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Synergistic effect of *Catha edulis* and smoking on exfoliated buccal cells in south west region of Saudi Arabia (Asser)

Kamel Ahmad Jaber Saleh

King Khalid University, Faculty of Science, Department of Biology, Abha, Zip code 61413, P.O. Box 9004, Saudi Arabia.

Abstract

The aim of this study is to use the micronuclei from exfoliated buccal cells to investigate the synergistic effect of *Catha edulis* (khat) chewing and cigarette smoking, as well as the interaction between two. To investigate the genotoxicity of *Catha edulis* (khat) and smoking combination on exfoliated buccal epithelial cells, a micronucleus (MN) test was carried out on south-west Saudi Arabia population. Twenty male individual with age 20-25 divided into four groups; first group non smokers and non khat chewers (control), second group was smokers but non khat chewers, third group included khat chewers but non smokers, whenever the last group contains both smokers and khat chewers. Three slides were prepared from every individual scored on 2000 epithelial cells. Results showed the incidence of MN ($p>0.05$) in the three groups (2, 3 and 4) compared to the control (group1). Moreover, the combination of khat and smoking almost duplicate genotoxicity effect. The obtained results confirmed that khat and smoking combination can induce micronucleus which might result in mouth cancer in advance.

Key words: clastogenic effects, *Catha edulis*, environmental genotoxicity, micronucleus test, exfoliated cells.

Introduction

Generally the chromosome fragments or entire chromosomes that failed to attach to the spindles formed by centeriol in human or animal cells will not include in the main daughter nuclei during nuclear division these fragments or whole chromosomes will known as micronucleus. So, Micronuclei due to chromosome fragments or entire chromosomes will form small cytoplasmic chromatin-containing bodies like satellite nucleus appears around the cell nucleus. In order to detect micronucleus, Heddle (1973) and Schmid (1975) were developed a new technique called micronucleus test. This i*n vivo* and *in vitro* technique is very suitable to identify both clastogenic (agents that induce chromosomal breaks mainly through interaction with the DNA, to form of acentric fragments) and an eugenic (chromosome lagging) and effects on spindle. In addition to that it is short-time screening, least expensive, reliable, simple, and rapid screening test, and it has been shown to be at least as sensitive an indicator of chromosome damage as classical metaphase chromosome analysis (Mukherjee et al. 1988, Muller-Tegethoff et al. 1995, Nesslany and Marzin 1999, Saleh and Zeytinoglu 2001, Fenech et al. 2009). Because of that it is a widely used method for assessing genotoxicity in organisms

***Corresponding author:** dr_ksaleh@yahoo.com

(Meier et al. 1999). The presence of micronuclei (MN) in cells is considered as a biomarker of damage of the DNA (Heddle 1973). In the light of these observations the micronucleus test has been well established in several systems i.e. ovary, bone marrow, epithelial tissue, peripheral blood, liver, exfoliated buccal cells and fetus cells of several laboratory animals or human (Higashikuni et al. 1992, Hernandez et al. 2006, Marques et al. 2009).

The carcinogenic risk for tobacco smoking has been extensively reviewed. The International Agency for Research on Cancer concluded that, "there is sufficient evidence for the carcinogenicity of tobacco smoke" (Fenech et al. 1999). Different epidemiological studies confirmed that cancer mortality and morbidity are increased in cigarette smokers. The frequency of micronucleus outcomes in different cancer types show significant increase from less than 2-fold to 10-fold comparison to controls (Baker et al. 2004, dos Reis Campos et al. 2008).

Khat (*Catha edulis* Forsk) is an evergreen plant that grows at high altitudes in East Africa and Arabian Peninsula. Fresh leaves of khat (*Catha edulis* Forsk) are customarily chewed to attain a state of stimulation, stamina and pleasure effects. These effects have been attributed to the khat's content in cathinone, a sympathomimetic amine with properties similar to those of amphetamine (Kalix 1992), although other less potent stimulant substances may also be present, namely norpseudoephedrine (cathine) and norephedrine (Al-Motarreb et al. 2002). The consumption of khat is related to esophageal cancer (Gunaid et al. 1995) disabling neurological illness (Morrish et al. 1999) incidence of acute coronary vasospasm and myocardial infarct (Alkadi et al. 2002, Al-Motarreb et al. 2002). In addition to that khat chewing genotoxicity was observed by micronucleus in exfoliated buccal and bladder cells of khat consumers (Kassie et al. 2001a and 2001b).

