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Correlation between expression of *CatSper* family and sperm profiles in the adult mouse testis following Iranian Kerack abuse

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SUMMARY

Illicit drug use can be an important cause of male infertility. The aim of this study was to investigate the effects of an Iranian illicit drug, Kerack, on sperm parameters, testicular structure and *CatSper* genes expression of mice. In this study, 25 male mice were divided into five groups consisting of control, sham and three experimental groups. All animal in experimental groups were addicted to Kerack for 7 days. These experimental groups include experimental I which was given Kerack at a dose of 5 mg/kg, experimental II, 35 mg/kg and experimental III, 70 mg/kg, intraperitoneally twice a day for a period of 35 days. Mice were then sacrificed and spermatozoas were removed from cauda epididymis and analyzed for count, motility, morphology (normal/abnormal) and viability. Right testes were removed, weighed and processed for light microscopic studies whereas left testes removed were subjected to total mRNA extraction for using in real-time PCR (RT-PCR). The results were analyzed by performing ANOVA (Tukey's tests) and Pearson correlation coefficient. Sperm parameters and seminiferous epithelium thickness were decreased in experimental groups (dose-dependently) vs. sham and control groups ($p < 0.05$). RT-PCR results showed that *CatSper 2, 3, 4* genes expressions were reduced with 35 and 70 mg/kg injected Kerack when compared with control testes ($p \leq 0.05$). However, *CatSper*1 expression was only reduced with high dose injected Kerack (70 mg/kg) in comparison to control testes ($p \leq 0.05$). This study shows the deleterious effects of Kerack used in Iran on testis structure and sperm parameters in general, and particularly sperm morphology in adult mouse. It could down-regulate the expression of *CatSper* genes, resulting in depression of sperm motility.

INTRODUCTION

Infertility is one of the most serious social problems facing the nations. In general, approximate half of all cases of infertility are caused by factors related to the male partner (Miyamoto *et al.*, 2011). Based on some national data sources have reported the number of drug abusers in Iran was found to be between 1 200 000 and 2 000 000 people (Razzaghi *et al.*, 2006; Shirinbayan *et al.*, 2010) and that they are generally young (Karbakhsh & Salehian Zandi, 2007). Then, infertility is an important issue in the country which can be of interest regarding the fact that illicit drug use includes use of anabolic steroids, marijuana, opioid narcotics, cocaine, and methamphetamines can be an important cause of male infertility (Fronczak *et al.*, 2011).

Iranian Kerack is a street name for a newer type of opiates, which is widely available and one of the most abused drugs in Iran (Narenjiha *et al.*, 2009; Akhgari *et al.*, 2012). Kerack is different from crack cocaine in other countries and heroin (Farhoudian *et al.*, 2014). Kerack analysis shows that its components consist of acetaminophen, acetylcodeine, caffeine, codeine, heroin, morphine, papaverin, tebain and some impurity (Akhgari *et al.*, 2012; Farhoudian *et al.*, 2014). It can be smoked, inhaled or injected by users (Razani *et al.*, 2007). The discontinuation of Kerack can produce withdrawal symptoms more rapidly in dependent persons, thereby requiring more frequent injection (Malekinejad & Vazirian, 2012).

Ion channel proteins generally, and calcium permeable channels in particular, play central roles during different stages of mammalian fertilization, including sperm capacitation (Yanagimachi, 1994), the acrosome reaction (Breitbart, 2002; Florman *et al.*, 2008) and sperm motility. These channel proteins consist

of voltage-gated Ca^{2+} channels (CaVs) (Trevino *et al.*, 2004), transient receptor potential channels (Castellano *et al.*, 2003; Aoshima *et al.*, 2013), cyclic nucleic gated channels (Wiesner *et al.*, 1998) and CATSPER *(1–4)* channels (Jungnickel *et al.*, 2001; Darszon *et al.*, 2005) in mammalian sperm. As a reminder, among CATSPER members CATSPER 1–4 are the ones which are exclusively expressed and plays major roles in sperm motility and sperm functions (Jin *et al.*, 2005; Li *et al.*, 2007; Qi *et al.*, 2007).

