Recombination homeostasis of meiosis during spermatogenesis under nicotine treatment

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Abstract: Cigarette smoking can affect male fertility via the quality of semen. To explore the effects of nicotine, a major component of cigarettes, on meiotic recombination during spermatogenesis, C57BL/6J male mice were injected with nicotine at a dosage of 0.2 mg/100 g body weight daily for 35 days (nicotine-treated group); mice in the control group were injected with isopycnic normal saline. According to previous expression profiles of mouse sperm, a subset of meiosis-related genes was pooled using bioinformatic analysis. Protein expression was compared between the two groups using by Western blotting and immunohistochemistry. Recombination frequency during the meiosis phase of spermatogenesis was estimated by combined use of chromosome spread and immunofluorescence staining in mouse testes. Data mining analysis indicated that 4 genes that express meiotic topoisomerase-like protein SPO11, MutS protein homolog 4 (MSH4), strand exchange protein RAD51 and MutL protein homologue 1 (MLH1), were associated with the meiosis recombination process. The results of Western blotting and immunohistochemistry further showed that the protein expression of SPO11 (0.73-fold) and MSH4 (0.73-fold) was downregulated in murine testes after nicotine treatment, whereas the protein expression of both RAD51 (2.06-fold) and MLH1 (1.40-fold) was upregulated. Unexpectedly, we did not detect a significant difference in recombination frequency in meiosis during spermatogenesis in the nicotine-treated group as compared to the control. Taken together, these results indicate that nicotine can affect the expression profile of restructuring-related genes, but it does not significantly change the recombination frequency during male meiosis. These findings suggest there is a self-regulating mechanism during meiotic chromosome restructuring in male mice that responds to environmental stress.

Key words: recombination; nicotine; meiosis; homeostasis; spermatogenesis

INTRODUCTION

According to the World Health Organization (WHO), there are more than one billion smokers in the world as of 2016, with more than six million people dying because of smoking every year [1]. Nicotine is one of the main harmful ingredients in cigarettes and is also the compound that causes smokers' addiction to cigarettes [2]. It has been previously established that nicotine acts on the acetylcholine receptor to stimulate the sympathetic and central nervous systems, which gives rise to a feeling of excitation and which damages the digestive and cardiovascular systems [3]. Nicotine not only leads to hypertension and apoplexy, but also affects reproduction, especially male reproduction. Much evidence has confirmed that nicotine can lead to lower sperm quality, including decline in sperm concentration, sperm motility and normal morphology [4-7]. Moreover, nicotine can induce dysfunction in sperm production in the testes, leading to reduced survival rates and birth number of offspring [8]. These studies suggest that the impact of nicotine may affect not only active and passive smokers but also future generations.

Although the effects of nicotine on male fertility have been investigated, the molecular mechanisms that underlie its actions have not been fully elucidated. It was reported that nicotine can cause DNA damage

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in sperm, resulting in abnormal sperm chromosome structure and aneuploidy of chromosomes [9]. Nicotine can induce apoptosis in murine testes by upregulating the expression of nucleoside diphosphate kinase 2 (Nme2) by promoter hypomethylation [1]. In addition, nicotine can induce murine spermatozoal apoptosis via the tumor necrosis factor (TNF) apoptotic pathway through upregulation of the deubiquitinated cobalt ion-binding protein (RIP1) by tripartite motif containing 27 (Trim27) promoter hypomethylation [10].

Meiotic recombination is a key step in the process of spermatogenesis. Meiotic arrest and reduced recombination frequency are considered to be underlying causes of infertility [11]. The frequency of meiotic recombination has been measured in response to changes in several extrinsic environmental conditions, such as temperature, chemicals, drought and nutrients [12-17]. We therefore asked what effect nicotine has on meiotic recombination. Transcriptome-associated recombination has provided evidence for alternative splicing after nicotine exposure [18]. We thus explored the potential effects of nicotine on homologous recombination and the molecular mechanisms in male meiotic recombination. We investigated the changes in expression of target recombination-related proteins and the meiotic recombination frequency of murine male reproductive cells under nicotine exposure.

MATERIALS AND METHODS

Experimental animals and nicotine treatments

Experimental mice were raised in an environment that was in strict accordance with the “Guide for the Care and Use of Laboratory Animals”. The use of these experimental animals was approved by the “Bioethics Committee of the School of Life Science and Biotechnology, Shanghai Jiao Tong University (Approval No. 2011-018)”.

