RESEARCH ARTICLE

Evaluation of Malondialdehyde, C-reactive Protein and DNA Damage Related with the Smoking Habit by Comet Assay in Iraq

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ABSTRACT

The purpose of the current investigation was to examine the relationship between inflammation marker C-reactive protein (CRP), an oxidative stress marker (Malondialdehyde; MDA), and DNA damage assessed by comet assay (single-cell gel electrophoresis, SCG) in smokers and non-smokers. The comet assay score was analyzed by image software using three parameters for measuring DNA damage which were tail length, tail DNA %, and tail moment. SCG is commonly used as an *in vitro* and *in vivo* genotoxicity test. Because of its ability to detect different forms of DNA damage and its simplicity of application, the technique is being increasingly used in human biomonitoring. The test was performed on blood samples from smokers and non-smokers to better characterize the comet assay suitability for biomonitoring. A total 123 males who were 21–30 years; they were randomly selected and distributed in three groups (40 non-smokers (consider as controls), 44 narghile-smokers, and 39 cigarette-smokers). The results showed there was a significant increase in both CRP and MDA levels in narghile and cigarette-smokers compared to non-smokers as well as the comet assay results showed augment in DNA damage in both smoker groups compared to non-smokers.

Keywords: Comet assay, CRP, DNA damage, Inflammation, MDA, Oxidative stress, Smoking.

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INTRODUCTION

In the recent past, narghile and cigarette smoking have become a common phenomenon in many countries. There are many terms used to refer to narghile, including waterpipe shisha, argileh, and hubble-bubble.[1] In tobacco, there are approximately 5000 harmful chemical materials that involve free radicals and oxidative gases; so, in addition to stimulation of intracellular production of reactive oxygen species, cigarette materials decrease intracellular antioxidant mechanisms, causing the oxidation stress.[2] As well as the smoking led to 20–25% increase in the white blood cells number[3] and an elevated in the level of different inflammatory markers like IL-1, IL-6, C-reactive protein, and TNF-α and others, has been identified as potential biomarker of tobacco effect.[4-7] Cigarette smoking, on the other hand, results to DNA damage that plays a crucial role in promoting lung cancer and other 13 types of cancer. It contains a mixture of many chemicals, but the substance of interest is more than 60 carcinogens contained in tobacco smoke that causes mutations resulting to cancer.[8] Cigarette smoking increases the mutation in all tissues as a result of DNA damage, and on the same note, the mutagens damage on DNA is detrimental to other tissues in the body and can result in various health problems.[9] Narghile and cigarettes have some effects on the human body and may affect its function in multiple ways; it is crucial to note that smoking narghile once a day will produce the amount of plasma nicotine as smoking 10 cigarettes a day.[10] Lung cancer is common among cigarette smokers, while other types of cancer caused by smoking such as mouth and tongue are common among narghile smokers.[11] DNA carries genetic materials because it is relatively stable compared to other macromolecules, including protein and RNA; DNA interacts with various substances that can be endogenous or extraneous, including environmental agents.[12] This interaction can lead to chemical modifications, which are detrimental as it can exert mutagenic and fatal effects upon replication; besides, damage to DNA arises in many ways, and it is inevitable.[13] In a day there is approximately 106 DNA damage in a single human cell.[14] The damage can be triggered by various factors, including exposure to ionizing radiotherapy and chemotherapeutic drugs, ultraviolet radiation, smoking, and spontaneous cleavage of chemical bond occurring in DNA.[15] Similarly, other factors have a significant effect on DNA integrity, including oxidative stress species like reactive oxygen species (ROS), which occur in the environment, and

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when the contents in the chromosome are altered, there will be chromosome damage or abnormalities.[16] As a result, DNA damage will lead to different types of breakage, including chromosome aberration and chromatid aberration.[17]

MATERIALS AND METHODS

Subjects

Participants in this study included 123 males of 21–30 years age, during September 2019 – January 2020; they were randomly selected and distributed into three groups (40 non-smokers, 44 narghile-smokers, and 39 cigarette-smokers). Smoker status was defined as self-reported smoking more than one cigarette/day, whereas, for narghile-smokers, they smoked more than twice/week. In addition, all participants enrolled in the study did not complain of any inflammatory or chronic disease.

