1. Introduction

Methamphetamine (METH) is a highly addictive hallucinogenic and psychoactive drug and next to marijuana, it is the second most widely used drug in the world, especially by young people [1]. Although the amount of METH use varies in different countries, statistics show that it has increased in North America, the Middle East, and East Asia [2]. The use of METH is associated with much damage to the physical and mental health of people [3]. The easy access to METH in some countries and its effect on increasing mortality makes it necessary to study more in the field of medicine, especially in cases related to genetics.

METH is classified as a stimulant of the central nervous system. The stimulating effect of METH on the central nervous system originates from the chemical structure similar to epinephrine and norepinephrine [4]. METH is an indirect agonist at noradrenaline, dopamine, and serotonin receptors. METH mimics the actions of adrenaline in the body, increasing the rate of breathing, heart rate, and blood pressure, and constricting blood vessels [5]. The risk of mortality increases with METH use disorder. It is also associated with the incidence of HIV and hepatitis C infection. Psychosis, depression, and suicidality are their mental health-related complications [6]. The endocrine system is also not spared from the destructive effects of these psychotropic substances.

METH also affects the hypothalamus-pituitary-adrenal axis, leading to increased secretion of ACTH and cortisol [7]. High doses of METH have been found to reduce the number of mature sperm in rats and have negative impacts on the fertility of individuals who use the drug.

METH can cause hypogonadism by reducing the levels of gonadotropin-releasing hormone and testosterone and may result in infertility in males [8]. Studies have shown that METH has effects on sex hormones and can lead to disturbances in the levels of male sex hormones, increasing the concentration of testosterone and reducing the levels of luteinizing hormone and follicle-stimulating hormone [9]. This may cause infertility in males. The ef-
ects of METH on brain and testis cells have been studied. There is limited research on the consumption of METH during pregnancy and its impact on the next generation.

METH use during pregnancy causes problems for the fetus, including growth restriction and low birth weight [10]. It can also increase the risk of preeclampsia, premature birth, stillbirth, and high blood pressure. METH affects the fetal brain and causes neurological and behavioral disorders [11]. Norepinephrine and serotonin transporters are highly expressed in the placenta. These transporters are essential in maintaining amniotic fluid homeostasis and fetal blood circulation. Previous studies suggest that following intrauterine exposure, sperm motility and number may decrease, and apoptosis may occur in the testis [12]. The mechanisms of apoptosis continue to be a focus of research, with recent studies investigating the role of miRNA in environmental effects on genes and changes in cell fate.

MiRNAs (microRNAs) are small RNA molecules that play a vital role in the post-transcriptional regulation of gene expression [13]. They function by binding to the untranslated portion of target mRNAs and suppressing their translation [14]. MiRNAs are involved in various biological processes, including cell differentiation, growth, and apoptosis [15]. Studies have indicated that a disruption in the synthesis of microRNAs significantly impacts spermatogenesis. One consequence of this is the complete loss of sperm, along with testicular degeneration [16]. Furthermore, deleting the microRNA gene in male germ cells results in disruptions during the differentiation of haploid spermatids, leading to apoptosis and failure of spermatogenesis during the haploid and meiotic stages. In mice, the absence of genes associated with microRNA synthesis is associated with reduced spermatogenesis and the inability of sperm cells to fertilize oocytes in vitro [16].

MiRNA-151-3p is located on chromosome 8 (q), and its expression is elevated in several types of cancer, including bladder, prostate, breast, stomach, rectum, and lung cancer [17]. MiRNAs have been implicated in various testicular diseases and their involvement in the pathogenesis of spermatogenesis has been demonstrated. For example, overexpression of miR-p5-34 in testes subjected to hyperthermia has reduced spermatid and spermatocyte activity [18]. On the other hand, calcium channels play a critical role in sperm functions such as motility, capacitation, and acrosome reaction [19].