CatSpers have a six-transmembrane-spanning repeat and a pore region resembling the voltage-dependent calcium channels, whereas they form a single-unit channel (Ren *et al.*, 2001). However, it seems that the four CATSPER proteins are indeed associated with each other to form a tetramer (Lobley *et al.*, 2003; Qi *et al.*, 2007), and principally localized to the membrane of sperm principal piece (Ren *et al.*, 2001; Quill *et al.*, 2003; Kirichok *et al.*, 2006; Qi *et al.*, 2007). Studies have suggested essential roles of *CatSper1* and *CatSper 2* for mouse sperm motility whereas *CatSper3* and *CatSper4* are crucial for acrosome reaction (Jin *et al.*, 2005), sperm hyperactivated motility, late motility and male fertility in mice (Jin *et al.*, 2007; Qi *et al.*, 2007). All four *CatSper* genes are required for male fertility as disruptions in any of these genes are also associated with male infertility in mice. This may also be because of incapability of hyperactivated sperm motility in zona pellucida penetration (Quill *et al.*, 2001; Ren *et al.*, 2001; Carlson *et al.*, 2005; Jin *et al.*, 2007; Qi *et al.*, 2007).

Sperm motility is one of the most important predictors of fertilizing ability (Yilmaz *et al.*, 1999; Rossato *et al.*, 2005; Fazelipour & Tootian, 2007; Badawy *et al.*, 2009) whereas several correlations between the *CatSper* gene expression and sperm motility have been reported in previous studies (Kidd *et al.*, 2001; Ren *et al.*, 2001; Jarow, 2002; Qi *et al.*, 2007). As *CatSper* is one of the most responsible genes for producing sperm motility, in the present study, we investigated the effect of Kerack on the level of *CatSper* gene expression and several reproductive parameters in Iranian Kerack-addicted mouse to assess any potential abnormality linked to sperm motility.

MATERIALS AND METHODS

Animals

Twenty-five 3- to 5-week-old male Balb/C mice were obtained from animal facility of Pasteur Institute (Tehran, Iran) and moved to the animal house of Iran University of Medical Sciences (Tehran, Iran). The mice were housed in polycarbonate cage in a room with 12 h dark/light cycle with a temperature maintained at 23 ± 2 °C during the study period, and the relative humidity ranged between 35 and 60%. They were fed a standard pelleted diet and allowed water ad libitum. The animals were acclimatized to the laboratory conditions for 2 weeks before the start of the experiments. All animal experimentation protocols were approved by Institutional Animal Care and Use Committee of Iran University of Medical Sciences, Tehran, Iran.

Preparation of addicted animal

To investigate the effect of Kerack on sperm parameters and *CatSper* family in clinic, we provided an addicted model to study drug abuse similar to patients who consume Kerack. So, all mice in experimental groups (I, II, III) were addicted to Kerack for 7 days. For the first 3 days they, respectively, received intra-peritoneal (IP) Kerack with doses of 20, 25 and 30 mg/kg of body weight. On the fourth/fifth, sixth and seventh day, they received Kerack at the 40, 60 and 80 mg/kg (IP) dosage twice a day, respectively.

Testing of the withdrawal syndrome

The withdrawal syndromes were measured by injection of Naloxone. Addicted mice were tested for previously identified behavioral characteristics of the mice opiate abstinence syndrome such as jumping, shaking and exploring (Rasmussen *et al.*, 1996) by injection of Naloxone HCl (5 mg/kg) 2 h after the first administration of Kerack on seventh day. Five addicted mouse was randomly selected and checked for withdrawal syndrome just for one time.

Experimental design

The animals were weighed and randomly divided into five groups ($n = 5$ in each group): (i) Control group received only water and food; (ii) Sham group received only 220 μL of normal saline and lemon juice (2.6 μL/mL); (iii) experimental group I received Kerack at a dose of 5 mg/kg after addiction to Kerack; (iv) experimental group II received Kerack at a dose of 35 mg/kg after addiction to Kerack and (v) experimental group III received Kerack at a dose of 75 mg/kg after addiction to Kerack (IP) for the remaining 35 days twice a day. After 6 weeks treatment with Kerack, the mice were sacrificed by cervical dislocation and the testes and epididymis in the control and experimental groups were immediately removed.

Measurement of the changes in body weight, testis weight and gonadosomatic index (GSI)

To determine the changes in body weight (at the beginning of the experiment); each of the male mice was weighed before being anaesthetized. After washing the testes, their weights were measured. The ratio of the weight of both testicles to the body weight was calculated and the percentage was determined and recorded as GSI.

