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Investigating intrauterine exposure to methamphetamine on serine-threonine kinase pathway in male rat testis

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Abstract

Today, methamphetamine (METH) is being used by adolescents and young adults. Our previous research demonstrated that intrauterine exposure to METH induces apoptosis in testicles and seminiferous tubes. However, based on available literature, the mechanism of this effect remains unidentified. This study aimed to investigate proteins involved in sperm growth and development pathways, such as testis-specific serine/threonine kinases (TSSK) and receptor-interacting protein kinases 2 (RIPK2), and to study the serine-threonine kinase pathway in the testes of rats whose mothers received intraperitoneal METH during pregnancy. In the present study, female rats during pregnancy received either 5 or 10 mg/kg of METH or normal saline for ten days. After reaching maturity, their testes were isolated and examined for histopathological and immunohistochemical mechanisms. Results were analyzed and reported using statistical software. Results revealed that following intrauterine exposure to METH, TSSK protein expression reduced from 52.68±2.4% in the control group to 48.04±2.29% in the 2 mg/kg/day group and 12.83±3.35% in the 5 mg/kg/day group with P=0.0029 and F=72.63. In addition, RIPK2 protein expression increased from 8.34±2.69% in the control group to 31.17±3.69% in the 2 mg/kg/day group and 98.49±4.66% in the 5 mg/kg/day group, with p=0.0037 and F=61.14. Histopathological findings indicated a reduction in the thickness of germ layers following intrauterine exposure to METH, with the seminiferous tubule's thickness decreasing. Inflammatory cell populations increased, and the number of vessels decreased due to intrauterine exposure to METH. Our study suggests intrauterine exposure to METH increases the prevalence of inflammatory cell populations, enhances RIPK2 protein expression, reduces the number of vessels, reduces the diameter of seminiferous tubes, decreases TSSK protein expression, and reduces the thickness of germ layers in testicular tissue. Apoptosis of spermatid cells observed in our previous study may be related to the signaling pathways of TSSK and RIPK2 proteins.

Keywords: Methamphetamine, RIPK2 protein expression, Testicle, TSSK protein expression

1. Introduction

Methamphetamine (METH) is an illegal psychoactive drug and its addiction is a global concern and often involves many neurological consequences, including anxiety, hallucinations, restlessness, and paranoia. Cardiovascular problems, renal failure, and, pulmonary arterial hypertension are also reported [1]. The primary users of METH are teenagers and young adults in their reproductive age. This issue has become a huge social problem [1-4]. According to studies, METH can also affect the reproductive system [5].

Abuse of METH in pregnant women can have irreparable effects on pregnancy, fetus, and newborn [6].

METH affects the mother's health by increasing pregnancy complications such as preeclampsia and high blood pressure [7]. It can easily cross the placental barrier, leading to premature birth, intrauterine fetal death, and overall higher rates of maternal and fetal mortality [8]. Prenatal exposure to METH has negative neurological consequences for children, including structural brain changes, memory loss, and increased cognitive and behavioral problems [9]. Additionally, METH can significantly impact the male reproductive system.

METH has been reported to be harmful to testicular functions and sperm quality [10]. METH reduces the proliferation/apoptosis ratio of seminiferous cells by reducing the number of proliferating cells and increasing apoptotic cells; therefore, this might alter seminiferous epithelium homeostasis [11].

These outcomes suggest that METH could reduce testi-

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cular function and potentially influence male sterility [12]. Besides increasing apoptosis and decreasing sperm count, METH affects sperm morphology, leading to abnormal sperm in male rats [13]. Despite these findings, the mechanisms by which METH disrupts normal spermatogenesis remain unclear, highlighting a gap that our study aims to address.

