Zygote

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Effects of cigarette smoke condensate on reproduction in mice *in vivo*

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Research Article

Cite this article: Banafshi O *et al*. (2023) Effects of cigarette smoke condensate on reproduction in mice *in vivo*. *Zygote*. **31**: 281–287. doi: 10.1017/S0967199423000072

Received: 14 November 2020
Revised: 8 April 2022
Accepted: 26 January 2023
First published online: 11 April 2023

Keywords:
Cigarette smoke condensate (CSC); Female; Fertility; Male; Mating

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Summary

Smoking has dangerous and sometimes irreversible effects on various body tissues, including the reproductive system. We conducted this research to determine the *in vivo* effects of cigarette smoke condensate (CSC) on reproduction in mice. In this experimental *in vivo* study, 32 male and female NMRI mice were divided into four groups. The mice were injected with CSC (CSC-1R3F) for 28 days. The mice were mated 1 day after the last injection and observed daily for 1 week for the presence of a vaginal plug to track mating. We evaluated mating success rate, and sperm and oocyte quality, pregnancy outcome, childbearing status, and *in vitro* fertilization (IVF). The results showed a decrease in successful mating in female mice that received the CSC injections. CSC significantly influenced the number of offspring born to males. When the CSC was injected into male mice, there was a significant increase in the number of offspring compared with the group in which only the females received CSC injections. According to the results, there was a negative effect of CSC on morphological parameters in male and female mice. Also, successful IVF after exposure to CSC was significantly decreased in the female mice treated group. The results indicated that CSC significantly affected the number of offspring and fecundity success in females.

Introduction

Infertility is a health problem observed in 10–15% of couples. Previously, most reproductive failures were presumed to result from problems with the female partner; however, numerous studies have shown that 30–50% of infertilities were caused by a male factor (Azad *et al.*, 2018).

Cigarette smoke is considered to be one of the biggest threats to human health. Cigarettes contains many mixtures of toxic chemical compounds that decrease reproduction or cause infertility in both males and females by reducing sperm quality and increasing reproductive failure (Oyeyipo *et al.*, 2011). Nicotine is an important component of cigarette smoke that adversely affects male and female reproductive function. In females, the toxic effects of cigarette smoke on the reproductive system decrease estradiol production and inhibit ovulation. (de Angelis *et al.*, 2020). It has been identified that nicotine affects spermatogenesis and epididymal count of sperm, motility, and capacity fertilization, decreases testosterone levels, impairs Leydig cell function, and leads to various histopathological changes in testicular tissue in experimental animals (Aydos *et al.*, 2001; Jana *et al.*, 2010; Nesseim *et al.*, 2011; Oyeyipo *et al.*, 2011). Cigarette smoke condensate directly affects white blood cells (WBC) in sperm, which leads to the production and release of a variety of reactive oxygen radicals or reactive oxygen species (ROS) that ultimately results in severely reduced sperm motility (Darbandi *et al.*, 2018). ROS and inflammation are interchangeable mechanisms mediated by cigarette smoke (Kaplan *et al.*, 2017).

Smoking affects body weight, food intake, and the reproductive system (Audi *et al.*, 2006). Ultrastructural changes such as rupture of the mitochondrial membrane, changes in the Golgi complex cisterns, and morphological changes in the nuclei of the attached glands of the reproductive tract and sex hormones (androgens, estrogens, and progestogens) have been reported after smoking cigarettes (Audi *et al.*, 2006; Florek *et al.*, 2008; de Souza *et al.*, 2009; Florescu *et al.*, 2009). Ultrastructural changes also occur in the germ cells of nicotine-exposed mice (Al-Mukhaini *et al.*, 2020). In our previous study, we reported that CSC affected the proliferation and expression of pluripotency genes in the embryonic stem cells of the mouse (Assadollahi *et al.*, 2019). The results of another study indicated that exposure to cigarette smoke induced oxidative stress, shortening of telomeres and apoptosis, and compromised development