Sperm parameters assessment

Following the mice sacrifices via cervical dislocation, the cauda epididymis were minced in 1 mL of phosphate-buffered saline and incubated at 37 °C in 5% CO_2 for 30 min. Then the clear fluid was used for the analysis of sperm profile including viability, concentration, motility and abnormality according to World Health Organization Criteria (fifth edition) with some modifications (Khaki *et al.*, 2009a,b; Awodele *et al.*, 2010; Eybl & Kotyzová, 2010).

Sperm viability was assessed by determining the percentage of sperms excluding vital dye (0.5% eosin B solution). Live spermatozoa remained colourless following staining whereas red sperm heads were considered and classified as dead ones. The sperm concentration was determined with a haematocytometer. The motility was assessed (as rapid linear progressive, slow linear progressive, vibratory and non-motile) and counted. The percentage of normal/abnormal spermatozoa which determined with the Papanicolaou staining procedure was also assessed under a light microscope. With the staining, the nuclei turned blue, whereas the acrosome and the tail became pink. The morphology of 200 sperms was observed under ×100 oil immersion

lenses. Abnormal morphology was randomly counted from each sample in five fields of vision and percentage of abnormal morphology was recorded and categorized in three groups including head, neck and tail abnormality.

Histopathological study of testes

Following cervical dislocation testes were removed and weighted. Right testis was fixed in Bouin's fixative for 48 h; dehydrated in various grades of ethanol, cleared in xylene, infiltrated and embedded in paraffin. Then 5 μm serial microscopic sections were prepared and at least five slides from each testis were stained with hematoxylin and eosin for histological assessment. The diameter of seminiferous tubules, diameter of lumen and height of seminiferous epithelium were randomly measured in 100 rounds or nearly round tubular sections at 400× magnifications per animal. The digitized images were captured utilizing the camera microscope (Olympus, Tokyo, Japan) and analyzed with Image J software (version 1.240; National Institutes of Health, Bethesda, MA, USA) for morphometric study.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA were extracted from mouse testes frozen in liquid nitrogen using RNeasy Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's recommendations. The purity and integrity of RNA were checked by a 260/280 nm ratio measurement. A minimum optical density (OD260/280) ratio of 1.80 was required for the following RT-PCR.

Quantitative PCR reactions were set up in triplicate with the Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, St. Leon-Rot, Germany) and analyzed with a Real-Time PCR system of Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia). For qRT-PCR, 300 ng of cDNA template was used in a 25 μL reaction volume with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas) and run on a Rotor-Gene 6000. Expression values were normalized to the average expression of the housekeeping gene (*ß2m*) and compared with a calibrator (control group) by the comparative CT method ($2^{-\Delta\Delta ct}$). The primer sequences of *CatSper* family genes were designed using http://www.ncbi.nlm.nih.gov site and Beacon Designer software as shown in Table 1.

Statistical analysis

The results were analyzed by performing one-way ANOVA and Tukey's test. $p < 0.05$ was considered as statistically significant and mean ± SD was also calculated for each value. Correlation between expression of *CatSper* family and sperm profiles was determined with Pearson correlation coefficient. Correlations were designated as small (0.10–0.29), medium (0.30–0.49) and large (0.50) (Cohen, 1988).

RESULTS

Establishment of addicted model in mice via intraperitoneal injection

Kerack administrated mice showed euphoria signs after substance injection including tail stiffness and twirling around the cage over 20 min (Video Clip S1). Withdrawal signs (jumping, shaking, exploring and also scratching and hand licking) were increased in naloxone-administered mice (Video Cilp S2). Therefore, the addicted model in mice via IP injection was established.

Body weight, testis weight and gonadosomatic index

For each animal, body weight was recorded at the beginning and after termination of treatment. No significant difference was observed in the mean weight of all groups at the beginning of the period. As shown in Table 1, at the end of the 42-day period, there was a decrease in body and testis weights in all experimental groups compared with control group ($p < 0.05$). The decrease in the weights of body and testes was in a dose-dependent manner and a maximum decrease was observed at a dose of 70 mg/kg in experimental group III. The mean GSI in experimental groups I and II was lower than control group, but there was no significant difference. However, a decrease was observed in mean GSI in experimental group III in comparison with control groups ($p < 0.05$) (Table 2).

Sperm parameters analysis

According to our data, there was a significant decline in sperm's viability and motility rates as well as in number of sperms following addiction to Kerack (Table 2). The mean sperm concentrations were 7.25 ± 0.29, 6.73 ± 0.14, 6.05 ± 0.25 and 5.80 ± 0.30 million/mL in control and experimental groups I-III, respectively. Treatment of mice with doses of 5, 35 and 70 mg/kg led to a decrease in sperm concentration when compared with control group ($p < 0.05$) (Table 3). Sperm concentration also decreased in a dose-dependent manner.

