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Paper

Testicular tissue response following a 90-day subchronic exposure to HTP aerosols and cigarette smoke in rats

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Background: Researches have shown that chronic inhalation of cigarette smoke (CS) disrupts male reproductive system, but it is unclear about the mechanisms behind reproductive damages by tobacco toxicants in male rats. This study was designed to explore the effects of heated tobacco products (HTP) aerosols and CS exposure on the testicular health of rats.
Materials and Methods: Experiments were performed on male SD rats exposed to filtered air, HTP aerosols at 10 μg/L, 23 μg/L, and 50 μg/L nicotine-equivalent contents, and also CS at 23 μg/L nicotine-equivalent content for 90 days in five exposure groups (coded as sham, HTP_10, HTP_23, HTP_50 and Cig_23). The expression of serum testosterone, testicular tissue inflammatory cytokines (IL-1β, IL-6, IL-10, TNF-α), reactive oxygen species (ROS), superoxide dismutase (SOD) and malondialdehyde (MDA), NLRP3 inflammasome-related mRNAs and proteins (NLRP3, ASC, and Caspase-1), the degree of pyroptosis and histopathology were investigated.
Results: The results demonstrated that HTP_50 and Cig_23 caused varying degrees of oxidative damage to rat testis, resulting in a decrease of sperm quantity and serum testosterone contents, an increase in the deformity rate, expression levels of proinflammatory cytokines, and NLRP3 inflammasome-related mRNA, and an increase in the NLRP3, ASC, and Caspase-1-immunopositive cells, pyroptosis cell indices, and histopathological damage in the testes of rats. Responses from the HTP_10 and HTP_23 groups were less than those found in the above two exposure groups.
Conclusion: These findings indicate that HTP_50 and Cig_23 induced oxidative stress in rat testes, induced inflammation and pyroptosis through the ROS/NLRP3/Caspase-1 pathway, and destroyed the integrity of thetesticular tissue structure.

Key words: heated tobacco products (HTPs); cigarette; testis; inhalation toxicology; NLRP3.

Introduction

Reproductive health can be adversely affected by CS, which can disrupt multiple biological systems, including the respiratory and cardiovascular systems.[1,2] Nicotine is the main addictive substance in smoke but is not generally considered the main carcinogen or neurotoxicant. However, some animal studies have demonstrated that it affects testicular cells and spermatogenesis, and has a negative impact on the reproductive system of males; therefore, further studies are needed to determine whether the use of new tobacco products leads to the decline of human fertility.[3] Nicotine concentrations of between 70 and 300 μg/L (0.43–1.85 μM) have been found in the seminal fluids of daily smokers,[4] which is significantly higher than in those who do not use cigarettes, suggesting that nicotine can penetrate the blood-testis barrier (BTB). Additional studies have demonstrated that cigarette smoking affects the normal arrangement of spermatogenic cells,[5] decreases semen quality,[6] induces sperm DNA double-strand breaks,[7] DNA adducts, chromosomal abnormalities, and affects abnormal plasma membrane integrity of Leydig cells.[8]

HTPs, also known as "heat-not-burn" products, enable processed tobacco to be heated rather than combusted in a controlled manner.[9] HTPs mainly heat tobacco flakes through a special heating source to heat the nicotine and aroma components in cut tobacco, producing a new type of aerosols that can be inhaled to meet the needs of smokers.[10] Compared with cigarettes, these new tobacco products can not only meet the sensory needs of consumers, but also reduce the content of toxic substances such as carbonyls in aerosols, mitigate the harm to human health and the environment. Related studies have demonstrated that the incidence rate of carcinogenic compounds in smoke decreased by more than 80%, the intake of mutagens decreased by 70%, and the incidence of asthma and pneumonia decreased by 36% ∼ 46%.[11] HTPs are odorless and less harmful,[12] a claim by tobacco companies that has been demonstrated in laboratory settings, but the mechanisms behind reproductive damages by tobacco toxicants of HTP aerosol in males remain unclear.

The male reproductive system, especially the testes and spermatozoa, are susceptible to oxidative damage, mainly because of their high content of polyunsaturated fatty acids in membranes, their limited antioxidant capacity, and the ability of spermatozoa to generate reactive oxygen species.[13] However, overproduction of reactive oxygen species can be detrimental to sperm and appears to be a common feature underlying male infertility. Harmful components in CS toxicity are correlated with pro-oxidant activity in several organs and tissues,[14,15] and more recently in male reproductive toxicity.[16–18] When orally administered to rats for 30 days, nicotine (0.5 and 1 mg/kg) has been reported to significantly decrease several indices of the antioxidant profile of rat plasma.[19]

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Studies have confirmed that testicular inflammatory injuries are an important pathological factor leading to male reproductive dysfunction. IL-1 β, IL-6, and TNF-α are proinflammatory factors, which are expressed in normal testicular tissue. Their main biological functions are to promote spermatogenesis and testosterone synthesis.[20] IL-10 is an anti-inflammatory factor, which inhibits inflammatory response in testicular tissue. IL-1β, IL-6, and TNF-α interact with IL-10 to maintain testicular immune homeostasis.[21] The generation and accumulation of reactive oxygen species (ROS) in biological systems are known to cause inflammation.[22] When testicular inflammatory response occurs, it can destroy testicular structure and BTB, inhibit spermatogenesis, and affect testosterone synthesis. The imbalance and accumulation of ROS are also considered to be the primary molecular mechanism inducing NLRP3 inflammasome activation. Activated NLRP3 inflammasome can catalyze the cleavage of Caspase-1 protein precursors to form active caspase-1, which induces maturation and release of inflammatory cytokine IL-1β, triggering an inflammatory cascade.[23]

HTPs are considered a novel tobacco category with the potential to reduce health risks caused by smoking. This study focused on comparing HTP aerosols and CS induced testicular inflammatory injury and male reproductive dysfunctions. The aim of this study was to investigate testicular morphological and functional changes associated with HTP aerosols and CS exposure, and to determine whether HTP aerosols and CS exposure affected male testicular injury by activating NLRP3 inflammasome, as well as other differences between the effects of the HTP aerosols and CS.

Materials and methods

Animals

Male Sprague–Dawley rats aged 6–7 weeks were procured from Charles River Laboratories (Beijing, China). At the beginning of the study, the rats were acclimatized for approximately 1 week. The rats were identified by number marks and cage-labels. Rats were singly housed in polycarbonate cages in an animal room at 23 ± 2 °C and 50 ± 10% humidity under an artificial lightning system of 12 h light and 12 h darkness (lights on from 07:00 to 19:00). Rats were able to access water and chow diets ad libitum during the experimental period. Rats used in this experiment, and their related disposal, strictly adhered to the guidelines for ethical review of experimental animal welfare (GB/T35892-2018), as required by national standards, and have passed the ethical review of the experimental animal management committee of the China National Tobacco Quality Supervision & Test Center (CTQTC-SYXK-2021008).

Experimental design

We conducted a 90 day HTP aerosol and CS exposure experiment according to OECD test guidelines (TG) 413; experimental groups consisted of ten male rats. Experimental groups were scheduled for 90 days of exposure for 5 days per week and 6 h per day to filtered air (sham), CS at 23 μg/L nicotine (Cig_23), HTP aerosols at 10 μg/L, 23 μg/L, and 50 μg/L nicotine (HTP_10, HTP_23, HTP_50).