of the embryo *in vivo* in mice (Huang *et al.*, 2009). Mice exposed to cigarette smoke had an increased pregnancy interval, decreased numbers of newborns per pregnancy, and increased infant mortality. These mice also had significant decreases in testicular and ovarian weight. The number of graafian follicles in the ovarian tissue also decreased significantly. Past studies have shown that cigarette smoke could decrease reproductive performance in both males and females (Audi *et al.*, 2006). In both *in vitro* and *in vivo* studies, nicotine resulted in the direct impairment of sperm motility and in apoptosis induction in male rat Leydig cells (Kim *et al.*, 2005). Pieces of evidence of the detrimental effect of smoke on specific domains of the female reproductive function are provided by experimental studies in animals. Overall, clinical studies have suggested that smoking is associated with decreased fertility, although causal inference should be demonstrated further (de Angelis *et al.*, 2020).

The results of numerous studies have shown that infants born to smokers have a large thyroid gland (Kapoor and Jones, 2005). Researchers at the American Heart Association reported that children whose parents smoke were at greater risk of developing advanced heart disease than other children (Farber *et al.*, 2015). Given the importance of the effect of smoking on health, this study aimed to research the cigarette smoke condensate (CSC) *in vivo* influence on reproduction in mice. Due to the increasing development of knowledge and more attention to detail, research such as the present study, which examines the effect of CSC on fertility in different ways, is needed.

Materials and methods

Animals

In this experimental laboratory study, thirty-two 2-month-old NMRI mice that weighed ~25 g were purchased from the Pasteur Institute, Tehran, Iran. The mice were kept at a temperature of 25 ± 2°C and a light/dark period of 12 h/12 h, with the onset of the light period beginning at 6 a.m. The animals were provided with water and food; the mouse feed was prepared by DamPars Livestock Factory, Tehran, Iran. The mice were assigned randomly to four experimental groups and samples were counted in each group (Table 1). None of the animals had any experience of disease or evidence of disease. The authors assert that all procedures contributing to this work complied with the ethical standards of the relevant national and institutional (Ethical project number: IR.MUK.REC. 1394/220) guides on the care and use of laboratory animals.

CSC treatments

The mice in group 1 (control) received injections of normal saline for 4 weeks. Mice in groups 2, 3, and 4 received injections of 10 µl CSC (CSC-1R3F; Murty Pharmaceutical Corporation, Lexington, KY, USA) diluted 10-fold with physiological normal saline, this amount reached 100 µl over 4 weeks. The day after the last injection, the mice were allowed to mate. In a recent experiment, we used CSC at a concentration of 10 µl (0.4 mg/ml of CSC-1R3F), which is comparable with the exposure of an individual who smokes more than one cigarette per day and a plasma nicotine concentration of more than 25 ng/dl (Mendelson *et al.*, 2003; Csiszar *et al.*, 2008). In addition, our preliminary experiments proved the sub-lethal effects of these concentrations in mice. The presence of a vaginal plug was checked daily for 1 week as confirmation of mating, and female mice were observed until the end of the pregnancy.

Table 1. Cigarette smoke condensate (CSC) treatments in NMRI male and female mice

	Injection	
Group	Male	Female
One (control)	Normal saline	Normal saline
Two	CSC	Normal saline
Three	Normal saline	CSC
Four	CSC	CSC

The numbers of newborns and the weights of the mothers and neonatal mice were assessed. The mice were weighed daily until the end of the seventh day and weekly until the end of the 42nd day.

Mating success rate

The animals were divided into four groups and treated with 0.4 mg/ml of CSC via intraperitoneal (i.p.) injection. The process was repeated four times and, after the last injection, the mice were allowed to mate, and females were monitored daily for the presence of a vaginal plug in a consecutive 7-day period. The rate was then calculated as the percentage of successful mating.

Number of offspring born

In a further study we measured the number born in each experimental group after mating. For this purpose, the number and weight of newborns were counted weekly.

Birth weight of offspring

The number of newborns was counted and the mothers and neonates were weighed daily until the end of the seventh day and weekly until the end of the 42nd day.