To simulate nicotine intoxication in heavy smokers, forty 5-week-old male C57BL/6J mice (Shanghai SLAC laboratory animal Co. Ltd) were randomly divided into two groups. One of these constituted the nicotine-treated (NT) group, and the other group was a control (CT) group. Mice in the NT group were injected intraperitoneally with nicotine at a dosage of 0.2 mg/100 g body weight (BW) daily (the body weight of each mice was approximately 20 g) to simulate the blood plasma level of nicotine in a heavy smoker [19]. For mice in CT group, this nicotine was replaced with isopycnic normal saline. Mice in both groups were treated for 35 days, which constituted a whole sperm production cycle.

Sample preparation

After the nicotine or saline treatment for 35 days, mice were killed by cervical dislocation. After phosphate buffer saline (PBS) perfusion, mice testes and spermatozoa samples were collected. To prepare the testes sample for paraffin sectioning, the mice were anesthetized by intraperitoneal injection with 300 μL 1% pentobarbital sodium. Then, 10 mL normal saline and 10 mL 4% paraformaldehyde (PFA) were used to perfuse the heart. To dehydrate, the fixed testes were steeped in 70%, 80%, 90%, and 100% ethanol in sequence for 2 h each, and then in xylene 3 times for 2 h each. After dehydration, mice testes were embedded in paraffin. The 5-μm testis paraffin sections were cut and fixed onto poly-L--lysine-coated slides.

To spread the chromosomes of the seminiferous tubule, the tubule was removed from the testes and hypotonically treated in a hypotonic extraction buffer (HEB) for 0.5 h and then torn repeatedly in 0.1 M isotonic sucrose solution to release germ cells. After the cell suspension was prepared, a drop of PFA solution was added to the pretreated slides, and the isotonic cell solution was added to make the cells spread evenly on the slides. The slides were kept in a humid chamber overnight to firmly attach the cells and chromosomes to the slides.

Determination of expression profiles: reverse transcript PCR and Western blotting

To explore whether nicotine can change the sperm expression profile in terms of genes related to meiotic recombination, sperm before and after nicotine treatment were collected to obtain reads per kilo-base per million (RPKM) of the two groups by next-generation sequencing. The Public Library of Bioinformatics was then used to generate a heat map of the selected recombination-related transcriptome to help visualize

the differences in gene expression in mouse sperm between the two groups.

Reverse transcript (RT) PCR

RT-PCR was performed to validate the level of expression of these transcripts. The RNA extract prepared using TRIzol reagent was reverse transcribed into cDNA with RevertAid™ Reverse Transcriptase (Fermentas, Canada). The cDNA served as the template for PCR amplification. In this article, the primers used for RT-PCR are as follows:

Spo11-F: 5′-TACTGCTGTGCCGACTAACA-3′,

Spo11-R: 5′-GTAGGGATCTGCATCGACCA-3′,

Rad51-F: 5′-ACCAGACCCAGCTCCTTTAC-3′,

Rad51-R: 5′-CAAGTCGAAGCAGCATCCTC-3′,

Msh4-F: 5′-TCTGTGCTAATGGAGGTGCA-3′,

Msh4-R: 5′-TCCCGCAGTCTTGGTGTAAT-3′,

Mlh1-F: 5′-ATTCTTCGACTGGCCACTGA-3′,

Mlh1-R: 5′-AATGCTTCGGAGGTAGGAGG-3′,

Gapdh-F: 5′-CTGCGACTTCAACAGCAACT-3′,

Gapdh-R: 5′-GAGTTGGGATAGGGCCTCTC-3′.

Western blotting

The protein samples obtained from mice testes were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membranes (bore diameter: 0.45 μm). The antibodies used for incubation were as follows: anti-SPO11 antibody ab81695 (Abcam, UK), anti-RAD51 antibody Cat. # 07-1782 (Millipore, USA), anti-MSH4 antibody Cat. #bs-17851R (Bioss, China), anti-MLH1 antibody Cat. #11697-1-AP (Proteintech, USA), and GAPDH Rabbit mAb (Cell Signaling Technology, USA). After sufficient rinsing with 100 mM Tris-buffered saline, pH 7.4, 0.1% Tween-20 (TBST), the membranes were incubated with corresponding secondary antibody HRP-labeled goat anti-rabbit IgG (H+L) at 37°C for 1 h. Millipore Immobile Western Chemiluminescent horseradish peroxidase (HRP) substrate was used to detect HRP, and the final blot was exposed to X-ray film in the darkroom. In each group, three parallel tests were performed. The expression levels of target genes were normalized against the internal control GAPDH.