Table 2 The effect of Kerack on body weight, testis weight and gonadosomatic index (GSI) in adult mice

Groups	Body weight	Testis weight	GSI
Control	27.37 ± 0.45	134 ± 4	0.49 ± 0.02
Sham	26.82 ± 0.24	132 ± 1	0.49 ± 0.01
Experimental I	24.05 ± 2.30[a]	116 ± 13[a]	0.48 ± 0.03
Experimental II	23.22 ± 2.02[a]	106 ± 9[a]	0.46 ± 0.002
Experimental III	22.87 ± 0.26[a]	102 ± 6[a]	0.45 ± 0.03[a]

Results from five separate experiments were used for all groups. Values are mean ± SD at different times. [a]Significant difference vs. control group in the same column ($p < 0.05$).

Table 1 Sequences of the designed primers used for real-time PCR

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product length
Catsper1	CGCTGCTTCACTGTCATGTT	CGGGTCCATGAGAAGTTGTT	134
Catsper2	TGTGCCTTGGTACGGCTTGGC	CCACGTGAGACATGGCACAAGAACA	132
Catsper3	AGCCATCAACCGTGGCCAAACT	ATCGCTGTGGGGGAGACGGT	184
Catsper4	GCAGCTGGAAGGCCGGATGG	CACCTGGGCCCTAAGAGAAAAGCC	166
β*2m*	CAGTCTCAGTGGGGGTGAAT	ATGGGAAGCCGAACATACTG	177

Table 3 The effect of Kerack on Cauda epididymal sperm count, viability, progresive motility and normal morphology in adult mice

Group	Sperm count ($\times 10^6$)	Viability (%)	Progressive motility (%)	Normal morphology (%)
Control	7.37 ± 0.21	83.62 ± 2.64	36.92 ± 3.44	79.93 ± 1.52
Sham	7.25 ± 0.29	83.79 ± 2.13	40.38 ± 4.12	81.09 ± 0.69
Experimental I	6.73 ± 0.14[a]	75.38 ± 1.98[a]	33.31 ± 3.67	63.26 ± 1.65[a]
Experimental II	6.05 ± 0.25[a,b]	66.27 ± 1.77[a,b]	30.59 ± 1.45[a]	50.02 ± 1.24[a]
Experimental III	5.80 ± 0.30[a,b]	61.85 ± 1.16[a,b,c]	29.26 ± 0.39[a,b]	43.03 ± 1.94[a]

Results from five separate experiments were used for all groups. Values are mean ± SD at different times. [a]Significant difference vs. control group in the same column ($p < 0.05$). [b]Significant difference vs. experimental group I in the same column ($p < 0.05$). [c]Significant difference vs. experimental group II in the same column ($p < 0.05$).

A statistically significant decline in the sperm viability was also observed following addiction to Kerack (Table 3). The mean percentage of viable spermatozoa in all experimental groups I-III (75.38 ± 1.98, 66.27 ± 1.77 and 61.85 ± 1.16, respectively) showed a decrease ($p \leq 0.05$), compared with control group (83.62 ± 2.64). The decrease in the sperm viability was also dose-dependent.

The mean percentage of progressive motile sperm in experimental group I (33.31 ± 3.67) was lower than control group (36.92 ± 3.44), but there was no significant difference. However, a dose-dependent decrease was observed in mean percentage of progressive motile sperm among experimental groups II (30.59 ± 1.45) and III (29.26 ± 0.39), compared with control group ($p \leq 0.05$) (Table 2). The mean percentage of morphologically normal sperm in all experimental groups showed a decrease when compared with control group ($p < 0.05$) (Table 3).

