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Methamphetamine Downregulates the Sperm-Specific Calcium Channels Involved in Sperm Motility in Rats

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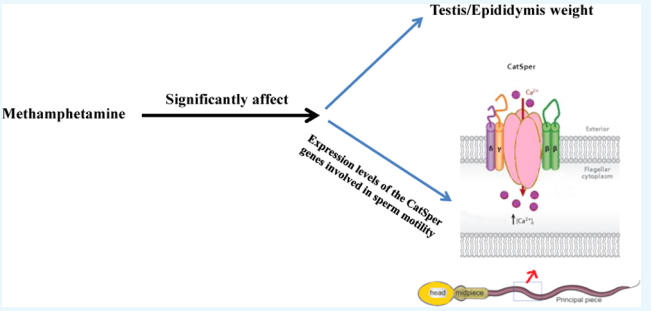
ABSTRACT: Calcium channels play essential roles in sperm motility. A family of sperm-specific cation channels including CatSper1−4 has been identified as voltage-dependent ion channels that act as sperm motility regulators. Methamphetamine is known to cause apoptosis in seminiferous tubules and affect sperm quality. This research was conducted to investigate the effects of methamphetamine on expression of the CatSper family and Mvh genes. Thirty-six adult Wistar rats were divided into four groups of nine rats each: the control and experimental groups 1, 2, and 3. The control group received no solvents or drugs, but experimental groups 1, 2, and 3 were daily given 0.2 mL of a solution by gavage that contained 0.5, 1, and 2 mg of methamphetamine, respectively, for 45 days. The rats were then anesthetized, and one testis removed from each rat was used in a reverse transcription-polymerase chain reaction (RT-PCR). Analysis of variance (ANOVA) and Tukey's posthoc test were used to analyze the data at the $P < 0.05$ significance level. Treatment with methamphetamine resulted in decreased testis and epididymis weights compared to the control rats. The results showed that the mRNA fold expression level of the CatSper family and Mvh genes decreased significantly in experimental groups compared to that in the control ($P < 0.05$). Methamphetamine decreased the expression levels of the CatSper and Mvh genes, and thus, it seemed that it can increase the probability of infertility through sperm motility reduction by lowering the expression levels of these genes.



INTRODUCTION

Infertility is one of the most important social problems in nations. Approximately half of the infertility cases are due to factors related to the male partner.[1] Male infertility can happen as a consequence of diverse pathological situations such as varicocele, cryptorchidism, mumps, testicular torsion, etc. In each case, precise diagnosis is of crucial significance.[2] Methamphetamine is one of the most frequently abused illicit drugs in Iran.[3] Methamphetamine is a stimulant and addictive drug that activates certain systems in the central nervous system immediately after ingestion. METH increases the release of dopamine and prevents its reuptake through its effects on the monoamine oxidase system.[4] It has been reported some effects of METH on the nervous system such as increases in body temperature, alertness, and euphoria and a decrease in appetite.[5] Methamphetamine is made underground and illegally in laboratories. It is commercially available in some countries, too. Street methamphetamine has various impurities and additives and is known as Speed, Chuck, and Matt.[6−10] Methamphetamine abuse among adolescents and young adults—the reproductive age groups—is on the rise, and it has turned into a social dilemma.[11,12] Many studies have been conducted on the effects of methamphetamine on the male reproductive system.[13−22] It has been shown that daily administration of methamphetamine in rats decreased the body, testicular, and epididymis weights.[16] Also, methamphetamine can cause apoptosis in seminiferous tubules and changes of serum testosterone levels in male mice and rats.[13,16−18,23] It can decrease the proliferation of spermatogonia and the ratio of proliferation/apoptosis in seminiferous tubules of rats.[18] Methamphetamine has been reported to reduce the normal sperm count and morphology and increase apoptotic cells in seminiferous tubules.[13,16] Nudmamud-Thanoi et al. reported that normal sperm motility, morphology, and concentrations were significantly decreased in animals receiving methamphetamine, In addition, progesterone receptors (PRs) and estrogen receptor α and β immunoreactive cells were significantly

