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Effects of concurrent chronic administration of alcohol and nicotine on rat sperm parameters

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Summary

The prevalence of cigarette and alcohol consumption is high among young adult males during the reproductive period. The current study aimed to evaluate the impact of concurrent chronic administration of nicotine and ethanol on the quality of sperm in the rat. Fifty healthy Wistar male rats were randomly divided into five groups ($n = 10$) and were given the following for a period of 50 days: ethanol (E), nicotine (N), ethanol and nicotine (E/N); the control group (C) and an intact (I) group. Body weight as well as the weight, volume and dimensions of the testes and the weight of the cauda epididymidis and vas deference were measured. The concentration, motility, viability and membrane integrity of sperm were also assessed. There were no significant differences between body weight and all testis parameters including weight, volume and dimensions. The concentration and motility of sperm in the E/N group was significantly reduced compared with the control group ($P < 0.01$). Nevertheless, only a marginally significant decrease in sperm viability was found in the E/N group compared with the control group. The study indicates that concurrent chronic administration of ethanol and nicotine may disturb male reproductive function.

Introduction

The prevalence of cigarette smoking is high among young adult males during the reproductive period (Trummer *et al.*, 2002; Hosseinzadeh Colagar *et al.*, 2007). Also, the majority of young adult males consume cigarettes and alcohol together. Some studies have indicated that consumption of either alcohol or nicotine encourages consumption of the other (Kouri *et al.*, 2004). In one estimation, 90% of Canadian smokers drank alcohol (Kozlowski & Ferrence, 1990). Furthermore, a positive correlation has been reported between the level of consumption of cigarettes and alcohol (Sobell *et al.*, 1990).

Nicotine is the most important alkaloid constituent of tobacco; the main metabolite of nicotine is cotinine (Zenzes *et al.*, 1996). Nicotine and its major metabolites have been found at significant levels in smokers' seminal plasma. It is evident that these materials can pass across the blood–testis barrier and intervene with male reproductive activity (Reddy *et al.*, 1995). A significant decrease in semen quality as demonstrated by reduced sperm count, motility and viability as well as altered morphology have been associated with smokeless consumption of nicotine (Lewin *et al.*, 1991). Ethanol, the most commonly abused substance, may also exert harmful effects on the reproductive system. Reduced fertility and sterility are among the probable side effects of alcohol drinking. Chronic ethanol consumption also impairs spermatogenesis (Srikanth *et al.*, 1999; Talebi *et al.*, 2011). Nonetheless, the existing information on the effects of alcohol on male reproduction is controversial.

It has been suggested that concurrent administration of nicotine and alcohol may intensify the effects of each other (Kouri *et al.*, 2004). A sharp decrease in sperm count was reported after concurrent chronic administration of 2 mg kg^{-1} s.c. nicotine and 3 g kg^{-1} p.o. ethanol in male rats (Dhawan & Sharma, 2002). Although some studies have addressed the impact of nicotine and alcohol

as well as concurrent administration of nicotine and alcohol on reproductive parameters and some sperm parameters in human and animal models (Rantala & Koskimies, 1987; Dhawan & Sharma, 2002), the impact of nicotine administration, at doses similar to those seen in moderate smokers, concurrent with alcohol administration, on sperm motility, viability and sperm count have not been reported yet. Therefore, the current study aimed to evaluate the impact of concurrent chronic administration of nicotine and ethanol on the quality of sperm in the rat.

Materials and methods

Experiments were carried out after institutional review board approval was obtained from Kerman University of Medical Sciences IRB committee, Kerman, Iran. Materials were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

Animals

Fifty healthy Wistar male rats (average weight 160–210 g, age 9–12 weeks) were maintained in a 12/12 h light/dark cycle at 21–24 °C with free access to rodent food and water. Animals were randomly divided to five groups ($n = 10$). Animals were given ethanol (Merck, Darmstadt, Germany) 2 g kg^{-1}, p.o. (oral gavage) in the E group; nicotine 0.1 mg kg^{-1} s.c. in the N group, nicotine 0.1 mg kg^{-1} s.c. plus ethanol 2 g kg^{-1} p.o. in the E/N group and normal saline 0.1 mg kg^{-1} s.c. and distilled water 2 g kg^{-1} p.o. were given to the vehicle control group. An intact group received no intervention and served to evaluate probable age-related changes in the reproductive system and to consider any stress induced by the treatments. Animals received the above interventions every 24 h for 50 days and the body weight was measured 30, 40 and 50 days after the onset of the experiment. At the end of the treatment period, rats were anaesthetised with an intra-peritoneal injection of 400 mg kg^{-1} chloral hydrate (Merck) (Field *et al.*, 1993) and the left cauda epididymidis and vas deferens were removed and kept for sperm quality analysis. Animals were perfused using a standard protocol of warm PBS followed by 4% paraformaldehyde (Biochemical Applichem, Chemica, Gatersleben, Germany) in PBS. The right testis, cauda epididymidis and vas deferens were carefully removed, weighed and their volume was measured as well. A standard calliper was used to measure testis dimensions.