Aerosol/smoke generation

The HTPs tested were purchased from the Korean market and produced by China Tobacco Hubei Industrial (Hubei, China). The reference cigarettes were procured from University of Kentucky and are widely used in scientific studies for comparison. CS was generated on a 10-port rotary smoking machine (Huironghe, Beijing, China). HTP aerosols were generated using a 50-port carousel smoking machine (Yizhong, Qingdao, China) equipped with stick holders and a temperature-controlled insulation kit (Tube Warming System) in the undiluted aerosol pathway to reduce aerosol condensation prior to diluting the aerosols. Rats were individually nose-only exposed in HRH-MNE3026-type (Huironghe, Beijing, China) flow-pass inhalation chambers and in plastic sleeves matching their body size. The relative humidity in the sham exposure chamber was 55 ± 1% (mean ± S.D.), and the temperature within the exposure chambers ranged from 21.7 to 22.4 °C.

Analytical characterization of the test atmosphere

To characterize the test atmosphere and check the generation and dilution of reproducible HTP aerosols and CS, we measured the particles size distribution (PSD) of HTP aerosols and CS produced at different times: before the exposure, during the exposure, and at the end of the exposure. To do this, the exposure port was connected with a TPI aerodynamic particle size spectrometer 3,321 (St. Paul, USA), and the particle size was measured. Determination of nicotine, acetaldehyde, acrolein and formaldehyde in HTP aerosols and CS by High-performance liquid chromatography.

Epididymis sperm morphology assay and assessment

After exposure, rats were anaesthetized with ketamine (24 mg/kg) and the testes were removed by dissection and washed using a saline solution. The epididymis of the rats was dissected and sperm cells were collected into 10 mL of 0.87% warmed normal saline. Cauda epididymal sperm suspensions were diluted 1:10 with 10% neutral buffered formalin in PBS. The sperm suspensions were diluted in 2% aqueous solution of eosin and sperm heads were stained, after which sperm morphology was evaluated. Two hundred spermatozoa per animal were evaluated by bright field microscopy (×40 magnification). Epididymal sperm cells were suspended with 1% Eosin Y stain (normal saline). The smear was prepared and the slides were allowed to air-dry and were coded for subsequent microscopic examination.

ROS, SOD, and MDA analyses in testes tissue

10% testicular tissue homogenate and single cell suspension were prepared using one testis. The supernatant of the testicular tissue homogenate was used to determine the MDA content and SOD activity, and the single cell suspension was used to determine the ROS contents. MDA content and SOD activity were determined by SOD and MDA assay kits (Jiancheng Bio, Nanjing, China), and the ROS contents were determined by an ROS detection kit (Beyotime Bio, Shanghai, China). The experimental process and measurement conditions were operated in strict accordance with the manufacturer's instructions.

Testosterone determination

At the end of the exposure, rats were anesthetized with pentobarbital, blood was collected from the abdominal aorta, the blood samples were centrifuged at 2,500 × g at 4 °C for 10 min and the resulting sera samples were stored in ice until they were used for testosterone determination. The resulting sera samples were analyzed to determine the concentration of testosterone using the Enzyme-Linked Immunosorbent Assay (ELISA) kit (Enzyme-linked Bio, Shanghai, China) based on enzyme-linked immunosorbent assay.

Table 1. Primer sequences used in q-RT PCR assay.

Genes	Primer sequence (5′–3′)	Size (bp)	Annealing temp (°C)
NLRP3 (NM_001191642.1)	F-TCCTGCAGAGCCTACAGTTG R-GTCCTGCTTCCACACCTACC	131	59
ASC (NM_172322.1)	F-CTGCAGATGGACCCCATAGAC R-ACAGCTCCAGACTCTTCCATA	152	58
Caspase-1 (NM_012762.3)	F-GGAGCTTCAGTCAGGTCCAT R-CTTGAGGGAACCACTCGGTC	139	59
GAPDH (NM_017008.4)	F-TCTCTGCTCCTCCCTGTTCT R-CCGATACGGCCAAATCCGTT	108	60

F forward, *R* reverse

Determination of inflammatory cytokines in testicular tissue

The levels of IL-6, TNF-α, IL-1β, and IL-10 in the testicular tissue were measured using ELISA kits (Enzyme-linked Bio, Shanghai, China). Samples, standards, and HRP-labeled detection antibodies were added to the micropores coated with corresponding antibodies in advance, incubated for 1 h at 37 °C and thoroughly washed 5 times. Color development used substrate TMB, as TMB would turn blue under the catalysis of peroxidase and yellow under the action of an acid. The color depth was measured and used to positively correlate the level of inflammatory factors in the sample. The absorbance was measured with a microplate reader (TECAN, Switzerland) at a wavelength of 450 nm, and the sample concentration was calculated.

Quantitative RT PCR analysis

RNA was isolated from testicular tissue using TRIzol reagent (Life Technologies, USA) according to the manufacturer's instructions. A total of 1 μg of RNA was converted into cDNA with oligoDT and random hexamer using PrimeScript RT Master Kit (Takara Bio, Japan) according to the manufacturer's instructions. Quantitative RT PCR (q-RT PCR) was performed in a 20 μL volume containing 10 μL of TB GreenTM Fast qPCR Mix (Takara Bio, Japan), 0.4 μL of each primer (10 μM), 2 μL template cDNA, and dH2O up to the final volume according to the manufacturer's instructions. Q-RT PCR was carried out on a LightCycler® 96 Real-Time system (ROCHE, Switzerland). Each sample was run in triplicates. The relative mRNA expression levels of selected genes in all groups were analyzed using the $2^{-\Delta\Delta CT}$ calculation method. The sequences of the primer pairs used for the q-RT PCR are listed in Table 1.

Histopathological examination of the testes

One testicle was fixed in the fixation solution, then subjected to histopathology, immunohistochemistry, and pyroptosis examinations. A portion of the testes excised from rats in each group was fixed in Bouin's fluid for 48 h and routinely processed for paraffin embedding. Sections of 5 μm thickness were taken serially with a Rotary Microtome (Leica, Germany), processed in alcohol-xylene series, and stained with hematoxylin and eosin (HE).

TUNEL

The assay was performed with the Fluorescein (FITC) Tunel Cell Pyroptosis Detection Kit (Servicebio, Wuhan, China) according to the manufacturer's instructions. Testis samples were treated as mentioned in the histopathology. Briefly, testicular tissue sections were pretreated with 20 μg/mL proteinase K for 15 min at 37 °C, washed with PBS, then stained by TUNEL reaction mixture (label and enzyme solutions) for 1 h at 37 °C. The FITC-labeled TUNEL-positive cells were imaged under a DMI 4,000 B fluorescent microscope (Leica, Germany). The cells with green fluorescence were defined as pyroptosis cells.