Spermatogenesis is a complex process that includes mitosis, meiosis, and spermiogenesis. This process is controlled by endocrine glands and genes [14]. In spermatogenesis, functional sperm are created from an undifferentiated primary germ cell. Recently, researchers have been attracted to a family of testis-specific protein kinases known as testis-specific serine/threonine kinases (TSSK) because of their critical roles in spermatogenesis [15, 16]. Researchers discovered Six TSSK subfamilies in mammals, which have the conserved serine/ threonine protein kinases catalytic (STKc) domain in TSSK1 through TSSK6 [15]. Different members of the TSSK gene family were expressed in various stages of spermatogenesis [17-19]. The TSSK1 expression in mature spermatozoa was also shown in earlier studies [3], and most are mainly expressed in sperm or spermatids [20]. For instance, in mice, TSSK1 and TSSK2 double-deletion caused infertility in males [21, 22]; the reason for infertility is the reduction of sperm's motility and number [21]. TSSK6 knockout mice showed sterility phenotype due to definite morphological defects in the sperm [23, 24]. But at the anatomical level, the reproductive system of the knockout mice was revealed to be expected. There was no meaningful difference in body weight and testis weight [21].

The RIPK family acts as essential intracellular and extracellular sensors involved in inflammation, induction of death, and immune responses [25]. The pathway that TSSK acts through includes receptor-interacting protein kinase 2 (RIPK2). It activates via protein-dependent polyubiquitination and tyrosine and serine phosphorylation. Active RIPK2 activates TAK1 (tank binding kinase 1). After a bit, IKK (I_B kinases) moved the NF-_B dimer (p65 and p50) to the nucleus to regulate gene transcription by turning them on [26].

As the fertility results of TSSK knockout mice are similar to the effect of amphetamine on mice treated with it, we suggested that amphetamine probably causes infertility through the impact on the TSSK genes. Therefore, in this research, we intend to investigate the pathway of serine-threonine kinase that can change due to methamphetamine. Also, according to the available sources, no study based on the mechanism of methamphetamine's effect on the testes of rats with mothers treated with methamphetamine has been found. Therefore, this research aims to investigate the pathway of TSSK and RIPK2 in the testes of rats with mothers who received methamphetamine intraperitoneally during pregnancy.

2. Materials and Methods

2.1. Animals and Experimental Design

Fifteen adult female and male Wistar rats were obtained from the Pasteur Institute in Tehran. All mice were in good health and had never been previously exposed to methamphetamine or any other drug. Food and water were fully available. Mice were given a 12-hour light-12-hour dark cycle (light starts at 7:00 AM). The room was maintained at approximately 20°C and 50% humidity. When females were sexually ready, they were caged with adult males overnight. There was always one female and one male in each cage. The following day, after being impregnated, the females returned to their normal state. The day when sperm was detected was determined as the first day of pregnancy. Females were randomly divided into one group treated with salt and two groups treated with methamphetamine (6 in each group). The investigation included intraperitoneal injection using methamphetamine hydrochloride (2 or 5 mg/kg) or normal saline (0.9% NaCl, 1 ml/kg) daily (10 days until the end of pregnancy). The doses of METH selected for this study were 2 mg/kg/day and 5 mg/kg/day, administered intraperitoneally to pregnant rats for ten days during pregnancy. These doses were chosen based on previous studies that demonstrated significant physiological and behavioral effects in rodents without causing acute toxicity or maternal mortality [27]. The 2 mg/kg/day dose represents a moderate exposure level to assess the threshold at which METH begins to affect fetal development, while the 5 mg/kg/day dose was used to evaluate dose-dependent effects on the testes.

Although direct extrapolation from animal doses to human exposure levels is complex, the selected doses aim to model significant exposure levels that could occur in pregnant women who abuse METH. We acknowledge that including a lower dose group could provide a more comprehensive understanding of the dose-response relationship. However, due to resource constraints and ethical considerations regarding the number of animals used, we focused on doses identified as impactful in previous literature. Future studies should include lower doses to explore the minimal effective dose and better characterize the dose-response curve.