Sperm analysis

The cauda epididymidis and vas deferens were placed in 5 ml of α-MEM supplemented with 2% BSA (Roche, Basel, Switzerland) and cut into several fragments to allow the spermatozoa come out from the reproductive ducts. Samples were incubated for 15 min at 37 °C and the following experiments were carried out on sperm suspensions from the various groups.

Sperm quality was determined by concentration, motility and viability. Sperm concentration was assessed by an improved Neubauer haemocytometer (cn-meditech, Jiangsu, China). Sperm suspensions were diluted 1 : 20 in a diluting solution (50 g l^{-1} Na_2HCO_3 and 1% formaldehyde; Merck) in distilled water. The diluted samples were put into the counting chamber and the number of sperm was counted under a light microscope (Nikon Ts100, Tokyo, Japan). The sperm concentration was expressed as number $\times$ 10^6 ml^{-1}. Sperm motility was analysed by counting 200 motile (sperm with slight movement without forward progression were also considered motile) and non-motile sperm in at least five microscopic fields at 400×. Sperm viability and membrane integrity were determined by three methods; the eosin (EO) dye exclusion test, the hypo-osmotic swelling (HOS) test and the propidium iodide (PI) exclusion test (Schrader *et al.*, 1986; Donoghue *et al.*, 1996). For the EO test, the sperm suspension was mixed thoroughly and 10 μl of the suspension was mixed with 10 μl of eosin-nigrosin dye. A thin smear was prepared after 1 min and the number of viable sperm was determined out of 200 sperm in at least 10 microscopic fields (400×). The live spermatozoa were white and the dead were stained red. For the HOS test, 30 μl of the sperm suspension was added to 1 ml of a solution containing 4.9 g l^{-1} sodium citrate and 9 g l^{-1} fructose in distilled water. The suspension was gently mixed with a pipette and kept for 20–30 min at 37 °C. An aliquot of 10 μl was placed on a clean slide, coverslipped and analysed under an inverted microscope with phase contrast apparatus (Olympus IX71, Tokyo, Japan). Two hundred sperm were counted and the sperm with curved or looped tails were considered to be viable. For the PI test, 10 μl of the sperm suspension was mixed with 1 ml α-MEM supplemented with 2% BSA. Six microlitres of 0.05 g l^{-1} PI in distilled water was added to the suspension and kept at 30 °C for 5 min, at which point a 10 μl aliquot was placed on a clean slide and coverslipped. The sample was evaluated under a fluorescent phase contrast microscope (IX71; Olympus). Two hundred spermatozoa were first counted under phase contrast conditions and the same fields were assessed for sperm with red fluorescence when excited by a green fluorescent light. Dead sperm were stained red under fluorescent light.

Statistical analyses

The data are presented as mean ± standard error of the mean (SEM) and were analysed using one-way analysis of

variance (ANOVA) followed by Tukey's *post hoc* test. Trend of weight gain in animals was assessed by repeated measure test. Differences at $P < 0.05$ were considered statistically significant.

Results

One animal in the ethanol group died due to problems with the gavage procedure. In addition, the results of sperm analysis in one sample in the intact group and one sample in the nicotine/ethanol group were excluded due to an error in sampling.

Effects of treatment with ethanol and nicotine on body weight

Body weight increased regularly in all groups, especially in the intact and ethanol groups (Table 1). There was only a marginally significant ($P = 0.054$) difference between groups. The poorest weight gain was seen in the animals which received nicotine. However, when weight gain was considered between the control and other groups, no significant differences were detected (Fig. 1).