Immunohistochemistry

Paraffin sections of 5 μm thickness were incubated in an oven at 56 °C overnight. After deparaffinization and rehydration, sections were boiled in citrate buffer, pH 6, (Invitrogen, USA) to retrieve antigens. Sections then were incubated in H_2O_2 (Abcam, UK) to block endogenous peroxidase activity. The sections were incubated in block solution (Invitrogen, USA) to prevent nonspecific binding of antibody. The sections then were treated with rabbit Anti-Caspase 1 antibody (Merck Millipore, USA) diluted 1:1000 in antibody diluent reagent solution (Invitrogen, USA), rabbit Anti-ACS antibody (Merck Millipore, USA) diluted 1:1,000 in antibody diluent reagent solution (Invitrogen, USA), and rabbit Anti-NLRP3 antibody (Merck Millipore, USA) diluted 1:1,000 in antibody diluent reagent solution (Invitrogen, USA) for 1 h at room temperature. The sections were incubated for 10 min in biotinylated secondary antibody (Invitrogen, USA) produced against the primary antibody. Staining reactions were visualized using DAB (Invitrogen, USA), then counterstained with hematoxylin. Sections were examined on a microscope (Olympus, Japan).

Statistical analysis

SPSS ver. 24 software was used for statistical analysis and GraphPad Prism ver. 6 was used for graph design. Quantification results were statistically described in terms of mean and standard deviation (mean ± SD). The Kolmogorov-Smirnov test ($P > 0.05$) was used to evaluate the normality and homogeneity of data. LSD's post hoc descriptive test and one-way analysis of variance (ANOVA) analyzed significant differences between the groups. Differences with $P < 0.05$ were considered statistically significant.

Results

Test atmosphere composition

The concentrations of nicotine and aldehydes were determined in the test atmospheres (Table 2). Nicotine concentrations achieved for HTP_23 and Cig_23 were very similar, and at the same nicotine concentration, the concentrations of acrolein, formaldehyde and acetaldehyde in HTP aerosols were lower than that in CS. The PSD measurements indicated that particle sizes and distributions were similar between the exposure groups, and were therefore similarly deposited in the respiratory tract. Based on the PSD data, our results indicate that particles were compatible with inhalation, and equally respirable between all exposure groups.

Table 2. Characterization of test atmospheres.

Chamber	Particle size distribution		Nicotine (μg/L)	Acetaldehyde (μg/L)	Acrolein (μg/L)	Formaldehyde (μg/L)
	MMAD (μm)	GSD				
Sham	NM	NM	NM	NM	NM	NM
HTP _10	0.76 ± 0.15	1.86 ± 0.31	10.64 ± 0.98	2.81 ± 0.56	0.15 ± 0.03	0.11 ± 0.02
HTP _23	0.80 ± 0.12	2.01 ± 0.40	23.62 ± 2.09	4.19 ± 0.71	0.22 ± 0.09	0.13 ± 0.05
HTP _50	0.86 ± 0.08	1.85 ± 0.23	48.73 ± 3.65	7.61 ± 1.07	0.35 ± 0.06	0.21 ± 0.05
Cig_23	1.02 ± 0.19	2.09 ± 0.24	24.54 ± 2.28	16.47 ± 1.82	1.23 ± 0.17	0.46 ± 0.11

Data represent mean ± SD, N = 5; MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation; NM, not measured.

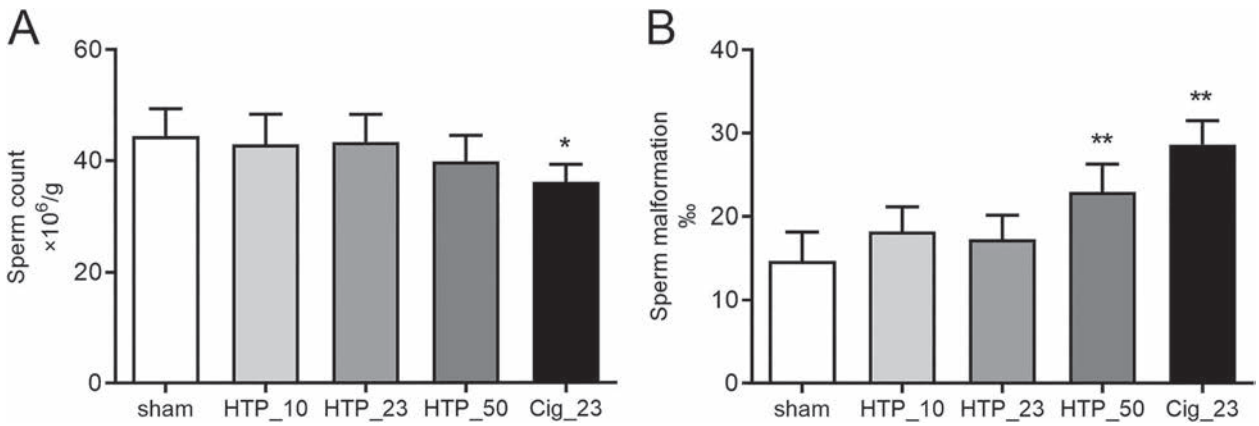

Fig. 1. Effects of HTP aerosols and CS on sperm quality. A) Changes in sperm number (n = 10 per group). Two hundred spermatozoa per animal were evaluated by bright field microscopy (×40 objective). B) Changes in sperm malformation rate (n = 10 per group). One thousand sperm cells were assessed for morphological abnormalities for each rat. Data were described as mean ± SD. *indicates $P < 0.05$ compared with sham group; **indicates $P < 0.01$ compared with sham group in the bar graph.

Changes in sperm quality

Sperm quality is the most important index for measuring male reproductive ability and any damage from environmental exposure. Cig_23 significantly reduced the number of sperm in the epididymis of rats and significantly increased the rate of sperm deformity. The sperm deformity rate of HTP_50 group was significantly higher than that of the sham group, however, the Cig_23 group had a more significant effect on sperm quality. The results demonstrated that HTP_50 and Cig_23 damaged the epididymal sperm of male rats, resulting in a decrease in sperm quantity and vitality, an increase in the deformity rate, and a decrease in sperm quality (Fig. 1).

Effect on the ROS, SOD, and MDA levels in rat testes

Examination of the biochemical changes, including oxidative and antioxidative markers, is of crucial importance when studying early signs of tissue aging since oxidative stress plays an important role in the damage of exogenous substances to the body. At the end of the exposure, detecting oxidative stress indexes in rat testicular tissue demonstrated that HTP aerosols and CS increased the contents of ROS and MDA in testicular tissue to varying degrees, decreased SOD activity, and caused oxidative damage in testicular tissue. The effects of HTP_50 and Cig_23 were statistically significant compared with the lower-dose HTP groups, and there was no significant dose-effect trend in the other groups. The results demonstrated that HTP_50 and Cig_23 caused oxidative damages to rat testis and affected the normal physiological function of testis (Fig. 2).

Changes of testosterone in rat serum

Compared with the sham group, the testosterone contents in the HTP_50 and Cig_23 groups decreased significantly, while there was no significant difference between the other groups and the sham group, and there was also no obvious dose-effect trend. The results demonstrated that HTP_50 and Cig_23 damaged the reproductive function of rats, affected testosterone synthesis, and reduced the content of serum testosterone (Fig. 3).