This study aimed to investigate the synergistic effects of the combination of smoking and (*Catha edulis* Forsk) using the micronucleus frequency in the exfoliated buccal cavity in the south-west Saudi Arabia population.

Materials and Methods

Samples

The study population includes 20 male volunteers. The volunteers were chosen carefully with aged 20-25 analyzed. All participants had not previously received non-surgical and surgical periodontal therapy. Subjects were medically healthy with no relevant medical or pharmacotherapy history that might influence the conduct of the study past 6 months and all of them were not alcohol consumers. It was important that they had no caries or dental restorations; because it is known that some dental materials increase the frequency of micronuclei.

Micronucleus Test

Epithelial cells were collected from oral mucosa and smear onto clean microscope glass slides. The cells were fixed with cold 100% methanol. The slides incubated at 37^{O}C overnight and then stained with Giemsa and May-grunwald (Sigma, USA). About 6000 nucleated cells were analyzed for the presence of MN on light microscope (Olympus) at a final 100x magnification for each participant. The numbers of cells harboring micronucleus were record.

Micronuclei where identified as DNA-containing structures in the cytoplasm, separated from the main nucleus, and of an area less than 1/3 of the area of the main nucleus, non-refractivity, not touching and

same the color as the nucleus or lighter. Only cells with intact cellular and nuclear membranes were scored. The following criteria were used as described by previous studies: (i) micronuclei should be one-tenth and one-third diameter of the main nucleus, (ii) they should be on the same plane of focus, (iii) they should have the same color, texture and refraction as the main nucleus, (iv) they should be clearly separated from the main nucleus.

Additionally micronuclei formation showed variation in their shapes and number per cell as shown in. (A) shows one, (B) tow, and (C) three micronucleus per cell.

Statistical analysis

Changing of MN incidence was calculated as percentage for each group and compared with control by Oneway ANOVA. Statistical significance was determined.

Results

This study of the clastogenic effect of synergistic effect of *Catha edulis* and tobacco smoking on human exfoliated cells using micronucleus revealed that there was a significant induction of micronucleus. Comparing the results showed that non smokers and non khat chewers had the lowest value of MN, while the MN frequency increased significantly in other three groups (Table 1-4). All groups were significantly increased comparing to control (f=178.9, df=3), whereas this increase was not very significant between group two (smokers) and group one (control; non smokers and non khat chewers) (f=184.9, df=1). The same slightly increase was detected between group two and group three (khat chewers) (f=45.5, df=1). Whereas it was more significant when group four (Smokers and chewers) comparing with group one (control) (f=319.3, df=1) (Table 5).

Table 1. Micronucleus at non smokers and non khat chewers (Control group)

Sample No.	Slide No.	Cell Type	MN. Type			
1		Counted Exfoliated Cell No.	A	B	C	Deformed Nucleus
	1	2000	3	0	0	-
	2	2000	3	0	0	-
	3	2000	1	0	0	-
2	(-----)					
	1	2000	2	0	0	-
	2	2000	3	0	0	-
	3	2000	1	0	0	-
3	(*****)					
	1	2000	2	0	0	-
	2	2000	1	0	0	-
	3	2000	1	0	0	-
4	(-----)					
	1	2000	3	0	0	-
	2	2000	1	0	0	-
	3	2000	0	0	0	-
5	(------)					
	1	2000	1	0	0	-
	2	2000	0	0	0	-
	3	2000	1	0	0	-

The microscopic investigation of Micronuclei also showed a variation in their shapes and number per cell as shown in Table 1-4. The micronucleus type (A) was found in all groups, while, type (B) and (D) micronucleus were not found in any group (Table 1-4). Where the deformed nucleus (irregular in shape and has loops) seen only in khat chewers and smokers group (group 4).