Immunohistochemistry and image processing

To show the localization of the expression of target proteins, we performed immunohistochemistry experiments on the testis paraffin sections. The testis paraffin section was immersed in xylene three times for 5 min each to dewax, and then immersed in 100, 95, 85 and 75% ethanol in sequence for 5 min each to hydrate. Next, the testis paraffin section was treated by 5% hydrogen peroxide for 10 min in a wet box, heated in antigen retrieval buffer (10 mM sodium citrate, pH 6.0) for 0.5 h, cooled down to room temperature, and blocked with sealing fluid at 4°C overnight. Then, we treated the section with primary antibody for 1 h. After the primary antibody was removed by washing with PBS, the goat anti-rabbit IgG was used to bind the primary antibody for 1 h at room temperature. Diaminobenzidine (DAB) was used to dye the target proteins marked by the second antibody. Hematoxylin was used to visualize the location of target proteins in brown. Photos were taken under fluorescence microscopy. For image processing, Adobe Photoshop CS6 was used to adjust brightness and contrast and to cut the picture obtained by immunohistochemistry (IHC) of the seminiferous tubule into 5 sections. Image-Pro Plus 6.0 was used for semiquantitative analysis to measure the positive signal of target proteins in different kinds of cells.

Immunofluorescence staining and image processing

To show the chromosome synapsis and recombination of primary spermatocytes, we performed immunofluorescence staining experiments on chromosomes that were spread on slides. The chromosome spread slides were treated by antibody dilution buffer (1*ADB) for 1 h. Three kinds of antibody were used to dye the cells. SYCP3 antibody was used to recognize the synaptonemal complex [20]. MLH1 antibody was used to detect the recombination repair sites [21]. CREST antibodies

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were used to mark the centromere. Three different fluorescently labeled secondary antibodies were used in combination with the primary antibodies. SYCP3 was dyed red, MLH1 was dyed green and CREST was dyed blue. Photos were taken under a fluorescence microscope. For image processing, Adobe Photoshop CS6 was used to adjust brightness and contrast, and to merge three different fluorescent photographs. We added random number tags to the synaptonemal complex, measured the length of chromosomes with ImageJ 1.48v, and at the same time recorded the number of corresponding recombination sites. After the measurement was completed, we sorted every synaptonemal complex in accordance to length, then renumbered XY and the 1-20 synaptonemal complex.

Statistical analyses

SPSS version 18 was used to analyze the data obtained from the experimental and control groups. The variance Lever test and an independent samples t-test equation were used to analyze the differences in CT and NT groups. The data are presented as the means±SD.

RESULTS

Meiotic-related genes were chosen from expression profiles of mouse sperm under nicotine exposure

According to the sperm expression profiles, a heat map (Fig. 1) of the selected recombination-related transcriptome was drawn. On the heat map we observed that nicotine changed the expression of genes involved in chromosome recombination and synapsis in mice sperm. Data mining analysis indicated that 4 genes (*Spo11*, *Msh4*, *Rad51* and *Mlh1*) were related to the meiosis recombination process. We then examined SPO11, RAD51, MSH4 and MLH1 expression levels after nicotine treatment [22-24]. Our results showed that after nicotine treatment, the expression of the four important genes varied as follows: SPO11 did not change significantly, RAD51 and MLH1 were both upregulated, MSH4 was downregulated. The RT-PCR result was in line with the heat map.