Effect on the level of inflammatory cytokines in rat testis

HTP aerosols and CS increased the contents of proinflammatory factors IL-1β, IL-6, and TNF-α in rat testes to varying degrees. Additionally, the content of anti-inflammatory factor IL-10 decreased; this change was more significant in the HTP_50 and Cig_23 groups. However, there was no significant dose-effect relationship between the HTP groups. These results suggested that HTP_50 and Cig_23 led to inflammatory reactions in rat testes, and Cig_23 was more significant than HTP_50 (Fig. 4).

Expression of NLRP3 inflammasome-related mRNA in rat testes

HTP aerosols and CS exposure increased the mRNA expression of NLRP3, ASC, and caspase-1 in rat testes. The expression levels of NLRP3 in the HTP groups were significantly higher than in the sham group; the expression of ASC was higher than in the sham group, but there was no significant difference. The expression of Caspase-1 was significantly higher than in the sham group, however, there was no obvious dose-effect trend. The mRNA expressions of NLRP3, ASC, and Caspase-1 in the exposure groups were significantly higher than in the sham group. This

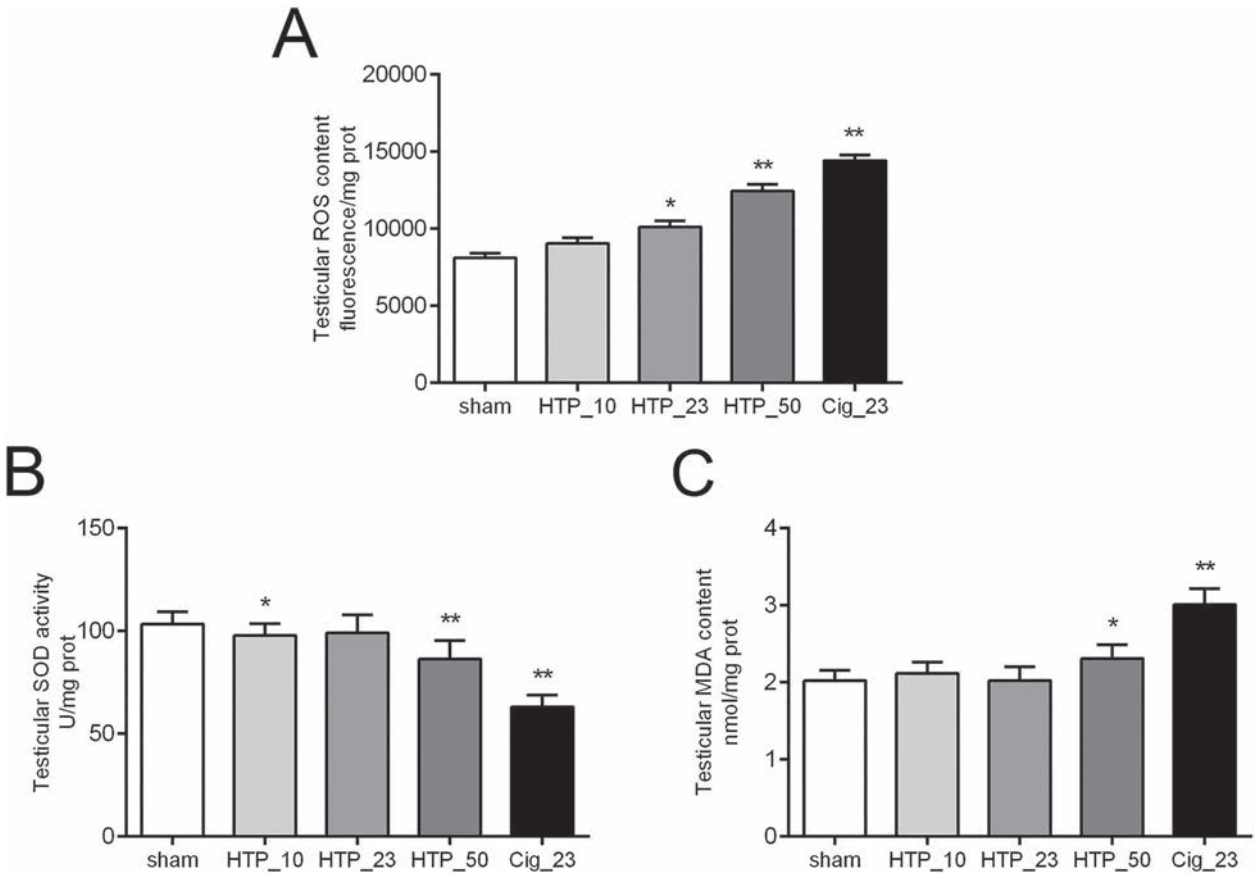

Fig. 2. Effects of HTP aerosols and CS on oxidative stress. A) Changes in ROS content (n = 10 per group). B) Changes in SOD activity (n = 10 per group). C) Changes in MDA content (n = 10 per group). Data were described as means ± SD. * indicates $P < 0.05$ compared with sham group; **indicates $P < 0.01$ compared with sham group in the bar graph.

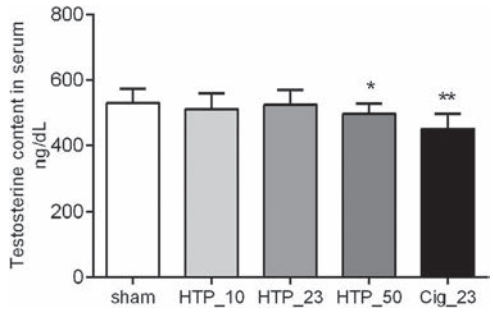

Fig. 3. Testosterone content in serum. Data were described as means ± SD. *indicates $P < 0.05$ compared with sham group; **indicates $P < 0.01$ compared with sham group in the bar graph (n = 10 per group).

demonstrated that HTP and CS exposure activated the expression of NLRP3 in rat testes. The activated NLRP3 inflammasome catalyzed the cleavage of the Caspase-1 protein precursor to form active caspase-1. Active caspase-1 induces inflammatory cytokine IL-1 β. Additionally, the maturation and release of IL-18 triggered an inflammatory cascade (Fig. 5).

Expression of NLRP3 inflammasome-related proteins in rat testes

Only a few NLRP3 immunopositive germ cells were observed in the seminiferous tubules in the testis of the sham and HTP_10 groups. The number of NLRP3 immunopositive cells in the seminiferous tubules of the HTP_23, HTP_50, and Cig_23 groups was significantly greater than in the sham group, especially in the Sertoli cells. ASC immunoreactivity was observed in a few Sertoli cells in the testes of the sham, HTP_10, HTP_23, and HTP_50 groups. The number of ASC immunopositive cells significantly increased in the Cig_23 group compared to the sham group. Caspase-1 immunoreactivity was observed in a few plasmalemmas and Sertoli cells in the testes of the sham group. The number of Caspase-1 immunopositive cells significantly increased in the HTP_10, HTP_23, HTP_50, and Cig_23 groups compared to the sham group, especially in the Sertoli cells (Fig. 6).

Pyroptosis in rat testis

We detected TUNEL reactions in only a few seminiferous tubules of the sham and HTP_10 groups sections and most of these cells were spermatogonia. However, in the HTP_23, HTP_50, and Cig_23 groups many TUNEL-positive cells could be observed among the spermatogenic cell series, which were significantly increased compared with the sham group (Fig. 7). Therefore, the evidence suggested that both HTP aerosols and CS caused pyroptosis in rat testicular tissue.