Studies have shown that METH affects calcium channels [20]. The presence of CACNA1C channels in the testis has been reported in previous research. However, the effects of METH on the expression of miRNA-151-3p and CACNA1C genes have not been studied in detail. Further research is needed to determine the impact of intrauterine METH exposure on the expression levels of miRNA-151-3p and CACNA1C genes in the testis. This article is a continuation of our previously published work in Paper [21]. The current manuscript is based on the same dataset and expands on the work identified in Article [21]. We have highlighted the updates. Additional analyses have been conducted since the previous submission to address the concerns raised [21].

2. Material and Methods
2.1. Selection of Animals and Experimental Protocol
Wistar rats of both sexes, weighing between 200-250 grams, were procured from the Pasteur Institute, Tehran, Iran. Ethical clearance for the experimental procedures was obtained from the Ethics Review Board of the Faculty of Pharmacy and Pharmaceutical Sciences at Islamic Azad Tehran Medical Sciences University, Tehran, Iran (Authorization Number: IR.IAU.PS.REC.1401.372), in alignment with the standards for Laboratory Animal Care and Use. The rodents were kept in standardized living conditions which included ad libitum access to nourishment and hydration and a 12-hour light/dark cycle maintained at a steady temperature of 22 ± 2°C. A period for adjustment was allowed before initiating mating protocols for inducing pregnancy in the female rodents. Verification of pregnancy was performed via examination of vaginal plugs and Papanicolaou staining tests post-copulation. Subsequently, the expectant females were categorized randomly into three distinct experimental units: a control group receiving intraperitoneal injections of saline; and two METH-exposed groups subjected to 2 mg/kg or 5 mg/kg of METH administered intraperitoneally. Post-weaning, the progeny were sorted into groups correlating with the prenatal exposure (each consisting of 6 subjects). The young were housed with their maternal figures in cages until day 28, which marked the weaning threshold. Male progeny were subsequently housed in octets within group-specific enclosures. The progeny were maintained in the predetermined environmental settings without further experimental manipulations.

Upon the experiment’s conclusion, the rats’ mass was cataloged. The animals were then humanely sacrificed under anesthesia with ketamine and xylazine, succeeded by the excision and weighing of the testicular and epididymal tissues.

2.2. Isolation of Total RNA from Testicular Tissue
2.2.1. Tissue Processing
The testes samples were thoroughly homogenized utilizing a tissue disruptor. Subsequently, to the homogenate, Qiazol Lysis Reagent (Kia Zist, Iran) was introduced in a ratio of 1 ml per 50-100 mg of tissue to achieve cellular lysis. Following this, for every 1 ml of Qiazol employed initially, 200 μl of chloroform (Dr. Majalli Chemical Industries Complex, Iran) was added. The containers were agitated manually for 15 seconds to ensure thorough mixing (eschewing the use of a vortex mixer). The samples were then left to stand at ambient temperature for a quarter of an hour before being subjected to centrifugation at 12,000 RPM for 15 minutes at a temperature of 4°C. The resulting upper aqueous phase, which contained the RNA, was carefully decanted into a new tube. To this phase, isopropanol (Dr. Majalli Chemical Industries Complex, Iran) was added in the measure of 500 μl for every 1 ml of Qiazol used, followed by gentle inversion of the tubes twice to mix. The tubes were then placed in a -20°C freezer for a 10-minute incubation to facilitate RNA precipitation. A further centrifugation step was carried out at 12,000 RPM for 10 minutes at 4°C (Hettich, Iran) to settle the RNA. The liquid above the pellet was then discarded, and the RNA pellets were cleansed with 75% ethanol, using 200 μl for each sample, vortexing briefly to ensure complete ethanol interaction with the pellets, and the removal of contaminants. This was followed by a centrifugation for 5 minutes at 75,000 RPM at 4°C. The excess fluid was then carefully drained, and the tubes were inverted and left to rest on
sterile absorbent paper for 15 minutes to allow the pellets to air-dry partially and the ethanol to evaporate. Once the pellets reached a semi-dry state, they were reconstituted in 20-30 μl of either DEPC-treated or RNase-free water (Thermo Fisher Scientific, USA), and the tubes were then placed in a thermal block set to 55°C for 15 minutes to facilitate the pellets dissolving. The RNA was finally aliquoted and stored at -80°C for prolonged preservation.