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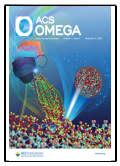

Table 1. Effects of Methamphetamine on the Body and Testis/Epididymis Weight in Rats Receiving Methamphetamine (0.5, 1, and 2 mg/kg for 45 days) and the Control Group

groups	control	0.5 mg/kg	1 mg/kg	2 mg/kg
body weight (g)	253.87 ± 9.48	256.45 ± 7.29	257.49 ± 9.86	260.8 ± 8.58
right testis weight (g)	1.67 ± 0.1[a]	1.26 ± 0.02	1.24 ± 0.07	1.21 ± 0.03
left testis weight (g)	1.72 ± 0.09[a]	1.29 ± 0.06	1.26 ± 0.02	1.24 ± 0.01
epididymis weight (g)	0.8 ± 0.015[a]	0.66 ± 0.012	0.59 ± 0.019	0.53 ± 0.013

[a]Significant difference with other groups in the same row. The data are shown as the means ± standard error of the mean (SEM) (n = 9). *P < 0.05.

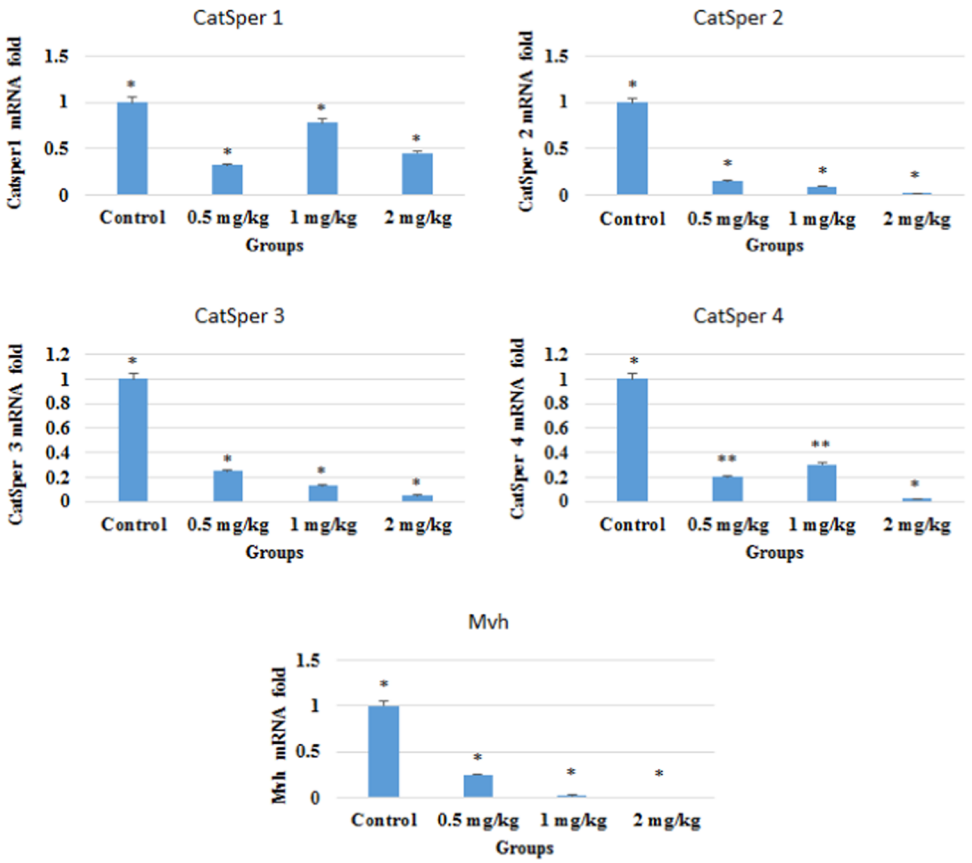

Figure 1. mRNA fold expression (mean ± SEM) of CatSper1−4 and Mvh genes relative to β-actin in the control group and in rats receiving methamphetamine (0.5, 1, and 2 mg/kg) for 45 days. (*) Shows significant difference with other groups and (**) indicates significant difference with control and 2 mg/kg groups (P < 0.05).