For this experiment, we used METH hydrochloride which was synthesized and analyzed by the central research laboratory of Shahid Beheshti University of Medical Sciences, Tehran, Iran. It was diluted in normal saline. On the 20th day of pregnancy, each pregnant mouse was placed in a separate cage. All mice gave birth during gestation days 21-22. One male child from each mother was randomly selected for the study. The children of mothers exposed to methamphetamine were used as the leading group, and the male children of mothers exposed to saline were used as the control group (6 in each group). All offspring lived in the cage with their mother until weaning at 28 days, after which male and female offspring were separated. Male offspring were kept in cages of the same group (2 mg/kg, 5 mg/kg methamphetamine or control). All male offspring were maintained until seven weeks of age, which corresponds to sexual maturity in rats [27]. At this age, the offspring were euthanized for testicular tissue collection and analysis. This time point was chosen to evaluate the long-term effects of intrauterine METH exposure on testicular development and spermatogenesis in mature male rats.

All groups were maintained under preselected conditions (food and water ad libitum, 12:12 h light/dark cycle, and room temperature) and did not receive methamphetamine, saline, or any other drugs. Texture processing and its steps

The tissue processing process included dehydration, molding, tissue fixation, and paraffin impregnation. In other words, with the aim of tissue processing, the pieces were ready to be cut by microtome and painted.

2.2. **Fixative of testicular tissue**

To penetrate the fixative solution, after measuring the volume of the testicular tissue with a needle, several holes were created in the testicular capsule, each of the testicles was placed in the MDF[2] fixative solution at the laboratory temperature for one week, and the fixative solution was replaced twice a week.

2.3. **IUR cutting of testicular tissue and preparation of trocar pieces**

The orientation method was used to cut the AUR of the testicular tissue after the tissue fixation stage. This way, three stages of theta θ and ϕ circular clock face (numbers 0-9) were used. In this way, the testicle was placed on the clock face ϕ, a random number between 0 and 9 was selected, and the testicle was cut in the direction of the chosen number, which resulted in 2 pieces. One of the pieces was placed on the clock face θ so that the cut surface was tangential and towards the bottom of the clock face, and again, a new random number was chosen from 0 to 9, and this time, at this angle, the fabric was cut equally and parallel. The cut surface of the other piece was placed at the 0-0 degree of θ plane, and a new random number was selected. The tissue piece was cut parallel and identically along the new angle, and finally, eight slabs with a thickness of about 1mm were prepared from each testicular tissue.

Spherical pieces were prepared by selecting 3 of the slabs randomly and using a trocar cylindrical rod. By vernier caliper, two perpendicular diameters of 3 pieces of trocar were measured and included in the estimation of testicular tissue shrinkage. Next, the prepared trocar parts and slabs were placed separately in unique baskets, and the characteristics of each cut were placed inside the baskets. In this way, tissue sections were prepared to continue the tissue processing.

2.4. **Processes and passages of testicular tissue**

The tissue processing was done by a tissue passage device (Leica), and to extract water from the tissue, baskets containing trocar parts and slabs were placed in alcohol glasses with increasing concentration. Next, xylene was used to remove alcohol and clarify. Finally, to be impregnated with paraffin, the baskets were placed in paraffin jars, and the tissue passage machine performed all these steps automatically.

2.5. **Texture molding**

A paraffin-blocking machine did tissue embedding after the tissue passage was completed. To do this, first, the device was turned on, solid paraffin was poured inside the device, and its temperature was set to 58°C (paraffin melting temperature).

L-shaped metal molds were used for block making, which adjusted the size of the blocks and saved paraffin consumption. To facilitate the separation of the molds, they were coated with glycerin. To make a block, after melting the paraffin, the mold was placed on a tiny glass to prevent the melted paraffin from coming out of the mold, and then every 3-4 pieces of the testicle cut were placed inside a mold. Each trocar piece was placed in a paraffin mold, as described above, and the characteristics of each tissue block were placed on it during molding. About 24 hours after the molds were cooled, the tissue blocks were removed from the molds and kept in the refrigerator until cut by a microtome machine.