2.2.2. Creation of Complementary DNA (cDNA)
Reagents from the Easy cDNA Synthesis Kit (Parstous, Iran) were taken from storage at -20°C and RNA specimens from -70°C, then allowed to thaw on ice. Before utilization, all kit components were given a brief vortex and centrifuge.

For RT mix preparation (containing RT buffer, MMLV RT enzyme, oligo dT, and random hexamer primers), oligo dT primer (1 μl) and diethyl pyrocarbonate (DEPC) treated water (3 μl) were combined for a total volume of 9 μl. This mixture was allocated to 0.2 ml PCR tubes. The tubes, now containing both RT mix and RNA, were positioned in a thermocycler or Dry block heater (Kiagene, Iran) to undergo the designated temperature sequence: an initial 10 minutes at 25°C, an elongation step at 47°C for 60 minutes, and a final denaturation at 85°C for 5 minutes. The synthesized cDNA was then stored at -20°C for future analysis.

2.2.3. Generation of cDNA for MicroRNA Analysis
Components from the cDNA synthesis kit were retrieved from -20°C and RNA specimens from -70°C, both being placed on ice to thaw. These components were also vortexed briefly and centrifuged prior to use.

For mixRT assembly, which includes RT buffer, MMLV RT enzyme, oligo dT, random hexamer, and stem-loop specific primers, these ingredients, along with DEPC water (3 μl), were blended to reach a 9 μl total in each PCR tube. The PCR tubes, now prepared with the RT mixture and RNA were transferred to a thermocycler or Dry block heater (Kiagene, Iran). The temperature protocol set was: 25°C for 10 minutes, 47°C for an hour, and a concluding phase at 85°C for 5 minutes. The cDNA for miRNA was then preserved at −20°C for subsequent use.

2.2.4. Evaluative Procedure for Gene Expression Variability
All components necessary for PCR analysis (Real-Time PCR System, ABI StepOne, USA) were removed from cold storage, briefly vortexed, then centrifuged and placed on ice. A PCR mix was prepared for each target gene including SYBR Green Master Mix (5 μl) from a high Rox (2X) source (Korea), forward primer (10μM) at 0.5 μl, reverse primer (10μM) at 0.5 μl (refer to Table 1), and H2O (3 μl) from Sinaclon, Iran. After thorough mixing and centrifugation, 9 μl of this mix was aliquoted into PCR-specific tubes. Subsequently, 1 μl of the appropriate cDNA was added to each tube to make the total reaction volume 10 μl. Following another mixing and vortexing step, the tubes containing the PCR mixtures were loaded into the thermocycler to undergo the temperature schedule listed in Table 2.

Each specimen was assayed three times, and the Ct values of qPCR experiments were averaged. The miR-151-3p level was determined by relative quantification, and the diversity of gene expression was expressed as 2^-ΔΔCT).

2.3. Statistical Analysis
The experiment results were analyzed using SPSS 16.0 software at a significance level of 0.05. The mean ± SEM

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
<th>Acquisition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95 °C</td>
<td>5 min</td>
<td>1X</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>95 °C</td>
<td>15 sec</td>
<td></td>
<td>Off</td>
</tr>
<tr>
<td>Amplification</td>
<td>95 °C</td>
<td>15-20 sec</td>
<td>40X</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>95 °C</td>
<td>15-30 sec</td>
<td></td>
<td>On</td>
</tr>
<tr>
<td>Melting curve</td>
<td>95 °C</td>
<td>15 sec</td>
<td>1X</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>65 – 95 °C, slop: 0.3 °C/ s</td>
<td>1 min</td>
<td></td>
<td>On</td>
</tr>
<tr>
<td>Cooling</td>
<td>95 °C</td>
<td>20 sec</td>
<td>1X</td>
<td>Off</td>
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</tbody>
</table>

Table 1. Sequence of used primers.

