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Heavy Smoking and Tears Protein Pattern

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In this research, the change in tear protein electrophoretic pattern in smokers, heavy smokers compared to non-smokers were investigated. The survey was conducted for 100 volunteers, out of which 50 were non-smokers, 25 smokers and 25 heavy smokers. The subjects, all male and aged 20–30 years old, filled a table in order to get some information about their health and smoking history. Total tear proteins and lysozyme concentration were measured and the proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed some alterations in tear protein pattern of smokers that was more noticeable in heavy smokers. The number of electrophoretic bands in tears of heavy smokers was about 25% more than of non-smokers. The changes observed in tear proteins of heavy smokers can explain the higher incidence of dry-eye syndrome observed in smokers.

Key Words: Smoking, Heavy smokers, Tear proteins, Eye disorders.

INTRODUCTION

Cigarette smoking has long been recognized as a strong risk factor for coronary heart disease and cerebrovascular disease[1–4], increased white blood cell count due to greater numbers of neutrophils[5, 6], higher levels of haemoglobin and hematocrit[7], higher level of cholesterol[8], lower levels of high density lipoprotein cholesterol (HDL-C)[9], lower blood pressures and less body mass compared to non-smokers[10]. It has been found that the prevalence of smoking among diabetic patients is significantly higher than that in the general population[11]. Smoking is also a well-known risk factor for the development of atherosclerosis[12, 13]. Recently, the problem of passive smoking as a health risk has widely been discussed[14–16]. Although detailed information on the role of passive smoking on hemostatic parameters is still limited[17–19], Schmid *et al.*[20] investigated the effect of passive smoking on platelet thromboxane and stated that passive smoking may activate thromboxane A_2 release from the platelets, contributing to the development of hemostatic imbalance.

Cigarette smoking may also be a risk factor in several eye diseases, including macula degeneration, glaucoma and cataract. Ischemic, toxic and oxidative effects of cigarettes play an important role in damaging ocular tissue. Tobacco is one of the most common toxic substances in everyone's life including non-smokers exposed to cigarette smoke. Smoking can cause symptoms of dry-eye disease, a common

eye disease especially among older individuals. Dry-eye syndrome has a high frequency of occurrence among cigarette smokers, even passive smokers, compared to non-smokers[21]. Stability and functionality of tear film plays an important role in ocular surface diseases. Dry-eye patients typically suffer from discomfort, burning, irritation, photophobia and blurred vision and have an increased risk of corneal infection and the resulting irreversible tissue damage[22, 23]. Analysis of tear film components, especially tear protein profiles, can play a significant role in the diagnosis of ocular surface diseases and in the development of new therapies.

The aim of this research was to measure the total tear proteins and the concentration of lysozyme, the most important tear protein, as well as the electrophoretic pattern of tear proteins in two groups of cigarette smokers in order to compare them with normal subjects.

EXPERIMENTAL

The following materials were purchased and used without further purification: Acrylamide (Sigma), N,N′-methylene-bis-acrylamide (Bis) (Sigma), tris-(hydroxymethyl) aminomethane (Tris) (Sigma), sodium dodecyl sulphate (BDH), ammonium persulphate (BDH); 2-mercaptoethanol, glycine, N,N,N′,N tetramethylene diamine TEMED, molecular weight markers for peptides, fixing solution, brilliant blue-G concentrate (all from Sigma); acetic acid, methanol, sodium chloride, radial immunodiffusion kit for human lysozyme; 'NL' NANORID™ (binding site Birmingham, GT-073.3); microprotein determination kit (Sigma).

100 male volunteers aged 20–30 years, 50 non-smokers as controls, 25 smokers and 25 heavy smokers were asked to fill in a form (Table-1).

TABLE-1
THE QUESTIONNAIRE FILLED BY VOLUNTEERS IN ORDER TO DECIDE WHICH INDIVIDUAL SHOULD BE EXCLUDED FROM THE STUDY

Question	Answer 1	Answer 2	Answer 2
Age	26	22	27
Sex	Male	Male	Male
Origin	South	Central Iran	North
Number of cigarettes/day	8–10	20–25	25–30
Type of cigarette used	Moderate	Cheap	Cheap
Meat food used/week	5	5	5
Itching	+	++	++
Foreign body sensation	+	+	+++
Burning	N	++	++
Tearing	N	+	++
Photophobia	N	+	++
Blurred vision	N	+	++
History of eye infections	+	+	++
Years of smoking	less than 3	3–5	more than 5

They were all students who lived in the university campus and used mostly similar foods provided by the university restaurant. Smoker group used less than 10 cigarettes, while heavy smokers used between 18–30 cigarettes per day. Some sample answers are stated in Table-1; the eye symptoms were indicated by N meaning no symptom and + to +++ meaning moderate to severe. Most of the volunteers used cheap to moderate price cigarettes. The type of their food was asked as the protein content of the food may affect the protein pattern in their tears.

