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INVESTIGATION OF THE POSSIBLE ROLE OF POLY (ADP-RIBOSE) POLYMERASE PATHWAY IN NICOTINE- EXPOSED TESTICULAR DAMAGE

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ABSTRACT

Although known adverse effects of nicotine exposure on general health, it is largely consumed as cigarette smoking. Smoking has negative effects on the fertility in males; however, the molecular mechanisms affected by nicotine are largely unclear. In this study, we aimed to investigate the effect of nicotine on poly(ADP-ribose) polymerase (PARP) pathway in testicular damage. Twenty-four male C57BL/6J mice were arbitrarily categorized into three subgroups: control, sham (subcutaneous, 0.9% sterile saline), and nicotine (subcutaneous, 3 mg/kg/body weight/day) groups. After 14 days of twice-daily subcutaneous injections, the weights of the body and testes were measured. The levels of testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), cotinine (main metabolite of nicotine), and 8- OHdG (oxidative DNA damage indicator) in serum were determined using enzyme-linked immunosorbent assay (ELISA) method. Light microscopy was used for assessing sperm count and motility, as well as the histopathological analysis of testes and seminiferous tubule degeneration. Immunohistochemical studies and real-time quantitative polymerase chain reaction (qPCR) were used for detecting the expressions of PARP-1 and caspase-3. The results showed that nicotine exposure significantly decreased the weight of mice and testes, reduced the count and motility of sperm, while increasing the damage to seminiferous tubules. Nicotine administration significantly lowered the levels of serum FSH, LH, and testosterone; however, it increased the levels of 8-OHdG and cotinine. Moreover, the expressions of caspase-3 and PARP-1 significantly increased. In conclusion, our results indicate that nicotine causes damage to testicular tissue by activating the PARP-1 pathway.

KEYWORDS:
Male infertility, PARP1, testis, apoptosis, 8-OHdG

INTRODUCTION

The most preventable reason for death is cigarette use, and the global frequencies of smoking for men and women older than 15 years were reported by World Health Organization (WHO) as 34% and 6%, respectively [1,2]. Cigarette smoke consists of a number of mutagenic, carcinogenic, teratogenic, and toxic compounds that have detrimental effects on the reproductive system [3]. Nicotine, a toxic alkaloid, is the primary addictive component of cigarette smoke and contributes to numerous tobacco-induced pathologies [4,5]. While nicotine is an important risk factor for cardiovascular, pulmonary, and chronic kidney diseases [6,7], it is reported to impair male fertility directly [8-12].

Epidemiological and clinical studies revealed that cigarette smoking was associated with decreased rates of success in couples experiencing in-vitro fertilization [13,14]. Moreover, the negative effects of nicotine on male reproductive functions, such as decreased sperm count, sperm viability and motility [15], disrupted Leydig and Sertoli cell functions [16,17], reduced the levels of sexual hormones [8,18,19] and increased DNA fragmentation and apoptosis in sperm cells [20-22]. Nevertheless, the molecular pathways disrupted by nicotine that led to testis destruction and dysfunctional sperms have remained unclear.

Oxidative stress (OS) is a major contributor to nicotine exposed testicular damage according to recent studies [23, 24]. OS is caused by the accumulation of reactive oxygen species (ROS) and/or decreased antioxidant capacity. The detrimental effects of excess ROS on sperm had been associated with the fragmentation of DNA and nucleotide base oxidation [25], lower mitochondrial membrane potential [26], lipid peroxidation in cell membrane and motility inhibition [27]. OS caused by nicotine is suggested as a mechanism that might lead to impairment of spermatogenesis. The activity of some antioxidant enzymes like superoxide dismutase (SOD) and glutathione was shown to be inhibited by nicotine leading to an excessive ROS production that, in turn, caused lipid peroxidation and damage to sperm DNA [23]. Ni et al. (2020) demonstrated that nicotine exposure resulted in the reduced activity of SOD

