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Yasuhisa Yamamoto · Eiko Isoyama
Nikolaos Sofikitis · Ikuo Miyagawa

Effects of smoking on testicular function and fertilizing potential in rats

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Abstract We evaluated the effects of smoking on testicular function and fertilizing potential in rats. Twenty rats (group A) were exposed to the smoke of 20 cigarettes for 1 h per day. Ten rats (group B) were exposed to the smoke of 40 incense sticks for 1 h per day, and an additional 10 rats served as a control group (group C). After 10 weeks of daily exposure, serum levels of nicotine and cotinine were assessed, and a mating test was conducted. Five days later, serum concentrations of testosterone before and after human chorionic gonadotropin (hCG) stimulation, gonadotropins, and epididymal sperm content and motility were evaluated. In addition, in vitro fertilization was carried out. Nicotine and cotinine were detected in group A, but not in groups B and C. Basal serum testosterone and gonadotropin concentrations did not differ significantly among the three groups, but the testosterone response to hCG stimulation was significantly lower in group A than in groups B and C. Group A showed significant reductions in epididymal sperm content and motility, and in fertility in vivo and in vitro. These findings suggest that smoking leads to a secretory dysfunction of the Leydig cells, and also a deficiency in sperm maturation and spermatogenesis. In addition, smoking has a detrimental effect on sperm fertilizing potentials in vivo and in vitro.

Key words Testicular function · Smoking · Fertility · Rat

Y. Yamamoto (✉) · N. Sofikitis · I. Miyagawa
Department of Urology,
Tottori University School of Medicine, 683 Nishimachi 36-1,
Yonago, Tottori, Japan; Fax: 81-859-34-8092

E. Isoyama
West Japan Railway Company, Yonago Health Center,
Yonago, Japan

Introduction

A large number of men of reproductive age in the United states and Japan smoke [8]. Many investigators have proposed a detrimental effect of smoking on sperm concentration, sperm motility and percentage of morphologically normal spermatozoa [8]. The adverse effects of cigarette smoke on Leydig cell function in animals have been reported [2, 4]. A review of the literature suggests that smoking may influence the ability of men to reproduce by causing impaired spermatogenesis secondary to various hormonal alterations [8, 11]. We recently demonstrated that smoking had direct effects on spermatozoa, including sperm acrosin activity, reaction to the hypo-osmotic swelling test, the ratio of single-stranded to double-stranded DNA, and results of the zona-free hamster oocyte penetration assay [5].

Although the detrimental effects of cigarette smoking on spermatogenesis and sperm motility are well known [2, 4, 8], there are no reports on the influence of smoking on the last events of the fertilization process (i.e., binding to the zona pellucida, or penetration through the zona pellucida). The objective of the present study was to evaluate the effects of smoke exposure on testicular function and sperm fertilizing capacity in vivo and in vitro.

Materials and methods

The Wistar rats used were 4-week-old males weighing 80–110 g each (Shimizu Experimental Material Company, Kyoto, Japan). There were three experimental groups: cigarette smoke-exposed (group A; $n = 20$; tar 9 mg, nicotine 0.8 mg), sham smoke-exposed control (group B; $n = 10$; incense stick), and control (group C; $n = 10$) groups. Rats in groups A and B were exposed to 1400 cigarettes and 2800 incense sticks over 70 days, at the rate of 20 cigarettes or 40 incense sticks per day. Ten weeks after the first exposure, blood samples were collected from the main tail vein to measure serum nicotine and cotinine, and then the fertility potential of each animal was assessed. Five days later, blood samples were collected from the left femoral vein of each rat for measure-

ments of basal serum testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) concentrations. Human chorionic gonadotropin (1500 IU; hCG; Teikoku Zoki, Tokyo, Japan) was then given intramuscularly and 3 h later another blood sample was collected from the right femoral vein for assessing testosterone responses to hCG. Epididymal sperm content and motility were evaluated. A sample of epididymal spermatozoa was processed for in vitro fertilization (IVF) of oocytes collected from 4-week-old female Wistar rats (10 oocytes per male rat).

The protocol of the present study was approved by the responsible ethical committee. Furthermore, The Principles of Laboratory Animal Care. (NIH publication No. 86-23, 1984) were followed. In addition, all regulations on experimental animal studies recommended by the Japanese Law were adopted.

