 with 20 ml of Buffer R2. Stable for 10 days when stored at +2 to +8°C.
2. Buffer (R2)
Contents ready for use. Stable up to the expiry date when stored at +2 to +8°C.
3. Xanthine Oxidase (R3)
Reconstitute the contents of one vial of Xanthine Oxidase R3 with 10 ml of distilled water. Stable for 14 days when stored at +2 to +8°C.
4.Standards (R4)
Reconstitute one vial of standard R4 with 10ml of distilled water. Subsequent dilutions of this standard must be made with the sample diluent (R5). The following dilutions are made of standard S6 to produce a standard curve.

	Volume of standard solution	Volume of Sample diluent
S6	UNDILUTED STANDARD	—
S5	5 ML OF S6	5ml
S4	5 ML OF S5	5ml
S3	5 ML OF S4	5ml
S2	5 ML OF S3	6ml

S1 = SAMPLE DILUENT
DILUTED STANDARDS ARE STABLE FOR 14 DAYS AT 2-8°C.
5. Sample Diluent (R6)
Contents ready for use. Stable up to the expiry date specified when stored at +2 to +8°C.

Sample:
Heparinised / EDTA whole blood samples. We recommend that red cells should be washed 4 times with 0.9% saline solution.
0.5ml of whole blood is centrifuged for 10 minutes at 3500 rpm and then aspirate off the plasma.
Wash red cells 4 times with 5 ml of 0.9% saline and centrifuged for 10 minutes at 3500 rpm after each run.
The washed centrifuged erythrocytes should then be made up to 2.0 ml with cold distilled water, mixed and left to stand at 4°C for 15 minutes.
The lysate is diluted with sample diluent so that the percentage inhibition falls between 30%-60%.
A 25 fold dilution of lysate is recommended for human samples (final dilution factor=100) and a 50 fold dilution for bovine samples (final dilution factor=200).

Manual Procedure:

Wavelength	Temperature	Cuvette	Measurement
505 nm	37°C	1 cm light path	Against Air or distilled water

Pipette into test tubes as follows:

	Sample Diluent	Standards S2-S6	Diluted Sample
Diluted Sample / Saliva	—	—	50 ul
Standard (R4)	—	50 ul	—
Sample Diluent (R6)	50 ul	—	—
Mixed Substrate (R1)	1.7 ml	1.7 ml	1.7 ml
Mix well. Then add:			
Xanthine Oxidase (R3)	250 ul	250 ul	250 ul

Mix incubate for 30 seconds at 37°C, then take the first reading. Read again after 1, 2 and 3 minutes.
Determine the mean absorbance change per minute (ΔA) and use it in the calculation.

Calculation:
To calculate the superoxide dismutase value

$$\frac{A_2 - A_1}{3} = \Delta A/\text{min of standard or sample}$$

sample diluent rate (S1 rate) = rate of uninhibited reaction = 100%

All standard rates and diluted sample rates must be converted into percentages of the sample diluent rate, and subtracted from 100% to give a percentage inhibition.

$$100 - \frac{A_{std/min} \times 100}{A_{s1/min}} = \%\ \text{inhibition}$$

$$100 - \frac{A_{sample/min} \times 100}{A_{s1/min}} = \% \text{ inhibition}$$

Plot percentage inhibition for each standard against Log_{10} (standard conc. In SOD units/ml)

Use percentage inhibition of sample to obtain units of SOD from standard curve.
SOD units/ml of whole blood = Sd units /ml from standard curve x dilution factor.

Conversion to SOD units/g Haemoglobin

SOD units/ml = SOD units/g Haemoglobin
g Haemoglobin/ml

Example:
1) A sample was diluted 1 in 300 times with SOD sample diluent. The diluted sample gave an inhibition of 33%.
From the standard curve:
Number of SOD units in sample = 0.575
2) Conversion to SOD units/ml of whole blood
0.575 x 300 = 172.5 SOD units/ml
3) Conversion to SOD units/g Haemoglobin
Sample Haemoglobin value = 0.118 g/ml

$$\text{(SOD units/ml)(g Haemoglobin/ml)} = \frac{172.5}{0.118} = 1461.9$$

Linearity:
Samples should be diluted to give an inhibition between 30% and 60% of the sample diluent rate i.e. the uninhibited reaction.

Normal Values:

1102-1601 U/g Hb	164-240 U/ml

These values are supplied as a guideline only.
It is recommended that a laboratory establishes its' own reference Range.

Use on Automated Analysers:
This reagent is suitable for use on a range of automated analysers. Specific instructions for these applications are available on request from our technical department.

Quality Control:
It is recommended that a laboratory uses normal and elevated reference control sera to verify the performance of the procedure, both performance of the reagent and any instrumentation employed in the determination. Results obtained should fall within the specified ranges.
Fortress SOD Control Cat No BXC0433A – 5 x 1 ml

Fortress QC materials of human source have been tested at donor level for HbsAg Antigen, HIV1&2 antibodies and HCV antibody and found to be negative. However no test can offer complete assurance to the absence of infectious diseases so all material should be handled and disposed of as if it is potentially infectious.