Table 1 Comparison of body weight between different experimental groups 30, 40 and 50 days after the onset of the experiment

		Weight (g) at day		
Groups	*n*	30	40	50
Intact	10	281.8 ± 10.4	288.3 ± 9.9	299.2 ± 9.6
Control	10	253 ± 10.4	257.8 ± 10.1	264.8 ± 10.2
Ethanol	9	278.2 ± 10.7	281.2 ± 11.2	290.2 ± 11.7
Nicotine	10	261.2 ± 6.8	264.3 ± 6.8	267.8 ± 6.2
Ethanol/nicotine	10	249.7 ± 10.2	269.7 ± 4.5	276.4 ± 4.6

Data are shown as mean ± SEM.

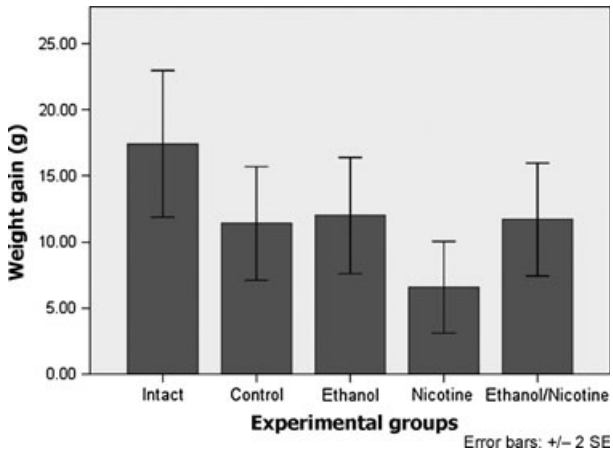

Fig. 1 Comparison of body weight gain between different experimental groups.

Effects of treatment with ethanol and nicotine on testis and epididymidis

Testis parameters including weight, volume and dimensions were greater in the intact group. However, when the T/B ratio (testis weight/body weight × 100) was calculated, the ethanol/nicotine group had the lowest value followed by the values in the ethanol, intact, nicotine and control groups. Small changes were detected in the long and short axes of the testis between groups. Although differences were observed between various treatments, especially in the ethanol/nicotine group compared to the intact group, none of the parameters were significantly different between groups (Table 2).

Effects of treatment with ethanol and nicotine on sperm parameters

Sperm concentration was comparable between the intact and control groups while a reduction in sperm concentration was observed in all treated groups (Table 3), especially in the ethanol/nicotine group compared to either the intact or control groups ($P < 0.01$). Motility was also significantly ($P < 0.01$) impaired in the ethanol/nicotine group compared to the control group. Although sperm viability was not significantly altered following treatment with ethanol, nicotine and ethanol/nicotine using any of the sperm viability tests (EO, HOS and PI), a marginally significant decrease in sperm viability was found in the ethanol/nicotine group compared to the control group, using both the HOS and PI tests (Fig. 2).

Discussion

Our major goal in the present study was to survey sperm quality including sperm count, motility and viability after concurrent administration of nicotine and ethanol by assessing various parameters in rat sperm. Whereas the sperm concentration in the ethanol and nicotine treated groups was nonsignificantly lower than in the control group, i.e. ethanol and/or nicotine alone did not significantly alter sperm concentration, a significant decrease in sperm concentration in the E/N group in comparison with the control group was detected (Table 3). We used a lower dose of nicotine and ethanol compared to those used in other studies (2 g rather than 3 g kg^{-1} ethanol (Srikanth *et al.*, 1999) and 0.1 mg kg^{-1} rather than 2–5 mg kg^{-1} nicotine. These levels are closer to the plasma concentrations seen in moderate smokers (Perkins

Table 2 Comparison of testis parameters as well as the weight and volume of the epididymis between different experimental groups

		Testis parameters					Epididymidis	
Groups	*n*	Weight (g)	Volume (μl)	Long axis (mm)	Short axis (mm)	T/B ratio	Weight (g)	Volume (μl)
Intact	10	1.41 ± 0.048	1360 ± 60	20.15 ± 0.24	11.97 ± 0.25	0.56 ± 0.02	0.699 ± 0.02	680 ± 33
Control	10	1.28 ± 0.047	1190 ± 48	19.25 ± 0.25	11.59 ± 0.30	0.67 ± 0.02	0.614 ± 0.03	590 ± 14
Ethanol	9	1.37 ± 0.037	1300 ± 33	19.87 ± 0.23	11.77 ± 0.20	0.47 ± 0.02	0.713 ± 0.02	700 ± 52
Nicotine	10	1.34 ± 0.042	1330 ± 45	19.66 ± 0.25	11.47 ± 0.12	0.59 ± 0.03	0.670 ± 0.01	670 ± 33
Ethanol/nicotine	10	1.27 ± 0.030	1230 ± 47	19.26 ± 0.26	11.43 ± 0.19	0.40 ± 0.01	0.633 ± 0.03	640 ± 34

Data are shown as mean ± SEM. T/B ratio, testis weight/body weight × 100.

