A preliminary study: matrix metalloproteinase expression as an indicator of the hazards of shisha (nargila) smoking

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Submitted: 3 November 2008
Accepted: 30 January 2009

Arch Med Sci 2009; 5, 4: 570-576

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Abstract

Introduction: There is a shift in the behaviour of many cigarette smokers to smoke tobacco by shisha because of a false belief that it is a safe way to smoke tobacco.
Material and methods: In this work we aimed to study the effect of smoking tobacco by shisha compared to cigarette smoking and to verify the hazardous effects of smoking shisha, by estimation of matrix metalloproteinase (MMP-2 and MMP-9) gene expression in BAL of COPD patients. From 32 COPD patients smoking cigarettes, shisha and combined cigarettes and shisha BAL fluid was obtained, and MMP-2 and MMP-9 were analyzed by reverse transcription polymerase chain reaction.
Results: Expression of MMP-2 and MMP-9 was as high in the shisha and combined cigarette and shisha smokers as the cigarette smokers, with a significant positive correlation between MMP-2 and MMP-9 only in the shisha smoker group. The mean FEV_1 (% predicted) of the shisha smoker group was significantly lower compared to the cigarette smoker group. In the shisha smoker group there was a significant statistical difference between severe cases and mild/moderate cases in both MMP-2 and MMP-9 expression. Also, MMP-2 and MMP-9 expression were lower in ex-smokers compared to those with an active smoking habit.
Conclusions: Smoking shisha induces expression of metalloproteinases in BAL as much as in smoking cigarettes; moreover, it has a highly deleterious effect on pulmonary function.

Key words: chronic obstructive pulmonary disease, matrix metalloproteinases, MMP-2, MMP-9, shisha smoking.

Introduction

There are an estimated 1.3 billion habitual cigarette smokers worldwide [1]. Cigarette smoke induces inflammation, oxidative stress, expression and/or function of several proteinases, including matrix metalloproteinase [2-4]. Matrix metalloproteinases (MMPs) constitute a broad family of more than 20 members, which share a significant structural homology (e.g. collagenases, elastases and gelatinases). Gelatinases, also named type IV collagenases, are two enzymes (MMP-2 or gelatinase A and MMP-9 or gelatinase B) which play a key role in lung extracellular matrix (ECM) biology, developmental processes, as well as in tissue repair and fibrosis [5, 6].

Matrix metalloproteinase-2 (gelatinase A, 72 KD type IV collagenase) is one of the ECM-degrading proteases and it has been recognized as a major

mediator of the degradation of basement membrane (which consists mainly of collagen IV, laminin and proteoglycans), parenchymal destruction and emphysema development [7, 8]. It has been found that cigarette smoke increases MMP-2 content in mice bronchoalveolar lavage (BAL) [9].

Matrix metalloproteinase-9 MMP-9 (gelatinase B, 92 KD, type IV collagenase) is present in low quantities in the healthy adult lung but in several lung diseases or cigarette smoking, many intrinsic lung and inflammatory cells produce MMP-9 [10]. In addition to digesting structural proteins MMP-9 can induce lung remodelling [11].

Awareness of the hazards of cigarette smoking has become evident and widespread, which has led to a shift of many cigarette smokers to another sort of smoking, such as shisha (nargila). There is a popular concept that shisha (nargila) smoking is safer than cigarette smoking, leading to increased prevalence of shisha (nargila) smoking and spread of its use worldwide.

A shisha (nargila) is a glass device for smoking tobacco. On its top tobacco leaves are arranged in a cone-shaped device with multiple holes at its base. The amount of tobacco leaves placed inside the cone is called Hagr. Burned coal or wood is placed on top of it. The cone is connected to a metal tube that ends under a water seal in a glass device. The sucked smoke passes through a mouthpiece connected to a side tube placed above the water seal of the glass device.

The aim of this work is to assess the hazardous effect of smoking tobacco by shisha (nargila) compared to cigarette smoking by estimation of MMP-2 and MMP-9 gene expression by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in BAL of chronic obstructive pulmonary disease (COPD) patients.