If results fall outside the acceptable range appropriate action as determined by the laboratory's internal quality procedures should be taken.
Some common reasons for incorrect results can be:
1. Wavelength used for the determination
2. Light source
3. Temperature
4. Cleanliness, e.g of cuvettes used in measurements
5. Bacterial contamination of reagent
6. Reagent expiry
7. Calibration frequency

Health & Safety:
This kit is designed for use by suitably qualified laboratory personnel only. Exercise the normal precautions required for the handling of laboratory reagents. Do not ingest the material. Dispose of material according to local guidelines.

References:
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Cu (29)

Standard Atomic Absorption Conditions for Cu

Wavelength (nm)	Slit (nm)	Relative Noise	Characteristic Concentration (mg/L)	Characteristic Concentration Check (mg/L)	Linear Range (mg/L)
324.8	0.7	1.0	0.077	4.0	5.0
327.4	0.7	1.1	0.17	8.0	5.0
216.5	0.2	7.2	0.117	20.0	20.0
222.6	0.2	5.9	1.1	50.0	50.0
249.2	0.7	1.7	5.8	300.0	100.0
224.4	0.2	6.0	14.0	650.0	---
244.2	0.7	2.2	24.0	1000.0	---

1. Recommended Flame: air-acetylene, oxidizing (lean, blue)
2. Data obtained with a standard nebulizer and flow spoiler. Operation with a High Sensitivity nebulizer or impact bead will typically provide a 2-3 × sensitivity improvement.
3. Characteristic Concentration with a N_2O-C_2H_2 flame at 324.8 nm: 0.45 mg/L

Standard Flame Emission Conditions for Cu

Wavelength (nm)	Slit (nm)	Flame
327.4	0.2	Nitrous oxide-acetylene

Stock Standard Solution

COPPER, 1000 mg/L. Dissolve 1.000 g of copper metal in a minimum volume of (1+1) HNO_3. Dilute to 1 liter with 1% (v/v) HNO_3.

Light Sources

With multielement lamps containing nickel or iron, a 0.2 nm spectral slit width should be used with the copper 324.8 nm line.

Zn (30)

Standard Atomic Absorption Conditions for Zn

Wavelength (nm)	Slit (nm)	Relative Noise	Characteristic Concentration (mg/L)	Characteristic Concentration Check (mg/L)	Linear Range (mg/L)
213.9	0.7	1.0	0.018	1.0	1.0
307.6	0.7	0.38	79.0	3500.0	—

1. Recommended Flame: air-acetylene, oxidizing (lean, blue)
2. Data obtained with a standard nebulizer and flow spoiler. Operation with a High Sensitivity nebulizer or impact bead will typically provide a 2-3 × sensitivity improvement.
3. Characteristic Concentration with a N_2O-C_2H_2 flame at 213.9 nm: 0.084 mg/L
4. Table contains HCL data. EDL sensitivity values approximately the same.

Standard Flame Emission Conditions for Zn

Wavelength (nm)	Slit (nm)	Flame
213.9	0.2	Nitrous oxide-acetylene

Stock Standard Solution

ZINC, 500 mg/L. Dissolve 0.500 g of zinc metal in a minimum volume of (1+1) HCl and dilute to 1 liter with 1% (v/v) HCl.

Light Sources

Both Electrodeless Discharge Lamps (EDLs) and Hollow Cathode Lamps are available for zinc. EDLs provide greater light output and longer life than Hollow Cathode Lamps. For zinc, both EDLs and Hollow Cathode Lamps provide approximately the same sensitivity and detection limit.

COD 11821 1 x 50 mL	COD 11521 1 x 200 mL	COD 11522 1 x 500 mL	COD 11540 1 x 1 L
STORE AT 2-8°C			
Reagents for measurement of uric acid concentration Only for *in vitro* use in the clinical laboratory			

URIC ACID

BioSystems
REAGENTS & INSTRUMENTS

CE

URIC ACID
URICASE/PEROXIDASE

PRINCIPLE OF THE METHOD

Uric acid in the sample originates, by means of the coupled reactions described below, a coloured complex that can be measured by spectrophotometry[1,2].

$$\text{Uric acid} + O_2 + 2\,H_2O \xrightarrow{\text{uricase}} \text{Alantoin} + CO_2 + H_2O_2$$

$$2\,H_2O_2 + 4\text{-Aminoantipyrine} + \text{DCFS} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\,H_2O$$

CONTENTS

	COD 11821	COD 11521	COD 11522	COD 11540
A. Reagent	1 x 50 mL	1 x 200 mL	1 x 500 mL	1 x 1 L
S. Standard	1 x 5 mL	1 x 5 mL	1 x 5 mL	1 x 5 mL

COMPOSITION

A. Reagent: Phosphate 100 mmol/L, detergent 1.5 g/L, dichlorophenolsulfonate 4 mmol/L, uricase > 0.12 U/mL, ascorbate oxidase > 5 U/mL, peroxidase > 1 U/mL, 4-aminoantipyrine 0.5 mmol/L, pH 7.8.

