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The Effects of Smoking on the Prostates of Rats which are Exposed to Cigarette Smoke

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ABSTRACT

Background: *Smoking is a hallmark preventable health issue worldwide. The role of smoking in the aetiology of prostate cancers remains a lack of consensus.* ***Methods and material:*** *100 male albino Wistar rats were used. The smoking group (n=40) was exposed to cigarette smoke for 2 hours per day for 8 weeks. The rest were set aside as a control group (n=60). At the end of 8 weeks, the rats were sacrificed and haemoglobin and serum cotinine values were measured. The prostate glands were excised and dorsolateral strips were prepared for in vitro examination. After application of 80 mM KCl, carbachol, and phenylephrine, respectively, contraction values were obtained and calculated in grams. Prostate tissues were stained using p53, bcl-2, cyclin D, and c-erb-B2 immunohistochemical and Hematoxylin-Eosin gram staining.* ***Results:*** *In the smoking group, the mean haemoglobin value was found out as 16.5 mg/dl, and 11.5 mg/dl in the control group respectively. The cotinine values were determined to be 76.43 ng/ml in the smoking group and <10 ng/ml in the control group. The prostate contractions in the smoking group were seen significantly lower than the contractions in the control group After immunohistochemical investigation of the prostate tissues, meaningful acinar dilatation and atrophy with Hematoxylin-Eosin staining were found out. Foam cells were observed in the smoking group.* ***Conclusions:*** *In our study, we determined that smoking significantly decreased prostate contraction. The findings after immunohistochemical investigation may help to clarify the effect of smoking on prostate cancer.*

Keywords: Prostate cancer, Smoking, *In vitro*, Immunohistochemical, Rat

INTRODUCTION

Smoking is a critical problem on the rise worldwide. Smoking causes health in the lungs and other organs. Smoking is an important etiologic factor for urological cancers and transitional cell carcinoma [1].

The relationship between smoking and various cancers has been thoroughly investigated. Its relationships with lung, larynx, bladder, oesophagus, pancreas, kidney, renal pelvis, oral, pharyngeal, stomach, and endometrial cancer has been well documented [2]. Many investigators have proposed a relationship between smoking, benign prostate hyperplasia and prostate cancer [3-5]. Some studies have proposed a positive correlation between prostate cancer and smoking [6-8], and in some it has been shown that it does not affect prostate cancer incidence but does increase mortality [9,10].

Carbon monoxide is formed by the partial oxidation of organic compounds in smoke. It is colourless and scentless. It is found in the smoke aerosol at levels approximating 2.9-5.1%. In the blood, haemoglobin binds to iron to form

carboxyhaemoglobin. This decreases the capacity of haemoglobin to release oxygen to tissues. It is 220 times more for the relation of haemoglobin to carbon monoxide than the relation to oxygen. About 0.5-1% carboxyhaemoglobin levels have been found in smoking people while reaching 1-20% in smoking people [11,12].

Nicotine is a colourless volatile base. It may be well dissolved in water. The consumption of tobacco per cigarette is generally 0.7-1.0 g, containing 17 mg of nicotine. Nicotine, when gotten from a cigarette, is rapidly absorbed into the lungs which have a very large absorption surface and passed to circulation. 80-90% of absorbed nicotine is metabolized in the liver while some is metabolized in the lungs and kidney. Around 10-20% of nicotine is extracted from the liver unchanged. It is transformed to its major metabolite cotinine in the liver. Nicotine exerts its effects by activating the nicotinic cholinergic receptors in the target cells. Nicotinic receptors have been divided into two subgroups, namely neuronal and muscular [13].

It has been shown that smoking causes damage to vascular endothelia, and tends to be the first cause of atherosclerosis development in endothelial impairment. The neutrophil number was proportionally increased in smokers due to exposure to cigarette aerosol [14-16].

Haemoglobin and haematocrit increased, also erythrocyte, leucocyte, neutrophil, lymphocyte, and thrombocyte numbers increased [17-21]. Principle thrombocyte potential increasing mechanisms of cigarette are an increase in ADP induced thrombocyte aggregation, increase in plasma fibrinogen levels, and a decrease in levels of plasminogen and plasminogen activators. This is caused to hypercoagulability [17]. Cigarettes are one of the leading vasospastic agent and also cause to atherosclerosis [22,23]. An increase in haemoglobin production is a reaction of the body to chronic smoking, leading to the production of other blood cells in the bone marrow. This is a cause of hypercoagulability [15].

The aim of this study was to investigate the effects of cigarette using potassium chloride, carbachol, and phenylephrine in vitro on prostate contraction in the rats. Secondly, we investigated morphological changes of prostatic tissue on smoking rats.

MATERIAL AND METHODS

Animals

Male Wistar-Albino rat (DETAB, Kocaeli, Turkey) weighing 200-250 g were used in this study. Animals (4-5 per cage) were kept in the laboratory at 21 ± 1.5ºC with 60% relative humidity under a 12 h light/dark cycle (lights on at 8.00 p.m.) for 2 weeks before experimentation. Tap water and food pellets were available *ad libitum*.