Table 2. Micronucleus at smokers but non khat chewers group

Sample No.	Slide No.	Cell Type	MN. Type			
Sample 1	(*****)	Counted Exfoliated Cell No.	A	B	C	Deformed Nucleus
	1	2000	9	0	0	-
	2	2000	9	0	0	-
	3	2000	7	0	0	-
Sample 2	(-----)					
	1	2000	8	0	0	-
	2	2000	11	0	0	-
	3	2000	10	0	0	-
Sample 3	(*****)					
	1	2000	6	0	0	-
	2	2000	8	0	0	-
	3	2000	6	0	0	-
Sample 4	(------)					
	1	2000	8	0	0	-
	2	2000	9	0	0	-
	3	2000	6	0	0	-
Sample 5	(------)					
	1	2000	7	0	0	-
	2	2000	8	0	0	-
	3	2000	10	0	0	-

Table 3. Micronucleus at khat chewers but non smokers group

Sample No.	Slide No.	Cell Type	MN. Type			
Sample 1	(*****)	Counted Exfoliated Cell No.	A	B	C	Deformed Nucleus
	1	2000	11	0	0	-
	2	2000	10	0	0	-
	3	2000	13	0	0	-
Sample 2	(-----)					
	1	2000	12	0	0	-
	2	2000	12	0	0	-
	3	2000	9	0	0	-
Sample 3	(*****)					
	1	2000	14	0	0	-
	2	2000	12	0	0	-
	3	2000	11	0	0	-
Sample 4	(------)					
	1	2000	14	0	0	-
	2	2000	10	0	0	-
	3	2000	13	0	0	-
Sample 5	(------)					
	1	2000	12	0	0	-
	2	2000	14	0	0	-
	3	2000	12	0	0	-

Table 4. Micronucleus at smokers and khat chewers group

Sample No.	Slide No.	Cell Type	MN. Type			
Sample 1	(*****)	Counted Exfoliated Cell No.	A	B	C	Deformed Nucleus
	1	2000	20	1	0	+
	2	2000	25	0	0	+
	3	2000	21	0	0	+
Sample 2	(-----)					
	1	2000	24	0	0	+
	2	2000	27	0	0	+
	3	2000	22	0	0	+
Sample 3	(-----)					
	1	2000	29	0	0	+
	2	2000	26	0	0	+
	3	2000	20	0	0	+
Sample 4	(-----)					
	1	2000	22	0	0	+
	2	2000	25	0	0	+
	3	2000	19	0	0	+
Sample 5	(-----)					
	1	2000	17	0	0	+
	2	2000	16	0	0	+
	3	2000	14	0	0	+

+= more than %2 of exfoliated cells have deformed nucleus.

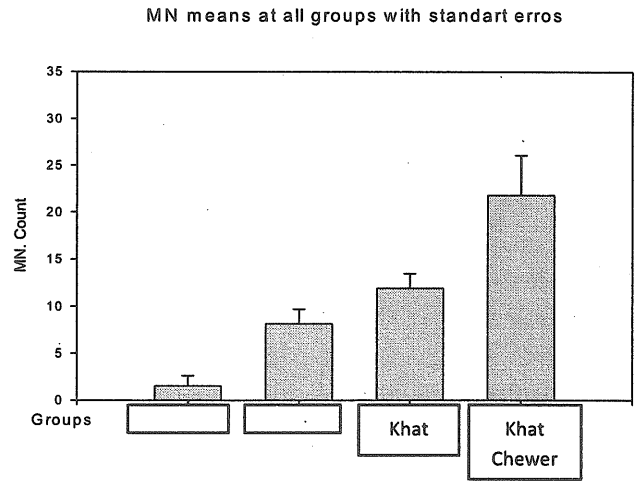
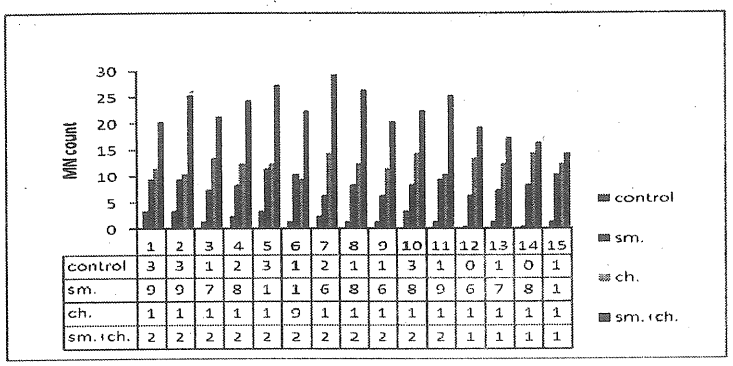