<table>
<thead>
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<tbody>
<tr>
<td>mir-151-3p-F</td>
<td>GGC TAG ACT GAG GCT CCT</td>
</tr>
<tr>
<td>mir-151-3p-R</td>
<td>GTGCAGGGTCCGAGGT</td>
</tr>
<tr>
<td>Stem-loop</td>
<td>5’- GTC GTA TGC AGT GCA GGG TCC GAG GTA TTC GCA CTG CAT ACG ACCCTC-3’</td>
</tr>
<tr>
<td>r-U6-F</td>
<td>TGCTTCGGCAGACCATATAC</td>
</tr>
<tr>
<td>r-U6-R</td>
<td>AGGGGCCATGCTAATCTTCT</td>
</tr>
<tr>
<td>r-cacna1c-F</td>
<td>CGACCATCTCTACGGTACGT</td>
</tr>
<tr>
<td>r-cacna1c-R</td>
<td>CTTCAGAGTCGGCAAGCAG</td>
</tr>
<tr>
<td>r-GAPDH-F</td>
<td>AGGTCCGGTGTAACCGGATTGT</td>
</tr>
<tr>
<td>r-GAPDH-R</td>
<td>TGAGACCATGTAGTTGAGGTCA</td>
</tr>
</tbody>
</table>

Table 2. Temperature program for PCR reaction.

**Table 1.** Sequence of used primers.

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</tr>
<tr>
<td>r-cacna1c-F</td>
<td>CGACCATCTCTACGGTACGT</td>
</tr>
<tr>
<td>r-cacna1c-R</td>
<td>CTTCAGAGTCGGCAAGCAG</td>
</tr>
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<td>AGGTCCGGTGTAACCGGATTGT</td>
</tr>
<tr>
<td>r-GAPDH-R</td>
<td>TGAGACCATGTAGTTGAGGTCA</td>
</tr>
</tbody>
</table>
of three separate experiments was calculated for each group and analyzed using a one-way analysis of variance. Tukey's post hoc test was then performed to compare the means. The normality of the distribution was checked using the Kolmogorov-Smirnov test.

3. Results

Figure 1 shows the gene expression changes between the control groups and the groups exposed to METH. The expression level of miRNA-151-3p gene expression in mice exposed to METH in the embryonic period has decreased compared to the control group. The P value obtained from the analysis is P≤0.001. There is a significant difference between the control and intervention groups.

Figure 2 shows the changes in gene expression between the control groups and the groups exposed to METH. Based on the present observations, the expression level of CACNA1C gene expression in mice exposed to METH in the embryonic period has decreased compared to the control group. The P value from the analysis is 0.005, and the CACNA1C gene expression decreases dose-dependently.

4. Discussion

The current experiment results showed that prenatal exposure to METH reduces the expression of miRNA-151-3p and CACNA1C genes in testicular tissue. The effects on the CACNA1C gene were dose-dependent, with higher METH use resulting in a greater decrease in CACNA1C levels. METH has been shown to cause apoptosis and structural damage in the testis [22], increase levels of Bax/BCL2, alter mitochondrial permeability and reduce spermatogonial proliferation and the proliferation/apoptosis ratio of rat spermatogenic tubules [23]. In addition to this, the harmful effects of METH are dose-dependent on sperm parameters and DNA/sperm chromatin integrity, and the negative impact of the molecule on the tissue structure of the testis has been reported [24].

Previous studies have shown that chronic intrauterine exposure to METH caused changes in the seminiferous tubules in the tunnel method and increased the expression of apoptotic proteins [25]. The mechanism of this pathway was not investigated in the studies. The results of the present study showed that exposure to METH during pregnancy causes changes in CACNA1C levels. Previous studies have discussed the importance and presence of L-type VDCC alpha-1c in the testis [26].

