

Regular Article

Effects of Fetal Exposure to Heat-Not-Burn Tobacco on Testicular Function in Male Offspring

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Several studies show that maternal conventional cigarette smoking during pregnancy has been associated with reduced sperm concentration in sons. The development of heat-not-burn (HnB) tobacco has gained a growing following. However, the effects of prenatal HnB tobacco smoking on male offspring are as yet unknown. Pregnant CD-1 mice were exposed to I-Quit-Ordinary-Smoking (IQOS) (HnB tobacco) aerosol from heat sticks, mainstream smoke from 3R4F (conventional cigarettes) or clean air, using a whole-body exposure system. Adult male offspring mice were divided into six groups: control (5- and 15-weeks-old offspring), IQOS (5 and 15-weeks-old) and 3R4F (5 and 15-weeks-old). Spermatogenesis, sperm characteristics, serum testosterone, and seminiferous tubule morphology were evaluated. Prenatal IQOS exposure increased abnormal seminiferous tubule morphology and decreased sperm production at 5 weeks, but 3R4F exposure did not. Prenatal exposure to IQOS aerosol delays sexual maturation of male offspring or adversely affects the male testicular function of the offspring more than smoke from a combustion cigarette.

Key words heat-not-burn tobacco (HnB tobacco); prenatal exposure; male offspring; daily sperm production

INTRODUCTION

The background research into the legacy of disease and death that cigarette smoking imposes on the public's health is extensive. It has become clear that smoking causes a variety of health effects, and not only smokers but also non-smokers are affected by secondhand smoke, which is a major problem in terms of public health.^{1–3} Furthermore, use of cigarettes during pregnancy can lead to significant developmental issues in the fetus.^{4,5} Many chemicals contained in cigarettes (such as nicotine, polycyclic aromatic hydrocarbons) can easily be transferred to the fetal development through the placenta.^{6–9}

The list of potential conditions that can occur in the children as a consequence of smoking during pregnancy is long and includes issues such as low-birth-weight infants, premature delivery, an increased risk of sudden infant death syndrome (SIDS), an increased risk of congenital deficiencies, and an increased risk for numerous developmental defects.^{10–14} Therefore, cigarettes use during pregnancy is the most important preventable cause of a wide range of adverse pregnancy outcomes. Moreover, several studies show that maternal smoking during pregnancy has been associated with reduced sperm concentration in sons later in life.^{15,16} Unfortunately, the majority of tobacco users who become pregnant either continue to smoke or, if they stop smoking during pregnancy, restart smoking within 6 months of delivery.¹⁷

In addition, the new heat-not-burn (HnB) type tobacco has been developed, which has added a new, less toxic possibility to smokers.¹⁸ After the rapid rise of novel tobacco products (such as Tobacco Heating System) over the past decade, the tobacco industry has launched its latest response to the documented harm of tobacco smoking: HnB tobacco products.¹⁹ HnB tobacco may be safer for both users and bystanders than conventional cigarette smoking, which is likely to lessen the burden of smoking-related diseases and deaths.^{20,21} However, the actual

health effects on users and the effectiveness of HnB tobacco for smoking cessation are still under discussion. Moreover, the effects of fetal HnB tobacco exposure on male offspring's testicular function have not yet been sufficiently elucidated.

In the present study, the aggravating effects of fetal HnB tobacco (IQOS: I-Quit-Ordinary-Smoking) exposure on the testicular function of male offspring were investigated. Additionally, another aim was to compare the adverse effects on the testicular function of male offspring exposed to aerosol from IQOS and a scientific reference cigarette (3R4F, combustion cigarette). In this study, we designed the study with a limited number of days of smoking, since pregnancy tends to reduce the number of cigarette. After pregnant mice were exposed to IQOS/3R4F, sex ratio at birth, spermatogenesis, sperm characteristics, serum testosterone, and seminiferous tubule morphology in these offspring mice were assessed at ages 5 and 15 weeks.