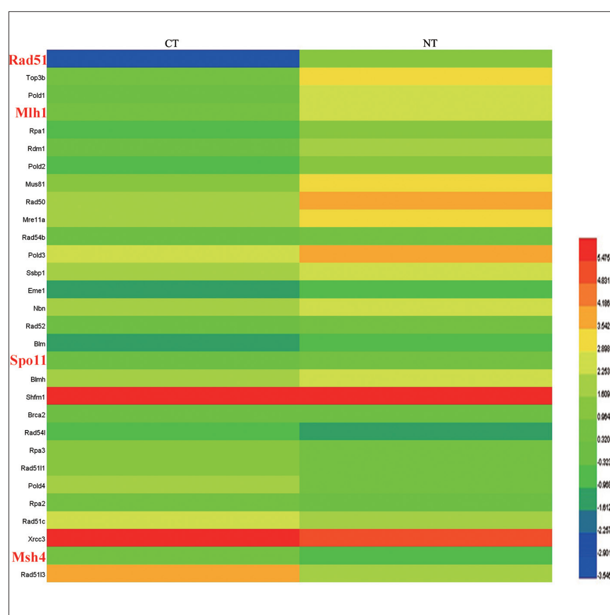

Fig. 1. Heat map of differentially expressed genes involved in meiotic recombination in mouse sperm after nicotine exposure. Expression signals are represented on a log scale in the heat map, where warmer colors correspond to higher levels of expression and colder colors correspond to lower levels of expression

Expression of meiotic recombination-related genes was regulated by nicotine in mouse testes

We further detected SPO11, RAD51, MSH4 and MLH1 protein expression in mouse testes after nicotine exposure by Western blotting and immunohistochemistry. The Western blot results (Fig. 2A) showed that the expression of both SPO11 (NT/CT:0.73-fold) and MSH4 (NT/CT:0.73-fold) was downregulated after nicotine treatment in murine testes, whereas the expression of RAD51 (NT/CT:2.06-fold) and MLH1 (NT/CT:1.40-fold) was upregulated (Fig. 2B).

To determine at which stage of meiosis the expression of SPO11, RAD51, MSH4 and MLH1 changed, an immunohistochemistry experiment was carried out on testis paraffin sections (Fig. 2C). The cells on the cross section of the seminiferous tubule were divided into five sections and numbered in order from 1 to 5 from the outside to the inside.

These results (Fig. 2C) showed that neither SPO11 nor RAD51 exhibited a significant difference in expression when the NT and CT groups were compared; SPO11 was expressed the highest in secondary sperma-

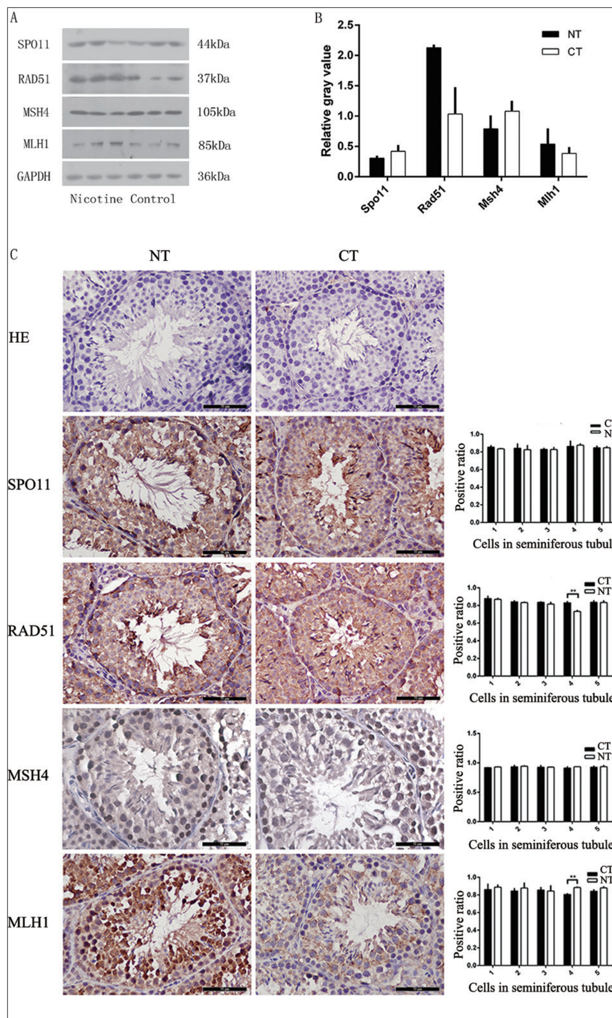

Fig. 2. Detection of reproductive cell expression levels in male mice from control (CT) and nicotine-treated (NT) groups. **A** – Western blotting performed to detect the expression of *Spo11, Rad51, Msh4* and *Mlh1* at the translational level. GAPDH served as an internal control. **B** – expression of *Rad51* (NT/CT:2.06-fold, P=0.01) was significantly upregulated. **C** – semiquantitative analysis by immunohistochemistry (IHC) on testis paraffin sections. For *Rad51*, the nicotine treatment significantly reduced protein expression in secondary spermatocytes and round spermatids. For protein Mlh1, the positive signal in whole seminiferous tubule tissue was upregulated significantly after the nicotine treatment, with the treatment significantly improving the expression of Mlh1 in secondary spermatocytes and round spermatids. In the histograms, "*" stands for P<0.05 and "**" stands for P<0.01.