Methods

C-reactive protein and Malondialdehyde Method

5 mL of venous blood was collected and divided into two parts 3 mL was dispensed into separator gel tubes and then it was c.f. at 3000 rpm to 15 minutes to have serum and kept at -20ºC for biochemical analysis. The remaining 2 mL was collected in EDTA tubes and used for assessing DNA damage. The evaluation of MDA (µmol/L) concentration in serum was determined depending to Buege and Aust procedure.[18] MDA come from the breakdown of polyunsaturated fatty acids serves as a convenient index of peroxidation reaction. The thiobarbituric acid method was used to determine the MDA, which interferes with thiobarbituric acid (TBA), giving pink color at ʎ max 535 nm.[19] while C-reactive protein was determined in serum by means of Automated Biosystem A15.

Alkaline Comet Assay (Single-cell gel electrophoresis)

Alkaline comet assay investigated the DNA damage in peripheral blood lymphocytes from both non-smokers and smokers that performed under alkaline conditions according.[20]

1. The agarose slides were prepared by submersion into normal agarose molten 1.5 % (w/v).
2. Agarose was drying by air to a thin layer.
3. Slides were labeled manually on end.
4. The centrifugation of the cells was for 2 minutes at 1500 rpm. The supernatant was ignored, and the pellet was washed with ice-cold PBS (without Mg2+ and Ca2+), then repeated the centrifugation at 1500 rpm for 2 minutes, followed by discarded supernatant.
5. Cell sample was mixed with a low melting point agarose of ratio 1:10 (V/V) then the mixture of 75 µL to each well was added into the comet slide.
6. The slides were kept in the dark container 4ºC for 30 minutes.
7. The slide put into a small tank containing precooled lysis buffer, then immersed the slide in the lysis buffer overnight in the dark at 4ºC.
8. Then, the slides were submerged in electrophoresis for 20 minutes.
9. Applied 24 Volt and 300 (mA) to horizontal electrophoresis chamber of slides for 18 minutes, filled with a cold TBE electrophoresis solution.
10. Electrophoresis solution was drawn from the chamber and changed with Neutralization Buffer, 0.4M of Tris-HCl solution (pH 7.5) for 5 minutes to neutralize the cells.
11. Ethidium bromide 50 µL was put in each well of the slide and incubated at room temperature for 15 minutes.
12. The slides were washed with distilled water to clear the slides from excessive stains.
13. Fluorescence microscopy used to view comet assay slides.

Statistical Analysis

The results were statistically analyzed using SPSS version 16.0. Their data were given as (mean ± standard error), and ANOVA assessed differences between groups.

RESULTS AND DISCUSSION

Statistical analysis for our data of group's levels (mean ± standard error) revealed important outputs. Regarding to MDA levels and as shown in Figure 1, there was significant ($p \leq 0.001$) differences between narghile-smokers and non-smokers (6.00 ± 0.28 vs. 3.94 ± 0.29 µmol/L) and between cigarette-smokers and non-smokers (4.79 ± 0.25 vs.3.94 ± 0.29 µmol/L) also between narghile and cigarette smokers. This result agrees with Safyudin and Subandrate,[21] studies of which revealed that toxic substances in cigarette smoke have the potential to increase malondialdehyde (MAD) levels. Furthermore, Bello *et al.*[22] reported that high and low levels of MDA and antioxidant vitamins subsequently in smoker's person related to increasing in levels of cigarette usage and suggested that smokers consuming more than 15 cigarettes each day are more susceptible to different diseases.

Regarding C-reactive protein, Figure 2 showed significantly elevated in CRP level in both narghile and cigarette smoker's groups compared to non-smokers. It was (10.41 ± 0.26 and 9.91 ± 0.26 mg/dl) respectively vs. (8.06 ± 0.27 mg/dL). In addition, there was an increasing but non-significantly

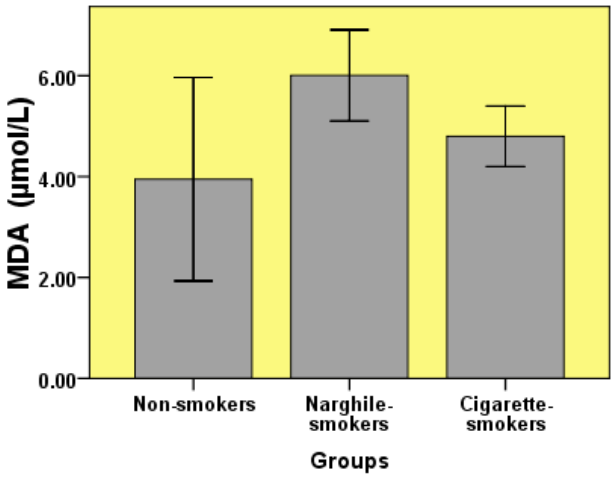

Figure 1: MDA concentration in non-smokers (healthy) and smokers (Narghile and Cigarette).