MATERIALS AND METHODS

Animals Pregnant CD-1 mice (30) were obtained from CLEA Japan Inc. (Tokyo, Japan). Induction took place at day five post-coitum (p.c.; 0: the day the plug appeared). Mice were randomly divided into three groups (10 mice each): the HnB tobacco (IQOS), 3R4F (filtered cigarettes, reference cigarette), and control groups. Up to day 16 of induction, each cage housed five mice, and then beginning on day 17 of induction, each pregnant female mouse was individually housed. The conditions for keeping the mice were as follows: temperature 23–25 °C, humidity 50–70%, and 12-h light–dark cycle. Mice had access to food (CE-2 solid food, CLEA Japan) and water *ad libitum*. The guidelines of Oita University of Nursing and Health Sciences for animal treatment were followed. The experiments were approved by the Committee of Research Ethics and Safety Commission of the Oita University of Nursing and

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Cigarette Smoke Exposure Mice were placed in a closed plastic box (6.5 L) connected to a tobacco smoke (TS) generator (SG-300, Shibata Scientific Technology, Tokyo, Japan) according to the Health Canada Intense (HCI) smoking regimes. The TS was generated at a stroke volume of 55 mL, 2 s puff duration, 30 s puff interval, and 10 puffs/heat stick or cigarette. The TS was diluted with compressed air (14.85 L/min). The pregnant mice ($n = 10$ per group) were exposed whole body to IQOS (IQOS Heat Sticks regular, Philip Morris International Inc., NY, U.S.A.) aerosol/3R4F reference cigarettes (University of Kentucky, Lexington, KY, U.S.A.) mainstream smoke of four heat stick/cigarettes a day with 20-min smoke exposures on days 7 and 14 of gestation. Exposure began after implantation and initial organ development had taken place. Control mice received filtered air according to the same procedure.

Offspring Pregnant mice were separated into single cages after their second inhalation and gestation length/litter size were recorded. Mice delivered after a gestation of 18.0 to 19.0 d were used for further testing. The first day of parturition was recorded as postnatal day 0. Gestation length was determined at the ± 0.5 d level of specificity (that is, births were recorded each morning and afternoon). At the age of 12 d, six male mice (from each mother mouse) were selected randomly. Finally, 24 dams—including 8 controls, 8 IQOS and 8 3R4F—were selected, and male offspring were randomly selected from each of these different dams. Each dam and her delivered offspring were housed at 25–26 d after birth in the nesting box, then male pups removed from their mothers' cages. All male offspring were anesthetized for examination at 5 weeks or 15 weeks ($n = 8$ per time point, 48 male offspring; including 8 controls, 8 IQOS and 8 3R4F at 5 weeks and 8 controls, 8 IQOS and 8 3R4F at 15 weeks). A schematic representation of our experimental protocols is shown in Fig. 1.

Testicle and Epididymis Removal The experimental male mice were anesthetized with pentobarbital sodium at age 5 (puberty; sexually immature) or 15 (adult; sexually mature) weeks. A blood sample was collected from the heart after body weight was measured. The testicular and epididymal tissues were removed and then both left and right testicle tissue samples were separately weighed. The right testis was stored frozen at -80°C and measured for daily sperm production (DSP). The right epididymis was evaluated for sperm characteristics (15-weeks-old only; there is no sperm in the epididymis of 5-weeks-old mice). The histological analysis was performed using the left testis.

Sperm Characteristics A sperm suspension was obtained from the cauda epididymis of a 15-week-old offspring. The removed cauda epididymis was minced using ophthalmological scissors in 1 mL of M199 (Sigma-Aldrich Japan, Tokyo, Japan) medium solution containing 10% fetal bovine serum (Sigma-Aldrich Japan) in a sterile 1.5-mL tube and then filtered using a 100-mesh membrane. Following a 5 min incubation

period at 37°C , the sperm suspension was loaded into a $20\mu\text{m}$ in depth Leja standard counting chamber (Leja, Nieuw Vennep, The Netherlands). The concentration of sperm in the epididymis was measured by a sperm analyzer (Sperm Class Analyzer [SCA] 6.0, Microptic, Barcelona, Spain). Approximately 800–1000 spermatozoa were analyzed in each sample. Parameters assessed were those recommended by the manufacturer for rodents sperm, and they are: MOT; motility (%), progressive motility (%), VCL; continuous line velocity ($\mu\text{m/s}$), VSL; straight line velocity ($\mu\text{m/s}$), VAP; average path velocity ($\mu\text{m/s}$), LIN; linearity (ratio of VSL/VCL) (%), STR; straightness (ratio of VSL/VAP) (%), WOB; wobble (%), ALH; amplitude of lateral head displacement (μm), BCF; beat cross frequency (Hz).