2.6. **Cutting and preparation of tissue sections**

Tissue sections were cut by microtome machine (Leitz1512), and cutting was done from the second cut surface of the slabs. To prepare the tissue sections, 20 and 5-micron slices were crafted from each tissue block. Four slices (each 5 microns) were prepared from each piece of trocar, and after cutting to make the sections completely flat, they were placed in a hot water bath of 40-35°C. Also, the characteristics of each tissue were recorded on the opaque part of the slide. The slides were kept in the laboratory until the staining step.

2.7. **Tissue staining by hematoxylin-eosin method**

Sections prepared from testicular tissue were stained with the hematoxylin-eosin (H&E) method. Coloring solutions were poured into special containers for colored glasses. First, the slides were placed at 60°C for 15 minutes to stain the tissues. Then, after a few minutes, their paraffin was melted, and we put the slides in small glass baskets. And according to the staining procedures, H&E staining was done.

2.8. **Staining of prepared sections with DAPI fluorescent dye**

DAPI dye is a fluorescent dye that strongly binds to adenine-thymine-rich regions in DNA. With this dye, polystyrene nanoparticles can be checked. The slides were incubated for 24 hours for this purpose. After passing this time, the cells were affected by DAPI dye for 5 minutes with 4% paraformaldehyde at room temperature, and then their morphology was checked with a fluorescent scope (Zeiss, Germany).

2.9. **Histopathological Analysis**

Histopathological evaluations were performed using hematoxylin and eosin (H&E) staining on testicular tissue sections. The assessment criteria included:

Seminiferous Tubule Diameter: Measured using image analysis software (ImageJ) by calculating the mean diameter from several tubules in randomly selected fields.

Germinal Epithelium Thickness: Determined by measuring the distance from the basement membrane to the lumen in multiple tubules.

Inflammatory Cell Count: Inflammatory responses were quantified by counting the number of inflammatory cells (e.g., macrophages, lymphocytes) in five randomly selected high-power fields (400× magnification) per slide.

Blood Vessel Count: Blood vessels in the interstitial tissue were counted in random fields.

All measurements were performed by two independent observers blinded to the group allocations to minimize observer bias. Inter-observer reliability was assessed using the intraclass correlation coefficient (ICC), which showed good agreement (ICC > 0.85).

After the tissues were paraffinized, the samples were sectioned and blocked on slides charged with polylysine, and finally, the IHC steps were performed as follows:

On the first day, washing with xylene was done twice for 3 minutes. In 50-7090-100 dilutions, cleaning with alcohol was done for three minutes each. Incubation was

Table 1. The name, manufacturer and catalog number of the monoclonal antibodies used in the IHC method.

The name of the antibody	Manufacturer	Catalog number
RIPK2	SANTA CRUZ	Orb382010
TSSK1	SANTA CRUZ	MBS9452495

done at 60°C for 1 hour, and the samples were placed in sodium citrate with pH=6. Washing was done twice with PBS for 5 minutes each time. For 10 minutes (1.5 ml in 300 ml PBS), the samples were placed in TRITON X_100. Then, washing was done twice with PBS for 5 minutes each time. For 30 minutes, the samples were placed in a 1% BSA2 blocking solution (Sigma, A3311). The samples were placed in the refrigerator for one day, and the primary antibody was added to the slices with a dilution of 1:500.

On the second day, washing was done three times with PBS for 5 minutes each, incubation was done for half an hour, and a secondary antibody was added to the samples. Washing was done three times with PBS for 5 minutes each time. A slide was placed on the samples covered with glycerol glue. Finally, the samples were observed with a fluorescent microscope.

2.10. **Ethical Approval**

All experimental procedures involving animals were conducted in strict accordance with ethical guidelines for laboratory animal care and were approved by the Institutional Animal Care and Use Committee. Throughout the study, efforts were made to minimize pain and distress in the animals. Methamphetamine administration and all handling procedures were performed by trained personnel to ensure the welfare of the animals. Animals were monitored daily for any signs of discomfort or adverse effects, and appropriate measures were taken to alleviate pain or distress when necessary. The code was approved by the Islamic Azad University of Medical Sciences (IR.IAU.PS.REC.1401.427).