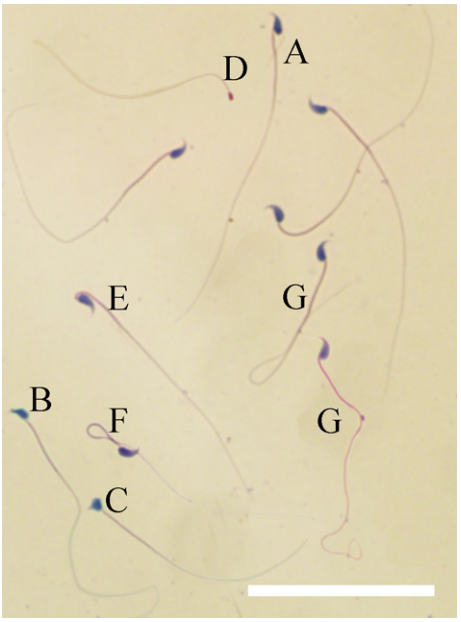
Sperm abnormalities

The counts of abnormal sperm showed an increase in the mean percentage of pin head and abnormal tail among all experimental groups when compared with control group ($p \leq 0.05$) (Table 4, Fig. 1). The mean percentage of quasi normal head, head and tail abnormality increased in experimental groups II and III, when compared with control group ($p < 0.05$). However, these abnormalities in experimental group I were higher than control group with no significant difference. Generally, the mean percentage of total morphologically abnormal sperm in all experimental groups increased when compared with control group ($p < 0.05$) (Table 4).

Histopathological study

Histological examination of seminiferous tubules cross-sections in mouse testes following Kerack treatment showed that the cycle of spermatogenesis was regular in all experimental and control groups (Fig. 2). As shown in Table 5, there was a mild non-significant decrease in the mean diameter of seminiferous tubules of the experimental mice when compared with control group. A dose-dependent decrease was also observed in the thickness of seminiferous epithelium and diameter of tubular lumens among the mice of all experimental groups when compared with control group ($p < 0.05$) (Table 5) Scale bar:100μm.

Figure 1 Morphology of epididymal sperm from Balb/C mice, as assessed by staining with Papanicolaou method. (A) Sperm with normal morphology. (B) Sperm with quasi-normal head. (C) Sperm with abnormal head. (D) Pinhead sperm. (E) Sperm with a bent neck. (F) Sperm with curved mid piece. (G) Sperm with twisted flagella.



Changes in *CatSper* genes expression following addiction

As shown in the Fig. 3, *CatSper 1* expression in the experimental group I and II increased, although decreased in experimental III (52.61 ± 5.63) when compared with the control group ($p \leq 0.05$). In addition, the expressions of all *CatSper* 2

Table 4 The effect of Kerack on percentage of Cauda epididymal sperm abnormal morphology in adult mice

Groups	Quasi normal head (%)	Head abnormality (%)	Pin head (%)	Mid piece abnormality (%)	Tail abnormality (%)	Total abnormality (%)
Control	3.19 ± 1.23	1.78 ± 0.89	0.27 ± 0.37	2.31 ± 0.31	12.52 ± 1.0	20.06 ± 1.52
Sham	2.36 ± 0.59	2.35 ± 0.34	0.7 ± 0.61	2.41 ± 0.37	11.09 ± 0.95	18.9 ± 0.69
Experimental I	4.3 ± 1.4	3.7 ± 0.28	2.6 ± 0.99[a]	2.66 ± 0.31	23.46 ± 0.96[a]	36.72 ± 1.64[a]
Experimental II	7.31 ± 1.0[a]	6.74 ± 1.09[a]	4.61 ± 1.36[a]	3.61 ± 0.42[a]	27.71 ± 0.77[a]	49.98 ± 1.24[a]
Experimental III	9.21 ± 1.35[a]	10.13 ± 0.91[a]	5.1 ± 0.52[a]	3.59 ± 1.21[a]	28.93 ± 1.26[a]	56.97 ± 1.94[a]

Results from five separate experiments were used for all groups. Values are mean ± SD at different times. [a]Significant difference vs. control group in the same column ($p < 0.05$).