Material and methods

Study population

A total of 32 male COPD patients were included in the study, which was conducted in the Chest Diseases and Biochemistry departments, Cairo University. COPD diagnosis was confirmed by medical history and the results of pulmonary function tests. Subjects with asthma or atopy were excluded from the study.

All the COPD patients were smokers and they were divided into three groups:

Cigarette smokers: consisting of 14 male patients; their age ranged from 20 to 76 years (mean 49.71 ±16.13 years); they were 4 smokers with severe COPD (GOLD III–IV) and 10 smokers with mild/moderate COPD (GOLD I–II).

Cigarette smoking history ranged from 0.5 to 160 packs/year (mean 45.46 ±45.52 packs/year) for a duration of smoking ranging from 1 to 60 years (mean 25.57 ±16.36 years). Five patients had stopped smoking for a period from 6 months to 8 years (mean 2.03 ±3.39 years).

Shisha smokers: consisting of 10 male patients; their age ranged from 47 to 70 years (mean 57.3 ±7.2 years). They were 4 smokers with severe COPD (GOLD III–IV) and 6 smokers with mild/moderate COPD (GOLD I–II).

Shisha smoking history ranged from 2 to 3 Hagr/day (mean 2.7 ±0.48 Hagr/day) for a duration of smoking ranging from 10 to 40 years (mean 22.9 ±12.46 years). Four patients had stopped shisha smoking for a period from 8 months to 5 years (mean 2.6 ±2.23 years).

Cigarette and shisha smokers: consisting of 8 male patients; their age ranged from 28 to 76 years (mean 18.50 ±15.80 years), all with mild/moderate COPD (GOLD I-II).

Cigarette smoking history ranged from 2.5 to 50 packs/year (mean 18.50 ±15.80 packs/year) for a duration of smoking ranging from 10 to 50 years (mean 17.63 ±13.81 years). Their shisha smoking history ranged from 2 to 3 Hagr/day (mean 2.38 ±0.52 Hagr/day) for a duration of smoking ranging from 10 to 40 years (mean 21.25 ±14.05 years). One patient had stopped smoking 4 years ago.

The study was approved by the Human Ethics Committee of Cairo University and all subjects gave written informed consent before BAL.

Bronchoalveolar lavage collection

Fibreoptic bronchoscopy (Olympas) was performed in all patients. Five 20 ml aliquots of sterile saline solution were instilled to the subsegmental bronchi of the right lower lobe and 15 ml of the recovered fluid was contained in a sterilized tube and stored at –70°C for RNA extraction.

Detection of MMP-2 and MMP-9 gene expression by RT-PCR

Bronchoalveolar lavage fluids of 15 ml in amount were centrifuged at 14,000 rpm for 10 min. Then the precipitate was examined for detection of MMP-2 and MMP-9 gene expression by RT-PCR.

1) RNA extraction: RNA was extracted by using the SV-total RNA isolation system (Promega-Madison, USA) according to manufacturer's recommendations and the extracted RNA was measured by a spectrophotometer at 280 nm.
2) RT-PCR: Five µg of RNA was reverse transcribed by using 12.5 µl of oligonucleotide primer denatured at 70°C for 2 min. The denatured RNA was placed on ice for 5 min. RT buffer (5 mmol KCl, 50 mmol tris HCL, at pH 8.3, 0.5 mmol of deoxynucleotide triphosphate (dNTPs) and 200 units of Moloney murine leukaemia virus (MMLV) reverse transcriptase) was used; the reaction conditions