while testicular levels of malondialdehyde increased, which led to OS in the testis [28]. Although a correlation between OS and nicotine exposed testicular damage was suggested, the exact cellular or molecular mechanisms have not been delineated yet. Poly(adenosinediphosphate[ADP]-ribose) polymerase (PARP) enzyme family consisting of 18 members (PARP1-18) has multiple roles in numerous cellular processes such as DNA repair, response to genotoxicity by maintaining the stability of the genome, chromatin remodeling, mitotic apparatus function, cell death including, apoptosis and necrosis [29,30,31]. In accordance with the published data, a major role player in intracellular processes causing OS has been shown to be the PARP pathway [32]. Under normal physiological conditions or when the DNA damage is low, PARP1, one of the most studied PARP family member, recognizes the damage to allow its repair for the survival of the cell. On the contrary,when the damage to the DNA is high, PARP1 plays a crucial role in cell death [31,33].

Based on the explained research results, we hypothesized that PARP pathway activation might have a role in testicular damage after nicotine exposure. In this study, we investigated the effect of activated PARP on the nicotine exposed testicular damage.

MATERIALS AND METHODS

Animals and nicotine treatment. The study protocol was approved (permit no. 2017/10), and procedures involving experimental animals complied with the regulations set by the Local Committee on the Ethics of Animal Experiments, Mersin University School of Medicine. Twenty-four 10-week old and 20-25 g C57BL6/J male mice were used. The mice were housed under controlled temperature and light conditions (23±2°C, 12 h light/dark, 50±5% humidity) with ad libitum access to food. Three groups each with eight mice were randomly formed. The control group of mice did not receive any treatments while the sham and nicotine groups of mice were administered subcutaneous injections of 0.9% sterile saline and 3 mg/kg nicotine (Sigma, ≥99%, N0267), respectively. The treatment continued for 14 days as twice daily (08.00 am, 12.00 pm) injections. The dose of nicotine administered was determined by taking its half-life in mice vs. human (6-7 min vs. 2 hrs.) into account, thus, targeting the comparable levels of nicotine in adult smokers [34]. Nicotine exposure is quantified by its primary metabolite, cotinine, which has a longer half-life than nicotine [35]. We measured serum cotinine levels to achieve comparable results with human studies [36]. The body weights of mice were recorded in all groups during the experimental period. All mice anesthetized by administering intraperitoneal ketamine hydrochloride/xylazine were sacrificed. The testes and epididymis were harvested, and the weight of the testes were measured. Blood samples collected by cardiac puncture centrifuged (12.000 rpm, 15 min, at 4 °C) and sera were obtained. Serum samples were stored at -80 °C until further studies.

Sperm count and motility analysis. Left cauda epididymis of each mice was harvested and rinsed in phosphate-buffered saline (PBS pH 7.4) three times. The tissue was placed on a petri dish containing 1 ml PBS at 37 °C cut into smaller pieces to release spermatozoa into the medium. One drop sperm suspension collected by pipetting was placed on a Makler chamber for calculating the total count and motile sperm ratio.

Histopathological analysis. After an overnight fixation in 4% paraformaldehyde, the right testicular tissue dehydrated using graded alcohol dilutions (70%, 80%, 90%, 96%) and rinsed in xylene was embedded in paraffin blocks. Cross-sections of 4 µm were obtained using a rotary microtome (Leica, RM2255). The tissue sections stained with Hematoxylin-Eosin were evaluated for morphology, and seminiferous tubule degeneration was assessed in 800 random samples from each group using Johnsen scoring as described previously [37].

Hormonal analysis. ELISA kits (Elabscience, E-EL-M0511, E-EL-M0057 and E-EL-0155, respectively) in accordance with the protocol supplied by the manufacturer were used to measure the levels of FSH, LH, and testosterone in serum.

Serum cotinine level measurement. Serum levels of cotinine were measured by ELISA (Calbiotech, CO096) following the manufacturer's instructions.

Serum 8-hydroxyguanosine (8-OHdG) level. The serum level of 8-OHdG, which is widely used as a marker of DNA damage due to oxidative stress, was measured using ELISA (ab201734, Abcam).