Fertility parameters

At 1-week intervals, 10 µl of CSC was injected into 12 male and 12 female NMRI mice. The mice were sacrificed at the end of day 28 to measure their fertility parameters. We measured some indices used for the evaluation of fertility including mating success, number of offspring, mouse weight, and testis weight, number of sperm, motility of sperm, morphology of sperm and survived sperm for male mice, and ovarian weight and cumulus–oocyte complexes (COCs) for female mice.

In vitro *fertilization*

Adult male mice were sacrificed and their epididymides were dissected, disrupted, and moved to *in vitro* fertilization (IVF) medium. IVF medium for capacitation of sperm consisted of human tubal fluid (HTF) medium complemented by 15 mg/ml bovine serum albumin (BSA) (equilibrated at 37.5°C in 5% CO_2). For sperm capacitation, an incubation period of ~1.5 h was considered sufficient.

Ovulation in female mice was induced with i.p. injections of 5 IU PMSG (Sigma, USA) followed by 5 IU hCG (Sigma) 48 h later. The ampullae were cut, and the mature oocytes (metaphase meiosis II) were transferred to 100 µl IVF medium droplets, followed by the addition of 1×10^6 sperm/ml to the IVF droplets. The zygotes were observed under an inverted microscope, and the percentage

https://doi.org/10.1017/S0967199423000072 Published online by Cambridge University Press

Table 2. Influence of cigarette smoke condensate (CSC) on mating success of male and female NMRI mice

	Injection		Day									
Group	Male ($n = 16$)	Female ($n = 16$)	1	2	3	4	5	6	7	Mating success (n)	Success (%)	P-value
One (control)	Normal saline	Normal saline	+	+	–	+	+	–	–	4	100	0.001
Two	CSC	Normal saline	–	+	+	+	–	+	–	4	100	
Three	Normal saline	CSC	–	–	+	+	+	–	–	3	75	
Four	CSC	CSC	+	–	+	–	+	–	–	3	75	

Values are means ± SD. The means with different letter codes are significantly different from each other (ANOVA, Tukey's test, $P < 0.05$) ($n = 32$) (mean ± SD).

Table 3. The effect of cigarette smoke condensate (CSC) on number of offspring

	Injection		Number of offspring born (iteration)						
Group	Male	Female	First	Second	Third	Fourth	Total	Mean	P-value
One (control)	Normal saline	Normal saline	13	11	13	14	51	12.7 (±3.44)	0.001
Two	CSC	Normal saline	12	9	10	9	40	10 (±2.35)	
Three	Normal saline	CSC	0	12	13	13	38	9.5 (±3.64)	
Four	CSC	CSC	12	0	12	11	35	8.7 (±3.73)	

Values are means ± SD. The means with different letter codes are significantly different from each other (ANOVA, Tukey's test, $P < 0.05$) ($n = 8$) (mean ± SD).

of 2-cell embryos that formed was recorded as an evaluation of the fertilization rate.

First, oestrous mice from the four groups were placed in a cage with vasectomized male mice. The next morning, the female mice that had vaginal plugs were considered to be day 0.5 pseudopregnant mice. We transferred the 2-cell embryos to the uterine tubes of these pseudopregnant mice to assess embryonic viability. After 19 days, the numbers of live-born mice were compared in the different groups.

Statistical analysis

Data analysis was performed using SPSS 16 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.2.1 software (GraphPad Prism Inc, San Diego, CA, USA). We used the mean ± standard deviation (SD) to represent the results. Then, for data comparison between the means of the studied subjects, Mann–Whitney test and one-way analysis of variance (ANOVA) analysis were performed, and P-values < 0.05 were considered statistically significant.

Results

Mating success rate

To study the effect of CSC on mating success rate, we measured this factor for 7 days consecutively. The results showed a significant difference between the groups in terms of successful mating ($P < 0.01$), as listed in Table 2. The effect of CSC on mating success showed that the rate of occurrence in the control group (100%) was equal to group 2 in which the male mice were injected with CSC (100%). Therefore, CSC did not affect the mating success rate (Table 2). However, the mating success rate decreased when female mice were injected with CSC (75%), as shown in groups 3 and 4. These results suggested that the effect of CSC on female mating success might be more significant than for males.