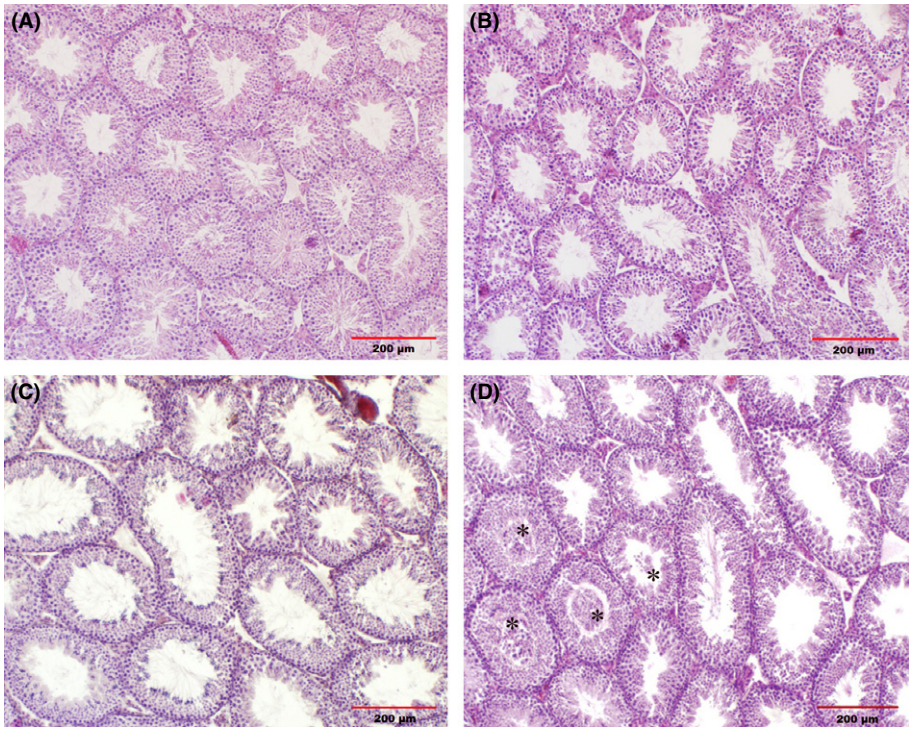


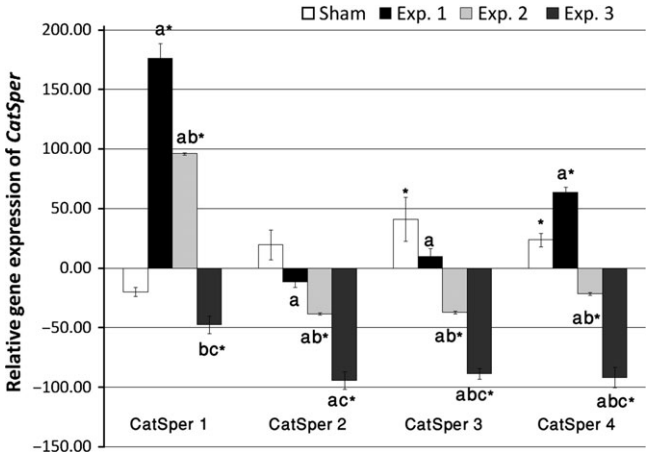
Figure 2 Seminiferous tubules cross-sections of mouse testis in control and experimental groups staining with H&E. (A) Regular seminiferous tubules with normal germinal epithelium morphology and sperm presence in lumen in the control group. (B) Testicular section from a treated mouse with Kerack (5 mg/kg twice a day). (C) Testicular section from a treated mouse with Kerack (35 mg/kg twice a day). (D) Testicular section from a treated mouse with Kerack (70 mg/kg twice a day). *Disorganized seminiferous epithelium with germ cells fallen down into the tubular lumen.

Groups	Diameter of seminiferous tubules	Diameter of tubular lumens	Seminiferous epithelium thickness
Control	195.014 ± 3.243	65.104 ± 2.382	129.91 ± 5.519
Sham	194.682 ± 6.592	62.319 ± 4.421	132.363 ± 2.409
Experimental I	191.262 ± 2.754	69.69 ± 2.074[a]	121.572 ± 0.938[a]
Experimental II	188.284 ± 8.279	84.989 ± 3.783[a]	103.295 ± 4.497[a]
Experimental III	186.057 ± 8.297	89.318 ± 4.941[a]	96.739 ± 3.417[a]

Table 5 The effect of Kerack on diameter of seminiferous tubules, lumens and seminiferous epithelium thickness (µm)

Results from five separate experiments were used for all groups. Values are mean ± SD at different times. [a]Significant difference vs. control group in the same column ($p < 0.05$).

Figure 3 Comparison between the relative gene expression of CatSpers and *β2m* in the treated mouse with Kerack. Values are shown as the mean ± SD. The experiments were replicated at least three times. *Significant difference vs. control group in the same gene ($p < 0.05$). [a]Significant difference vs. sham group in the same gene ($p < 0.05$). [b]Significant difference vs. experimental group I in the same gene ($p < 0.05$). [c]Significant difference vs. experimental group II in the same gene ($p < 0.05$).