Ethics

All procedures involving animals were in compliance with the European Community Council Directive of 24 November 1986, and ethical approval was granted by the Kocaeli University Ethics Committee (Number: AEK 139/12-2005, Kocaeli, Turkey).

Experimental group and drugs

We had two groups, namely smoking (n=40) and control (n=60) groups. Smoking group animals were exposed to smoking aerosol for 8 weeks before euthanization. Control group animals were not exposed to smoking aerosol for 8 weeks, but were kept in the same conditions as the smoking group. In the smoking group, 1 rat was omitted from the experiment since the rat died during smoking exposure.

Apparatus of smoking exposure

For the smoking aerosol exposure, a special apparatus was built as defined by Chen and predicated on the Walton's modified smoking exposure setup [24,25]. There were three compartments which were connected with each other in the apparatus as defined by Chen. The first compartment was the production section, in which smoking aerosol was formed and accumulated, while the second compartment was the dilution section in which the smoking aerosol was diluted, and the third compartment was the exposure section in which the rats were exposed smoking exposure. If the volumes of the first and third sections were 1, the volume of second section was 10. First of all, the smoking aerosol burning in the first section was collected and when it was passed to the second section, it was diluted with room air at a ratio of 1/9, such that 10% smoking aerosol was obtained. The rats were exposed to 10% smoking aerosol in the exposure section. Air pumps were used between these three sections to pass the aerosol from one to another. Chen

used one cigarette per subject in his study [25]. The apparatus was built according to the volumes and permanent flow in this setup. The exposure room was built according to in which 6 rats could be kept in once a time, and the mean weight of the animals could reach 250 g. Four inhalation rooms of 28 litres were also prepared, along with smoke burning rooms of 28 litres as well as 280 litre dilution rooms providing 10% dilution (Figure 1).

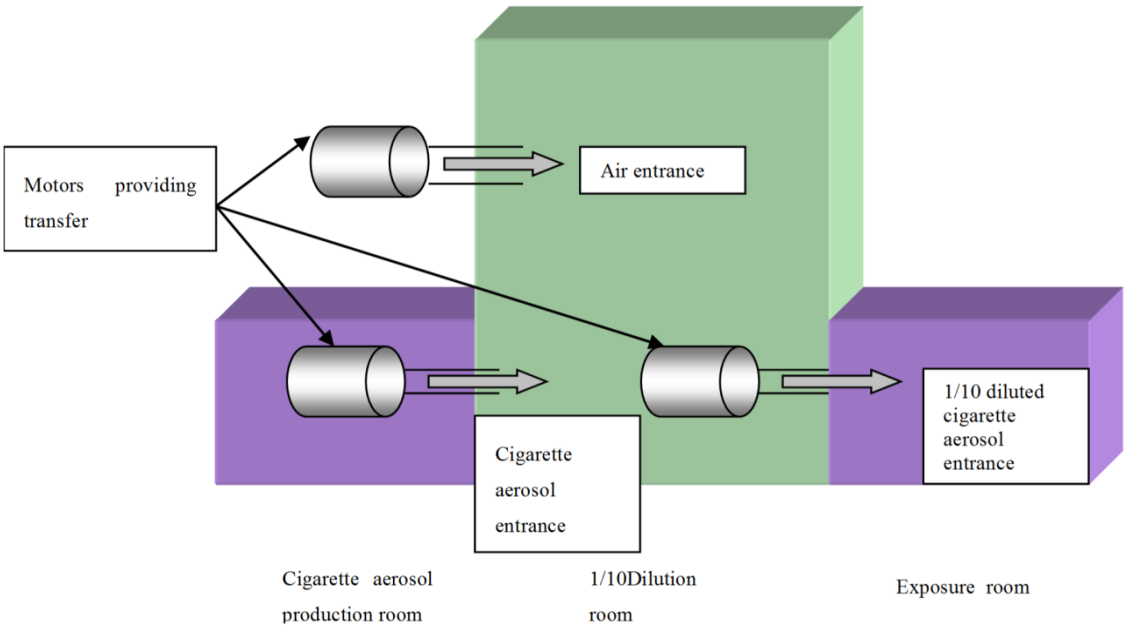

Figure 1 Drawing of the "Modified Walton smoking aerosol exposure apparatus" which was built for this study

Smoking aerosol production and exposure rooms were designed with a height of 20 cm, width of 28 cm, and length of 50 cm. The dilution room had a width and length of 65 cm, a height of 66.2 cm, was also sealed to prevent air leakage. All sections were designed to avoid air pass-through, and higher parts had window covers to provide cleaning, air conditioning, and to provide a clear view of the inside of the apparatus. Permeant aerosol between the sections and aquarium ventilation motors was used to allow for air circulation. This current was transferred from one part to other by hoses used in aquarium air ventilation system (Figure 2).