The presence of these channels in rat testis, seminiferous epithelium, germ cells, and Sertoli cells has been confirmed [27]. The role of these channels in the acrosomal reaction has been reported in previous studies. Insufficient acrosomal reactions cause infertility [28]. Reports concurrent with the present study indicate the effects of heavy metals and lipopholic toxins on calcium channels that cause reproductive toxicity [28]. The toxicity of ethylene glycol on the reproductive system is probably due to the effect on calcium channels [18], which has been determined in vivo and in vitro. Along with these studies, the reproductive toxicity of METH on calcium channels was determined. Because it can be involved in infertility by affecting acrosomal reactions, more studies on METH users are needed to substantiate this claim. Contrary to the present observation report, Andres et al. showed that METH in nerve cells in case of acute exposure causes calcium channels to close and ultimately increases the expression of CACNA. Our observations were in testicular tissue, which differs in number and function from CACNA1C in brain cells. This aligns with our findings that METH downregulates the sperm-specific calcium channels (CatSper1−4) involved in sperm motility. [29] This suggests that exposure to METH could hurt male fertility by altering the normal functioning of sperm-specific calcium channels. However, as this is a single study conducted on rats, more research is needed to understand the impact of METH on human fertility fully and to confirm these findings in other species. Exposure to METH has a significant effect on testicular tissue, reducing the expression of the CACNA1C gene and causing various detrimental effects.

The current study demonstrated that exposure to METH during pregnancy results in changes in miRNA levels. Previous research has shown that disruptions in miRNA synthesis have a substantial impact on sperma-
togenesis[30]. Björkgren and Papaioannou showed that changes in miRNA levels result in testicular degeneration and sperm loss [16, 31]. Other researchers have verified that removing genes associated with miRNA causes problems in developing haploid spermatids and results in cell death [32, 33]. The current study showed that the levels of miR-151-3p were significantly decreased in the testis of rats after intrauterine administration of METH, suggesting a link between miR-151-3p levels and METH. In a study of rats with hyperuricemia, a decrease in miRNA levels was accompanied by a reduction in sperm motility [25]. In addition, microRNAs were decreased in azoospermic patients and increased in asthenozoospermic patients in a study of 457 patients [34]. Another study showed that miR-19b and let-7a were significantly increased in seminal plasma samples of patients with non-obstructive azoospermia compared to control samples of fertile men [35]. These microRNAs may have potential as diagnostic biomarkers for idiopathic male infertility and in developing new targeted therapeutic tools [36-38]. Also, a possible mechanism for the destructive effects of METH on spermatogenesis is the reduction of miRNA-151-3p expression. Given the widespread use of METH and its potential impact on reproductive health, further research is needed to understand better the mechanisms by which exposure to METH affects the regulation of these genes and the potential implications for human health.

5. Conclusion
In this study, it was observed that intrauterine exposure to METH decreases the expression of miRNA-151-3P and CACNA1. Therefore, regulatory pathways related to voltage-dependent calcium channels involved in the CACNA1 gene are disrupted and endanger the health of the male reproductive system. It is suggested that more studies be conducted in this field on miRNA-151-3P polymorphism in testicular tissue, the expression level of CACNA1, and miRNA-151-3P genes in men with azoospermia.

Abbreviation
METH: Methamphetamine; LH: luteinizing hormone; FSH: follicle-stimulating hormone

Conflict of interests
The authors affirm they have no conflict of interest.

Statements of disclosure
The authors attest there are no competing financial interests or personal affiliations that might be perceived as influencing the outcomes presented in this publication.

Consent for publications
All contributing authors have reviewed and consented to the publication of the manuscript in its current form.

Ethical clearance
The investigative procedures received authorization from the Ethics Committee of Shahid Beheshti Medical University, Tehran, Iran, with the endorsement code: IR.SBMU. AEC.1402.095. This research adhered to the relevant global and local animal welfare guidelines.

Availability of data and materials
The data supporting this research's conclusions can be furnished upon a justified request.

Authors' contributions
RB was responsible for the study's framework, execution of the research, data analysis, and initial manuscript draft. NZ provided significant contributions to the ideation, planning, operationalization, and manuscript preparation. BGY offered strategic guidance, insights for the experimental setup, interpretational analysis, and revision of the manuscript. ZBZ was involved in the interpretation of data and critical revision of the manuscript. All authors granted their approval for the final version to be published.

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