(61.65 ± 16.27, 5.91 ± 0.04), *CatSper* 3 (62.97 ± 3.92, 11.45 ± 2.61) and *CatSper* 4 (78.64 ± 8.42, 8.39 ± 0.70) were declined in the experimental II and experimental III mice testes, when compared with the control group ($p \leq 0.05$). The results showed that there is a correlation between the sperm progressive motility and *CatSper* 2–4 expressions in the groups. Pearson correlation coefficient between expression of *CatSper* 1–4 and sperm profiles was −0.322, 0.723, 0.688 and 0.373, respectively. The results showed that there is a correlation between the sperm progressive motility and *CatSper* 2–4 expressions in the groups. Pearson correlation coefficient between expression of *CatSper* 1–4 and sperm profiles was −0.322, 0.723, 0.688 and 0.373, which showed medium, large, large and medium correlations respectively.

DISCUSSION

Considering the critical roles of *CatSper* family members in sperm motility and male fertility, we have investigated the expression level of the *CatSper* genes as well as their relationships to sperm profile in adult mouse testis following Kerack abuse. So, we used a RT-PCR amplification to provide valuable new information on the relationship of expression level of *CatSper 1–4* in mouse testis following substances abuse. The present study revealed that testicular weight, epididymal sperm parameters and thickness of seminiferous tubules epithelium and the relative expression of the *CatSper* genes were decreased among the mice addicted to Kerack. Also the effects of higher doses of Kerack on assessed sperm parameters dramatically increased.

o confirm the mouse addicted model, naloxone was administrated after 7 days of treatment with Kerack. Withdrawal signs were appeared following administration of naloxone. As a conclusion, it may be related to the opioid activity of Kerack which is a subject to be confirmed in a further study. Other researchers showed that administration of naloxone can reverse morphine effects in decreasing fertility in male rats (Packman & Rothchild, 1976).

Our study showed that the Kerack could change the body weight and testicular weight, but there was no significant difference in the GSI of control and experimental groups I and II. An inhibition in androgen production has already been expected to be associated with its progressive decrease in metabolism, and body and testicular weights (Yilmaz *et al.*, 1999). Yamamoto *et al.* (2002) also suggested that apoptosis in seminiferous tubules can decrease testicular weight. Our finding is concordant and consistent with others addressing decreased body and testicular weight upon administration of heroin (Fazelipour & Tootian, 2007) or chronic exposure to morphine (Yilmaz *et al.*, 1999).

In the present study, epididymal sperm parameters (sperm count, viability, progressive motility and normal morphology) decreased following Kerack treatment in adult mice in a dose-dependent manner. Our findings supported previous reports of heroin (Fazelipour & Tootian, 2007) and nicotin (Oyeyipo *et al.*, 2011) consumption in animal and opioids in human (Khan *et al.*, 2003) that showed decreases in sperm parameters. The mechanism by which illicit drugs deteriorate the sperm profile has been elucidated. Other studies have shown a decrease in testis cellular function which may be because of decreased serum testosterone levels after heroin and chronic morphine administration (Yilmaz *et al.*, 1999; Fazelipour & Tootian, 2007). Endogenous opioids can suppress gonadotropin release (Blank *et al.*, 1994) and likely to influence testosterone secretion by the hypothalamo-hypophyseal-gonadal axis or putative opioid receptors in the testis (Margioris *et al.*, 1989; Wittert *et al.*, 1996). Another study also suggested that exogenously administered opioids may have a similar effect on the hypothalamic-pituitary axis (Rajagopal *et al.*, 2004). Endogenous opioid receptors are present in Sertoli cells of testis (Orth, 1982), as well as central and peripheral nervous system (Gnessi *et al.*, 1997). These cells also play an important role for development of a normal spermatogenesis in the adult and the production of normal numbers of germ cells (Orth, 1982; Orth *et al.*, 1988). We conclude that a reduction in some sperm profile (e.g. sperm count, testis weight and epithelium height of seminiferous tubules) may result from decreased serum testosterone level.

In our study, the sperm morphology assay showed that sperm abnormality in general and abnormal sperm tail, in particular, increased following Kerack administration in mice. These findings are supported by another cross-sectional study that showed, long time addiction to heroin, morphine and other narcotics increase amount of morphologically abnormal spermatozoa with head and neck abnormalities and immature forms (Singer *et al.*, 1986). The most prominent morphological abnormalities observed in the Kerack-treated mice were curve tail, curve midpiece forms, pin head and abnormal head. Tail abnormalities usually occur during epididymal transit, maturation and storage of sperm during which period the spermatozoa develop motility (Tulsiani *et al.*, 1998).