Testicular Daily Sperm Production (DSP) A lysis buffer solution containing 0.05% Triton-X 100, 0.88% sodium chloride and 0.02% eosin-Y was prepared for DSP measurement. The testes were placed into 1 mL of the lysis buffer solution and homogenized for 2 min at 4°C . The number of cells (spermatozoa) in the solution was counted four times using an improved Burkert-Turk counting chamber. DSP was calculated according to the following formula²²⁾:

$$\begin{aligned} \text{Sperm count/mL} \times \text{volume of lysis buffer} &= \text{testicular sperm count} \\ \text{Testicular sperm count}/4.84^* &= \text{sperm produced/day (DSP)} \\ (\text{Sperm produced/day})/\text{testis weight} &= \text{DSP/g testis} \end{aligned}$$

*: 4.84 is the number of days for a spermatid to develop through stages 14 to 16 in mice, *i.e.*, the stages where spermatids are resistant to homogenization.

Serum Hormone Measurement Serum testosterone, estradiol and follicle-stimulating hormone (FSH) were measured using the Testosterone EIA Kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.), the Estradiol EIA Kit (Cayman Chemical Co.) and the Follicle-Stimulating Hormone enzyme-linked immunosorbent assay (ELISA) Kit (CUSABIO TECHNOLOGY LLC. (CUSABIO), Wuhan, China).

Histological Analysis A testis prepared in Bouin's solution was embedded in paraffin and then thin sliced. After each slice was stained using hematoxylin and eosin (H&E), it was observed under a Nikon ECLIPSE light microscope (Nikon Corporation, Tokyo, Japan). All slides were anonymized, examined blind, and only identified after measurement. To compare the extent of damage in the testes, the ratio of damaged seminiferous tubules was calculated per a median cross section of each testis. Variables assessed included seminiferous epithelium damage and vacuolation of seminiferous tubules.

Statistical Analysis KyPlot version 5 (Kyens Lab Inc., Tokyo, Japan) was used for all statistical analyses. Analyses of fertility were conducted using a chi-square test. Analyses of gestation length, litter size, gender ratio, body weights, organ weights, sperm production, and hormone concentration were performed by an ANOVA test followed by Dunnett's test. A

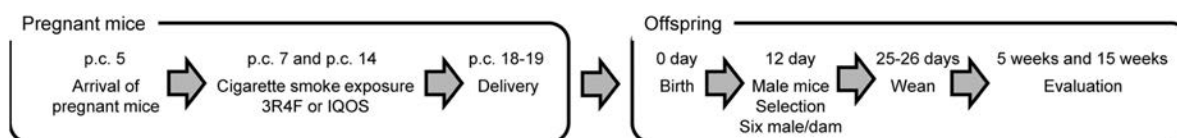


Fig. 1. Experimental Design

p.c.; Post-coitum.

p -value of <0.05 was considered statistically significance.

RESULTS

Effects of IQOS and 3R4F Exposure on Dams' Fertility

The effects of IQOS aerosol/3R4F mainstream smoke inhalation on fertility parameters and pups were determined by

comparing gestation length, litter size, fertility, and gender ratio between the three groups. No significant differences were observed in fertility, gestation length, litter size, or sex ratio at birth (Table 1).

Table 1. Effects of IQOS and 3R4F Exposure on Dams and Fertility

	Control	IQOS	3R4F
Fertility	100%	100%	90%
Gestation length (d)	18.9 ± 0.5	18.9 ± 0.2	18.8 ± 0.3
Litter size	13.5 ± 2.1	14.8 ± 3.8	12.4 ± 4.1
Male ratio	0.51 ± 0.11	0.50 ± 0.11	0.56 ± 0.20

Data are mean \pm standard deviation (S.D.) values.