2.12. Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS software version 21. Normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated with Levene's test. One-way analysis of variance (ANOVA) was used to compare means among groups. Tukey's post hoc test was applied for pairwise comparisons when ANOVA indicated significant differences. A p-value less than 0.05 was considered statistically significant.

3. Results

The study showed that administration of METH in male rats at doses of 2mg/kg and 5mg/kg resulted in significant changes in various parameters compared to the control group. The results showed a decrease in the average seminiferous tubule diameter, an increase in the average inflammatory cell population, a decrease in the average number of vessels, a reduction in the average germinal layer thickness, and a decrease in the average percentage of TSSK1 expression. On the other hand, the average rate of RIPK2 expression showed a significant increase. The differences between the control group and the 2mg/kg and 5mg/kg groups were statistically significant ($p<0.05$ or $p<0.01$ or $p<0.001$). Figure 1 shows the obtained histopathological results.

The average seminiferous tubule diameter in the control group,2mg group, and 5mg group were 233.7 ±27.02 micrometer,161.0±35.55 micrometer, and 83.33±8.622 micrometer, respectively. There was a significant reduction in seminiferous tubule diameter in control vs 2mg groups($p<0.05$), control vs 5mg groups($p<0.01$) and 2mg vs 5mg groups($p<0.05$).

Our results show that intrauterine exposure to METH decreased the tubule size in the seminiferous tubules in the groups exposed intrauterine to METH compared to the control group. So the thickness of the seminiferous tubules from 233.7±27 micrometers in the control group to 161±35.5 micrometers in the 2 mg/kg per day group and 83.33±8.62 micrometers in the 5 mg/kg group in The day decreased and p=0.0013 and F=59.24. Figure 2 shows the

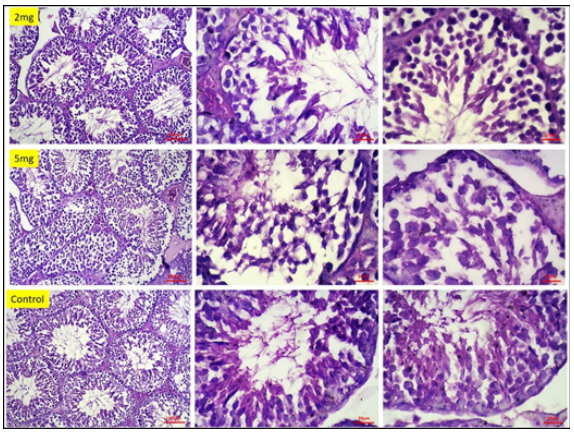

Fig. 1. Histopathological results were obtained after intrauterine exposure to doses of 2 and 5 mg/kg/day METH compared to the control group. These histopathological graphs show a decrease in the diameter of the seminiferous tubules, an increase in inflammatory cells, a decrease in the number of vessels, and a decrease in the number of germ cells.

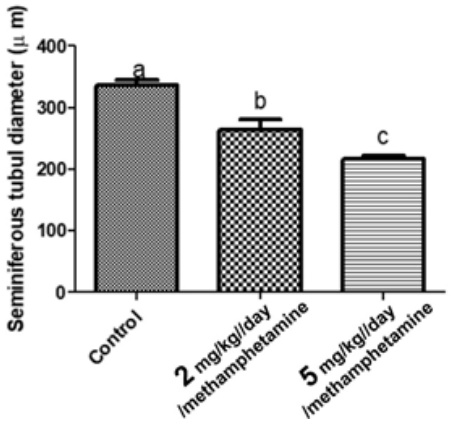

Fig. 2. Chart of examination and comparison of mean and standard deviation of diameter of seminiferous tubules in micrometers in the control group and test group. b Difference between the group (2mg/kg/day) and the control group c shows the difference between the group (mg/kg/Day 5) and the control group.

mean and standard deviation (SD) of the diameter of the seminiferous tubules.