Histopathological examination of rat testes

The testes of rats in the sham, HTP_10, and HTP_23 groups were covered by a well-defined capsule formed of tunica albuginea and tunica vasculosa (Fig. 8). The testicular parenchyma was composed of several seminiferous tubules with narrow interstitial

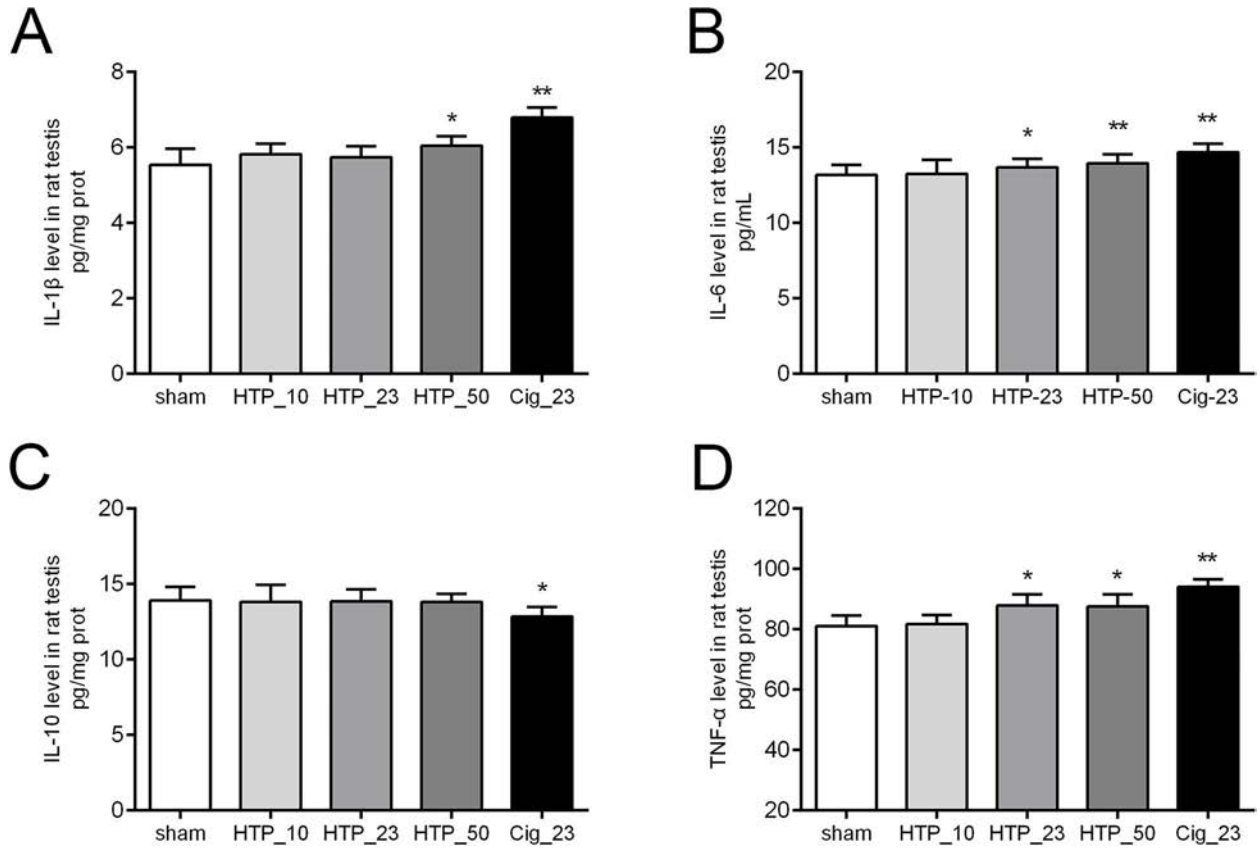

Fig. 4. HTP aerosols and CS increase testis pro-inflammatory cytokines and decrease anti-inflammatory cytokines. The IL-1β, IL-6, IL-10, and TNF-α in the HTP, Cig, and sham groups are shown in the figures. A) Regarding the IL-1β levels (n = 10 per group). B) Regarding the IL-6 levels (n = 10 per group). C) Regarding the IL-10 levels (n = 10 per group). D) Regarding the TNF-α levels (n = 10 per group). Data were described as means ± SD. *indicates $P < 0.05$ compared with sham group; **indicates $P < 0.01$ compared with sham group in the bar graph.

spaces containing loose areolar tissue in between. The seminiferous tubules were lined by stratified epithelium with two types of cells: the supporting Sertoli cells, and the spermatogenic cells. The spermatogenic cells included spermatogonia, spermatocytes, round early spermatids, and elongated late spermatids, arranged in that order from the basal compartment to the adluminal compartment of the seminiferous tubule (Fig. 8A–C). In the HTP_50 group and Cig_23 group, testicular tissue was slightly atrophied, spermatogenesis of a few seminiferous tubules decreased, interstitial edema was slight, slight structural changes were produced, a small number of spermatogenic cells remained in the atrophied seminiferous tubules, and the lumen increased (Fig. 8D and E).

Discussion

Despite well-known health hazards associated with tobacco use, cigarette smoking is still highly prevalent in many parts of the world. Globally, approximately 36% of current smokers are men,[24] and epidemiological investigations have established a clear association between cigarette smoking and the impairment of male reproductive function.[25] For example, previous studies report that heavy smokers (>20 cigarettes per day) had 29% fewer sperm and 13% fewer motile sperm than non-smoking men. Attention has recently focused on the damage cigarette smoking can cause to testicular germ cells.[26] Despite the claims of reduced harmful effects of HTPs compared to cigarette smoking, their effects on the reproductive system are not well-documented. Based on a series of experimental investigations on oxidative stress, biochemical, molecular, histomorphometric, pyroptosis, and immunohistochemical responses, this study demonstrated the systemic toxic effects of HTP aerosols and CS on rat testes following the OECD 90 day exposure for the first time. Our results indicate that HTP aerosols and CS induced oxidative stress responses in rat testes and activated NLRP3 inflammatory bodies, leading to the occurrence and development of orchitis, and destroying normal tissue structure and function.