Figure 2 Smoking aerosol production and exposure rooms

In this study, 0.9 mg nicotine and 12 mg tar including long LM (Philsa A.Ş.) cigarette was used. An ash tray was placed to collect the ashes of the cigarettes burning inside and to push the cigarette inside it when the cigarettes burning is finished. Holes were opened around the sections to release the carbon dioxide which was produced by rats and also to release the 1/10 diluted aerosol provided to exposure rooms.

All groups were exposed to smoking aerosol every day at the same time, in a well ventilated and well bedded exposure sections for two hours. Exposure to smoking aerosol was one cigarette per rat. The rats which were not exposed to cigarette aerosol were also placed every day at the beginning of the daily study in the ventilated apparatus for two hours to simulate the effects of similar stress in the other groups.

Surgical process

Ether anaesthesia was given on the 8th week, and the rats exposed to smoke aerosol were decapitated and 5cc blood was removed from their vena cava inferior after the abdomen and thorax was opened. Prostate tissue was removed. Prostate dorsolateral strip was prepared for the *in vitro* experiments. The remaining prostate tissue and the dorsolateral strip was taken to pathology hood in 10% formaldehyde solution after the *in vitro* study.

Measurement of serum cotinine and haemoglobin levels

The immulite nicotine metabolite kit is a solid phase chemiluminescence enzyme immunometric assay designed in immulite automatize analyzer for the quantitative measurement of basic nicotine metabolites and cotinine in the serum. Value of 25 ng/ml and above was the borderline to separate smokers. The variation coefficient of the method was less than 16%. The prior determination limit was 2 ng/ml.

In blood samples removed from the rats, serum was removed by centrifuge in lower speed (1000 g for 5 minutes). Serum cotinine levels were measured by immulite nicotine kit (apparatus immulite one). The prior determination limit was defined as 10 ng/ml to increase the sensitivity of the procedure.

Using a random selectivity method, haemoglobin levels of 12 subjects from the control group and 10 subjects from the smoking exposure groups were examined.

The pathological examination of prostate (Histopathological studies)

After the prostate tissue was dissected and detected in 10% formaldehyde, standard samples were taken and passed through formalin, alcohol, xylene, and kerosene tracks and 5-micron thickness sections were taken. After all the tissues were painted with haematoxylin and eosin, immunohistochemical examinations were made by p53, cyclin D, Bcl-2, and c-erbB-2 antibodies. All of the sections were examined by microscope to investigate prostatic intraepithelial neoplasm, prostate carcinoma and other morphological changes.

Examination of in vitro prostate contraction responses

Prostate tissue was removed immediately and put into Krebs-Henseleit solution (in millimole scale. It was prepared as follows: 1L distilled water, NaCl: 118, KCl: 4.7, $NaHCO_3$:25, KH_2PO_4: 1.2, $MgSO_4$: 1.2, $CaCl_2$: 2.5, and glucose: 11.1. The dorsolateral ribbon was removed. After that, the prostate dorsolateral ribbon was attached to a hook diagonally by its lower end as detected with yarns in both sides at the base of isolated organ bath, then gassed with a mixture of 95% O_2 and 5% CO_2, after which 20 ml Krebs-Henseleit solution was applied. The upper side of the prostate dorsolateral ribbon was connected to micrometric player, which was attached to an isometric force transducer (FTO_3) connected to a polygraph (MP_3O).

After the prostate dorsolateral ribbon was hooked up, 1 g pretension was applied and it was incubated for 60 minutes. During this period, the tissue was bathed every 15 minutes with fresh solution a total of three times. In the end of incubation period, 80 mM KCI contraction response was taken. After that, it was bathed and for 60 minutes incubation

period it was bathed 3 times more. After that, carbachol at 10^{-8}-10^{-4} M concentration was applied to the tissues and cumulative dose-response curves were obtained. After that the tissue was bathed again it was taken to 60 minutes incubation period. During this period, bathing continued at 15 minutes time intervals. Lastly, phenylephrine was given in 10^{-8}-10^{-4} M concentrations and a cumulative dose-response curve was obtained. The contraction responses to applied KCI, carbachol, and phenylephrine were calculated using gram scales.

Statistical analyses

The obtained data was analysed using a Mann-Whitney U test for the binary comparison of groups and by a chi-square test for the evaluation of pathological results. $p<0.05$ was accepted as statistically significant.

RESULTS

Haemoglobin and cotinine values measured in the end of the experiment

Serum cotinine levels were measured in all subjects. In non-smoking group, cotinine levels were determined to be lower than 10 ng/ml which was accepted as the base border (Table 1). Haemoglobin levels were measured at *in vitro* experiment performed subjects (Figure 3).