The data of the H&E test and H&E tissue sections showed that the number of inflammatory cells increased significantly as a result of intrauterine exposure to METH, so in the control group, the average number of inflammatory cells was 6±2, but in the group, (2mg/kg/day) of METH was 21 ± 2 and in the METH (5mg/kg/day) group it was 37.3 ± 2.5. Therefore, as a result of intrauterine exposure to METH, the number of inflammatory cells has increased. Also, the one_way Anova test shows the difference between the groups is significant (P = 0.0013 and F = 24.59). Therefore, intrauterine exposure to METH increased the number of inflammatory cells.

Intrauterine with METH causes the number of vessels to decrease. The results show that the number of vessels in the seminiferous tubules in the control group was 37.3±2.5, but after administration of 2 mg it was 29±4.3 mg/kg/day, and after administration of 5 mg decreased to 6±2 mg/kg/day (P < 0.0001 and F = 80.81). Our results show that the ratio of the thickness of the germinal layers in the tubule from 155±13.08 in the control group to 117.3±8.5 and 85.3±5.6 in the group exposed intrauterinely to METH 2 and 5 mg/kg. The day has come. Statistical tests show that this difference is significant (P = 0.0003, F = 39.7)

In our study, the expression level of TSSK1 proteins in the control group was 52.68±4.2%, which was increased to 29.48±2.04% after the administration of 2mg/kg/day of METH and to 29.48±2.04% after administration of 5mg/kg/day. It has decreased by 12.83±3.35%. One-way analysis of variance test with P-value = 0.0029 and F = 63.72 confirms this decrease. Figure 3 shows the images obtained from the optical microscope.

Our results show that the expression level of RIPK2 proteins in the control group was 8.34±2.69%, which was increased to 31.17±3.69% by administration of 2mg/kg/day methamphetamine and by administration of 5mg/kg/day. Day of methamphetamine has increased to 98.49±66.4%. Also, the One-way analysis of the variance test with P-value = 0.0037 and F = 61.14 confirms this increase. Figure 4 shows the images obtained from the light microscope with a magnification of 400 for the analysis of RIPK2 proteins in the test group and the studied groups.

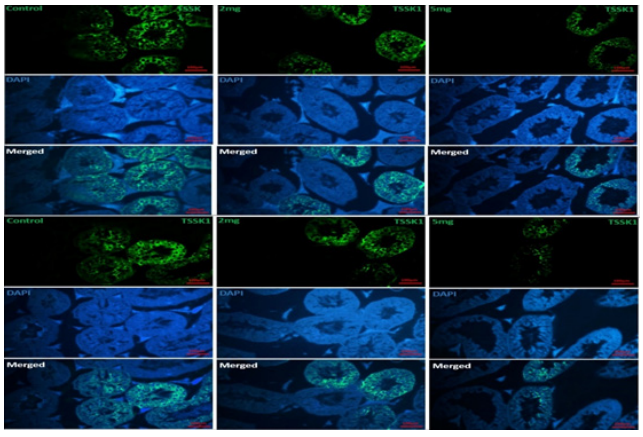

Fig. 3. The images were obtained from the light microscope with 400 magnification for the analysis of TSSK1 proteins in the test group and the studied groups. In the first row, it shows antibodies labeled with Tssk1, in the second row, the blue color shows the stained nuclei of cells, and in the third row, it shows the integration of the other two rows. As a result of intrauterine exposure to methamphetamine, TSSK1 protein expression has decreased.