Oxidative stresses were found to be involved in the general inflammatory response that occurs because of metabolic imbalance caused by ROS and/or a decreased action of host antioxidant defense mechanisms.[27] During inflammation, oxygen is utilized at a high rate, releasing mitochondrial superoxide free radicals, which impairs mitochondrial function and leads to cell and organ dysfunction. CS contains large quantities of ROS and RNS,[28] which have been found to induce oxidative and nitrosative stress, and inflammation in both humans and animals.[29] In the present study, HTP aerosol and CS exposure increased oxidative stress in the testes, as evidenced by an increase in ROS generation and MDA levels (Fig. 2). At the same time, this exposure also led to a decrease in SOD activity. ROS levels among the testes of rats in the HTP_10 and HTP_23 groups had a certain dose-effect trend, while there was no obvious dose-effect relationship between SOD and MDA, and the effects of the HTP_50 and Cig_23 were significant compared to the other HTP groups. The oxidative stress was behind the inflammatory process in the testis of rats exposed to the HTP_50 and Cig_23 groups. This result supports the hypothesis that tobacco smoke and nicotine can cause oxidative stress.[30]

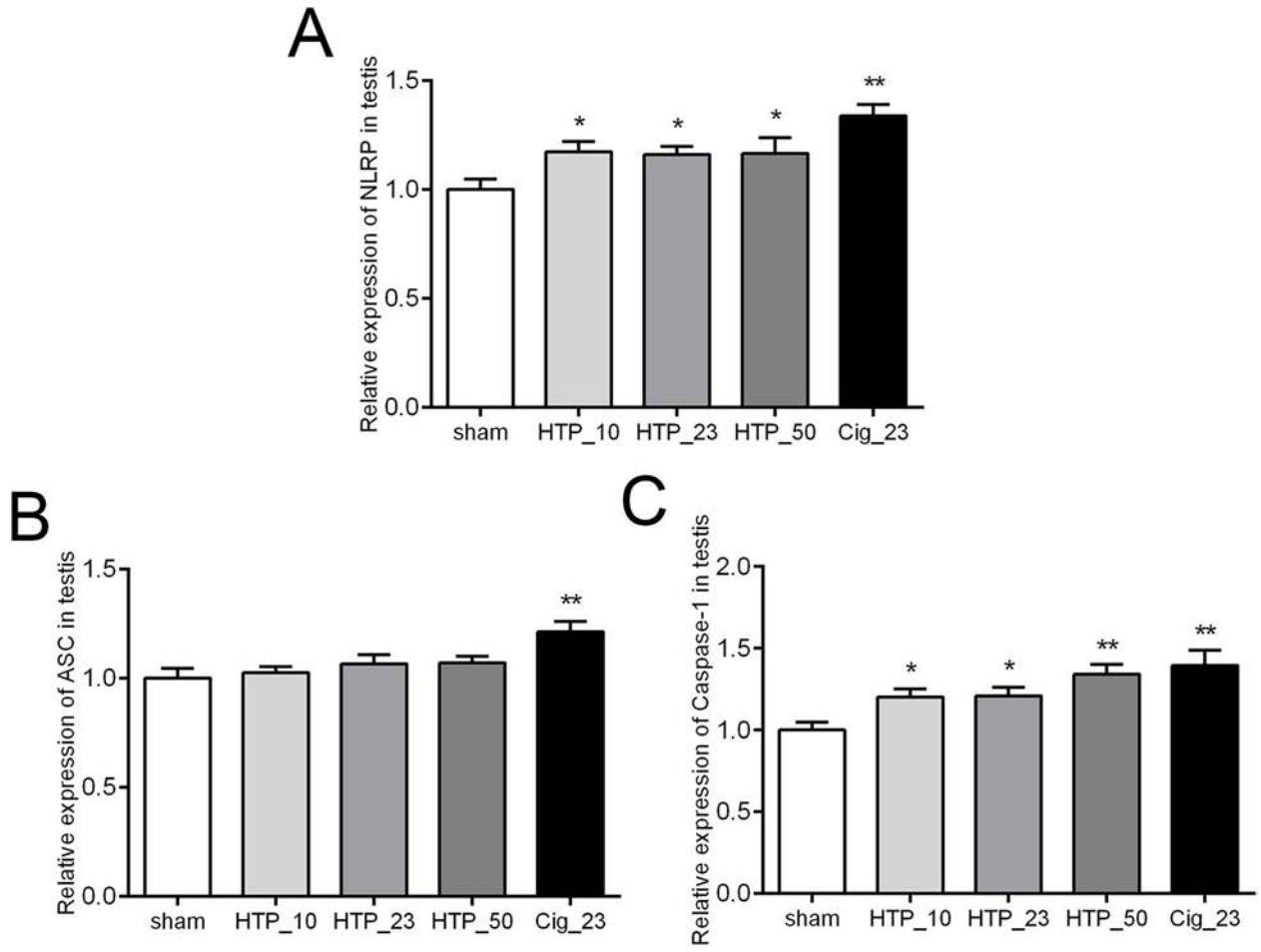

Fig. 5. HTP aerosols and CS activate the expression of NLRP3 inflammasome-related mRNA in rat testes. A) Relative expression of NLRP3 in testes (n = 10 per group). B) Relative expression of ASC in testes (n = 10 per group). C) Relative expression of Caspase-1 in testes (n = 10 per group). Data were described as means ± SD. *indicates $P < 0.05$ compared with sham group; ** indicates $P < 0.01$ compared with sham group in the bar graph.

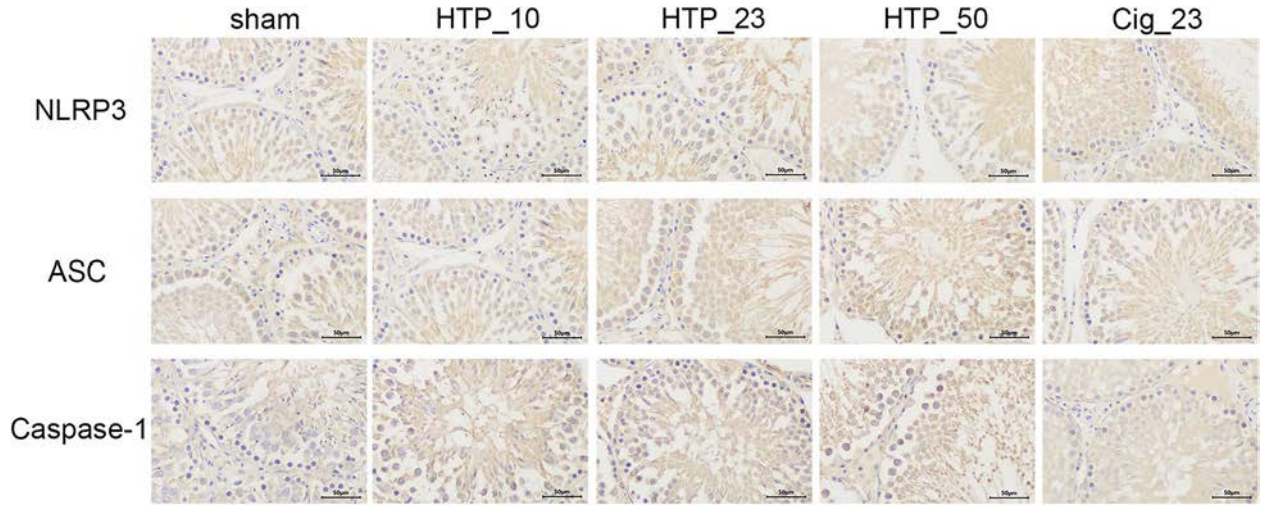

Fig. 6. Immunohistochemical staining results of rat testis (×200). Inhalation to filtered air (sham) and HTP_10, HTP_23, HTP_50, and Cig_23 (n = 10 per group).