The number of offspring

The results showed a statistically significant difference between the groups based on the number of offspring ($P < 0.01$). The highest number of offspring was observed in the control group (mean: 12.7). The lowest number of offspring (mean: 8.7) was observed in group 4. CSC had a significant effect on the number of offspring born to females. However, when CSC was injected into males, the number of offspring significantly decreased compared with the control group. Injections of CSC into females also decreased the number of offspring (Table 3).

The condition of the newborn offspring

Offspring born from CSC-injected mice (groups 2–4) and control mice (group 1) were weighed according to the schedule presented in Table 4 until the day of puberty (day 42). The results showed a statistically significant difference in birth weight between the groups, except for days 3, 4, and 5 at the 5% levels. There was a significant difference between the days of study in the different groups. The effect of CSC on the weights of the offspring showed that the highest birth weight was recorded on day 42 in the control group (mean: 22.8 g). This difference was statistically significant compared with the group 4 (Table 4). The lowest birth weight was observed in group 3 (mean: 20.8 g). CSC had a significant effect on male birth weight.

Fertility parameters

The findings indicated a considerable difference between male mice in the intervention and control groups in terms of weight, sperm count, sperm motility, sperm morphology, and survival at

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Table 4. Influence of cigarette smoke condensate (CSC) on the weights of the offspring

Group		One (control)	Two	Three	Four	*P*-value
Injection	Male	Normal saline	CSC	Normal saline	CSC	
	Female	Normal saline	Normal saline	CSC	CSC	
Offspring (*n*)		51	40	38	35	
Adults (*n*)		46	31	34	30	
Adult/offspring (%)		90.2	77.5	89.4	85.7	0.015
Average weight (g)	Days					
	1	1.6	1.7	1.7	2.1	0.018
	7	3.7	4.1	4.2	4.7	0.047
	14	5.1	6.1	6.9	8.6	0.024
	21	13.0	11.9	11.5	14.1	0.001
	28	17.4	15.6	15.3	18.4	0.001
	35	20.2	18.7	17.8	20.8	0.049
	42	22.8	21.8	20.8	22.7	0.015
P-value						0.01

Values are means ± SD. The means with different letter codes are significantly different from each other (ANOVA, Tukey's test, $P < 0.05$) ($n = 8$) (mean ± SD).

Table 5. Influence of cigarette smoke condensate (CSC) on reproductive parameters in NMRI male mice

			Sperm			
Group	Mouse weight (g)	Testis weight (g)	Number ($\times 10^6$)	Motility (%)	Morphology (%)	Survived (%)
CSC injections	43.25 (±4.32)	0.26 (±0.08)	36.77 (±3.12)	40.90 (±3.66)	49.67 (±4.52)	68.00 (±5.35)
Control	46.58 (±4.27)	0.27 (±0.023)	60.30 (±5.26)	72.83 (±6.39)	63.00 (±5.90)	75.50 (±6.72)
P-value	0.001	0.415	0.001	0.001	0.001	0.001

Significant differences between treatment groups compared with the control group. (ANOVA, Tukey's test, $P < 0.05$) ($n = 4$) (mean ± SD).

the 1% level. However, there was no significant difference between male mice in the intervention groups and control mice in testicular weight. Mean comparison results showed that the highest weight was observed in male control mice (Table 5). Also, the highest numbers of sperm, sperm motility, sperm morphology, and survival were observed in control male mice. The results showed a negative effect of CSC on morphological parameters in male mice.

The findings showed a marked difference between female mice in the intervention and control groups regarding weight and cumulus–oocyte complex (COC) number at the 1% level. However, there was no significant difference between female mice in the intervention and control mice in terms of ovarian weight. Mean comparison results showed that female control mice had the highest mean weight (Table 6). The highest number of COC was observed in the female mice from the control group.