Table 1: Results of comet assay on non-smokers and smokers (Narghile and Cigarette) peripheral blood lymphocytes

		Tail length	*DNA in tail (%)*	*Tail moment*
Groups	*No.*	*(Mean ± SE.)*		
Non-smokers	40	4.40 ± 0.40	0.29 ± 0.16	0.02 ± 0.01
Narghile-smokers	44	27.4 ± 6.85*	12.34 ± 4.87*	4.71 ± 1.90*
Cigarette smokers	39	22.80 ± 1.59*	9.41 ± 1.51*	2.07 ± 0.24

(*) Means with significant ($p \leq 0.05$) correlation between smoker and non-smokers groups

Table 2: DNA damage percentage in peripheral blood lymphocytes of non-smokers and smokers (Narghile and Cigarette)

Scores mean % / *Groups*	*No.*	*No damage (ND) %*	*Low damage (LD) %*	*Medium damage (MD) %*	*High damage (HD) %*
Non-smokers	40	45.21	40.50	8.46	5.85
Narghile-smokers	44	25.62	23.34	24.59	26.45
Cigarette-smokers	39	26.72	24.34	24.82	24.12

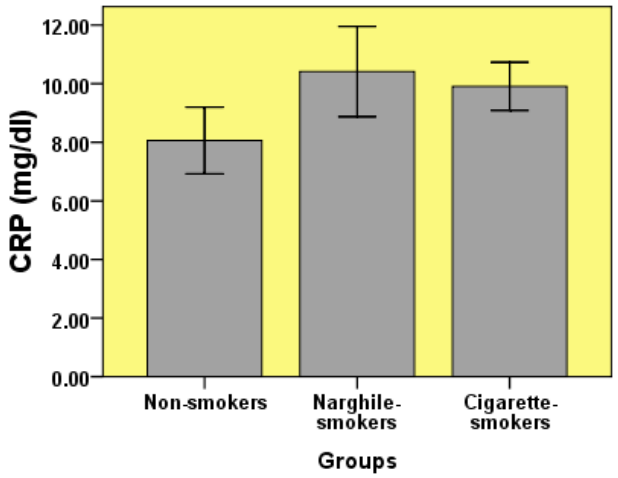

Figure 2: CRP levels in non-smokers (healthy) and smokers (Narghile and Cigarette)

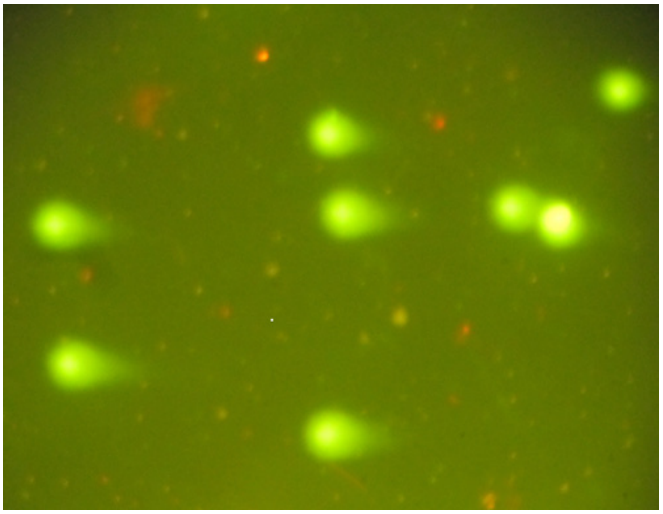

Figure 3: Illustrate fluorescent spheres without DNA damage (no tail) in non-smokers in peripheral blood lymphocytes examined by fluorescent microscope (400X)

($p > 0.063$) in CRP level between narghile and cigarette smokers. Numerous studies have shown that tobacco smoking is associated with inflammation. Chung *et al.*[23] studies demonstrated a significant positive relationship was noticed between hs-CRP and cotinine (an alkaloid found in tobacco and used as a biomarker for exposure to smoke) levels indicating an association between cigarette smoking and inflammation. Diab *et al,*[24] reported that hs-CRP levels were slightly increased (non-significant) in both waterpipe and cigarette smokers than non-smokers. In addition, Aldahm and colleagues[25] showed that smokers had significantly higher levels of serum IL-6 (pro-inflammatory biomarker) compared to male former smokers also, they reported that CPR is significantly associated with IL-6 regardless of smoking status.

