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Nicotine induced autophagy of Leydig cells rather than apoptosis is the major reason of the decrease of serum testosterone

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ABSTRACT

A new report has shown that nicotine exposure can decrease serum testosterone by apoptosis in Leydig cells; however, in our previous studies, we have almost never observed apoptosis there. The purpose of this study is to ensure whether apoptosis or autophagy in Leydig cells occurred. Our results confirmed again that the concentration of testosterone in the sera of nicotine-treated mice statistically decreased ($P < 0.05$). Furthermore, the data of single cell transcriptome indicated that the expression of autophagy-related genes was increased after nicotine exposure. Likewise, chemical and immune-histological staining demonstrated that autophagy of the Leydig cells increased after nicotine treatment rather than apoptosis. Apoptosis mainly exists in spermatids. Further, the expression of autophagy-related genes, such as *Beclin1* and *LC3*, were up-regulated after nicotine exposure ($P < 0.05$). Additionally, the data of transmission electron microscopy showed more autophagosomes in the Leydig cells of the nicotine-exposed groups than the cells of the control groups. Moreover, immunofluorescent staining of LC3 in the TM3 Leydig cell line indicated that rapamycin and nicotine exposure up-regulates the autophagy phenotype/process and down-regulates their testosterone synthesis. In addition, the methylation level of the promoter region of *TCL1* is increased in the nicotine-treated group compared to the control group, consequently decreasing the expression of *TCL1*. In conclusion, the autophagy in Leydig cells induced by nicotine, which is set by the hyper-methylation of the *TCL1* promoter region via the *TCL1-mTOR*-autophagy signaling pathway.

1. Introduction

Among the most widespread addictive behaviors, cigarette smoking is the leading cause of cancer death worldwide (Bialous and Sarna, 2017), and is responsible for the increased risk for at least 15 types of cancer (Zon et al., 2009). In addition, smoking can accelerate aging and death (Watanabe, 2016), reduce fecundity rates, lead to adverse reproductive outcomes and a higher risk of *in vitro* fertilization failures (Dechanet et al., 2011; Kawazoe and Shinkai, 2015). Previous studies have shown that smokers, compared to non-smokers, had significantly lower semen volume, sperm concentration, sperm motility, total sperm count and sperm morphology (Asare-Anane et al., 2016). Moreover, negative alterations in the functional quality of the sperm by decreasing acrosome integrity and mitochondrial activity have been attributed to cigarette smoking (Antoniassi et al., 2016).

As to sperm production within the acrosome, cigarette smoking can alter the protein expression profile of mouse sperm (Zhu et al., 2013). In addition, the ERK pathway can be activated and spermatogenesis can be impaired through the epigenetic inactivation of *Pebp1* caused by cigarette smoking (Xu et al., 2013). Nicotine, the main addictive component of cigarettes (De Biasi and Dani et al., 2011), it has been shown to prevent the proper maturation and capacitation of sperm (Chen et al., 2015; Dai et al., 2016). Furthermore, nicotine can induce apoptosis in cells of murine testes through the inhibition of telomerase activity, resulting in the down-regulation of *Nme2* expression due to its promoter's hypomethylation (Gu et al., 2016). Another study demonstrated that apoptosis in murine spermatozoa can be induced by nicotine, and can be attributed to the up-regulation of deubiquitinated RIP1 through the hypomethylation of the *Trim27* promoter (Nie et al., 2016).

Nicotine has also been shown to hormonally promoting the

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production of testosterone by the testes. This process starts by nicotine stimulating the hypothalamus to release Gonadotropin-Releasing Hormone (GnRH). This then activates the Hypothalamic-Pituitary-Gonadal axis (HPG), which stimulates the release of both Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH). The release of FSH and LH stimulates the testes to produce more testosterone (Tweed et al., 2012).

Despite nicotine's ability to increase the production of testosterone through the hormonal pathway discussed, it is able to reduce serum testosterone. In this research, we attempt to draw a connection, between reduced serum testosterone levels and increased apoptosis in murine spermatozoa as induced by nicotine, using a molecular pathway within Leydig cells of the testis where nicotine decreases the concentration of testosterone in serum, to explain how nicotine influences male infertility *in vitro* and *in vivo*.

2. Materials and methods

2.1. Ethics

All the animal experiments were carried out in compliance with The Guidelines for The Care and Use of Laboratory Animals established by the Chinese Council on Animal Care and approved by the Shanghai Jiao Tong University Ethics Committee.

2.2. Cell culture and drug treatment

TM3 Leydig cells were plated on cover slips in DMEM medium supplemented with 5% FBS. The cells were placed in an incubator containing 5% CO_2 at 37 °C. Cultured TM3 cells were treated with hCG (1U/ml) (Zhao et al., 2011), and hCG (1 U/ml) plus Rapamycin (0.1 μM, 1 μM, 10 μM), Nicotine (1 μM, 10 μM, 100 μM), BTX (20 μM), and Bafilomycin A1 (50 nM) respectively. The cells were used for Immunofluorescence, Western blot and the cell culture medium supernatants were collected for hormone detection.