Table 1 Blood Cotinine Values in smoking aerosol exposure group

Number	Mean	Standard Deviation	Minimum Value	Maximum Value
39	76.43	48.01	36.10	238.00

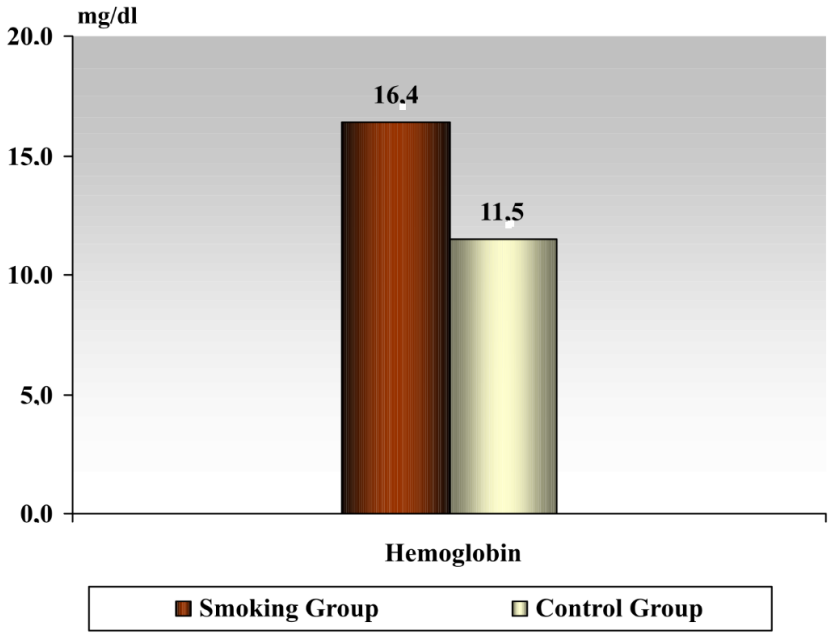

Figure 3 Smoking aerosol exposure group and control group mean haemoglobin values

Pathological examination

In both the control group and the group exposed to smoking aerosol, there was no tumour development or intraepithelial neoplasia in prostate tissue. In both groups, there was common and focused cystic expansions and epithelial atrophy in the prostatic sinus. In some of the sinuses, bubble cells were noticed. After that, immunohistochemical examinations were made with p53, cyclin D, bcl-2 and c-erbB-2 antibodies.

There was no significant difference between the control group and smoking aerosol exposed group when the immunohistochemical photomicrograph features were investigated (Figures 4-9).