Table 6. Influence of cigarette smoke condensate (CSC) on reproductive parameters in female NMRI mice

Group	Mouse weight (g)	Ovarian weight (g)	COC (*n*)
CSC injection	29.75 (±2.62)	0.02 (±0.012)	12.67 (±1.92)
Control	36.38 (±3.77)	0.02 (±0.007)	15.17 (±3.03)
P-value	0.001	0.758	0.001

Values are means ± SD. The means with different letter codes are significantly different from each other (ANOVA, Tukey's test, $P < 0.05$) ($n = 4$) (mean ± SD).

In vitro *fertilization*

The results showed a statistically significant difference between the groups in the rate of IVF success and the number of 2PN ($P < 0.001$). The success rate of IVF was calculated based on the number of 2PN created.

The highest 2PN number was observed in the control group (mean: 107) (Table 7). The lowest 2PN number (mean: 73) was observed in group 3. The effect of CSC on the rate of IVF success showed that the highest percentage was observed in the control group (mean: 89.1%). The lowest rate of IVF success (mean: 60.8%) was observed in group 3. The findings showed that CSC had a significant effect on the 2PN number and rate of IVF success in females. Therefore, CSC injections in females could decrease both the 2PN number and the rate of IVF success.

Birth rate after In vitro *fertilization*

The findings revealed a statistically notable difference between the groups in the number of offspring and fecundity success ($P < 0.01$). The effect of CSC on the number of offspring showed that the highest number of offspring was observed in the second group (mean: 23) (Table 8). The lowest number of offspring (mean: 15) was observed in group 3. The effect of CSC on fecundity success

https://doi.org/10.1017/S0967199423000072 Published online by Cambridge University Press

Table 7. Influence of cigarette smoke condensate (CSC) on *in vitro* fertilization (IVF) parameters in NMRI mice

	Injection				
Group	Male	Female	Oocytes (*n*)	2PN (*n*)	IVF success (%)
One (control)	Normal saline	Normal saline	120	107	89.1 (±9.46)
Two	CSC	Normal saline	120	96	80 (±7.02)
Three	Normal saline	CSC	120	73	60.8 (±7.71)
Four	CSC	CSC	120	87	72.5 (±8.12)
P-value					0.001

Values are means ± SD. The means with different letter codes are significantly different from each other (ANOVA, Tukey's test, $P < 0.05$) ($n = 8$) (mean ± SD).

Table 8. Influence of cigarette smoke condensate (CSC) on birth rate after *in vitro* fertilization (IVF) in NMRI mice

	Injection						
Group	Male	Female	2-cell (*n*)	Pseudopregnant mice (*n*)	2-cell/mice (*n*)	Offspring (*n*)	Fecundity success (%)
One (control)	Normal saline	Normal saline	60	4	15	21	35 (±3.31)
Two	CSC	Normal saline	60	4	15	23	38.3 (±5.47)
Three	Normal saline	CSC	60	4	15	15	25 (±4.09)
Four	CSC	CSC	60	4	15	19	31.7 (±3.28)
P-value							0.001

Values are means ± SD. The means with different letter codes are significantly different from each other (ANOVA, Tukey's test, $P < 0.05$) ($n = 8$) (mean ± SD).

showed that the highest value was observed in group 2 (mean: 38.3). The results indicated that CSC had a significant effect on the number of offspring and fecundity success in females. Therefore, injection of CSC in females would decrease both the number of offspring and fecundity success.

Discussion

Results of the current study suggested that smoking may cause devastating effects on mouse fertility and reproduction. This study showed that CSC negatively affected pregnancy and successful mating. The results of animal studies have shown that modified gonadotropin secretion, decreased luteinizing hormone surge, inhibited prolactin release, altered tubular motility, and impaired blastocyst formation and implantation may be the mechanisms of reproductive defects in smokers. (Woodward and Mehta, 2019).