were 42°C for 1 h followed by heating at 95°C for 5 min to stop the reaction. PCR reaction was performed by adding the PCR mix to 5 µl of cDNA; the mixture contained 10 mmol/LHcl pH 8.3, 50 mmol KCl, 100 mmol dNTPs, 2.5 units of Taq polymerase and 10 µmol of each of sense and anti-sense primer of MMP-2 and MMP-9 with the following sequences, respectively: sense primer 5'GTGCTG GGCTGCTTTGCTG-3' and anti-sense primer 5'GTCGCCCTCAAAGGTTTGGAAT-3', sense primer 5'TTCACCCGGTTGTGGAAACT-3' and anti-sense primer 5AAATGTGGGTGTACACAGGC-3'. The amplification products of MMP-2 and MMP-9 were 457 and 562 bp. β-Actin was used as an internal control. The primers for β-actin were: sense primer 5'-CTATCGGC- AATGAGCGGTTC-3', and anti-sense primer 5'-CTTAGGAGT- TGGGGGTGGCT-3'. The amplification products were 734 bp. The PCR cycling conditions were 94°C for 1 min for denaturation followed by 60°C for 1 min and 72°C for 45 s, for 40 cycles with final extension at 72°C for 12 min.

3) Gel electrophoresis: PCR product of 10 µl was analyzed on 2% agarose gel with ethidium bromide staining and the product was visualized on an ultraviolet transilluminator, and then gel documentation was performed. Optical density of bands was measured by the automatic image analysis system (Bio Doc Analyze) supplied by Biometra [13]. The relative expression level of MMP-2 and MMP-9 was calculated using β-actin as an internal control.

Statistical methods

Data were statistically described in terms of range, mean ± standard deviation (± SD), frequencies (number of cases) and relative frequencies (percentages) when appropriate. Comparison of quantitative variables between the study groups was done using Mann-Whitney *U* test. Correlations between various variables were done using Spearman rank correlation equation. A probability value (*p* value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL, USA) version 13 for Microsoft Windows.

Results

Characteristics of subjects

The age distribution was similar between the cigarette smokers and the combined cigarette and shisha smokers, while the shisha smokers were older in age. All the studied cases were males due to the higher prevalence of the different smoking habits in the male gender in oriental countries (Egypt) (Table I).

The smoking history of the 3 groups revealed that the mean amount and duration of cigarette smoking between those smoking cigarettes alone and those smoking both shisha and cigarettes showed no statistical difference. Also, the same items (the mean amount and duration of shisha smoking) did not show a statistical difference between shisha smokers and both cigarette and shisha smokers (Table II).

The mean FEV_1/FVC% ratio, the FEV_1 (% predicted) and the FEV_1 reversibility (% predicted) values in the cigarette smoker group were 62.07 ±6.44, 63.14 ±17.26 and 67.57 ±16.39, in the shisha smoker group they were 56.90 ±5.97, 49.80 ±13.21 and 54.60 ±14.00, whereas in the cigarette and shisha smoker group they were 64.25 ±3.28, 66.63 ±5.95 and 69.38 ±5.97, respectively. All of the values were decreased in the 3 groups, confirming the presence of airflow obstruction.

However, the FEV_1/FVC% ratio and the FEV_1 reversibility (% predicted) were significantly lower in the shisha smoker group compared to the two other groups, whereas the mean FEV_1 (% predicted) of the shisha smoker group was significantly lower compared to the cigarette smoker group (Table I).

Also, the correlations between the amount (Hagr) of shisha smoking in the shisha smoker group showed a significant negative correlation with FEV_1 (% predicted) and FEV1 reversibility (% predicted) values [0.037 and 0.035 respectively]. (Figures 1, 2).

Concerning the gene expression of MMP-2 and MMP-9 in BAL

The MMP-2 gene expression in the cigarette smoker group was in the range 31.6-117.0 µg/ml (mean 74.97±30.88 µg/ml), in the shisha smoker group it was in the range 27.9-110.6 µg/ml (mean 63.5 ±29.48 µg/ml), and in the cigarette and shisha smoker group it was in the range 44.142.6 µg/ml (mean 87.65 ±30.88 µg/ml).