2.3. Immunofluorescent staining of Beclin1 in TM3 cells

The TM3 cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, then punched with 0.2% Triton X-100, and blocked with 5% BSA. Next, the cells were immunolabeled with rat anti-Beclin1 primary antibody (diluted 1:100 in PBS) overnight at 4 °C. After three washes with PBS, the cells were incubated with fluoresce in isothiocyanate-conjugated anti-rat IgG (1:200) at room temperature. The slides were finally analyzed by observation under a Super-Resolution Multiphoton Confocal Microscope (TCS SP8 STED 3X, Leica, Germany).

2.4. Animal model set-up

Forty 7-week-old male C57BL/6 J mice were randomly divided into two groups: control-treated group (CT) and nicotine-treated group (NT). The latter group was injected with nicotine while the former group was injected with saline. Nicotine was injected at a dose of 0.2 mg/mouse/day diluted with phosphate buffered saline (PBS). The concentration of intraperitoneal injection is in accordance with the definition of "heavy smoking" by WHO (≥20 cigarettes daily) (Matta et al., 2007). The injection time was five weeks, which corresponds to the duration of the spermatogenic cycle.

2.5. Hormone detection

After 5 consecutive weeks of treatments with saline or nicotine, blood samples were collected by retro orbital bleeding of each mouse before sacrifice. To separate the serum, blood was kept at 37 °C for 1 h, followed by 4 °C 1 h then subjected to centrifugation for 10 min at 5000 *g* at 4 °C. The serum samples were kept at 4 °C until the hormonal assay was performed. Analysis of serum testosterone was performed by the electrochemiluminescence method according to the manufacturer's instructions (ADVIA Centaur XP, Siemens, Germany).

2.6. Analysis of expression profiles of Leydig cells

Leydig cells were isolated from the mouse testis from both groups by flow cytometry using anti-HSD3B2 antibody. Samples of Leydig cells were sent to the Chinese National Human Genome Center at Shanghai for next-generation analysis. Then, the Public Library of Bioinformatics was used to construct heat maps of the autophagy related-genes, to help comprehend the differences in gene expression in the testis between the two groups.

2.7. Terminal deoxynucleotidyl transferase-mediated dUTP Nick End labeling (TUNEL) assay

TUNEL assay was used to detect the apoptotic cells by using the Apoptosis Detection Kit III (QIA33, Merck Millipore, Germany). Testis tissues were digested with proteinase K (20 μg/mL PBS) for 15 min at room temperature. The endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. Then, digoxin deoxyuridine triphosphate nucleotides (DIG-dUTP) and terminal deoxynucleotidyl-transferase (TdT) were added to the sections to bind the 3′-hydroxyl termini of DNA. The sections were then blocked with the blocking reagent for 30 min. After incubating with biotinylated mouse anti-DIG (1:100 diluted in antibody dilution buffer), the sections were incubated with Cy3-conjugated goat anti-mouse immunoglobulin G (IgG) (SA00009-1; Proteintech Group, 1:100 diluted in TBS). The tissue sections were then incubated with fluoresce in isothiocyanate-conjugated peanut agglutinin (PNA-FITC, 1:100 diluted in TBS; Sigma, USA). Finally, the slides were mounted with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured with a fluorescence microscope (DM2500, Leica, Germany).

2.8. Monodansylcadaverine (MDC) staining

The typical methods for monitoring autophagy are MDC staining. Detection of the autophagy-related proteins LC3 and MDC can be used to specifically detect the formation of autophagic vacuoles in macrophages and acidic organelles (Su et al., 2013). Chemical staining with MDC was used to detect acidic vesicular organelles. Testicular tissue sections of the mice were used to locate and detect where autophagy was occurring. The kit (Cat.No. KGATG001) was bought from KeyGenBiotech, China. Detection was performed according to the manufacturer's instructions. The nuclei were stained with DAPI, and the sections were observed with a fluorescence microscope (DM2500, Leica, Germany).

2.9. Immunofluorescence

To detect the expression ofLC3 and Beclin1 in the testis by immunofluorescence, paraffin-embedded sections of the testes were baked at 85 °C for 10 min, dewaxed with xylene and then rehydrated in graded concentrations of ethanol. The rehydrated sections were washed under running water, and 0.01 M citrate buffer (pH 6.0) was used for 15 min for antigen retrieval in a pressure cooker. Then, each section was treated with 1% Triton X-100 for 15 min and incubated with 5% bovine serum albumin, BSA (Solarbio; catalog no. A8010), dissolved in PBS for 30 min. This was followed by incubation with rabbit anti-Beclin1 antibodies (1:150, Catalog No. 2983; Cell Signaling Technology, Beverly, Massachusetts, USA) overnight at 4 °C. After being washed three times in PBS, the sections were incubated in Cy3-conjugated goat anti-rabbit IgG (SA00009-2; Proteintech Group, 1:100) at 37 °C for 1 h. Finally, the sections were then mounted with 4,6-diamidino-2-phenylindol (DAPI,