decreased in spermatogonia, spermatogenic cells, and especially in Sertoli cells in methamphetamine-treated groups. Furthermore, mRNA expression of the above-mentioned genes was significantly decreased in all methamphetamine-treated animals.[14] Moreover, detrimental effects of methamphetamine in a dose-dependent manner on sperm parameters and sperm chromatin/DNA integrity and the negative effects of this molecule on testicular histoarchitecture have been reported.[19,21,22] Previous studies showed that methamphetamine has significant results in reducing sperm motility.[14,24] Decreased sperm motility was reported at 24 h in a study using mice that received a single 15 mg/kg dosage of methamphetamine.[24] GABAergic alterations in the rat testis were also shown after methamphetamine exposure.[20] Several studies showed the inhibitory effects of methamphetamine on testosterone production by the Leydig cells in the testes. This effect results from increased AMP production and reduced activity of calcium channels and the enzymes related to AMP synthesis.[13,16,22] Also, it has been reported the reduction of testis index and mRNA and protein expressions of glucose transporter 1 (GLUT1), hexokinase 1 (HK 1), and lactate dehydrogenase C (LDHC) in the testes, and thereby, this stimulant also inhibits spermatogenesis by damaging glycolysis.[25] Barenysa et al. reported that prenatal exposure to methamphetamine led to a significant delay in sexual maturation of female rats, poor sperm quality, and increased sperm DNA damage in the male offspring.[26] Calcium channels play important roles in sperm functions such as sperm motility, capacitation, and the acrosome reaction.[27] Many calcium-permeable cation channels have been identified in mammalian sperms including the voltage-gated calcium, cyclic nucleotide-gated, and transient receptor potential (TRP) channels.[28] A family of sperm-specific cation channels including CatSper1−4 has been identified. These proteins are voltage-dependent ion channels that act as sperm motility regulators.[29] The expression of spermatic protein Mvh (VASA) associates with

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sperm motility too, implying that Mvh may be a candidate marker for evaluation of spermatic motility.[30] No fundamental studies have been conducted on the effects this substance has on CatSper and Mvh gene expression. Therefore, this research intended to determine the impacts that methamphetamine has on expression levels of Mvh and CatSper family genes in adult rat testes.

RESULTS

Daily Injections of Methamphetamine Reduced Testis/Epididymis Weights. Continuous exposure of the rats to methamphetamine in all doses resulted in decreased testis and epididymis weights over time relative to the control rats (Table 1). However, despite the reduced testis/epididymis weights, the relative weight of the body in the methamphetamine-treated groups did not statistically differ from the control rats (Table 1).

Gene Expression Analysis. In the current study's analysis, the relative expression level of the CatSper1, 2, 3, and 4 and Mvh genes decreased significantly in experimental groups compared to that in the control. Also, the relative expression levels of the CatSper2 and 3 and Mvh genes in methamphetamine receivers significantly decreased in a dose-dependent manner, and these reduced expressions in each of the experimental groups were significantly different among all other groups.

The relative expression of CatSper1 indicates the significantly decreased level in all experimental groups separately relative to all other groups.

Our results also showed that 2 mg/kg methamphetamine receivers had significantly lower CatSper4 gene expression levels compared to other groups. Similarly, decreased levels in 0.5 and 1 mg/kg receivers were statistically significant relative to control and 2 mg/kg treated groups (Figure 1).