The MMP-9 gene expression in the cigarette smoker group was in the range 115.9-608.3 µg/ml (mean 278.53 ±163.09 µg/ml), in the shisha smoker group it was in the range 103.8-412.8 µg/ml (mean 250.93 ±119.3 µg/ml), and in the cigarette and shisha smoker group it was in the range 102.6-432.7 µg/ml (mean 279.14 ±134.54 µg/ml).

The MMP-2 and MMP-9 gene expression values were increased with no statistical difference among the 3 groups (Table I).

The correlation between all the studied parameters in the 3 groups showed that only in the group of shisha smokers is there always a significant positive correlation between MMP-2 and MMP-9 gene expression values. The correlation coefficient was 0.673 (Figure 3).

Table I. Description and comparison of data of the 3 studied groups

		No. of patients	Mean	Standard deviation	Minimum	Maximum	Significance*
Age [year]	Shisha	10	57.30	7.20	47.00	70.00	A
	Cigarette	14	49.71	16.13	20.00	76.00	A
	Both	8	50.25	17.56	28.00	76.00	A
	Total	32	52.22	14.35	20.00	76.00	
MMP-2 [μg/ml]	Shisha	10	63.50	29.48	27.90	110.60	A
	Cigarette	14	74.97	30.88	31.60	117.00	A
	Both	8	87.65	30.88	44.70	142.60	A
	Total	32	74.56	30.84	27.90	142.60	
MMP-9 [μg/ml]	Shisha	10	250.93	119.30	103.80	412.80	A
	Cigarette	14	278.53	163.09	115.90	608.30	A
	Both	8	279.14	134.54	102.60	432.70	A
	Total	32	270.06	139.80	24.70	608.30	
FEV_1 (% predicted)	Shisha	10	49.80	13.21	30.00	68.00	A
	Cigarette	14	63.14	17.26	20.00	78.00	AB
	Both	8	66.63	5.95	57.00	76.00	B
	Total	32	60.81	14.18	20.00	78.00	
FEV_1/FVC %	Shisha	10	56.90	5.97	49.00	68.00	A
	Cigarette	14	62.07	6.44	48.00	69.00	B
	Both	8	64.25	3.28	60.00	68.00	B
	Total	32	61.00	6.23	48.00	69.00	
FEV_1 reversibility (% predicted)	Shisha	10	54.60	14.00	25.00	69.00	A
	Cigarette	14	67.57	16.39	22.00	80.00	B
	Both	8	69.38	5.97	60.00	77.00	B
	Total	32	64.28	14.57	22.00	80.00	

**Common letters mean no significant difference; different letters mean significant difference at the level of 0.05*

Table II. Comparison of mean amount and duration of cigarette and shisha smoking of the 3 groups

	Groups	No. of patients	Mean	Standard deviation	*P* value
cig_pack/y	Cig. alone	14	45.46	45.52	
	Cig. & shisha	8	18.50	15.80	0.15 (NS)
cig_duration/y	Cig. alone	14	25.57	16.36	
	Cig. & shisha	8	17.63	13.81	0.62 (NS)
cig_stopped/y	Cig. alone	5	2.03	3.39	
	Cig. & shisha	1	4.00	–	–
shisha_hagr/y	Shisha alone	10	2.70	0.48	
	Cig. & shisha	8	2.38	0.52	0.19 (NS)
shisha_duration/y	Shisha alone	10	22.90	12.46	
	Cig. & shisha	8	21.25	14.05	0.80 (NS)
shisha_stopped/y	Shisha alone	4	2.60	2.23	
	Cig. & shisha	1	4.00	–	–

Also in the shisha smoker group there was a significant negative correlation between the MMP-9 gene expression, FEV_1 (% predicted) and FEV_1 reversibility (% predicted) values. The correlation coefficients were –0.794 and –0.801 respectively (Figures 4, 5).