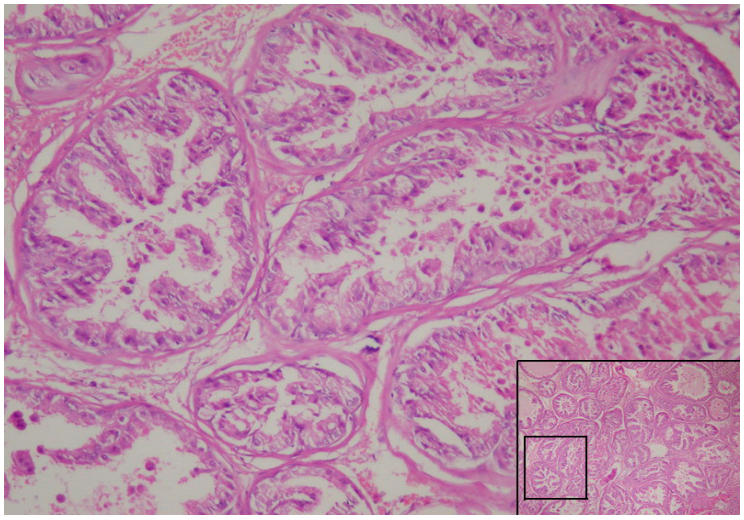

Figure 4 Control group Hematoxylin and Eosin staining

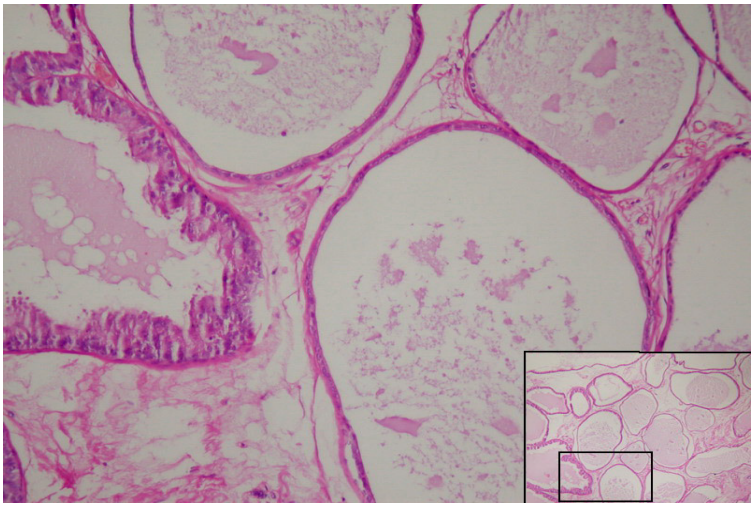

Figure 5 Smoking group Hematoxylin and Eosin staining

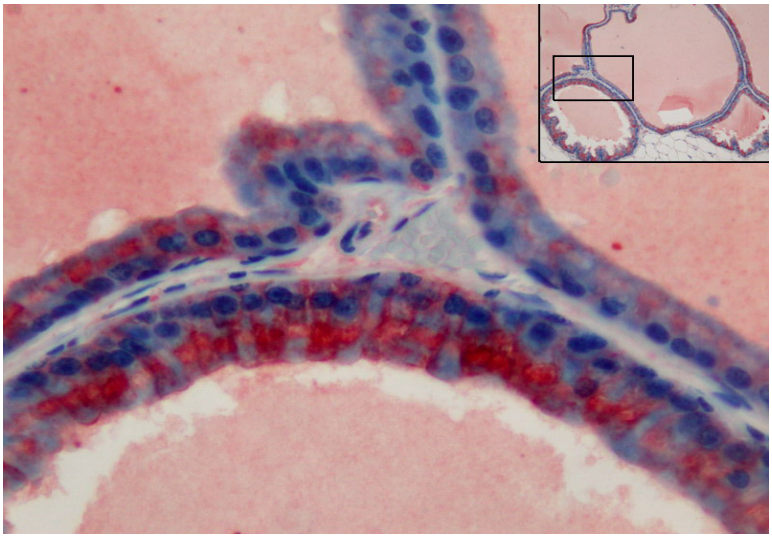

Figure 6 Smoking group p53 immunohistochemical photomicrograph of a section of prostate tissue

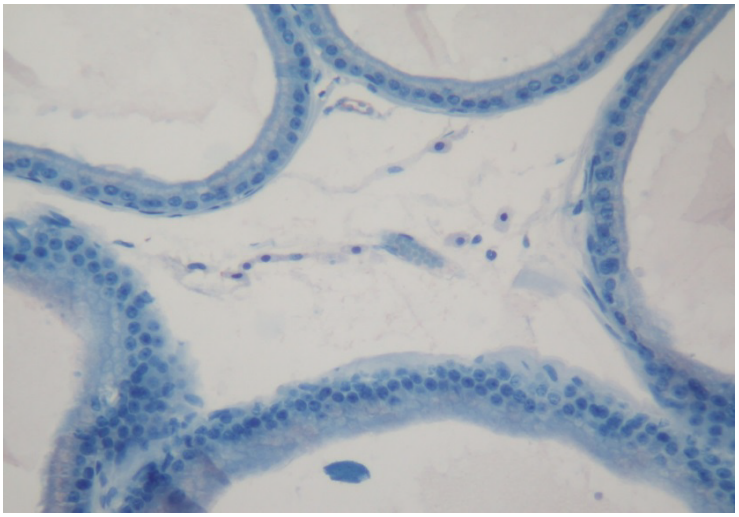

Figure 7 Smoking group c erb B2 immunohistochemical photomicrograph of a section of prostate tissue

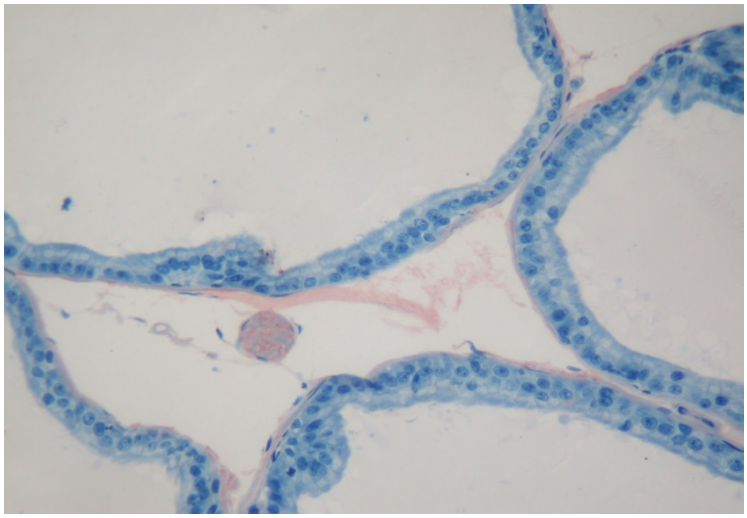

Figure 8 Smoking group bcl-2 immunohistochemical photomicrograph of a section of prostate tissue

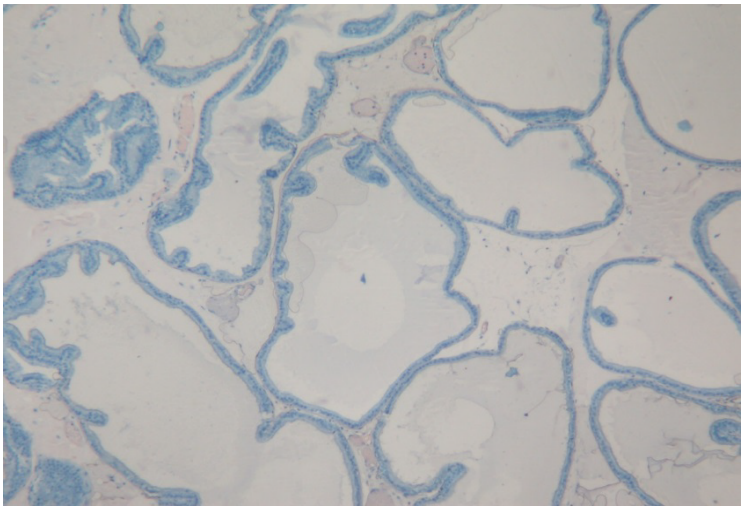

Figure 9 Smoking group cyclin D immunohistochemical photomicrograph of a section of prostate tissue