DISCUSSION

The results of this study showed that the use of methamphetamine significantly reduced the expression of Mvh and CatSper family genes in a dose-dependent manner. Adjustment of intracellular calcium concentration is necessary for spermatogenesis, sperm maturation, acrosomal reaction,[31,32] capacitation,[31,33] and sperm motility.[34]

Calcium channels are one of the mechanisms for calcium to enter the sperm from the extracellular space. Among them is the voltage-dependent calcium channel of the CatSper family genes, which plays a key role in sperm motility and fertility and includes the CatSper1 to CatSper4 family genes. CatSper1−4 genes are sperm-specific calcium channels that are expressed only in the testes and are required for motility and fertility.[35,36] These genes are expressed within the acrosome of late spermatids and spermatozoa and play an essential role in the acrosome reaction and male fertility.[37] The CatSper protein is a channel protein that is located specifically in the plasma membrane of the principal piece of the tail of human and mouse sperm and plays a vital role in the regulation of sperm hyperactivation.[38,39] The activity of this channel is regulated by pH and cAMP, and its function is to transport calcium ions, which are necessary for sperm motility and capacitation.[29,40] The CatSper gene is an important gene in mammalian fertilization, and based on our results, the use of methamphetamine downregulates the expression of these genes. So far, no research has been done on the effect of methamphetamine on CatSper gene expression, but a review of various studies shows that methamphetamine use affects calcium channels.[41,42] In this line, Zhou reported that increased intracellular calcium in neurons causes neurotoxicity from various pathways.[41] In fact, calcium is a major mediator of neurotoxicity used by methamphetamine.[41−43] Anders et al. showed that the expression of voltage-dependent calcium channels is increased by amphetamine use and facilitates the entry of calcium ions into the cell by increasing the number of channels and disturbing the calcium balance within the cell, which is affected by amphetamine and causes cell death.[42] Methamphetamine-induced cell death occurs via processes that resemble apoptosis and is associated with DNA strand breaks, chromatin condensation, and nuclear dissolution.[16,44] Because the CatSper family of genes is present as a channel protein in the male fertility process, the expression of CatSper genes could be another cause of extinction in males if the expression is reduced.

In this research, methamphetamine treatment resulted in decreased testis and epididymis weights relative to the control rats. A review of previous research suggested that using methamphetamine influenced spermatogenesis and body, testicular, and epididymis weights.[13−20] Studies by researchers on sperms in cases who took 3,4-methylenedioxymethamphetamine (MDMA) (Ecstasy) showed serious injury to sperm DNA, interstitial edema in the testes, and tubular degeneration.[19,45] In another study, Taghavi et al. investigated the effects of methamphetamine on sperm characteristics of adult rats and reported that repeated use of high methamphetamine doses decreased the number of mature sperms in the epididymis and negatively affected the reproductive power of the rats addicted to methamphetamine.[46] In this line, Nudmamud-Thanoi reported that normal sperm motility was significantly decreased in animals receiving methamphetamine.[14]

Previous studies showed that there were CatSper ion channels in the principal piece of the sperm tail with an essential role in creating strong asymmetrical tail motion called hyperactivation, which enabled the sperm to move forward in the fallopian fluid. In other words, sperms that do not have CatSper lack the beating movements required to travel long distances and enter the ovule wall.[47,48] In spite of flagellar localization, CatSper mediates calcium influx that propagates within seconds through the head and midpiece after channel activation.[49] The lack of any of the four CatSper subunits disturbs the process of sperm hyperactivation in mice.[50] The role of CatSper in the final steps of murine sperm journey is shown in Moran's study in which targeted disruption of murine CatSper3 or 4 prevented sperms from hyperactivation but not from basic motility.[48] In humans, deletions in CatSper2[51,52] or an insertion in the CatSper1 gene[53] was diagnosed in patients with asthenoteratozoospermia. Also, interrupted structural continuity of CatSper location along the flagellum was seen in mice with the targeted disruption of CatSperζ that made the proximal piece of sperm tail inflexible and caused males subfertile.[54] Similar results were seen in Efcab9 knockout mice by Hwang et al.[55] Recently, Nowicka-Bauer showed that the EFCAB9 subunit is important for pH-dependent and Ca^{2+}-sensitive activation of the CatSper channel.[56]

Another gene involved in sperm motility is Mvh (Vasa).[30] It has been claimed that this gene express in germ-cell line derived from undifferentiated embryonic cells.[57] The expression of Mvh is limited to the gonads and is unremarkable