Concerning the grade of severity in the cigarette smoking group, the MMP-2 gene expression mean value in the 4 patients with severe COPD [GOLD III-IV] was 93.12 ±27.07 μg/ml, while in the 10 patients with mild/moderate COPD [GOLD I-II] it was 67.71 ±30.47 μg/ml, whereas the MMP-9 gene expression mean value in COPD (GOLD III-IV) was 397.35 ±190.27 μg/ml and in COPD (GOLD I-II) was 231.0 ±132.54 μg/ml. Although both MMP-2 and MMP-9 gene expression mean values were higher in the severe cases than in mild/moderate cases, there was no statistically significant difference (Table III).

In the shisha smoker group, the MMP-2 gene expression mean value in the 4 patients with severe

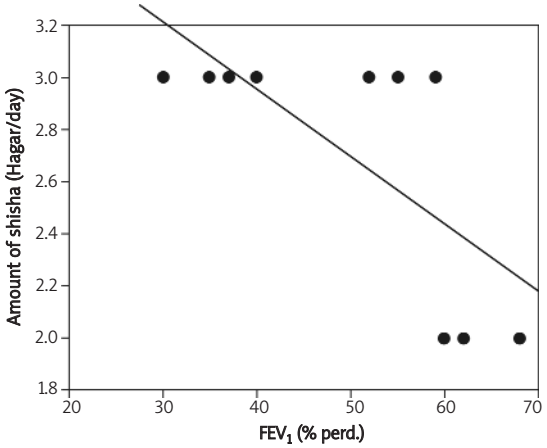

Figure 1. Shows the significant negative correlation between the amount of shisha smoking and FEV_1 (% predicted)

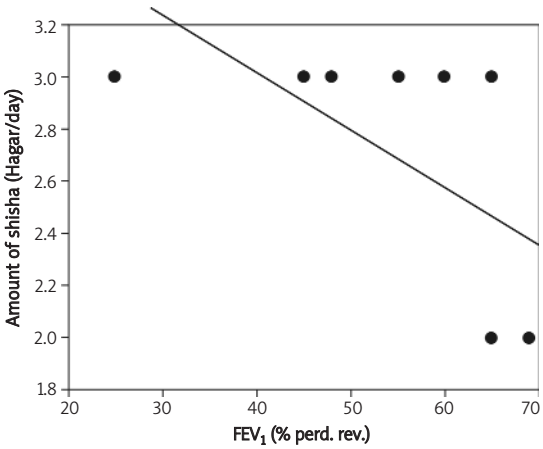

Figure 2. Shows the significant negative correlation between the amount of shisha smoking and FEV_1 reversibility (% predicted)

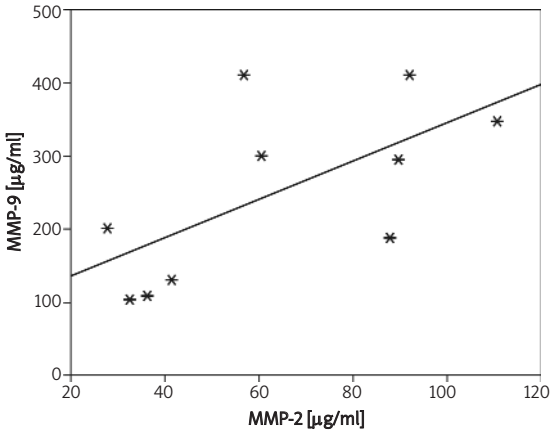

Figure 3. Shows the significant positive correlation between MMP-2 and MMP-9 gene expression values in the shisha smoker group

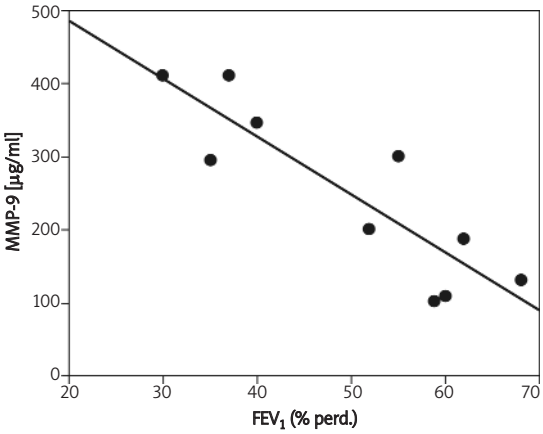

Figure 4. Shows the negative correlation between MMP-9 and FEV_1 (% predicted)