Immunohistochemistry. Expressions and localizations of caspase-3 and PARP1 protein were examined by the immunohistochemical method. For antigen retrieval, testicular tissue sections were deparaffinized, rehydrated, and boiled in citrate buffer (pH 6, at 100°C, 20 min). Next, the endogenous peroxidase activity in samples was blocked using 3% H2O2 for 10 min and rinsed in PBS for 5 mins and repeated three times. The epitopes were stabilized using a blocking solution (Abcam, ab93677) for 10 mins. Testicular tissues were exposed to rabbit monoclonal anti-caspase-3 (Cell Signaling, #9664S, 1/200) and rabbit polyclonal anti-PARP-1 (Abcam, ab218132, 1/200) primary antibodies and were incubated overnight at +4 °C in humidified conditions.

The incubation of negative control samples was performed using PBS alone. The samples were washed in PBS three times, each for 5 mins. For detecting the binding of primary antibody, the samples were exposed to biotinylated secondary antibody (Abcam, ab93677) followed by streptavidin peroxidase for 10 mins each and rinsed in PBS at room temperature for 5 mins each for three times. The immunoreactivity was observed after '3' diaminobenzidine tetrahydrochloride (DAB), (Abcam, ab64238) incubation, followed by methyl green counterstaining. Next, light microscopy (Olympus BX51, Japan) was used to evaluate the slides.

A blinded histologist scored the relative staining intensity for caspase-3 and PARP1 in testis sections as follows: absent (-, no staining), weak (+), moderate (++) and strong (+++). For each group, 800 immunopositive germ cells were evaluated.

Total RNA isolation and quantitative real-time PCR. Left testis tissue was used for the isolation of total RNA by TRIzol Reagent (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer. RNA quality was assessed by spectroscopy. The isolated RNA samples were treated with DNase (Applied Biosystems, Darmstadt, Germany) before the real-time quantitative polymerase chain reaction (RT- qPCR). The reverse transcription of total RNA was performed via first-strand cDNA synthesis kit (Qiagen, 205311) and the qPCR reaction was set up in a total volume of 25 µl with 12.5 µl of 2X SYBR Green master mix (Qiagen) and primer pairs for mouse caspase-3 (Mm_Casp3_1_SG, QuantiTect Primer Assay, Cat No: QT00260169, Qiagen) and mouse PARP-1 (Mm_Parp1_1_SG, QuantiTect Primer Assay, Cat. No: QT00157584, Qiagen) and run on a Rotor-Gene Q (Qiagen). 2-ΔΔCt method using beta-actin (Mm_Actb_1_SG, QuantiTect Primer Assay, Cat. No: QT00095242) as internal control was used for relative gene expressions.

Statistical analysis. Data obtained from experiments repeated three times were analyzed using Graph Pad 3.0 (GraphPad Software, San Diego, CA) and reported as mean±SEM. The Shapiro-Wilk test was used to assess normal distribution. Differences among groups were tested by the analysis of variance ANOVA followed by the Tukey posthoc test. The significance level was set at $p<0.05$.

RESULTS

Body and testis weights. Mice body weights in the Nicotine group were significantly decreased compared to those of the control and sham groups. The testis weight in Nicotine group compared to the control and Sham groups decreased significantly and indicated damage to the testis (Table 1).

The levels of hormones in serum. The serum levels of FSH, LH, and testosterone in the nicotine group were significantly lower than those in the other groups (Table 2).

Epididymal sperm count and motility. The mean sperm motility in caudal epididymis and the mean sperm count of mice in the Nicotine group were significantly reduced compared to the other groups (Table 3).

Serum cotinine level. The mean serum cotinine level of mice nicotine group was found to be 306.12±0.3 ng/mL whereas cotinine was undetectable in the serum of mice in the remaining groups (p=0.001).

TABLE 1
Body and tests weights of mice in control, sham and nicotine group.