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Table 2. Primer Sequences of the Mvh and CatSper Family Genes

gene	forward	reverse
CatSper1	5′TCGGAGAACCACAGAGAAGAG3′	5′CACACACCGGGAATATCTTC3′
CatSper2	5′TGGCCACAGAGCAGTATTTG3′	5′TGTCAGGCTGTTGCTTTGTC3′
CatSper3	5′TCTTCCAACATCAGGCTCAG3′	5′GCTCTTCCTCCTCATGTTTG3′
CatSper4	5′TATTCCAGCCATCCTTCCAG3′	5′AAGGGGACACAGCAAAGATG3′
Mvh	5′GCT CAA ACA GGG TCT GGG AAG3′	5′GGT TGA TCA GTT CTC GAG3′
β-actin	5′AAGGCCAACCGTGAAAAGAT3′	5′ACCAGAGGCATACAGGGAC3′

in somatic tissues.[58] The Mvh protein is specifically expressed in polar granules and then especially shown during all germ cell stages.[59] Abd Razak determined the level of VASA gene expression in samples of testis cells of nonobstructive azoospermic (NOA) by testicular sperm extraction (TESE) to obtain testicular biopsies and compare it with sperms of healthy men. No Mvh expression was discovered in spermatogonial-like stem cells cultured on days 1, 7, 14, and 21 in the azoospermic samples.[60]

A study on the sperm chromatin and its parameters in addicts indicated that parameters such as the semen volume and number and motility of sperms decreased in these people.[61] A study by Taghavi et al. also suggested that the proliferation to apoptosis ratio changed in testis tissues of adult rats treated with methamphetamine. This is also considered an effective factor in fertility problems of methamphetamine-dependent men.[62] Research has shown that methamphetamine consumption decreases sperm motility.[24] Thus, based on our results, it seems that repeated methamphetamine use, even at low doses, considerably decreases expression levels of the Mvh and CatSper family genes, thereby increasing the probability of male sterility and infertility. An important limitation associated with this study is that the exact mechanism(s) through which methamphetamine exerts reduction in sperm-specific calcium channels involved in sperm motility remains to be elucidated.

CONCLUSIONS

This study fills the existing gap of knowledge about the adverse effects of methamphetamine on the expression levels of genes, e.g., Mvh and CatSper1−4 family, involved in sperm motility and fertilization. Decreased levels of these genes can increase the probability of male sterility and infertility.

MATERIALS AND METHODS

Ethical Statements. This experimental study was conducted at the Cellular and Molecular Research Center in Fasa University of Medical Sciences on 36 adult Wistar rats (8 weeks of age and weighing about 200 ± 20 g). They were bought from Shiraz University of Medical Sciences and transferred to the Laboratory Animals Center, Fasa University of Medical Sciences. The rats were kept for 2 weeks at 23 ± 2 °C and in a 12 h light/dark cycle with free access to food and water in polycarbonate cages with wood shavings for bedding to acclimatize to the environmental conditions. All ethical principles for experiments on laboratory animals were performed. Ethical approval was taken from the institutional review board of NAMS (IRB reference no. E-9311). All trials were done in agreement with relevant guidelines and protocols.

Sample Size Estimation. The sample size was calculated based on Nudmamud-Thanoi et al.[14] with the first-type error α equal to 0.05 and a power β of 0.90. The minimum sample volume was obtained for the study of 6. For the prediction of the probability of collapse and the total power increase in the study group, nine rats were chosen for each group of the study.

Experimental Design. Since methamphetamine was the active ingredient in this research, its doses were selected based on a previous study.[63] Mortality rates due to injections were also recorded. The rats were divided into four groups of nine rats each (one control and four experimental groups). Experimental groups 1−3 received 0.2 mL of a solution by a gavage that contained 0.5, 1, and 2 mg of methamphetamine/kg body weight for 45 consecutive days.[23] Methamphetamine was obtained in tablets from the Antinarcotic General Administration, Deputy of Food and Drug, Shiraz University of Medical Sciences, Iran. The control group received no drugs.