tocytes and round spermatids; RAD51 was expressed the highest in spermatogonia and primary spermatocytes. Nicotine treatment significantly reduced the expression of RAD51 in secondary spermatocytes and round spermatids. The expression of MSH4 and MLH1 was upregulated significantly in whole seminiferous tubule tissue after the nicotine treatment. MSH4 was expressed the highest in primary spermatocytes and secondary spermatocytes, while there was no difference between the NT and CT groups' cell sections. MLH1 was expressed the highest in spermatogonia and primary spermatocytes, and the nicotine treatment significantly increased the expression of MLH1 in secondary spermatocytes and round spermatids.

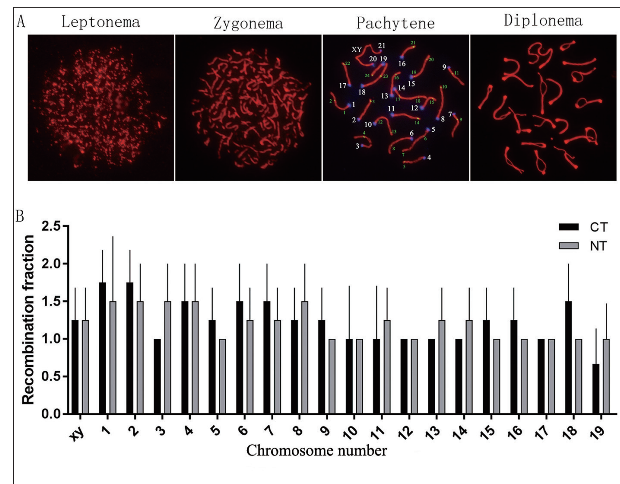

Fig. 3. Detection of meiotic recombination frequency in mice spermatocytes from CT and NT groups. **A** – four different stages in meiotic prophase I of mice germ cells: leptonema, zygonema, pachytene and diplonema. **B** –statistical analysis of every recombination complex did not reveal significant differences (P=0.050) among the 30 recombination complexes.

Nicotine had no significant effects on meiotic recombination frequency

To investigate the effects of nicotine on meiotic recombination frequency, we first spread the chromosomes of the seminiferous tubule. We detected recombination in testicular primary spermatocytes by fluorescence staining with SYCP3, MLH1 and CREST as primary antibodies (Fig. 3A). SYCP3 was used to show the outline and structure of the whole synaptonemal complex, MLH1 was used to show the recombination site on every synaptonemal complex [25], and CREST was used to show the centromeres. After analyzing the results from 30 male mice with statistics, we found that there was no significant difference (P=0.950) between the NT (22.6 recombination sites on average) and CT (24.1 recombination sites

on average) groups. According to statistical analysis of every recombination complex, we found that there was no significant difference (P=0.050) among the 30 recombination complexes (Fig. 3B). The results showed that nicotine had no significant effects on the meiotic recombination frequency in male mice.

DISCUSSION

In this research, we considered the relationship between nicotine and meiotic recombination of *Spo11*, *Rad51*, *Msh4* and *Mlh1*. Recombination of male reproductive cells occurs in the first division of meiosis of primary spermatocytes [26]. The first meiotic prophase is divided into four periods. In pachytene, the synaptonemal complex presents a compact structure under the microscope, and recombination occurs during this period. Many proteins participate in mammalian meiotic recombination, from which we chose four important recombination-related proteins (SPO11, RAD51, MSH4 and MLH1) to explore the potential changes associated with nicotine. The double-stranded break (DSB) that initiates meiotic recombination is catalyzed by SPO11 (topoisomerase-like meiotic recombination enzyme) [27]. After DSB formation, two strand-exchange proteins, RAD51 and DMC1 (which is the meiosis-specific homolog of RAD51), catalyze strand invasion [27]. MSH4 then participates in the process of intermediate processing [27]. The resolution of recombination intermediates is the final step of recombination. MLH1 (a participator in the process of resolution) foci mark the crossover sites [27]. When there is interference in the expression of SPO11, there is a loss of DSB generation, correlating with a defect in synapsis [27]. The accumulation of RAD51 foci and a strong synapsis defect occur when mice carry mutations in Msh4, which both indicate defects in DSB repair [27]. The other mechanism remains unclear.