Table 3 Sperm characteristics

				% Viable sperm		
Groups	*n*	Concentration ($\times 10^6$ ml^{-1})	Motility (%)	EO	HOS	PI
Intact	9	49.9 ± 2.47^{d}	41.1 ± 3.33	44.8 ± 3.16	53.4 ± 1.70	34.4 ± 2.13
Control	10	46.3 ± 4.16^{a}	46.6 ± 2.09^{a}	49.5 ± 1.80	54.6 ± 3.21	38.8 ± 2.18
Ethanol	9	36.3 ± 1.64^{e}	38.3 ± 2.04	44.2 ± 1.91	47.4 ± 2.82	31.9 ± 2.31
Nicotine	10	37.2 ± 1.53^{e}	39.8 ± 2.71	47.3 ± 2.84	47.4 ± 3.25	34.0 ± 3.08
Ethanol/nicotine	9	31.4 ± 2.91^{b,c}	31.0 ± 3.13^{b}	40.7 ± 2.05	41.8 ± 4.39**	28.7 ± 2.55*

EO, eosin dye exclusion test; HOS, hypo-osmotic swelling test; PI, propidium iodide exclusion test.
Values are representative of the mean ± SEM.
In each column, figures with different superscripts are significantly different; $^{a,b}P < 0.01$, $^{c,d}P < 0.001$, $^{d,e}P < 0.05$.
A nearly significant ($^{*}P = 0.051$, $^{**}P = 0.063$) difference was detected between ethanol/nicotine group and the control group.

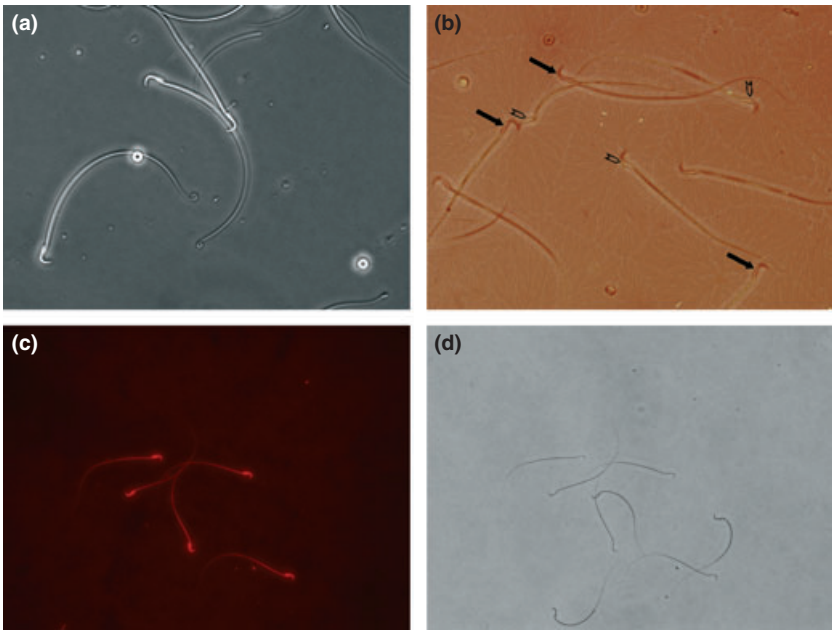

Fig. 2 Sperm evaluated for viability by the hypo-osmotic-swelling (HOS) test (a) were stained by the Papanicolaou method (b); arrow: dead cells, arrow head: viable cells, and were analysed by propidium iodide using a fluorescent microscope (c) and a light microscope (d) to determine the number of viable sperm.