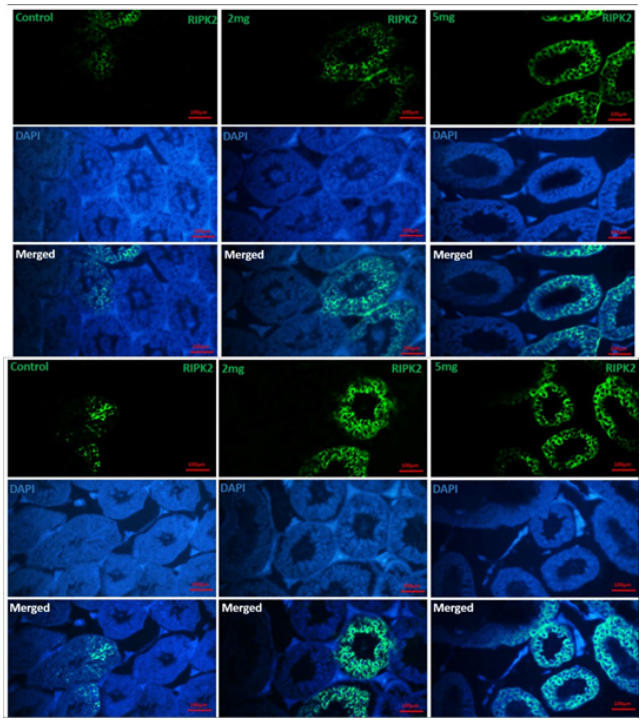

Fig. 4. The images were obtained from the light microscope with 400 magnification for the analysis of RIPK2 proteins in the test group and the studied groups. As a result of intrauterine exposure to methamphetamine, RIPK2 protein expression has increased.

4. Discussion

As we mentioned, METH exposure during pregnancy affects male offspring's testis by several pathways, including reducing TSSK1 expression. The impact on RIPK2 expression is increased significantly in mice exposed to METH prenatally. Also, we found many histopathological changes in the testis, such as increasing inflammatory cell population, reducing the number of vessels, decreasing tubule diameter, and reduction in germinal layer thickness. The results of our research were consistent with some previous studies on the effect of methamphetamine on the seminiferous tubules.

Nudmamud-Thanoi and Thanoi reported that METH increased apoptosis significantly in the seminiferous tubules in the testes of male rats in a dose-dependent way [28]. Yamamoto et al. confirmed that METH caused apoptosis in seminiferous tubules [29]. Alavi et al. appeared that repeated administration of METH (10 mg/kg/14 days) induced a critical impact on the spermatogenesis process such as significant impairment in primary spermatocytes and spermatogonia and increasing apoptotic cells in seminiferous tubules of rat testis [11]. Our results revealed METH caused a reduction in many blood vessels, which induced hypoxia and other researchers reported that hypoxia-induced apoptosis [11]. So it could be concluded that a reduction in blood vessels induced apoptosis in males exposed to METH prenatally. The administration of METH is related to ROS production, and consequently autophagy, DNA damage, and apoptosis [30].

Exposure to METH during pregnancy can negatively affect the testes of male offspring through various pathways, including decreased expression of TSSK1 and

increased expression of RIPK2. This can cause several histopathological changes in the testes, such as inflammation, reduced blood vessel count, decreased tubule diameter, and reduced germinal layer thickness. Several studies have reported that exposure to METH can lead to increased apoptosis in the seminiferous tubules of rats, which can impact spermatogenesis and result in an increase in apoptotic cells [11]. Additionally, we observed a reduction in blood vessels caused by METH exposure, which can induce hypoxia and further lead to apoptosis. It can be concluded that a decrease in blood vessels induced by METH exposure during pregnancy can result in apoptosis among male offspring. Furthermore, administration of METH is related to the production of reactive oxygen species, which can cause autophagy, DNA damage, and apoptosis.

METH exposure during pregnancy caused significant impairments in neurodevelopmental parameters including alteration of behavioral development and pinna unfolding [8]. Exposure to METH during pregnancy is revealed to cause toxicity in other organs [7]. However, our information concerning METH-induced toxicity to the reproductive system remains constrained. Previous studies showed the effect of METH on sperm motility, abnormal sperm morphology, and decreasing in testosterone plasma concentration and low sperm concentration in male rats [29]. However, Taghavi et al. reported that acute exposure to METH didn't affect on morphology and motility of sperm [11]. It is reported that TSSK1 haploinsufficiency caused an alteration in spermatogenesis in the seminiferous such as a lack of testicular sperm in the lumen of tubules, changing the shape of spermatozoa to round cells in the epididymal lumen and an absence of elongated spermatids [22]. Shang, Peng, et al discovered that TSSK gene expression is reduced in METH-exposed mice and sterility [21]. This research shows that exposure to METH during pregnancy caused reduction in TSSK1 expression in offspring.