COPD [GOLD III–IV] was 87.23 ±22.36 µg/ml, while in the 6 patients with mild/moderate COPD [GOLD I-II] it was 47.68 ±22.66 µg/ml, whereas the MMP-9 gene expression mean value in COPD (GOLD III-IV) was 367.43 ±56.45 µg/ml and in COPD (GOLD I-II) was 173.27 ±74.90 µg/ml. There was a significant statistical difference in both MMP-2 and MMP-9 gene expression mean values between the severe cases and the mild/moderate cases in the shisha smoker group (Table III).

The MMP-2 gene expression mean value of the 10 ex-smoker patients was 68.54 ±28.63 µg/ml and of those who continued smoking was 78.17 ±32.26 µg/ml, while the MMP-9 gene expression mean value of the ex-smoker patients was 245.28 ±133.43 µg/ml and of those who continued smoking was 285.92 ±144.78 µg/ml (Table IV). The difference between MMP-2 and MMP-9 gene expression values in the ex-smoker patients was lower than in those who continued smoking; however, the values were not significant.

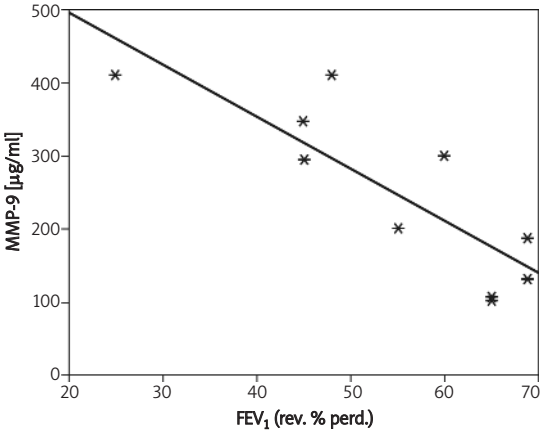

Figure 5. Shows the negative correlation between MMP-9 and FEV_1 reversibility (% predicted)

Discussion

It is now an established fact that the major risk factor for the development of fixed airflow

Table III. Comparison between patients with mild/moderate COPD and those with severe COPD in the cigarette and shisha smoker group

	Smoker groups	No. of patients	Mean	Standard deviation	*P* value*
MMP-2 [μg/ml] (cigarettes)	Mil/mod. COPD	10	67.71	30.47	0.19
	Severe COPD	4	93.12	27.07	
MMP-9 [μg/ml] (cigarettes)	Mil/mod. COPD	10	231.00	132.54	
	Severe COPD	4	397.35	190.27	0.14
MMP-2 [μg/ml] (Shisha)	Mil/mod. COPD	4	47.68	22.66	
	Severe COPD	6	87.23	22.36	0.033
MMP-9 [μg/ml] (Shisha)	Mil/mod. COPD	6	173.27	74.90	
	Severe COPD	4	367.43	56.45	0.019

**Mann-Whitney U test (p value > 0.05 = significant)*

Table IV. Comparison between patients who gave up and those who still smoke cigarettes and/or shisha

	x habit	No. of patients	Mean	Standard deviation	*P* value
MMP-2 [μg/ml]	continuing	22	78.17	32.26	0.24
	x-habit	10	68.54	28.63	
MMP-9 [μg/ml]	continuing	22	284.92	144.78	0.52
	x-habit	10	245.28	133.43	
FEV_1 (% predicted)	continuing	22	61.10	14.22	0.30
	x-habit	10	60.33	14.74	
FEV_1/FVC [%]	continuing	22	60.35	6.67	0.44
	x-habit	10	62.08	5.52	
FEV_1 reversibility (% predicted)	continuing	22	63.55	14.71	1.0
	x-habit	10	65.50	14.89	

obstruction and emphysema development in patients with COPD is cigarette smoking [14]. The major hypothesis for the pathogenesis of emphysema is the protease-antiprotease hypothesis. Among various proteases that have been proposed to damage connective tissue components in lung parenchyma, there is now increasing evidence that matrix metalloproteinases play a role in the pathogenesis of COPD and emphysema development [15].