Parameter	Control	Sham	Nicotine
Body weight (g)	22.64±1.19	22.29±0.59	20.85±0.13*
Testes weight (g)	0.12126±0.005	0.12098±0.006	0.11352±0.009*

Data are presented as±SEM. n=8 for each group.
*p=0.01 compared to the control and sham groups.

TABLE 2
Effects of nicotine on reproductive hormone levels (ng/ml) among groups.

Parameter	Control	Sham	Nicotine
FSH	2.79±0.41	2.67±0.44	1.73±0.38*
LH	4.38±0.17	4.34±0.12	2.36±0.11*
Testosterone	3.73±0.09	3.71±0.12	1.86±0.16*

Data are presented as±SEM. n=8 for each group.
*$p<0.01$ compared to the control and sham groups.

TABLE 3
Effects of nicotine on sperm count, motility and Johnsen Score among groups.

Parameter	Control	Sham	Nicotine
Sperm count (10^6 /mL)	4.41±0.06	4.35 ±0.04	2.16±0.5*
Sperm motility (%)	63.75±1.25	61.24±1.37	40.1±1.51*
Johnsen score	9.1±0.05	9±0.04	6.3±0.08*

Data are presented as±SEM. n=8 for each group.
*p<0.01 compared to the control and sham groups.

Serum 8-OHdG level. The serum level of 8-OHdG in the nicotine group of mice was significantly increased (4.76±0.12 ng/mL) when compared to control (0.40±0.09 ng/mL) and sham groups (0.39±0.08 ng/mL), (p<0.05).

Histopathological evaluation of the testes. The morphology of the seminiferous tubules and spermatogenesis in control and sham mice appeared normal whereas mice in the nicotine group revealed a significant damage of testicular tissue (Figure 1). The layers of germ cells and spermatozoa were observed as irregular in most of the seminiferous tubule of mice in the nicotine group, and the lumen contained only a few germ cells. The Johnsen score, which indicated the tubular degeneration, was significantly lower in the nicotine group than that in the other groups (p<0.01) (Table 3).

Immunohistochemistry and quantitative real-time PCR findings. Caspase-3 and PARP-1 were used to stain the apoptotic germ cells. Caspase-3 expression in the spermatogonia nuclei in control and sham groups was significantly decreased compared to that of the Nicotine group. Semi-quantitative analysis of Caspase-3 expression was shown in (Figure 2). The expression of PARP-1 in the spermatogonia nuclei and Sertoli cells in control and sham groups was significantly lower than that of the Nicotine group. Figure 3 demonstrated the semi-quantitatively evaluated levels of PARP-1 expression (Figure 3). The data from quantitative assessment by RT-PCR confirmed the results of the immunohistochemical analysis. The mRNA levels of caspase-3 and PARP-1 were significantly more in the Nicotine group than those in the other groups (Table 4).

TABLE 4
Relative mRNA expressions among groups.

Parameter	Control	Sham	Nicotine
Caspase-3	0.26±0.08	0.27±0.07	1.01±0.014*
PARP1	0.48±0.07	0.47±0.05	1.62±0.018*

Data are presented as±SEM. n=8 for each group.
*p<0.05 compared to the control and sham groups.

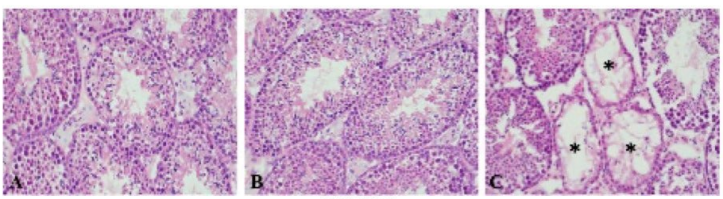
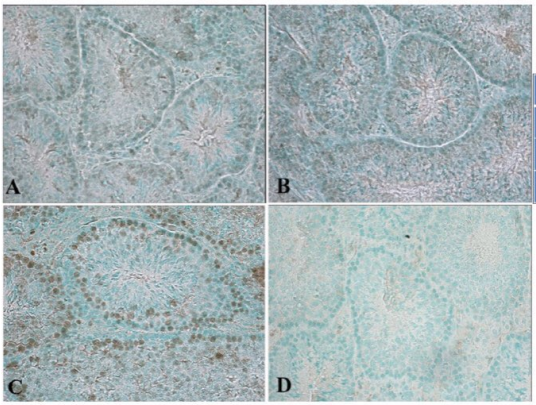