Table 2. Effects of Fetal IQOS and 3R4F Exposure on Offspring Body, Testis, and Epididymis Weights

		Control	IQOS	3R4F
Body weight (g)	5 weeks	32.5 ± 0.6	32.5 ± 1.2	32.5 ± 1.0
	15 weeks	43.8 ± 1.2	47.2 ± 1.6	47.8 ± 1.6
Testis (mg)	5 weeks	113.1 ± 4.5	107.6 ± 5.9	115.6 ± 2.9
	15 weeks	164.9 ± 8.7	170.7 ± 7.1	153.1 ± 5.9
Epididymis (mg)	5 weeks	29.8 ± 0.8	28.7 ± 0.9	30.2 ± 0.9
	15 weeks	62.9 ± 2.4	66.6 ± 2.2	61.3 ± 3.3

Data are mean \pm S.D. values. $n = 8$.

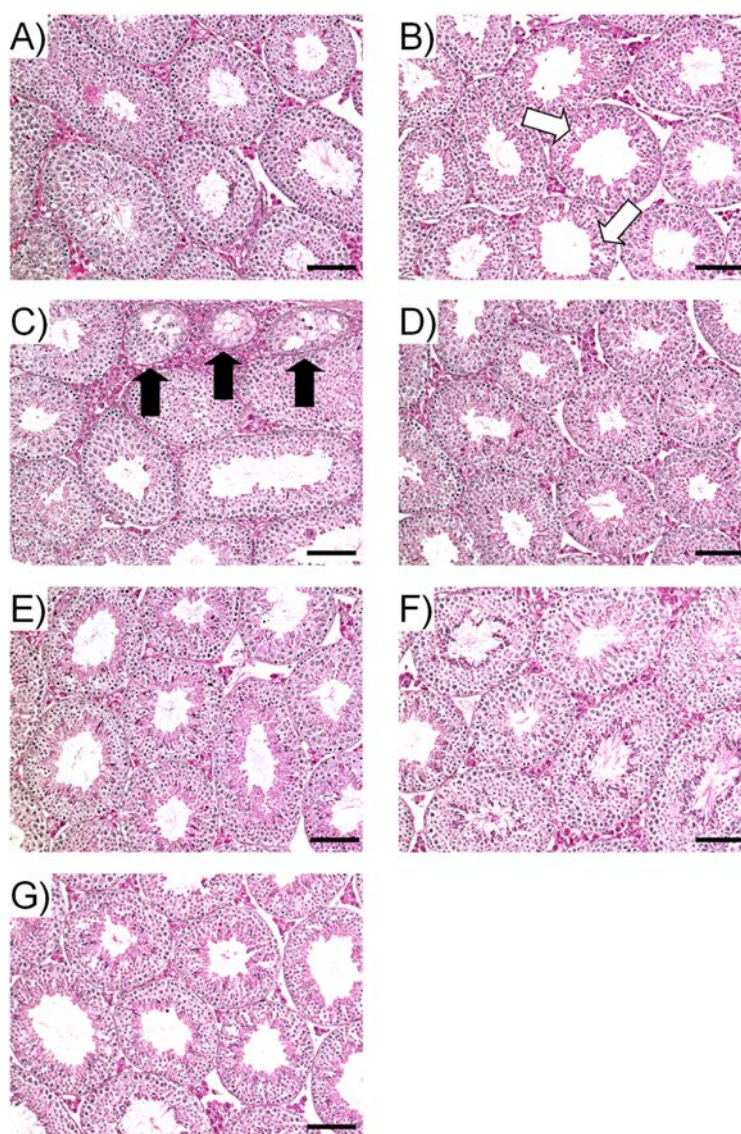


Fig. 2. Seminiferous Tubule Morphology Analyzed with H&E Staining by Light Microscopy of Control Mice and of Fetal IQOS/3R4F Exposure Mice

A): control, 5-week-old mouse, B), and C): IQOS, 5-week-old mouse, D): 3R4F, 5-week-old mouse, E): control, 15-week-old mouse, F): IQOS, 15-week-old mouse, G): 3R4F, 15-week-old mouse, A)–G): $\times 200$ magnification. Bar = $100\mu\text{m}$. At 5 weeks of age, mice in the IQOS exposed group showed seminiferous epithelial damage (white arrows). Vacuolation was seen with some seminiferous tubules (black arrows). These observations were found in other animals ($n = 8$, each group). (Color figure can be accessed in the online version.)