In the smoking aerosol exposed group, acinar atrophy and acinar dilation in prostate tissue was significantly increased compared to control group ($p<0.05$) (Tables 2 and 3). There was no significant difference between groups when the focal atrophy was investigated ($p>0.05$).

Table 2 The statistical acinar atrophy in smoking aerosol exposure rats and control group

Group		Acinar Atrophy		
		Absent	**Present**	**Total**
Smoking	Number (n)	1.0	38.0	39.0
	Percentage (%)	2.6	97.4	100.0
Control	Number (n)	23.0	37.0	60.0
	Percentage (%)	38.3	61.7	100.0
Total		24.0	75.0	99.0
Chi-square=16.5; p=0.01				

We found significant acinar atrophy in smoking aerosol exposure rats compared to control group ($p<0.05$) (Table 2). There was no significant difference for focal atrophy between two groups ($p>0.05$).

Table 3 The statistical acinar dilatation table in smoking aerosol exposure rats and control group

Group		Dilatation		
		Absent	**Present**	**Total**
Smoking	Number (n)	1.0	38.0	39.0
	Percentage	2.6	97.4	100.0
Control	Number (n)	17.0	43.0	60.0
	Percentage	28.3	71.7	100.0
Total		18.0	81.0	99.0
Chi-Square= 10.6; p= 0.01				

In Table 3, we found significant acinar dilatation in smoking aerosol exposure rats compared to control group ($p<0.05$). In the group exposed to smoking aerosol, there were bubble cells in the sinuses which were not seen in the control group but were statistically insignificant ($p>0.05$) (Table 4).

Table 4 The statistical bubble cell table in smoking aerosol exposure rats and control group

Group		Bubble cell		
		Absent	**Present**	**Total**
Smoking	Number (n)	37.0	2.0	39.0
	Row percentage (%)	94.9	5.1	100.0
Control	Number (n)	60.0	0.0	60.0
	Row percentage (%)	100.0	0.0	100.0
Total		97.0	2.0	99.0
Chi-Square=3.2; p=0.076				

In smoking aerosol exposure group bubble cells were seen which were not seen in the control group but it was statistically insignificant ($p>0.05$).

***In vitro* prostate contraction responses**

Carbachol (10^{-8}-10^{-4} M), Phenylephrine (10^{-8}-10^{-4} M) and 80 mM KCI were applied cumulatively to prostate and contraction responses were obtained and calculated in a gram scale. Results were evaluated using a Mann-Whitney U test. In the group that was exposed to smoking aerosol, there was a significant decrease in prostate contractions compared to control group ($p<0.05$) (Figures 10-12).

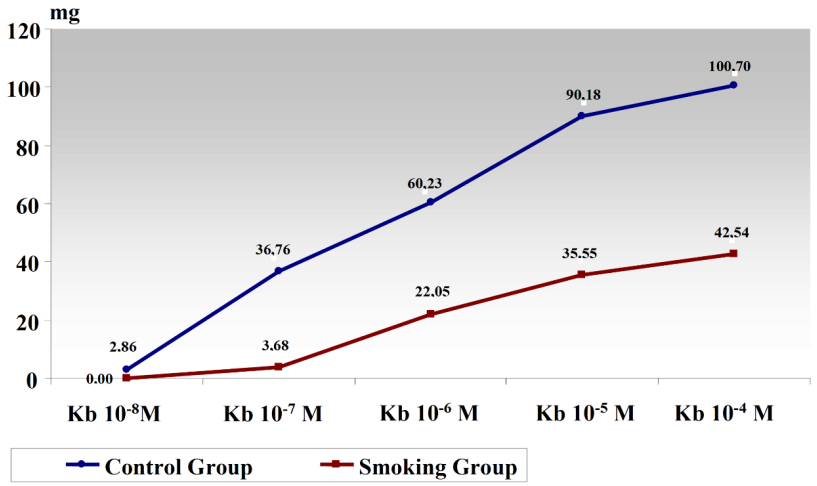

Figure 10 Prostate dorsolateral strip contraction response to 10^{-8}-10^{-4} M Carbachol in smoking aerosol exposure rats and control group