Evaluate the genotoxic effects of tobacco smoking was determined using the comet assay. Three marks were dependent as an indicator of DNA damage percentage of DNA in the tail, tail length, and tail moment. As shown in Table 1, the mean of tail lengths of comet assay in narghile-smokers and cigarette-smokers were significantly increasing ($p \leq 0.05$) as compared to non-smokers (27.4 ± 6.85 and 22.80 ± 1.59 vs. 4.40 ± 0.40). The same observation in DNA in the tail (12.34 ± 4.87 and 9.41 ± 1.51 vs. 0.29 ± 0.16) while in tail moment the significant difference was observed in the narghile group as compared to non-smokers (4.71 ± 1.90 vs. 0.02 ± 0.01). As well as shown in Table 2, the lowest percentage of high damage (HD) was observed in non-smokers (5.85%) Figure 3 as compared with smokers (narghile and cigarette) (26.45% and 24.12% respectively) Figure 4 & 5. These findings were agreed with Söylemez *et al.*[26,] who revealed that smoking cause DNA damages and females are more sensitive to the effect of smoking than males.

In smoker's person, the high levels of serum of xanthine oxidase enzyme have amplified secretion of reactive oxygen species, which might have a role in speed up the lipid peroxidation that is characterized by high levels of MDA in smoker's person as differentiate to no-smokers person.[27] Smoking in such a manner could lead to a huge effect on the cells by increased cell turnover causing an increased in purine catabolism which in turned increased ROS secretion.[28,29]

Oxidative stress of smoker cells increases by elevating the xanthine oxidase and minimizing the antioxidant vitamins.[30] In this manner, smokers encounter a sustained free radical load, which could enhance LDL oxidation

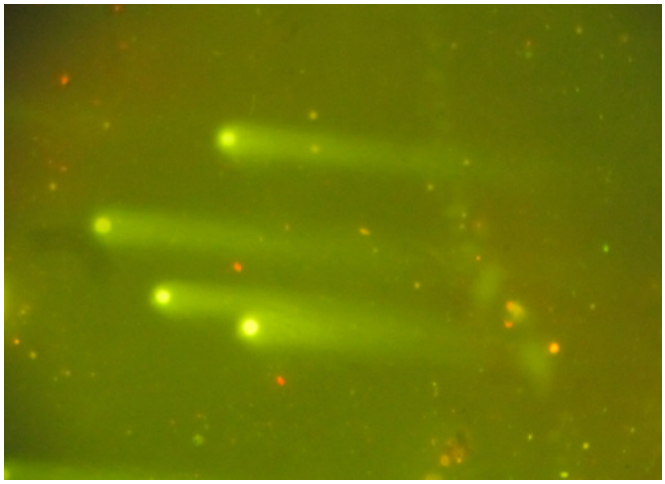

Figure 4: Illustrate fluorescent heads with tails indicating DNA damage in narghile smokers in peripheral blood lymphocytes examined by fluorescent microscope (400X)

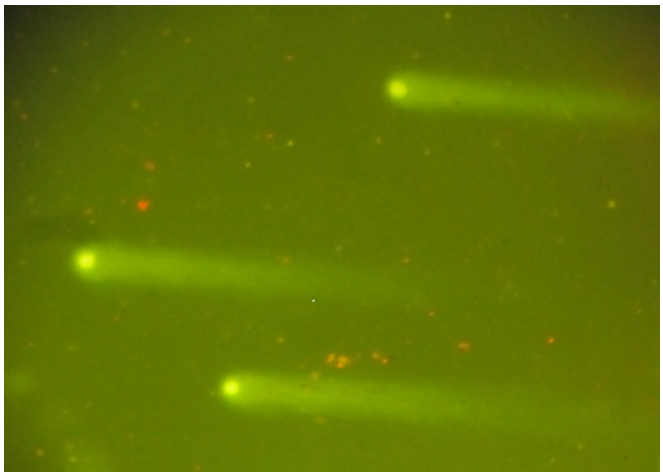

Figure 5: Illustrate fluorescent heads with tails indicating DNA damage in cigarette smokers in peripheral blood lymphocytes examined by fluorescent microscope (400X)

and hence facilitate the development of the atheromatous plaque.[31]

Chronic infections cause immune system activation, thus secreting the C-reactive protein in the bloodstream.[32] Tobacco is mark in decrease the blood supply to gums, depriving them of oxygen and nutrients, departing them vulnerable to bacterial infections.[33] Smoking increases the CRP by its effect on the accumulation of plaque and effects on the host response.[34]

CONCLUSION

the results of the current study showed a significant relationship between the serum levels of MDA, CRP and cytotoxicity, and the ratio of the relationships depends on the number of narghile or cigarette smoke/day, the duration of exposure to toxic substances, gases emitted from cigarette smoke and the genetic predisposition of the individual.

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