On the other hand, our study showed that intrauterine exposure of mice to methamphetamine increases the expression of RIPK2. It mediates pro-inflammatory signaling through activation of signaling pathways [31]. On the other hand, simultaneously with the increase of RIPK2, inflammatory cells also increased in the spermatogenic tubes, which seems to be in line with other studies on the role of RIPK2 in the field of inflammation mechanism. The current study shows the increase of RIPK2, which is associated with the increase in the activity of the kinase cascades related to it. This issue can lead to the disturbance of the balance of phosphorylation/dephosphorylation, which can affect sperm motility.

Therefore, it seems that the increased expression of RIPK2 is effective in methamphetamine-dependent intracellular mechanisms. Also, the current study has investigated the histopathological changes in the testicles of male rats whose mothers were exposed to methamphetamine during pregnancy, which has been proven to be the diameter of the spermatogenic tubes, the thickness of the germ layer, and the number of blood vessels in the tube decreases.

Studies indicate that exposure to METH can influence the expression of TSSK and RIPK2 proteins through several interconnected pathways. METH induces the production of reactive oxygen species (ROS), leading to oxidative stress, which may result in cellular damage and apoptosis[7]. Oxidative stress might reduce TSSK expression by affecting transcription factors or signaling pathways associated with spermatogenesis [32]. Additionally, METH-induced oxidative stress can activate inflammatory pathways, increasing RIPK2 expression, a key mediator of innate immune responses [33]. Elevated RIPK2 expression may amplify inflammatory responses, contributing to the observed inflammation and damage in testicular tissue.

Moreover, METH might disrupt the hypothalamic-pituitary-gonadal axis, altering the levels of hormones essential for spermatogenesis, which could affect TSSK expression [34]. METH may also impact other signaling pathways, such as MAPK/ERK and JNK, which are involved in the proliferation, differentiation, and apoptosis of testicular germ cells [35]. Disruption of these pathways can lead to impaired spermatogenesis and increased apoptosis of germ cells, providing a broader perspective on the effects of METH on male reproductive health.

As the study Limitations, in our study, the control group received normal saline injections to account for stress related to handling and injection procedures. However, we acknowledge that the act of injection itself may induce a stress response that could affect physiological parameters. Including an additional control group that does not receive any injections (sham control) could help distinguish the effects of injection stress from those of METH. Future studies should consider including such a control group to enhance the validity of the findings and reduce potential confounding variables[36].

5. Conclusion

Intrauterine exposure to methamphetamine has various destructive effects on testicular tissue. These include increased presence of inflammatory cells, altered protein expression levels (especially increased Ripk2 and decreased Tssk), decreased number of vessels, decreased diameter of seminiferous tubules, and decreased thickness of spermatogenic layers. The observed apoptosis of spermatid cells in previous studies may be related to the signaling pathways of TSSK and RIPK2 proteins. In future studies, it is suggested to investigate the TSSK/TSSE pathway and other apoptosis pathways such as BCL2 and BAX.

Abbreviation

METH: Methamphetamine; TSSK: Testis-specific serine/threonine kinases; RIPK2: receptor-interacting protein kinases 2

Competing interests

The authors declare that they have no conflict of interest.

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publications

The final manuscript was approved and read by all authors.

Ethical Approval

The experiments were approved by the Research Ethics Committees of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Code: IR.SBMU.AEC.

1402/08/07). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Availability of data and materials

Data and materials will be made available if there is a reasonable request.

Authors' contributions

SDP designed the study, conducted experiments, analyzed data, and drafted the manuscript. NZ contributed to the study's conception, design, execution, and manuscript preparation. BGY provided conceptual advice, experimental design input, analytical interpretation, and manuscript review. ZBZ participated in data analysis and manuscript review. All authors read and approved the final manuscript**.**

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