With the increased interest and research on MMPs, we selected both MMP-2 and MMP-9 gene expression in BAL of COPD patients to evaluate the effect of different ways of smoking tobacco mainly by shisha (nargila) and to test the popular belief that it is a safer way of smoking. MMP-2 and MMP-9 explored in this work are known to have the capacity to degrade type IV collagen, the main protein of basement membranes damaged in COPD [15]. Also, both seem to be involved in pulmonary fibrosis [16] and cigarette smoke induced pulmonary vascular remodelling [17].

With the increased awareness of the hazards of cigarette smoking, the old device for smoking tobacco in oriental countries which is the shisha (nargila) is emerging again and is spreading worldwide because of the idea that it is safe. Smoking shisha (nargila) is now a very frequent practice even in Europe. Its smoke includes a cocktail of tobacco leaves in addition to smoke of burned coal or wood and other biomass such as honey, molasses or other plant extracts to give the flavour of fruits (for example apple, mint etc.). Respiratory alterations associated with smoking of this cocktail of materials are not known yet.

A few studies have covered the effect of smoke of materials other than tobacco. Montano *et al.* reported that the clinical respiratory alterations associated with long-term exposure to wood smoke during cooking have the same effect as cigarette smoking [18]. Also, domestic exposure to the smoke from firewood increases the prevalence of respiratory diseases such as chronic bronchitis and emphysema [19, 20].

In our work, the age of the patients and the mean amount and duration of smoking tobacco in the form of cigarettes, shisha (nargila) and both cigarettes and shisha (nargila) were comparable, with no statistical difference. We found that there is an increase in the expression of MMP-2 and MMP-9 gene in the BAL fluid of all of our patients, with a significant positive correlation between MMP-2 and MMP-9 gene expression values in the group of shisha smokers only, which indicates that smoking shisha (nargila) is as hazardous as smoking cigarettes and has the same risk of inducing an increase in MMP-2 and MMP-9 gene expression.

In our study, the mean FEV_1 (% predicted) of the shisha smoker group was significantly lower compared to the cigarette smoker group. Also, there was a significant negative correlation between MMP-9 gene expression, FEV_1 (% predicted) and FEV_1 reversibility (% predicted) values in the shisha smoker group, which confirms that the notion that smoking shisha is safe is a myth.

We found that in the cigarette smoker group although MMP-2 and MMP-9 gene expression were higher in severe cases of COPD than in mild/moderate cases, there was no statistically significant difference. This could be explained by the difference between individuals in the response of special genes to smoking [21]. However, in the shisha smoker group there was a significant statistical difference between severe cases and mild/mo-derate cases of COPD concerning MMP-2 and MMP-9 gene expression. Moreover, we found that the difference between MMP-2 and MMP-9 gene expression values in the ex-smoker patients was lower than in those who continued smoking, although the values were not significant. This is due to the fact that habitual smoking has a distinctive and reproductive effect on macrophage activation, inducing a dramatic and highly consistent change in macrophage gene expression patterns. This suggests that COPD risk may relate to the cumulative effects in macrophage gene expression in smokers [21].

Our data demolish the false belief that smoking shisha is safer than smoking cigarettes because its smoke is purified and diluted as it is washed when it passes through water. On the contrary, its smoke carries many hazardous substances, as its tobacco is usually mixed with other biomaterials. So, smokers suffer not only the effect of non-filtered tobacco but also the smoke of burned coal, wood, glycerin, molasses, dried plants and other modified fragments that add flavour to the smoke.

In conclusion, although the specific scientific work on shisha is limited, our data confirm the incorrectness of the belief that shisha is safer than cigarette smoking.

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