About 10 μL of un-stimulated tear samples were collected without touching lid margins or eyelashes using glass capillaries from each individual. The samples were marked and stored at 4°C until analysis for less than one week. It has been shown that no significant change in protein concentration is observed when stored up to one week at 4°C, up to two months at –20°C and up to 4 months at –70°C[24].

Total tear proteins, the concentration of lysozyme and the electrophoretic pattern were obtained using the following procedures.

Determination of total tear proteins

The Biuret reagent[25] and Lowry *et al.*[26] procedures have been used for the determination of proteins for clinical assays from a long time ago. However, the more sensitive Lowry method has the disadvantage of poor stability of combined reagents, non-reproducibility of the color especially at low protein concentrations and non-linear chromogenic response with protein concentration. A more accurate and simpler method, *i.e.*, the use of Biuret reagents for Lowry method[27], was used in our study. The principle is that the Biuret reagent reacts first with proteins followed by phenol reagent. The colour is developed after about half an hour and the absorbance is read at 700 nm.

All tear samples (from smokers and non-smokers) were diluted with sodium chloride (0.85%) solution, so that the final protein concentration was between 0.015 and 0.1 mg/mL. Diluted samples (0.2 mL) (sodium chloride in the case of blank) were added to biuret reagent (1.2 mL), mixed and allowed to stand at room temperature for 10 min. Folin and Ciocalteu's phenol reagent (0.1 mL) was then added to the tube, and left at room temperature for 30 min. Standard protein solutions provided by the kit were diluted to give concentrations between 0.025–0.01 mg/mL and treated in a similar way. The calibration curve was obtained by plotting the total protein (mg/mL) against the absorption at 700 nm and the total protein of tear samples was calculated using this curve.

Concentration of lysozyme

To specifically measure the biological activity of lysozyme, a radial immuno diffusion kit for human lysozyme was used[28]. The tear samples were diluted as recommended by the kit to values of about 0.01–0.017 mg/mL. The plates were left while open at room temperature for 10 min to warm up before the application of the samples. Each well was checked for the presence of any moisture or dust

and 10 mL of samples were applied to each well very carefully to avoid contamination. The lid was closed tightly and the plate was stored flat at room temperature (not more than 22°C) for 96 h. After the diffusion was complete the ring diameters were measured to the nearest 0.1 mm using a jeweller's eye-piece (Binding Site, code: D040). The sample concentrations were then calculated directly from the calibration curve obtained by plotting the ring diameter squared against the concentrations of the standards, the accurate concentration of each sample was then calculated by taking into account the dilutions made. A calibrator was used each time to check the accuracy of the test. The ring diameter was measured for a series of standard lysozyme solution (Table-2). The calibration curve was plotted using the ring diameter squared and the concentrations of lysozyme standard provided by the kit (Fig. 1). The quantity of lysozyme was calculated in tear samples using this calibration curve.

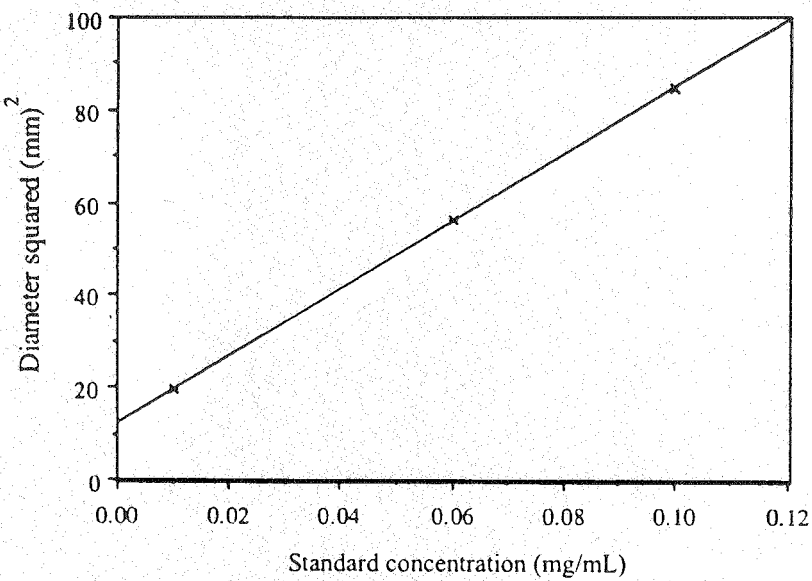

Fig. 1. The calibration curve for the NANORID™ kit

TABLE-2
THE RELATIONSHIP BETWEEN THE RING DIAMETER AND LYSOZYME CONCENTRATION

Concentration (mg/mL)	Ring diameter (mm)	Diameter square (mm^2)
0.01	4.4	19.36
0.06	7.5	56.25
0.10	9.2	84.65

Electrophoresis of tear proteins

A separating gel (10%) was prepared and the stacking gel (5%) poured over

it after being set[29]. Tear samples were diluted (1 : 3) with sample buffer and injected into each well after removal of the combs and rinsing the wells with distilled water. The syringe used to apply the samples was washed five times with distilled water between each sample application to avoid cross-contamination with the different proteins. This stage of the preparation was performed as quickly as possible, because the samples diffuse sideways if they stand for a long time in the wells before the electrophoresis run. A current of 25 mA/1 cm of gel was applied and the dye front reached 1 cm above the bottom of the separating gel in about 4 h, when the run was stopped. The gel was then stained with colloidal brilliant blue-G.