Within the testes, numerous factors, including tumor necrosis factors and interleukins, coordinate immune cell function and promote sperm production. In certain pathologic conditions, an increase in pro-inflammatory cytokines shifts the balance in favor of immune and inflammatory responses.[31] Immune cells are either recruited to the testis or activated in the testis germ cell, which can result in apoptosis, and spermatogenesis is affected.[32] In this work, after exposure to HTP aerosols and CS, the contents of the pro-inflammatory factors IL-1, IL-6, and TNF in rat testes increased to varying degrees, while the content of

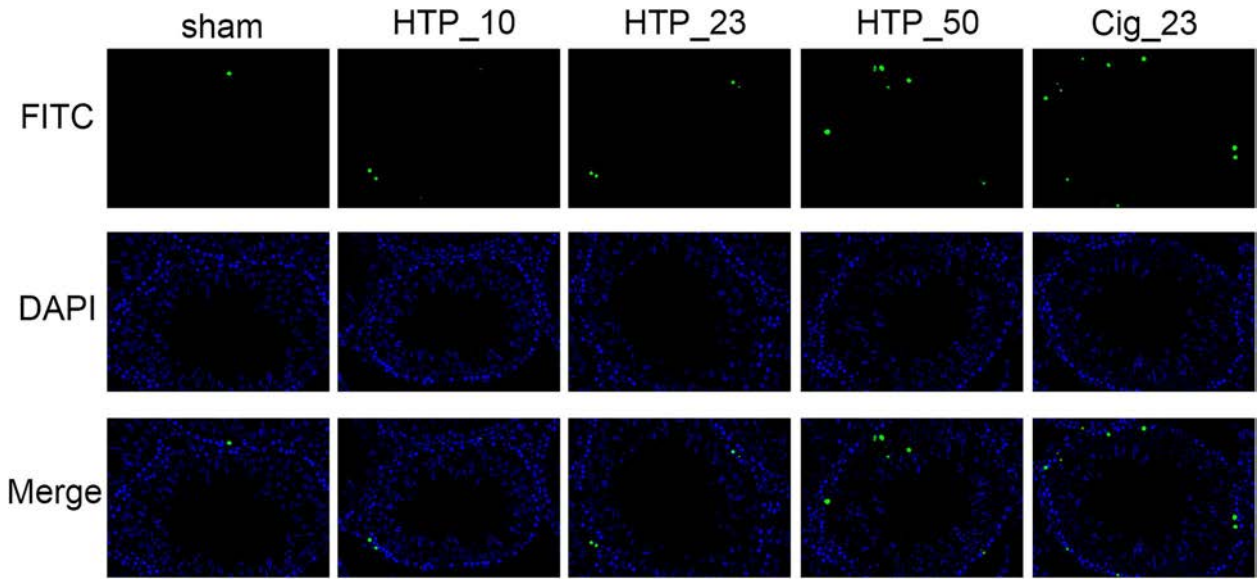

Fig. 7. TUNEL staining results of rat testes (×400). Inhalation to filtered air (sham) and HTP_10, HTP_23, HTP_50, and Cig_23 (n = 10 per group).

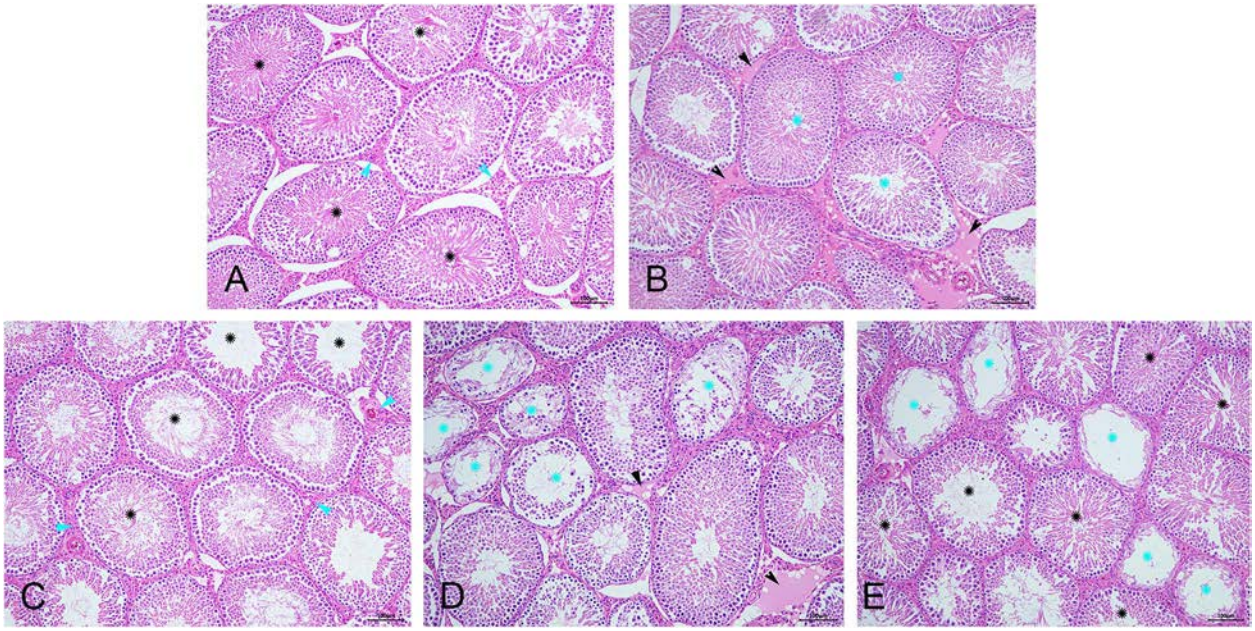

Fig. 8. Changes in testes histology (×100). A) Testicular histopathology of sham (n = 10 per group). B) Testicular histopathology of HTP_10 (n = 10 per group). C) Testicular histopathology of HTP_23 (n = 10 per group). D) Testicular histopathology of HTP_50 (n = 10 per group). E) Testicular histopathology of Cig_23 (n = 10 per group).

anti-inflammatory factor IL-10 decreased. There was no significant dose effect trend in the HTP groups, but this change was more significant in the HTP_50 and Cig_23 groups. These results indicated that exposure to HTP aerosols and CS leads to the inflammation in rat testes through oxidative stress.

Previous studies confirm that Sertoli cells play a key role in controlling germ cell development and spermatogenesis, and damage to these somatic cells can severely impair spermatogenesis.[33] Some studies reported increased levels of total testosterone and androstenedione in smokers, which suggest that androgen secretion by Leydig cells is affected.[34] Our results suggested that the sperm quality of HTP groups had a significant dose-response trend with the increase of HTP aerosol concentration, and the HTP_10 and HTP_23 groups had no significant effect on rat sperm quality in addition to that observed in the sham, while HTP_50 and Cig_23 exposure could damage the epididymal sperm of male rats, resulting in a decrease of sperm quantity and vitality, an increase in the deformity rate, and a decrease in sperm quality. Therefore, HTP aerosols at high doses and CS should be considered hazardous to male reproductive health based on the animal model used here. Ninty days of HTP aerosol and CS exposure clearly impaired sperm quality, as observed by suppression of sperm production and count reduction followed by motility and morphological abnormalities in the sperm of rats. This functional impairment appeared together with the observed redox imbalance, i.e. increased ROS production, lipid peroxidation, and altered antioxidant capacity. In addition, exposure to HTP_50 and Cig_23 led to a significant decrease in serum testosterone levels,

which means that HTP aerosols and CS affected the structure and function of the testes of rats, which is consistent with the understanding that testicular oxidative stress will damage testicular structure and BTB, damage spermatogenesis, and affect testosterone synthesis.