The diameter and germinal epithelium height of the seminiferous tubules are two important parameters for assessment of the spermatogenesis defects and its pathology (Osinubi *et al.*, 2005). In this study, the diameter and epithelium height of seminiferous tubules reduced in treated groups. The tubular diameter and the epithelium height can indicate spermatogenesis activity in experimental and toxicological analysis (Berndtson & Thompson, 1990; Russell *et al.*, 1990). Some researchers reported that reduction in the diameter and epithelium height of seminiferous tubules may be because of a decrease in the number of type A spermatogonia, spermatocyte, spermatid and Sertoli cells (Mohammad-Ghasemi *et al.*, 2006; Soleimani-Mehranjani *et al.*, 2009). The results of our study are in accordance with the one recently showed that methadone administration prevented spermatogenesis, sloughed (degenerated) the germinal epithelium in seminiferous tubules of rat testis (Heidari *et al.*, 2012). Therefore we suppose that Kerack administration may reduce diameter of germinal epithelium in the seminiferous tubules.

The major role of *CatSper* four family members in sperm motility and male fertility (Fattahi *et al.*, 2009) as well as potential targets for screening of male infertility and ideal targets for contraception (Quill *et al.*, 2001; Ren *et al.*, 2001; Nikpoor *et al.*, 2004; Katz *et al.*, 2013) has been previously demonstrated. A decrease in *CatSper* expression has also been reported in subfertile men with immotile sperm (Nikpoor *et al.*, 2004). Here we demonstrated that Kerack treatment in a dose-dependent manner result in a synchronous down-regulation and decrease in *CatSper*s *(1–4)* and the epididymal sperm parameters (sperm count, viability, progressive motility and normal morphology), respectively. Also administration of 5 mg/kg Kerack caused an increase in expression of *CatSper 1 and 4*, however, motility analysis showed no significant difference when compared with control sperm. As previously described, elimination of any *CatSper* gene (*CatSper1, CatSper2, CatSper3 and CatSper4*) decreases sperm progressive motility over time (Quill *et al.*, 2003; Qi *et al.*, 2007). So, we have assessed only progressive motility sperm as an indicator for hyperactivated motility. It seems that Kerack has a wide range of targets and can independently influence either *CatSper* levels and sperm parameters. Probably *CatSper* family is not the alone genes or factors damaged and other factors might be affected in Kerack treatment model and will be addressed in future studies. On the other hand, it may suggest that level of RNA transcript does not necessarily correlate with protein level or channel activity. For this reason, although there is no particular association between *CatSper*1 and 2 expressions among the experimental groups (I, II, III), rather a decrease is observed in the *CatSper* 3 and *CatSper* 4 expressions of all experimental mice testes, compared with the control group. Our finding is consistent with the reduction in all sperm parameters and down-regulation of *CatSpers 1* and *2* as two critical genes in sperm motility following spinal cord injury (SCI) in a mouse model (Rezaian *et al.*, 2009). Of course, as *Castper* genes encode proteins that constitute calcium channel in the principal piece of flagellum, only motility could be correlated with a decreasing in down-regulation of *CatSpers* 1 and 2 in SCI model.

On the other hand, Selenium (Se) as an antioxidant has a profound effect on *CatSper* expression in the ageing male mice. A synchronous *CatSpers* over-expression and improved sperm parameters (especially its morphology and viability rates) has

also been reported (Mohammadi *et al.*, 2009). Previous studies have shown that usage of some illicit drugs including heroin may result in ROS formation (Pan *et al.*, 2005; Xu *et al.*, 2006). Therefore, Kerack – a heroin derivative – may also increase the production of ROS in testis. Then excessive free radicals may inhibit the expression of proteins such as CatSper family which are involved in flagellar movement and ciliary beating of spermatozoa.

CONCLUSIONS

In summary, the present study showed that Kerack used in Iran decreases epididymal sperm profile, down-regulate the expression of *CatSper* genes and changes testes morphology in male adult mice. It is also shown that administration of Kerack at high doses is much more destructive than its lower doses.

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AUTHOR CONTRIBUTIONS

MA, PS performed the research; PS, MK analyzed the data, MK, BB, MTJ, MR wrote the paper and MK, PS AF designed the research study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Video Clip S1. Addicted model to Kerack abuse: Euphoria signs were observed after substance injection including tail stiffness and twirling around the cage.

Video Clip S2. The withdrawal syndromes by injection of Naloxone: Withdrawal signs (jumping, shaking, exploring and also scratching and hand licking) were increased in naloxone-administered mice.