RESULTS AND DISCUSSIONS

Total tear proteins showed a decrease in the case of smokers and an increase in heavy smokers compared to normal volunteers (Fig. 2). Fig. 2 also shows that the concentration of lysozyme (mg/mL) has been increased in both smokers groups compared to non-smokers.

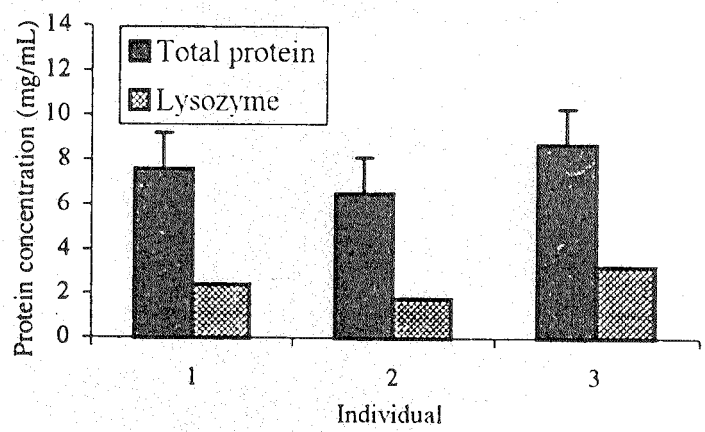

Fig. 2. Mean protein concentration measured by modified Lowry method and lysozyme activity in tear samples. The mean values are taken as average protein concentration (±SE) of 25 individuals. The numbers on the x-axis refer to non-smokers, smokers and heavy smokers respectively

Table-3 compares the number of bonds found by electrophoresis in a normal group (non-smokers) with those of smokers. The electrophoretic pattern of all groups showed most of the tear proteins (lactoferrin, lysozyme, immunoglobulin, tear specific pre-albumin and serum albumin). However, it was found that the total number of electrophoretic bonds decreased in smokers and this is mostly due to the loss of bonds related to immunoglobins (A and G) as well as lysozyme. In the case of heavy smokers, the number of bonds showed an increase compared to smokers and non-smokers. This can be related to a decrease in their tear volume due to the toxic effects of nicotine in tobacco.

TABLE-3
THE TYPE, MOLECULAR WEIGHTS AND MEAN NUMBER OF ELECTROPHORETIC BONDS (±SE) OBSERVED IN TEARS OF SMOKERS, HEAVY SMOKERS AND NON-SMOKERS

Protein	MW (KD)	Number of bands		
		Non-smokers	Smokers	Heavy smokers
GP1	2000	1	1	1
GP2	1300	1	1	1
IgA	260–360	1	—	2
Gp3M	200	1	—	1
IgG	150, 310, 350	3	2	3 dense
Transferrin	75	2 dense	1 dense, 1 faint	2 dense
Lactoferrin	74, 81	2 dense	1 dense	2 dense
Albumin	65	1	1	1
Secretory component	59, 78, 260–360	3–4	2 faint	4
Alpha globulin	>40	2–3	2	3
Serum pre-albumin	31, 70	2 faint	2 faint	2 dense
Tear pre-albumin	15–19	2–3	2	3 faint
Lysozyme	14.5, 23, 24	1 dense, 2 faint	1 dense, 1 faint	2 dense, 1 faint
Total	—	22–24	17	28

Conclusions

Although much literature indicates the effect of smoking on a number of systemic diseases, there is almost no report about the influence of smoking on tear protein pattern. The change in total protein concentration and pattern may be a cause for development of dry-eye syndrome leading to its consequences such as burning and itching. We showed some noticeable variations in electrophoretic patterns of tear proteins, especially in the case of heavy smokers. The total protein concentration decreases in smokers compared to non-smokers, although in the case of heavy smokers a total increase may be observed due to the reduced break up time and tear volume caused by dry eye like symptoms. The most evident variation in tear protein pattern is in the regions related to immunoglobins, lysozyme and lactoferrin. It is known that these proteins are mainly involved in the defense mechanism of eye. It can be concluded that the defense mechanism in the eye changes in response to smoking. Smoking increases the chances of occurrence of dry eye symptoms. The change in protein pattern can be explained in terms of direct toxic effect of smoking on proteins leading to oxidative damage on them. The damaged proteins may show new electrophoretic bands or some displaced bands.

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