Effects of Fetal IQOS Aerosol/3R4F Mainstream Smoke Inhalation on Pups' Body, Testis, and Epididymis Weights There were no significant differences in body weight, testicular weight, or epididymis weight between the IQOS, 3R4F and control group pups at any age (Table 2).

Histological Changes in the Testes of Mice Exposed to IQOS Aerosol/3R4F Mainstream Smoke during the Fetal Period The testes of 5-week-old male mice exposed to IQOS as fetuses exhibited low cellular adhesion of seminiferous epithelia (Fig. 2B), white arrows) and vacuolation of the seminiferous tubules (Fig. 2C), black arrows). When compared to the control group, the ratio of damaged seminiferous tubule in the IQOS group was significantly higher (5-week-old mice: $p < 0.05$) (Fig. 3).

Effects of Fetal IQOS Aerosol/3R4F Mainstream Smoke Exposure on Sperm Production and Sperm Characteristics DSP and DSP/g testis were significantly decreased in the IQOS-exposed group at 5 weeks (respectively 63.0%, $p < 0.05$ and 65.8%, $p < 0.05$, each control group) (Fig. 4). On the other hand, there were no significant differences observed in DSP and DSP/g testis between the 3R4F and control groups.

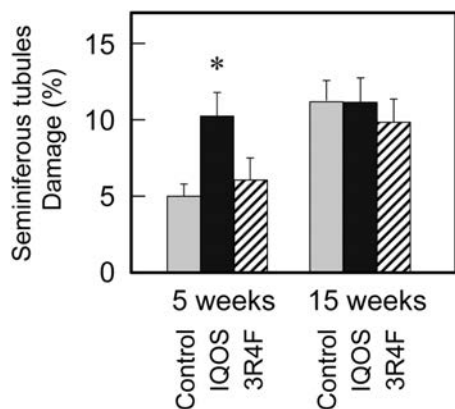


Fig. 3. Percentage of Degenerated Seminiferous Tubules in Cross Sections of Fetal IQOS/3R4F Exposed Mice and Control Mice

Estimation of testicular damage was conducted by counting the number of tubular cross sections and determining the percentage of total degenerated tubules in approximately 300 seminiferous tubules per testis. Means \pm standard error (S.E.) ($n = 8$); * $p < 0.001$, vs. controls.

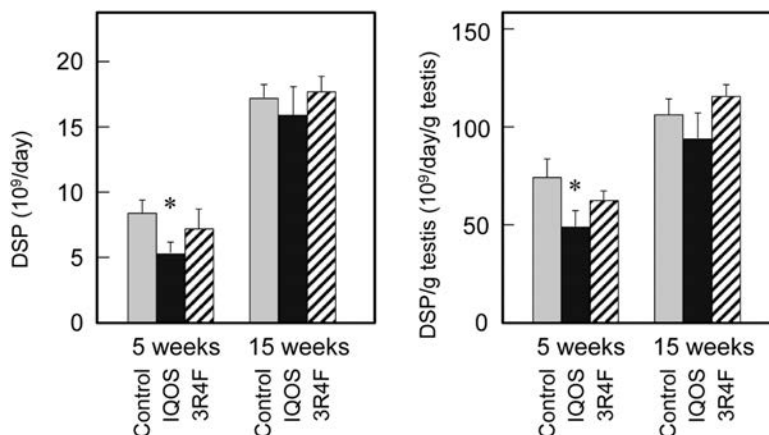


Fig. 4. Effects of Fetal IQOS/3R4F Exposure on Spermatogenesis

A right testis sample was weighed, placed into 1 mL of 0.05% Triton-X 100 and homogenized for 2 min using the Polytron System PT 2500E. The homogenates were kept on ice for 30 min. The homogenates diluted to the desired volume, and a homogenate of the diluted suspension was taken to count sperm heads or sperm cells in a Burkert-Türk counting chamber. DSP and DSP/g testis were calculated as described in Materials and Methods. ($n = 8$); * $p < 0.05$, vs. controls.