There are many inflammasome family members,[35] however, NLRP3 inflammasome is the best characterized. The mechanisms driving the activation of NLRP3 include NLRP3 oligomerization, ASC recruitment, and caspase-1 activation. The canonical pathway is based on the recognition of general cellular stress, such as oxidative stress induced by I/R, bacterial toxins, and particulate substances.[36] Recent studies have demonstrated that NLRP3 could sense the presence of ROS produced in the same cell by normal or malfunctioning mitochondria.[37] In particular, following an increase in cellular ROS concentration, thioredoxin-interacting protein-thioredoxin (TXNIP-TRX) dissociates and TXNIP binds to the LRR region of NLRP3, leading to NLRP3 activation.[38] Activated NLRP3 binds to a caspase recruitment domain (CARD) and a pyrin domain. In this way, inflammasomes can activate inflammatory caspases.[39] The representative member, caspase-1, stimulates the secretion of cytokines such as IL-1β and IL-18,[40] which are crucial mediators of the inflammatory response. These cytokines start or amplify different downstream signaling pathways and drive pro-inflammatory processes,[41] leading to cell damage. Studies have demonstrated that CS stimulates ROS production and activates the NLRP3 inflammasome and ultimately leads to pyroptosis of the bladder urothelial cells.[42]

Our laboratory has recently demonstrated that both HTP aerosols and CS exposure increased the mRNA expression of NLRP3, ASC, and caspase-1 in rat testes, but there was no significant dose effect trend in the HTP groups. NLRP3, ASC, and caspase-1 immunopositive cell counts increased in the HTP_50 and Cig_23 groups compared to those of the sham group. This demonstrated that HTP and CS exposure activated the expression of NLRP3 inflammasome in rat testes. The activated NLRP3 inflammasome could further catalyze the cleavage of the Caspase-1 protein precursor to form active caspase-1. Active caspase-1 is known to induce the maturation and release of inflammatory cytokines IL-1β and IL-18, triggering an inflammatory cascade, which seemed to agree with the observations of this study (Fig. 4).

Activated NLRP3 inflammasome can cause an inflammatory form of cell death called pyroptosis.[43,44] Pyroptosis is programmed lytic cell death characterized by rapid plasma membrane rupture, DNA damage, and the release of proinflammatory intracellular contents, where caspase-1 specifically cleaves the linker between the amino-terminal gasdermin-N and carboxy-terminal gasdermin-C domains in GSDMD, leading to rapid cell lysis.[45] Our results demonstrated a significant increase in testicular pyroptosis following HTP_50 and Cig_23 exposure (Fig. 7), and there was no significant change in other groups. These results support the hypothesis that apoptosis could underlie testicular defects in rat testes following chronic exposure to CS.[46] Specifically, we identified an association between extensive CS inhalation and a decrease in the number of generative cells.

Recent research found that testicular atrophy and associated histopathological lesions were associated with the administration of several drugs, including cyclophosphamide, methotrexate, and various industrial chemicals.[47] CS itself contains more than 5,000 chemical constituents, including nicotine, tar, carbon monoxide, polycyclic aromatic hydrocarbons, several radioactive substances, and heavy metals, and most of these chemicals are recognized as carcinogens or mutagens.[48] Significant morphological damages to the testicular germ cells of rats were evident in animals exposed to the HTP_50 and Cig_23 over a 90 day period. This phenotype was especially exacerbated in the Cig_23 group, where spermatogenetic cells were completely lost and seminiferous tubules were no longer arranged in a network structure, and there was no significant change in other groups. Therefore, inhalation of Cig_23 and HTP_50 can seriously damage testes architecture and spermatogenesis in the experimental animals, which is consistent with previous experimental results. These animal results highlight that more research is needed to fully characterize the health risks associated with HTP usage, since they can adversely affect the body's reproductive system.

By analyzing the above results, we found that Cig_23 and HTP_50 had a significant impact on the testicular tissue of rats, and Cig_23 was more significant compared to HTP_50. While HTP_23, which had the same nicotine content as Cig_23, did not show a similar effect. Combined with our detection results of HTP aerosols and CS chemical composition content, we found that Cig_23 had a much higher content of aldehydes than other groups. Studies have shown that, exposure to formaldehyde vapor can destroy testicular structure and decrease percentages of concentration, viability, normal morphology, and progressive motility, in addition to increasing the percentage of immotile sperm.[49] Maternal exposure to high doses of acrolein inhibited fetal testosterone synthesis.[50] Acrolein can impairs the cytoskeleton of Sertoli cells which is caused by induction of the oxidative stress response through up-regulation of ERK and p38MAPK expression.[51] Acetaldehyde is the main metabolite of ethanol, and studies have observed lower testosterone concentrations in alcoholics, chronic alcohol intake can increase serum prolactin, causing hypogonadism, reduced sperm production, and impotence.[52] In rats, decreased sperm motility was observed after exposure to ethanol, as well as changes in the meiotic divisions, reduced gametes viability, and a higher rate of sperm with poorly condensed chromatin.[53] However, a wide range of carcinogens and mutagens, including radioactive polonium, benzopyrene, dimethylbenzanthracene, naphthalene methylnaphthalene, polycystic aromatic hydrocarbons, and heavy metals such as cadmium, also have been found in CS.[48] Some of these chemicals have been proven to be extremely detrimental to male fertility, while some have not been studied yet. This indicates that the observed results may not be influenced by a single nicotine, and other components in the smoke also play a role in this process. Therefore, this provides new goals for our subsequent research.

Conclusion

Following the OECD 90 day subchronic inhalation toxicological protocol, this work focused on comparing the effects of HTP aerosol and CS exposure on the reproductive health of rats. The results demonstrated that higher equivalent nicotine dosages of HTPs (HTP_50) and cigarette smoke (Cig_23) could induce observable oxidative stress in rat testes, inhibited the production of testosterone by rat Leydig cells, induced inflammation and pyroptosis through the ROS/NLRP3/Caspase-1 pathway, and destroyed the integrity of testicular tissue structure. On the other hand, HTP aerosol exposure at lower nicotine-equivalent dosages exhibited reduced adverse effects, and some responses were statistically indistinguishable from the sham exposure.

Author contributions

Hongjuan Wang and Huan Chen conceived of the study. Yushan Tian, Shuhao Ma, Wenming Wang, Xiaoxiao Xu, Xianmei Li, Fengjun Lu and Pengxia Feng performed data collection, data

analysis, and produced the figured and scripts, with overall guidance from Hongwei Hou and Qingyuan Hu. Yushan Tian, Shuhao Ma, Wenming Wang, Xiaoxiao Xu, Xianmei Li and Fengjun Lu wrote the manuscript. Hongjuan Wang and Huan Chen directed the writing and revision of the manuscript. Yaning Fu, Shulei Han and Chuan Liu deposited the data. All the authors approved the final version of the manuscript prior to submission.

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Availability of data and materials

All the data are contained in the manuscript.

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