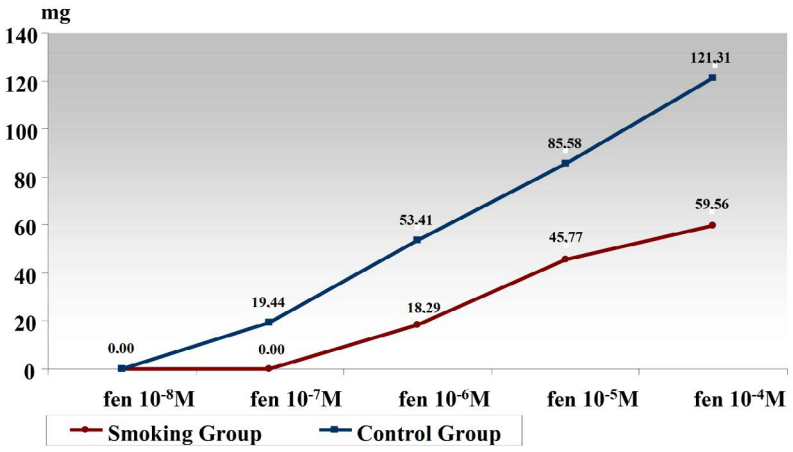

Figure 11 Prostate dorsolateral strip contraction response to 10^{-8}-10^{-4} M Phenylephrine in smoking aerosol exposure rats and control group

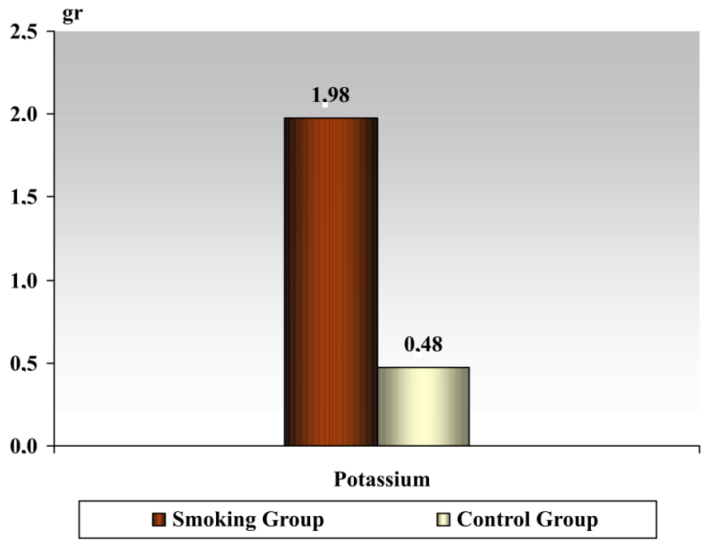

Figure 12 Prostate dorsolateral strip contraction response to 80 mM KCl in smoking aerosol exposure rats and control group

DISCUSSION

Prostate cancer is the highest cancer incidence in men in America, and the second leading cause of death due to cancer. For prostate cancer, besides some environmental factors, there are other certain risk factors such as old age, history of the illness in close family members, and being African-American [26].

Most of the effects of smoking on human body were investigated and miscellaneous results were obtained. Carcinogenic and mutagenic effects in particular are seen from the carcinogenic materials inside cigarettes, which number about 55 nowadays. These are polyaromatic hydrocarbons, nitrosamines, aromatic amines, aldehydes, and other organic and inorganic compounds [24].

The carcinogenic effects of cigarette have been investigated for the prostate as well as other organs. There is no consensus on the positive relationship between cigarette smoking and the aetiology of prostate cancer. There are prospective studies that telling the positive and negative effects between cigarette and prostate cancer [6-10]. Hickey, et al. investigated the relationship by analysing the available literature, and determined that there was a significant relationship between cigarette and prostate cancer in the 5 of 15 case-control studies [27].

Smoking can change the levels of steroid hormones in circulation. The bioactive testosterone levels increased in man by smoking and bioactive oestrogen levels decreased [28]. In some of the performed epidemiologic studies, there was significant positive relationship between smoking in men and total or free testosterone which was like serum total androstenedione [11,29]. Some of the studies showed no significant increase between smoking and hormone levels.

In a study by Küpeli, et al., it was shown that cigarette did not significantly change hormone levels, but was a factor increasing prostate level [4]. Seitter and Barrett-Connor, Glynn, et al. found no relationship between smoking and benign prostate hyperplasia (BPH). In a retrospective study by Daniell on prostatectomy patients, it was observed that prostate volume was smaller in smoking patients [29-31]. In prostate development, androgen is not isolated. Testosterone, androstenedione, oestrogen, and prolactin all affect the development of prostate. Oestrogens exert a synergistic effect on androgen-induced prostate growth [29].

Nicotine exerts different and variable effect in many organs. Nicotine causes relaxation in striated muscles, and in lower doses bradycardia and hypotension, while in higher doses causing tachycardia and hypertension. It exerts its effects in the central nervous system by nicotinic receptors. It stimulates the sympathetic nervous system and it induces catecholamine release from adrenal medulla [13]. Kester, et al. determined alpha-1 adrenergic, dopaminergic, muscarinic cholinergic, serotoninergic and H1 histaminergic neuroreceptors in human prostate. There have been many studies investigating the effect of smoking on prostate volume and lower urinary system. Küpeli, et al. noted that smoking causes an increase in prostate volume [4]. Roberts, et al. in Olmsted country study mentioned that in low or moderate smoking people lower urinary system problems were little and also in moderate or higher smoking people lower urinary system problems were same with non-smoking people [32].