There were no significant differences observed in sperm characteristics between the IQOS or 3R4F and control groups at 15 weeks (Table 3).

Effects of Fetal IQOS Aerosol/3R4F Mainstream Smoke Exposure on Serum Hormone Concentration There were no significant differences in serum testosterone, estradiol or FSH between the IQOS or 3R4F and control groups at either age (data not shown).

DISCUSSION

The present study was designed to examine the effects of maternal HnB tobacco products (IQOS) on male offspring testicular by examining its effects on pups' number, spermatogenesis, sperm characteristics, serum testosterone and seminiferous tubule morphology. Prenatally IQOS-exposed male offspring mice exhibited changes in testicular morphology and decreased spermatogenesis, suggesting that generalized IQOS aggravates the male testicular function or delays sexual maturation.

Tobacco smoking is known to cause a risk of spontaneous abortion.²³⁾ Prenatal tobacco exposure is the leading cause

Table 3. Effects of Fetal IQOS and 3R4F Exposure on Sperm Parameters of Male Offspring

	Control	IQOS	3R4F
MOT (%)	59.8 \pm 4.6	68.1 \pm 4.2	63.4 \pm 4.9
Progressive (%)	38.6 \pm 5.1	46.7 \pm 6.0	41.4 \pm 5.3
VCL (μ m/s)	61.0 \pm 7.6	76.5 \pm 11.9	67.1 \pm 8.0
VSL (μ m/s)	17.8 \pm 7.2	23.4 \pm 4.0	20.9 \pm 2.6
VAP (μ m/s)	29.8 \pm 4.0	39.3 \pm 6.1	35.6 \pm 4.5
LIN (%)	29.8 \pm 3.0	33.7 \pm 0.6	34.4 \pm 1.8
STR (%)	54.6 \pm 2.4	57.6 \pm 0.6	57.9 \pm 1.1
WOB (%)	50.1 \pm 2.7	54.8 \pm 0.8	55.1 \pm 1.7
ALH (μ m)	1.8 \pm 0.2	2.1 \pm 0.3	1.9 \pm 0.2
BCF (Hz)	4.6 \pm 0.5	5.8 \pm 0.6	5.4 \pm 0.6

Data are mean \pm S.D. values. $n = 8$. MOT; sperm motility (%), progressive motility (%), VCL; continuous line velocity (μ m/s), VSL; straight line velocity (μ m/s), VAP; average path velocity (μ m/s), LIN; linearity (ratio of VSL/VCL) (%), STR; straightness (ratio of VSL/VAP) (%), WOB; wobble (%), ALH; amplitude of lateral head displacement (μ m), BCF; beat cross frequency (Hz).

of premature morbidity and mortality in the United States.²⁴⁾ Moreover, the population of newly born human males declined significantly in Canada,²⁵⁾ the United States,²⁶⁾ and Japan.²⁷⁾ Our previous research has shown that fetal exposure to Asian sand dust significantly decreased the secondary sex ratio (SSR) of mice.²⁸⁾ However, in this study, fetal IQOS/3R4F exposure did not affect litter size or SSR. However, these results do not indicate that IQOS use is safe during gestation. In the next study, it will be necessary for the pregnant mice to be exposed whole body to a large amount of IQOS aerosol.