FIGURE 1
Testicular morphology in control group (A), sham group (B) and nicotine group (C), (H&E, 400X). Normal seminiferous tubule morphology is shown in control and sham groups. Sertoli cell only tubules were seen in nicotine group (asterisks).

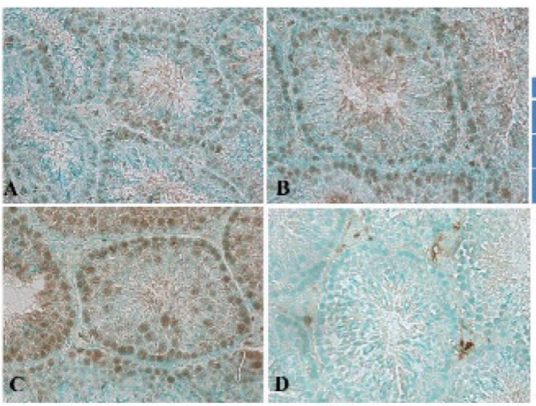


E

Caspase-3 protein expression level	
Control	+
Sham	+
Nicotine	+++

FIGURE 2

Immunohistochemical analysis of caspase-3 protein in control (A), sham (B) and nicotine (C) groups. Increased caspase-3 expression can be seen in nicotine induced group. Figure D represented as negative control. Caspase-3 protein expression evaluated semiquantitatively has been presented in E. Magnification X400.



E

PARP1 protein expression level	
Control	++
Sham	++
Nicotine	++

FIGURE 3

Representative photomicrographs for PARP1 immunohistochemistry in control (A), sham (B) and nicotine (C) groups. Spermatogonial cells were PARP1 immunopositive in control and sham groups. Spermatogonia and spermatocytes were strong PARP1 immunostained germ cells in nicotine group. Figure D represented as negative control. PARP1 protein expression evaluated semiquantitatively has been presented in E. Magnification X400.

DISCUSSION AND CONCLUSIONS

In this study, the effect of the PARP pathway on the testicular damage caused by nicotine exposure was investigated. To date, several cellular or molecular pathways have been suggested to cause the damage to the testes by the nicotine. Our study represents the first example of research in which the effect of the PARP pathway on the testes damage in nicotine-exposed mice has been investigated.

It is critical to consider the differences in nicotine metabolism between mouse and human while determining the experimental dose. As the half-life of nicotine in mice is much shorter than it is in humans, higher doses of nicotine were required to reach plasma cotinine levels comparable to smokers. In this study, the serum cotinine level in mice of the Nicotine group was found as 306.12±0.3 ng/mL, which correlated with adult human smokers [35].

In toxicology studies, the weight of the organism and organs are important determinants for detecting the potential toxicity of a substance. Our results demonstrated a significant reduction in the weight of the body and testes in the Nicotine group of mice. Additionally, testicular weight is a valuable index of reproductive toxicity. Studies have shown that the testes and body weights were reduced in the groups treated with nicotine [11,38,39]. Similarly, clinical studies have shown that lower body weights observed in cigarette smokers started to increase when smoking was quitted [40].