Carmines *et al.* (2003) showed in female mice exposed to the high concentrations of CSC, weight gains during pregnancy and mean uterine weight decreased significantly. The results of this study showed that smoking could have detrimental effects on sperm.

In the current study, we observed the negative impacts of CSC on sperm parameters in male NMRI mice. Cigarette smoking negatively affected all conventional semen parameters in addition to sperm chromatin condensation and sperm viability. These abnormalities are also proportional to the number of cigarettes smoked per day and to the duration of smoking. (Mostafa *et al.*, 2018). ROS is an important factor in male infertility and is produced from two different sources of sperm fluid-damaged sperm cells and activated WBC. ROS production in healthy and active sperm cells is a physiological process known as 'the fog' that causes an acrosomal reaction in sperm. The positive effect of ROS depends on the presence of antioxidants or excess ROS eliminators in the sperm operation medium. In sperm, any imbalance between ROS production and elimination causes oxidative stress in the cell. Disorders in the structure of ROS lead to decreased sperm motility and, ultimately, binding of sperm to zygote surfaces (Gavriliouk and Aitken, 2015). Numerous studies have reported that smoking can cause abnormal sperm parameters (Harlev *et al.*, 2015), Smokers have a lower density of sperm, natural morphology, and motility than non-smokers (Budin *et al.*, 2017). In another study, Zhang *et al.* (2000) reported that smoking males had a lower density of sperm and motility movement than non-smokers. Ozgur *et al.* (2005) concluded that no significant difference existed in sperm density, motility, shape, and quality between smokers and non-smokers. Smokers had less semen volume and total spermatozoa than non-smokers. Nadeem *et al.* (2012) reported that smoking reduced sperm motility and viability; these effects are directly related to cigarette smoke. Chohan and Badawy (2010) attributed the effects of cigarette smoke to the aerobic metabolism of sperm.

The initiation of apoptotic cell death in ovarian follicles and granulosa cells by specific stimuli is due to increased ROS (Lim and Luderer, 2011). Mai *et al.* (2014) suggested that exposure to CSC is associated with reduced oocyte size and low quality.

In our study, we observed the negative effects of CSC on reproductive parameters in female NMRI mice. Numerous studies have reported an association between smoking and reduced fertility. Smoking cigarettes affects the physical health of women in the late reproductive stage through negative influences on lipid and hormone metabolism, among other factors (Szkup *et al.*, 2018); Olooto *et al.* (2012) reported the increased chance for delayed conception, and primary and secondary infertility in female smokers. Researchers who conducted a literature review concluded that there was sufficient evidence to conclude a causal relationship between smoking and reduced fertility in women. Many studies found a significant reduction in smoking-related fertility and evidence of dose–response patterns (Onor *et al.*, 2017). In a later qualitative review of 22 studies, 18 studies reported harmful effects of smoking on fecundity in women (Wilks and Hay, 2004). The

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Practice Committee of the American Society for Reproductive Medicine (PCASRM) also released a statement that strongly endorsed evidence of a connection between smoking and infertility and estimated that smoking could account for 13% of infertility (Rogers, 2009).

Studies in humans indicated that men's smoking behaviour can rely more on nicotine's pharmacological effects. For women, this behaviour can depend more on smoking-related non-pharmacological factors. In line with human research, numerous animal studies that have evaluated sex differences have noticed different sensitivities to the pharmacological effects of nicotine in both male and female rats. These reports highlight the importance that researchers should take into consideration both the species of the animal and their genetic backgrounds when examining nicotine-response sexual differences (Isiegas *et al.*, 2009).

It may be concluded from the results that CSC reduced mouse weight, the successful mating percentage, parental status, and the quality of sperm and oocytes, in addition to IVF fertilization changes. The results showed a negative effect of CSC on morphological parameters in male and female mice. CSC had a significant negative effect on the number of offspring and fecundity success in female mice.

Acknowledgements. The results of this paper are part of a PhD dissertation (research project number: 1394/220), which was financially supported by the School of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.

Conflict of interest. The authors declare that they have no conflict of interest.

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