Hypothesis, Primary, and Secondary Outcome Measurements. The hypothesis was that the use of methamphetamine would be associated with a significant reduction in reproductive tissue weights and expression levels of Mvh and CatSper family genes as primary and secondary outcomes, respectively.

Body and Reproductive Tissue Weight. At the end of the experiment, the body weights were recorded. The rats were sacrificed under deep anesthesia by ketamine and xylazine. The testes and epididymides were removed surgically and weighed.

Total RNA Extraction from Testes. Total RNA was extracted from testes using the RNX Plus solution (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. The RNX Plus solution (1 mL) was poured onto the testes followed by incubation at room temperature for 5 min after which 200 μL of chloroform (Merck, Germany) was added to the mixture. The mixture was shaken vigorously and then centrifuged at 12 000g for 15 min at 4 °C. The supernatant was transferred to another microtube to which its equal volume of isopropanol (Merck, Germany) was added. The microtube was incubated in ice for 15 min and centrifuged again under the conditions of the previous centrifugation. Ethanol (75%, 1 mL) was poured on the obtained precipitate, and, following vortexing, the mixture was centrifuged at 7500g for 5 min. The resulting pellet was dissolved in 50 μL of water that was treated with 0.01% DEPC (Sigma-Aldrich, St. Louis, MO) and stored at −80 °C.

Qualitative and Quantitative Analyses of the Extracted RNA. RNA (1%) was prepared and its absorbance read at 260 and 280 nm. The RNA concentration (μg/L) was calculated using the following equation: (RNA) = A 260 × dilution × 40/1000. The A 260/A 280 ratio was also calculated to determine RNA purity.

Reverse Transcription. Total extracted RNA was reverted to complementary deoxyribonucleic acid (cDNAs) by the PrimeScript reagent kit (Cat. no.: RR037Q, Takara, Japan) per the manufacturer's instructions as follows: Oligo (dT) primer (1 μL) was added to 1 μg of extracted RNA and the volume was increased to 12 μL by adding water. The solution was

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incubated for 5 min at 70 °C in a normal thermal cycler and transferred to be placed on ice. Four microliters of amplification buffer (×5), 1 μL of RNasin, and 2 μL of dNTP mixture (20 mM) were added to denatured RNA. The reaction mixture was incubated for 5 min at 37 °C, 1 μL of MMLV reverse transcriptase was added, and the microtube was incubated first at 37 °C for 60 min and then at 70 °C for 10 min. The reaction mixture was stored at −20 °C to be used in PCR.

Polymerase Chain Reaction (PCR). PCR was done using SYBR green (Cat. no.: RR820Q, TaKaRa, Japan) on a StepOnePlus Real-Time PCR System (Applied Biosystems) using primers adapted from articles[64−66] and NCBI Primer Blast and made by Cinnagen (Tehran, Iran) (Table 2). PCR was performed as follows: Five microliters of amplification buffer (×5) (Cinnagen, Tehran, Iran), 1 μL of dNTP mixture (20 mM), 0.25 μL of Taq DNA Polymerase (Cinnagen, Tehran, Iran), 1 μL of upstream and downstream primer (20 μM), 3 μL of $MgCl_2$ (25 mM) (Cinnagen, Tehran, Iran), and 35 μL of deionized water were added to 5 μL of the RT reaction product. The microtube was then put into a normal thermal cycler, and 25−30 denaturation cycles (95 °C for 30 s), annealing cycles (58.5 °C for 30 s), extension cycles (72 °C for 2 min), and one final extension cycle (72 °C for 2 min) were run. Melt curves were checked, and the ratio of gene expression was determined using the comparative CT (cycle threshold) method.

Data Analysis. The results of the experiment were analyzed in SPSS 16.0 software at the 0.05 significance level. The mean ± SEM of three separate experiments in each group was analyzed using one-way analysis of variance followed by Tukey's posthoc test. The Kolmogorov−Smirnov test was used to examine the normality of distribution.

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Notes

The authors declare no competing financial interest.

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