RAD51 and MLH1 expression were both upregulated after nicotine treatment, but SPO11 and MSH4 expression were downregulated in the NT group. The protein expression results from Western blotting were consistent with the known trend of expression. *Spo11* was mostly highly expressed in secondary spermatocytes and round spermatids. After nicotine treatment, there could be upregulation of *Rad51* protein in Leydig cells and Sertoli cells. *Rad51* was most highly expressed in spermatogonia and primary spermatocytes, and nicotine treatment significantly reduced the expression of Rad51 in secondary spermatocytes and round spermatids. *Msh4* was most highly expressed in primary spermatocytes and secondary spermatocytes. *Mlh1* was most highly expressed in spermatogonia and primary spermatocytes, and the nicotine treatment significantly increased the expression of Mlh1 in secondary spermatocytes and round spermatids.

Research shows that the recombination process is tightly regulated. Such regulation confirms that each pair of homologous chromosomes has at least one recombination site, and recombination loci on each pair of chromosomes generally number no more than two. The specific molecular mechanisms that control the number of recombination foci remain unclear, but there is no doubt that this mechanism is an important means of guaranteeing genetic stability and offspring's health. Nicotine has a wide range of effects on the human body. By changing the methylation of certain genes' promoter regions, nicotine can affect multiple signaling pathways, resulting in a variety of physiological effects. The effects of nicotine on synapsis and recombination is a new field of study. We hypothesized that nicotine could have an impact on the recombination regulation pathway, and could thus change the number of recombination loci overall, or bring about changes to specific homologous chromosomes. We therefore decided to start from the phenotype by counting the number of recombination foci in primary spermatocytes after nicotine injection in mice.

The evolution of meiotic recombination is conservative. During the meiotic prophase, cell-to-cell variability in recombination intermediates decreases. Since it is the Spo11 locus number, not the crossover number, that indicates early recombination, we can infer that the nicotine treatment did not play an important role in early recombination. Regardless of whether the early recombination intermediates decreases or increases, the cytological interference will not change. At the zygonema and pachynema stages, the DSBs generated by Spo11 eventually return to a relatively constant middle value [27]. Mouse spermatocytes have homeostatic control of recombination, which finally guides crossover control that can reconcile the differences between absolute DSB number and the kinetics of DSB formation and repair [27].

We hypothesize that there is a homeostatic mechanism in meiotic recombination that occurs under nicotine exposure. There are many other examples supporting the homeostasis mechanisms in organisms under environmental pressure. There is a homeostatic mechanism in mammals related to hormones and nutrients that ensures that blood glucose levels remain stable in a narrow range [28]. Most environmental factors cannot change DNA sequences but can affect the epigenome [29]. With increasing temperature, there is an increase in recombination frequency in some species, while in other species there is a decrease [12,30-31]. The frequency of meiotic recombination is under homeostatic control in yeast [32]. The mechanisms that accounts for meiotic recombination frequency in response to extrinsic factors are presently unknown. Possible mechanisms include the synaptonemal complex, DNA methylation, chromatin modifications and RNA splicing [13,30,33-35]. In these experiments, although nicotine affected the expression of many synapsis restructuring-related genes, we did not observe a change in recombination frequency in the sperm cells of nicotine-treated mice. The effect of nicotine on meiosis recombination is complex. It points to a balance adjustment mechanism in the process of meiotic chromosome restructuring in male mice. Nicotine does influence the expression of some genes, but it does not appear to exceed the tolerance threshold. Therefore, while nicotine could have some effect on epigenetic mechanisms in male mice, it did not affect meiosis recombination frequency. The ability of organisms to respond to nicotine in a plastic way is significant, as this ability may have adaptive consequences. Because nicotine is the addictive substance and one of the main harmful ingredients in cigarettes [2], it is reasonable to assume that cigarette smoking can have a similar effect on male mice. These studies contribute to an improved understanding of the effects of nicotine and cigarette smoking on animal reproduction, genetics and development. Specific homeostatic and potential epigenetic mechanisms will have to be explored in future experiments.

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Conflict of interest disclosure: The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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