Serum assays

Serum testosterone was measured by radioimmunoassay using kits from Nihon DPC (Tokyo, Japan). Serum LH and FSH were measured at the Japanese Special Reference Laboratory by means of specific homologous double-antibody radioimmunoassays, using kits supplied by Nihon DPC (Tokyo, Japan). Intra- and inter-assay coefficients of variation for testosterone, LH, and FSH were 5% and 9%, 8.5% and 11.3%, and 7.3% and 10.1%, respectively. The sensitivities of assays for testosterone, LH, and FSH were 0.1 ng/dl, 200 ng/l, and 400 ng/l, respectively. Serum nicotine and cotinine were measured by high-performance liquid chromatography as described by Pacifici et al. [3]. The sensitivities of the assays were 8 ng/ml and 4 ng/ml, respectively. Whenever one of these substances was not detected (concentration less than the sensitivity of the assay), its serum concentration was reported as 0 ng/ml.

Assessment of fertility in vivo

Each rat was placed in the same cage for 5 days with three healthy female rats with regular cycles as determined by vaginal smears. Twenty-one days after the beginning of the fertility test, all the females were checked for evidence of parturition. The number of offspring was recorded. A male rat was considered fertile if matings resulted in at least one pregnancy.

In vitro fertilization

Preparation of sperm suspension

Each male rat was killed by cervical dislocation and its left cauda epididymis was removed aseptically. After the epididymal ducts had been cut with a pair of sharp scissors, five or six drops of the dense mass of spermatozoa were introduced into 5 ml of Toyoda and Chang medium [9]. Spermatozoa from cigarette-exposed rats were filtrated via Sperm Prep II tubes (ZBL, Lexington, Ky.) as described previously [6] to isolate sperm fractions showing strong forward motion. Final sperm suspensions containing 0.5×10^7 spermatozoa/ml were used.

Collection of oocytes and in vitro fertilization

The Wistar rats used were 4-week-old females weighing 80–100 g each (Shimizu Experimental Material, Kyoto, Japan). Fourteen rats were injected subcutaneously with 25 IU pregnant mare serum gonadotropin (PMSG; Sigma, St. Louis, Mo.) 54 h before an intraperitoneal injection of 25 IU hCG (Teikoku Zoki, Tokyo, Japan) and were killed 16 h after hCG injection. The oviducts were removed and the ampullary portions placed in a plastic dish containing Toyoda and Chang medium. The oocytes in the cumulus mass were dissected out of the oviducts and introduced into the Toyoda and Chang medium. A volume of 0.05 ml of sperm suspension was introduced to 0.45 ml of Toyoda and Chang medium containing the oocytes. The dishes were kept in a incubator (37 °C, 5% CO_2 and 5% O_2).

Examination of oocytes

Eighteen hours after insemination, the percentage of oocytes with two pronuclei was evaluated using an inverted microscope (Olympus IX-70, Tokyo, Japan).

Caudal epididymal sperm content and motility

Each right epididymis was carefully separated from the testis and divided into head, body, and tail. The epididymal tails were trimmed and minced in 5 ml of Toyoda and Chang medium [7] titrated to pH 7.4 at 37 °C with sodium hydroxide. The minced epididymal tissue was then separated from the liberated spermatozoa by filtration through a steel wire mesh with pores of 80–100 μm. Twenty droplets of the filtrate were used to assess the sperm count (number of spermatozoa per milliliter of Toyoda and Chang medium), and the average number was calculated. The chamber was placed on the slide of a light microscope, ×200 magnification. Ten droplets were counted to calculate the percentage of motile spermatozoa in the filtrate immediately after its preparation. Average motility parameters were calculated. Samples used for motility evaluation were stored on a warming tray at 35 °C between viewings.

Statistical analysis

Values are expressed as the mean SD. Analysis of variance and the Duncan multiple range test were used to analyze differences in the results. The chi-squared test with Yates' correction was used to analyze differences in fertility, the number of litters per fertile male rat, and the percentage of oocytes with two pronuclei. A probability level of less than 0.05 was considered statistically significant.

Results

Assessment of serum nicotine and cotinine

Peripheral serum levels of nicotine and cotinine in group A were 246 ± 11 and 274 ± 19 ng/ml, respectively. Nicotine and cotinine were not detected in groups B and C.

Hormonal profiles

Basal testosterone concentrations did not differ significantly among the three groups. However, the serum testosteone level after hCG stimulation was significantly

Table 1 Serum concentrations of testosterone and the testosterone response to human chorionic gonadotropin (hCG) (*1* group A vs B, *2* group A vs C, *3* group B vs C)

Group	Testosterone (ng/dl)	Testosterone response to hCG (ng/dl)
A (cigarette smoke)	182 ± 23	466 ± 45*
B (sham smoke)	178 ± 30	758 ± 53
C (control)	181 ± 25	735 ± 57
P values		
1	0.6498	0.0001
2	0.8547	0.0001
3	0.8229	0.3484