Previous reports have indicated that prenatal combustion tobacco smoking may affect male offspring's decreased semen volume and low sperm count in adult life.^{15,29)} Maternal combustion tobacco (3R4F) smoke exposure reportedly caused defects in male offspring's fertility in mice.³⁰⁾ However, the effects of maternal exposure to HnB tobacco on male mice's subsequent testicular of offspring has not been investigated yet. Our results demonstrate that maternal HnB tobacco (IQOS) aerosol exposure during organogenetic period induces juvenile delay of spermatogenesis and increase of abnormal seminiferous tubule morphology, and these effects may be the result of delayed sexual maturation or these effects were based on development delay. In a different experiment (unpublished data), we examined the effects of exposure of pregnant mice to IQOS on male fetus development. Fetal exposure to IQOS reduced fetal weight at 14.5 d of fetal age by 23% compared to the controls. On the other hand, our results suggest that there are no adverse effects of prenatal 3R4F exposure on spermatogenesis or increases in the incidence of seminiferous tubule damage in male offspring in this experimental condition. Our experimental conditions were four IQOS/3R4F heat sticks or cigarettes per exposure day on the 7th and 14th days of gestation (a total of 8 cigarettes). The 7th day of gestation is important as an organ formation stage, and the 14th day of gestation is an important period for male sexual differentiation. Therefore, in this study, we planned to carry out cigarette exposure on those two days. In contrast, the conditions of the Sobinoff study³⁰⁾ are 3R4F 24 cigarettes per exposure day for five times per weeks during the gestational/weaning period (7-weeks, a total of 840 cigarettes; 24 cigarettes/d \times 5 d/week \times 7-weeks). Our experimental design, with a much lower level of exposure in comparison with the Sobinoff study, showed that maternal 3R4F exposure is not associated with negative effects on male offspring's reproductive capacity. Despite the small number of heat sticks, however, maternal exposure to IQOS aerosol causes negative effects on spermatogenesis in male offspring mice even at this low level.

One of the HnB tobacco products, IQOS is sold in 52 countries,³¹⁾ and is used by almost 10.6 million adult smokers.³²⁾ Philip Morris declares that IQOS emit no secondhand smoke or tobacco specific smell. It has been shown that the concentration of tobacco specific nitrosamines in IQOS aerosol are much lower than those in combustion cigarettes (3R4F).³³⁾ HnB tobacco products have been advertised to smokers as an alternative to conventional cigarettes that is less harmful to both users and bystanders.³⁴⁾ Almost all smokers think that IQOS has the potential to be less harmful than combustion tobacco.³⁵⁾ Even in smoking conditions that are not affected by 3R4F, however, the same exposure conditions were affected by IQOS. Moreover, the preclinical data indicated that IQOS had an even stronger hepatotoxicity than tobacco.³⁶⁾ According to

the aerosol chemistry data, IQOS reduced user exposure to some compounds compared with 3R4F. However, concentrations of some constituents, such as 4-amiobiphenyl (4-ABP), are higher in IQOS than in 3R4F.³⁷⁾ There is no information on the effects on male offspring of administering 4-ABP to pregnant mice. However, 4-ABP, which has carcinogenesis, has been shown to cross the placenta, and it has also been shown that 4-ABP binds to fetal DNA.³⁸⁾ These results do not indicate that HnB tobacco, such as IQOS, is the safer tobacco.

We conclude that fetal IQOS aerosol exposure can delay sexual maturation or impair spermatogenesis of male mice offspring more than when the gestating mother is exposed to smoke from a conventional combustion cigarette. While these findings do not prove the adverse effects of maternal inhalation of IQOS aerosol on a spermatogenesis disorder or delayed sexual maturation among men and the information offered by tobacco companies did not clearly show adverse health effects, at least some of the adverse health effects of cigarettes may not be avoided by using HnB tobacco. Further research is needed to determine the chemicals most centrally contributing to the fetal IQOS exposure effects on male testicular function of offspring.

On the whole, this study raises concerns about the safety of HnB tobacco. Tobacco control actions have led to some reduction in conventional smoking; however, policy makers should be aware of the impacts of HnB tobacco on pregnant tobacco users and their offspring.

Several studies show that maternal conventional cigarette smoking during pregnancy has been associated with reduced sperm concentration in sons. In this study, we investigated the effects of maternal HnB tobacco products (IQOS) on male offspring testicular function. We showed that prenatal exposure to IQOS aerosol delayed sexual maturation of male offspring and adversely affects the male testicular function of the offspring more than smoke from a combustion cigarettes.

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Conflict of Interest The authors declare no conflict of interest.

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