The histopathological findings of the current study revealed that nicotine caused testicular damage, which was characterized by the degeneration of seminiferous tubules, tubular arrest at different stages of spermatogenesis, and the presence of Sertoli cells only in the tubules. We used Johnsen scores to assess the tubular degeneration and spermatogenesis, and found that it was significantly lower in Nicotine group. Previous studies demonstrated that nicotine exposure caused spermatogenic disorder, represented by a reduction in the germ cell numbers, germ cell degeneration [8], decreased spermatogenic cell mass thickness, vacuolation in Sertoli cells, and increase in basal lamina thickness [9]. Furthermore, numerous human and animal studies revealed that nicotine impaired the reproductive system in males by decreasing the count, concentration, motility, and viability of the sperm, and the quality of the semen [10,11,39]. Sperm motility is a critical component of fertility in males and significantly affects fertilization [41]. Our results demonstrated that the motility of sperm was lower in the Nicotine group compared to those in the other groups. Condorelli et al. (2013) reported that sperm motility was suppressed by nicotine and had a deleterious effect on sperm DNA integrity and spermatozoa apoptosis [21]. In a previous study, nicotine was proved as a potential oxidant agent by impairing the sperm plasma membrane, breaking double-stranded DNA in the nuclei, altering the metabolism of GSH, and morphology and motility of the sperm [42]. Moreover, a study revealed that nicotine reduced testosterone levels, suggesting an inhibition of testicular androgenesis by nicotine [8]. The testosterone is required for spermatogenesis and the preservation of the testicular cytoarchitecture. Previous studies have indicated that low serum testosterone levels adversely affected testicular morphology, testis weight, and sperm functions. The significant reduction in testes weight and sperm motility might be due to a decrease in the serum testosterone levels, which in turn adversely affected the number of Leydig cells responsible for testosterone synthesis [43]. Our ELISA results showed that the serum testosterone, FSH, and LH levels were significantly reduced in nicotine administered mice. Guo et al. (2017) reported that the markedly reduced testosterone levels in the nicotine-induced mice was the result of down-regulated testicular steroidogenic acute regulatory protein (StAR) and reduced enzyme activities of 3β-HSD and17β-HSD, which were the key enzymes of testicular androgenesis [16]. Gonadotropin hormones, FSH and LH, control the functions of Sertoli and Leydig cell, which are crucial for the initiation and preservation of the maintenance of spermatogenesis and steroidogenesis. In the current study, the lower levels of serum FSH and LH following nicotine exposure might be caused by hypothalamic-pituitary-testicular axis impairment.

In our study, serum 8-OHdG level was significantly increased after nicotine exposure, which indicated an oxidative DNA damage as the toxic effect of nicotine. It is well established that OS induces single-stranded breaks in DNA, apoptosis, and activation of PARP [30]. Furthermore, OS has been reported to rapidly induce the intracellular depletion of ATP and to lead to axonemal damage with reduced sperm motility and viability in addition to detrimental effects on acrosome reaction and capacitation [44]. The immunohistochemical results in our study showed that the testicular expression pattern of the PARP1 was similar in control and sham mice. Immunolocalization of PARP1 was seen in spermatogonia and spermatocyte in control and sham groups. In the Nicotine group, the expression of PARP1 was increased, leading to an overactivation of PARP1. It is clearly demonstrated that the overactivation of PARP1 caused the depletion of NAD+ and ATP and slowed the rates of glycolysis and mitochondrial respiration resulting in the dysfunction and ultimately death of the cell [45]. Similarly, the immunolocalization of caspase-3 was present in spermatogonia in the testes of the nicotine-exposed mice. Confirming the immunohistochemical results, quantitative analysis of PARP1 and caspase-3 by RT-PCR revealed an increase of expression for those two genes in the nicotine-exposed group compared to the other groups. The number of studies that have researched the relationship between infertility in males and the

PARP pathway is scarce. A clinical study in a varicocele patient reported a high expression in PARP1 [46]. Similarly, in an experimental varicocele rat model, the apoptotic index and PARP-1 expression of germ cells were increased [47]. According to a previous study, damage to testis caused by doxorubicin was found to be due to an increase in the expressions of the apoptotic pathway and PARP1 proteins [48].

In conclusion, this study showed that the PARP pathway affects the damage to testis in mice induced by nicotine; and this result might be associated with PARP overactivation via caspase- dependent pathways.

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