* Versus B or C; significant difference

Table 2 Epididymal sperm content, motility, and outcome of mating test and in vitro fertilization (*1* group A vs B, *2* group A vs C, *3* group B vs C. *SC* sperm count, *MS* motile sperm, *2 PN* two pronuclei)

Group	SC ($\times 10^6$/ml)	%MS	Fertile male rats (%)	No. of litters per fertile male rat	Oocytes with 2 PN (%)
A (cigarette smoke)	54.5 ± 6.3*	42.8 ± 5.7*	40*	6.1 ± 0.8*	45*
B (sham smoke)	81.1 ± 5.5	79.3 ± 5.1	100	11.0 ± 1.1	72
C (control)	81.0 ± 6.7	82.5 ± 6.8	100	10.9 ± 1.2	75
P values					
1	0.0001	0.0001	0.0008	0.0001	0.0001
2	0.0001	0.0001	0.0008	0.0001	0.0001
3	0.9713	0.2515	–	0.8451	0.3306

* Versus B or C; significant difference

lower in group A (466 ± 45 ng/dl) than in groups B (758 ± 53 ng/dl) and C (735 ± 57 ng/dl) (A vs B, $P = 0.0001$, A vs C, $P = 0.0001$) (Table 1).

In contrast, peripheral serum LH and FSH concentrations were not significantly different among the three groups (0.6 ±0.2 ng/ml and 12.1 ± 2.2 ng/ml, 0.5 ± 0.2 ng/ml and 13.3 ± 3.0 ng/ml, and 0.5 ± 0.2 ng/ml and 14.3 ± 3.5 ng/ml, respectively).

Quantitative, qualitative, and functional sperm parameters

Caudal epididymal sperm content (SC) and motility (%MS) were significantly lower in group A (54.5 ± 6.3 × 10^6/ml and 42.8 ± 5.7%) than in groups B (81.8 ± 5.5 × 10^6/ml and 79.3 ± 5.1%) and C (81.0 ± 6.7 × 10^6/ml and 82.5 ± 6.8%) (SC: A vs B, $P = 0.0001$, A vs C, $P = 0.0001$; %MS: A vs B, $P = 0.0001$, A vs C, $P = 0.0001$) (Table 2). The percentage of male fertile rats and the number of litters per fertile male rat were significantly lower in group A (40% and 6.1 ± 0.8) than in groups B (100% and 11.0 ± 1.1) and C (100% and 10.9 ± 1.2)(% fertile male rats: A vs B, $P = 0.0008$; A vs C, $P = 0.0008$; number of litters per fertile male rat: A vs B, $P = 0.0001$, A vs C, $P = 0.0001$) (Table 2). The percentage of oocytes with two pronuclei was significantly lower in group A (45%) than in groups B (72%) and C (75%) (A vs B, $P = 0.0001$; A vs C, $P = 0.0001$) (Table 2).

Discussion

Nicotine, the major psychoactive substance in cigarette smoke, is a highly toxic alkaloid that stimulates the autonomic ganglia and central nervous system. Nicotine, and its major metabolite cotinine, have been implicated in the detrimental effects of smoking on sperm parameters [3, 8, 11]. This study was successful in establishing a smoke-exposed rat model as demonstrated by significant elevations in nicotine and cotinine.

There were no significant differences in the peripheral levels of testosterone, LH, and FSH between rats in the smoke-exposed group and rats in control groups. However, significantly lower levels of testosterone after hCG stimulation were seen in group A, indicating a detrimental effect of smoke exposure on Leydig cell secretory function. The lower values for caudal epididymal sperm content and motility in group A may be caused by disturbances in spermatogenesis or in the epididymal sperm maturation process secondary to secretory dysfunction of the Leydig and Sertoli cells, since testosterone is necessary for normal spermatogenesis, and testosterone and androgen binding protein have important roles in the epididymal sperm maturation process [7]. However, the present data are not sufficient to show whether or not exposure to cigarette smoke affects Sertoli cell function.

In addition, the significantly lower percentage of fertile rats and the percentage of oocytes with two pronuclei suggest that cigarette smoke has detrimental effects on fertilizing potentials both in vivo and in vitro. A reduction in fertility potential in vivo after exposure to smoke in rats may also be attributed to a decrease in libido and/or impotence. Smoking is a risk factor for decreased libido and/or impotence in humans [1, 10]. However, the decrease in fertilizing capacity in vitro does not have any relation to a reduction in libido and/or to impotence. This result suggests that smoking may affect the sperm fertilizing process; for example, sperm capacitation, the acrosome reaction, binding to the zona pellucida, and penetration through the zona pellucida. This effect is probably mediated through alterations in spermatogenesis and the epididymal sperm maturation process, and/or direct influences on spermatozoa induced by cigarette smoke.

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