In our study, one cigarette per rat was used per studies by Nolan and Chen. This amount equals the amount of smoking by a smoker in a single day. The study by Van Adrichem and Craig was the longest study for the exposure to smoking aerosol between the studies made on this subject in the literature. In our study, even a period of time above this duration, an 8-week smoking exposure was applied.

When we investigated the apparatus choices in which rats were exposed to smoking aerosol in the literature, the modified Walton smoking aerosol exposure apparatus, which was effective and detailed, was preferred. There are two main reasons why we selected this apparatus. Firstly, this technique has been accepted in the literature, and secondly it was easy to ask for technical help because this apparatus has been used in Ankara University and in our university previously [33,34].

We measured the haemoglobin and cotinine values to show that smoking exposure level is enough or not. Cotinine is a metabolite of nicotine, and 70-80% of nicotine which enters the body is converted to cotinine. Value of 25 ng/ml serum cotinine level was the baseline value border chosen to determine smoking people. Cotinine is excreted in the urine as a gluconate conjugate. The elimination half-life of cotinine is 16-18 hours [35]. Cotinine is a good agent to show short-term smoking exposure. The mean value of cotinine in smoking group was found to be 76.43 ng/ml [36].

Smoking increases haemoglobin and haematocrit values and these values are stated at high level for 120 days during

erythrocyte life [16,17]. We measured haemoglobin and haematocrit levels with cotinine to determine the long-term exposure. We measured the haemoglobin and haematocrit values of 10 rats in the smoking group and 12 rats in the control group through a random selection method. Haematocrit values were very high and difficult to measure in the smoking group, so we checked the haemoglobin levels. We determined 16.4 mg/dl (16-17.5) haemoglobin value to be the mean value in the smoking group and measured 11.5 mg/dl (10.4-12.6) mean haemoglobin value in the control group.

We determined that when 80 mM KCI, Carbachol (10^{-8}-10^{-4} M) and Phenylephrine (10^{-8}-10^{-4} M) were applied in these doses, smoking significantly decreased prostate contractions (p=0.001). We thought that different results determined in previous studies were probably related with the changeable effect of nicotine. Nicotine in lower doses affects parasympathetic ganglions and in higher doses stimulated sympathetic ganglions [13,35]. Besides, nicotine increases nitric oxide release and can cause variety in results by its direct effect on smooth muscle relaxation via nitric oxide [13]. Smoking decreases prostate contraction, so lower urinary system symptoms are seen in older smoking people compared to non-smokers. Operation morbidity is increased when patients see doctors due to the negative effects of nicotine on other organs. In a study on American men by Rohrmann, et al., nicotine did not affect lower urinary system symptoms, although it increased lower urinary system symptoms in 50 package/year or more smokers [36]. In this area, prospective studies are required in which there are no effects of nicotine on lower urinary system symptoms in men.

For the p53 gene mutation in prostate cancer values between 0% and 80% have been noted. In most studies it is about 50%. Macoska, et al. determined that there was a ratio of 70% in the deletion of chromosome 8p in primary prostate cancer [37]. Cher, et al. showed that chromosome 8p loses its short arm in 80% of metastatic prostate cancer patients. Also, in various studies deletions in different regions of chromosome were introduced, so there may be more than one tumour suppressor genes in this area [38].

Craft, et al. showed that there is a relationship between her-2/neu (c-erbB2) oncogene and androgen receptor. This supports the theory that tyrosine kinase receptors may play a role in prostate cancer progression [39].

In our study, we performed immunohistochemical colouring with p53, bcl-2, c-erbB2, and cyclin D antibodies in prostate tissues. There was no significant difference between smoking and control groups. This may indicate that more smoking exposure is required for the formation of mutation in tumour suppressor genes. In staining with haematoxylin-eosin, we found statistically significant acinar dilatation and atrophy (p=0.001). We also found bubbling cells in the smoking group which were not in the control group. We did not encounter prostate cancer in this study's subjects.

A study by Humprey, et al. determined that prostate cancer may be defined as pseudohyperplastic prostatic adenocarcinoma and is similar to low grade, hyperplastic prostate tissue. This study supports the claim that there is a variant prostate cancer type which wraps up the papillary structures, containing branching and cystic dilatations in complex large glands [40]. Arista-Nasr, et al. found 1-3% pseudohyperplastic adenocarcinoma in TUR-P patients [41].

CONCLUSION

An increase in acinar dilatation, atrophy, and bubble cells as found in our study may be the starting stage of pseudohyperplastic prostatic adenocarcinoma. Future studies are required to shed more light on our findings.

DECLARATION

Conflict of interest

The authors declare that they have no conflict of interest.

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