Figure 1. Micronucleus frequencies and standard errors in all groups

Discussion

Because of its pleasurable and stimulating effects a large number of people in East Africa and Southern Arabia chew khat leaves. According to the World Health Organization, khat classified as a "Substance of Abuse". But many investigators confirm that khat chewing has many side effects on different organs and systems in human body. (Soufi et al. 1991, Nasr and Khatri 2000) mentioned that the people who chew khat for prolonged periods have been found to have a higher incidence of head and neck cancer when compared with those who do not chew. The genotoxic effect of khat on human oral cells has been shown (Kassie et al. 2001a and 2001b),

also confirmed that there was an evidence that khat is able to decrease the systemic capacity of the body to handle reactive oxygen species in animals (Al-Qirim et al. 2002).



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
control	3	3	1	2	3	1	2	1	1	3	1	0	1	0	1
sm.	9	9	7	8	1	1	6	8	6	8	9	6	7	8	1
ch.	1	1	1	1	1	9	1	1	1	1	1	1	1	1	1
sm.+ch.	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1

Sm.= Smokers. Ch.= Khat chewers.

Figure 2. Comparison table between all groups

Table 4. Comparison data between groups

Groups	control	smokers	chewers	Smokers+chewers
Smokers	f=184.9, df=1	-------	f=45.5, df=1	f=136.1, df=1
Chewers	f=466.7, df=1	f=45.5, df=1	--------	f=71.1, df=1
Smokers+chewers	f=319.3, df=3	f=136.1, df=1	f=71.1, df=1	----------
control	-----	f=184.9, df=1	f=466.7, df=1	f=319.3, df=3
Significance	------	P>0.05	P>0.05	P>0.01

Reports from *in vitro* studies have shown that khat is able to inhibit *de novo* synthesis of proteins, RNA and DNA (Al-Ahdal et al. 1988), and to induce apoptosis in human cells ((Dimba et al. 2004, Lukandu et al. 2008). In spite of a lack of knowledge on the specific mechanism(s) involved in khat induced cytotoxicity (Carvalho 2003) and the mechanisms leading to the development of khat-related pathological changes, including cancer of oral mucosa are not yet fully elucidated (Carvalho 2003). There were many publications explained the chemical structure of khat extract. These different publications confirmed that *Catha edulis* leaves contain some primary amines, such as cathinone, cathine, and norephedrine (Geisshusler and Brenneisen 1987, Dimba et al. 2004) and secondary amines, such as ephedrine and pseudoephedrine (Caveney et al. 2001), which may be considered to be precursors of nitrosamines (potent carcinogens) in the presence of nitrite. Significant concentrations of nitrite are present in the dietary intake added either as a preservative or formed by the action of nitrifying bacteria as an intermediate stage in the formation of nitrates. (Gunaid et al. 1995) found indications of an increase in cancer in the cardia and gastroesophageal junction in individuals from Yemen who chewed khat and smoked water pipes, but the number of cases (in total 20) was insufficient to identify independent effects of the two factors. (Al-Ahdal et al. 1988, Kassie et al. 2001a and 2001b) suggested that khat consumption, especially when accompanied by alcohol, and tobacco consumption might be a potential cause of oral malignancy.

(Bonassi et al. 2009) explained that the exfoliated cells can be used as an endogenous dosimeter in tissues that are specific targets of genotoxic and carcinogenic agents, where carcinomas will

develop. In the light of these data the present study was designed to investigate the synergistic genotoxicity effect of khat and smoking in exfoliated buccal cells using MN frequency at Asser region population in south west Saudi Arabia.

Age, gender, health and nutrition were the most commonly factors may affect the MN frequency. So to eliminate the affect of these factors the present study choose only young, active and non medicated healthy peoples finally they feed almost same.

The results shown that the collecting data taken from samples in Asser region population shows that the synergistic effect of khat and smoking not only folded for many times the clastogenic effect but also induce the nuclear defragmentation. These results are agree with the results observed by another investigators in different populations (Tice et al. 1998, Kassie et al. 2001a and 2001b).

Fortunately, it is good to mention that khat chewing is prohibited by low in Saudi Arabia so the number of chewers are very small which my decrease the number of oral cancer cases, but unfortunately smokers number still increase day by day.

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