S. Uric Acid Standard: Uric acid 6 mg/dL (357 µmol/L). Aqueous primary standard.

STORAGE

Store at 2-8°C.

Reagent and Standard are stable until the expiry date shown on the label when stored tightly closed and if contaminations are prevented during their use.

Indications of deterioration:

- Reagent: Presence of particulate material, turbidity, absorbance of the blank over 0.200 at 520 nm (1 cm cuvette).
- Standard: Presence of particulate material, turbidity.

REAGENT PREPARATION

Reagent and Standard are provided ready to use.

ADDITIONAL EQUIPMENT

- Thermostatic water bath at 37°C
- Analyzer, spectrophotometer or photometer able to read at 520 ± 10 nm

SAMPLES

Serum, plasma or urine collected by standard procedures. Dilute urine 1/10 with distilled water before measurement

Uric acid in serum or plasma is stable for 7 days at 2-8°C. Heparin, EDTA, oxalate and fluoride may be used as anticoagulants.

Uric acid in urine is stable for 4 days at room temperature if pH is adjusted to > 8 with NaOH. Do not refrigerate.

PROCEDURE

1. Bring the Reagent to room temperature.
2. Pipette into labelled test tubes: (Note 1)

	Blank	Standard	Sample
Distilled water	25 µL	—	—
Uric Acid Standard (S)	—	25 µL	—
Sample	—	—	25 µL
Reagent (A)	1.0 mL	1.0 mL	1.0 mL

3. Mix thoroughly and incubate the tubes for 10 minutes at room temperature (16-25°C) or for 5 minutes at 37°C.
4. Measure the absorbance (A) of the Standard and the Sample at 520 nm against the Blank. The colour is stable for at least 30 minutes.

CALCULATIONS

The uric acid concentration in the sample is calculated using the following general formula:

$$\frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times C_{\text{Standard}} \times \text{Sample dilution factor} = C_{\text{Sample}}$$

If the Uric Acid Standard provided has been used to calibrate (Note 2):

	Serum and plasma	Urine
$\frac{A_{\text{Sample}}}{A_{\text{Standard}}}$	x 6 = mg/dL uric acid x 357 = µmol/L uric acid	x 60 = mg/dL uric acid x 3570 = µmol/L uric acid

REFERENCE VALUES

Serum and plasma[3]

Men: 3.5-7.2 mg/dL = 210-420 µmol/L
Women: 2.6-6.0 mg/dL = 150-350 µmol/L

Urine[3]

250-750 mg/24-h = 1.5-4.5 mmol/24-h

These ranges are given for orientation only; each laboratory should establish its own reference ranges.

QUALITY CONTROL

It is recommended to use the Biochemistry Control Serum level I (cod. 18005, 18009 and 18042), level II (cod. 18007, 18010 and 18043) and the Biochemistry Control Urine (cod. 18054) to verify the performance of the measurement procedure.

Each laboratory should establish its own Internal Quality Control scheme and procedures for corrective action if controls do not recover within the acceptable tolerances.

METROLOGICAL CHARACTERISTICS

- Detection limit: 0.02 mg/dL = 1.19 µmol/L
- Linearity limit: 25 mg/dL = 1487µmol/L. For higher values dilute sample 1/5 with distilled water and repeat measurement.
- Repeatability (within run):

Mean Concentration	CV	n
5.00 mg/dL = 298 µmol/L	0.4 %	20
8.22 mg/dL = 489 µmol/L	0.5 %	20

- Reproducibility (run to run):

Mean Concentration	CV	n
5.00 mg/dL = 298 µmol/L	2.1 %	25
8.22 mg/dL = 489 µmol/L	1.9 %	25

- Sensitivity: 33.3 mA·dL/mg = 0.56 mA·L/µmol
- Trueness: Results obtained with this reagent did not show systematic differences when compared with reference reagents (Note 2). Details of the comparison experiments are available on request.
- Interferences: Hemoglobin (2 g/L), bilirubin (2.5 mg/dL) and lipemia interfere. Other drugs and substances may interfere.

These metrological characteristics have been obtained using an analyzer. Results may vary if a different instrument or a manual procedure are used.

DIAGNOSTIC CHARACTERISTICS

In humans, uric acid is the major product of the catabolism of the purine bases which are obtained partly from the diet and partly from *in vivo* synthesis.

Increased uric acid concentration in serum and urine maybe attributable to an overproduction of urate (increased purine synthesis) or to a defective elimination of urate[3].

Hyperuricemia is commonly associated with gout, decreased renal function, dehydratation, myeloproliferative disorders, and other conditions not well known[3,5].

Clinical diagnosis should not be made on the findings of a single test result, but should integrate both clinical and laboratory data.

NOTES

1. These reagents may be used in several automatic analysers. Specific instructions for application in many of them are available on request.
2. Calibration with the provided aqueous standard may cause a matrix related bias, specially in some analyzers. In these cases, it is recommended to calibrate using a serum based standard (Biochemistry Calibrator, cod. 18011 and 18044).

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