et al., 1989; Dhawan & Sharma, 2002; Mahanem *et al.*, 2006) and alcohol drinkers. Dhawan & Sharma (2002) reported a sharp reduction in sperm concentration in rats after concurrent administration of 3 g kg^{-1} p.o. ethanol and 2 mg kg^{-1} s.c. nicotine. These results confirm that co-administration of nicotine and ethanol intensifies the

adverse effects of nicotine on sperm count. We used an intact group which provided the baseline values for our study. Sperm parameters in this group were nearly identical with those of the control group, but the level of significance was greater when the data from E/N group were compared to those of the intact group. Also, when the sperm concentrations found in the ethanol and nicotine groups were compared to those of the intact group, a significant decrease was noted. One of the presumptive indirect effects of concomitant administration of nicotine and alcohol is disturbed testosterone production due to high levels of oestrogen (Sadeu *et al.*, 2010; Manning & Fink, 2011). Nicotine and its main metabolite, cotinine, can inhibit steroidogenesis (Patterson *et al.*, 1990). Testosterone alterations can lead to decreased sperm concentration and maturation in males (Dhawan & Sharma, 2002). Both clinical and experimental studies have shown the hampering effects of ethanol on the hypothalamo-hypophysis-testicular axis (Srikanth *et al.*, 1999). In addition, high levels of nicotine and its major metabolites cotinine and trans-3-hydroxycotinine in human seminal plasma have been negatively correlated with sperm parameters (Pacifici *et al.*, 1993). Long-term exposure to bioactive materials such as tobacco smoke, coffee and alcohol that can move across the blood–testis barrier and can exist at high residual concentrations in the testes may influence male reproductive function (Reddy *et al.*, 1995). Nicotine and cotinine have been found at significant levels in smokers' seminal plasma and may inhibit human sperm creatine kinase activity by competitive inhibition of NADP (Ghaffari *et al.*, 2008). We found a significant decrease in sperm concentration when ethanol was given separately, but the motility and viability remained unchanged compared with control.

The results of the present study indicate a significant decrease in sperm motility in the E/N group compared with the control group, whereas administration of ethanol and nicotine alone did not significantly alter sperm motility. Moreover, a borderline significant difference ($P = 0.051$) was observed in sperm viability in the E/N group compared to control by the PI exclusion test. Although some reports have shown a positive relationship between increased sperm motility/viability and moderate drinking of some alcoholic beverages such as red wine due to its antioxidant effect (Telisman *et al.*, 2000; Auger *et al.*, 2001; Bianchini *et al.*, 2001; Marinelli *et al.*, 2004), Hung *et al.* (2007) indicated decreased sperm motility following tobacco smoking. The mechanism by which nicotine and ethanol can alter sperm motility and viability is not clear yet. Shen *et al.* (1997) suggested that cigarette smoke damages sperm DNA. Some studies have shown that high levels of ROS are present in the seminal plasma of 25–40% of infertile men (de Lamirande & Gagnon, 1995; Padron *et al.*, 1997). Increase in seminal plasma ROS is reinforced by smoking as seminal oxidative stress is higher in infertile men who smoke cigarette than nonsmoker infertile men. The increase in seminal ROS can be attributed in part to an increase in seminal leucocyte as well as morphologically abnormal spermatozoa (Kessopoulou *et al.*, 1992). Agarwal & Saleh (2002) showed that smoking can increase seminal leucocyte concentration as high as 48%. Increase in seminal leucocyte and ROS may affect DNA integrity and produce DNA strand breaks in sperm cells (Potts *et al.*, 1999).

A survey of studies conducted so far has indicated that sperm motility is affected by several extracellular and intracellular factors such as ion concentration, cell volume and pH. Increased calcium, cAMP and pH levels could affect sperm motility (Darszon *et al.*, 1999; Luconi & Baldi, 2003; Luconi *et al.*, 2006). Some researchers have suggested that nicotine can potentiate the effects of alcohol (Kouri *et al.*, 2004). One more valuable point is the potential of nicotine to enhance alcohol absorption in the small intestine; this is especially important because the greatest part of alcohol absorption is conducted via the small intestine. Although some studies have indicated that smoking can delay stomach emptying (Gritz *et al.*, 1988; Wong *et al.*, 1999; Kadota *et al.*, 2010), another study has conversely indicated that nicotine speeds up stomach emptying (Qiu *et al.*, 1992). Although our study showed a reduction in sperm parameters following concomitant administration of ethanol and nicotine in rat, application of these data to human subjects remains controversial (Vine *et al.*, 1997).

In conclusion, our results demonstrate that simultaneous chronic administration of ethanol and nicotine significantly decreases sperm concentration and motility. These results may disturb male reproductive function.

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