ORIGINAL ARTICLE
Effects of curcumin on sperm parameters abnormalities induced by morphine in rat

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Opioids are the most potent and effective analgesics available and have become accepted as appropriate treatment for acute, cancer and non-cancer. Morphine, which is commonly used for the treatment of severe pain, gastrointestinal tract and kidneys. Curcumin petals consist of, glycosides, flavonoids, and anthocyanin.

The study aims at evaluating curcumin effect and morphine on sperm parameters, testis tissue and serum testosterone level in rat. In this experimental study, 48 male rats with 28 weeks of age and limited weight of 270 to 300g were selected. They were divided into eight groups of 6, untreated control group; morphine–treated group (20 mg/kg/day); curcumin–treated groups (10, 30, 60 mg/kg/day); and morphine and curcumin treated group intraperitoneal administration for successive 28 days. After 24 hours animals were killed.

Sperm motility was measured using WHO protocols. The sperm parameter such as motility, sperm count, morphology, seminiferous tubules diameter, weight testis, and serum testosterone level were analyzed (one-way ANOVA). Curcumin (10, 30 and 60 mg/kg) significantly increased mean percentage of sperm motility, count, testis weight, and serum testosterone level compared to control group (p<0.05). Testosterone level decreased significantly in rats treated with morphine. Co-administration of curcumin to morphine-treated rats improved the histopathological alterations induced by morphine in testis and increased the sperm count. Curcumin has a very strong antioxidant effects at applied doses and it can probably be used as an antioxidant and food supplement in reproductive disorders.

Keywords: curcumin, sperm, morphine, antioxidant, rat

INTRODUCTION
White or light brown crystalline morphine is extracted from opium (the main ingredient of morphine) or directly from poppy straw (Jonsson et al., 1988). Opioids are used to regulate gonadal functions by binding to the opioid receptors in hypothalamus and testicles because of their endogenous and exogenous properties. Direct effects of opioids on testis including reductions in testosterone generation and semen liquid. Opioids increases release of prolactin in the pituitary (Jordan et al., 1996; Drolet et al., 2001).

Studies on heroin addicts indicated a decline of testosterone levels in these men accompanies with lower levels of LH and FSH compared to the normal men (Bliesener et al., 2005). Spermatozoa as well as all living cells in aerobic conditions are facing a contrast on oxygen. This means that it requires oxygen to survive besides oxygen metabolites such as reactive oxygen species (ROS) which can damage the cell survival. ROS results in oxidative stress in sperm (Saleh and HCLD, 2002).

Curcumin is the active ingredient of turmeric rhizome with chemical name of methane diferuloyl which is useful in protecting the brain and body organs, preventing the growth of cancer cells and in treatment of Alzheimer’s disease, rheumatoid arthritis, diabetes, respiratory diseases, atherosclerosis and neurodegenerative diseases (Verghe, 1993; Goel et al., 2008). This material has a high antioxi-
Curcumin effects on sperm parameters in rats
Roshankhah et al.,

Dant potential.

In addition, curcumin have a protective effect on lipid oxidation of rats in oxidative stress and acts as a free radical scavenger and increasing intracellular glutathione. In traditional medicine, turmeric is mentioned as the plant laxative, bile and liver protector (Kurzrock and Li, 2005; Bisht and Maitra, 2009). Currently, there is paucity of data on the effects of curcumin on morphine-induced damages. Due to the toxic effects of morphine and antioxidant properties of curcumin, the study aims determining the effect of curcumin on morphine-induced damages on sperm parameters in male rats.

MATERIALS AND METHODS
In this study, adult male rats weighing 300-270g were purchased from Razi Institute of Iran. They were kept in Animal House for a week before the start of the experiment under laboratory conditions and at a temperature of 20 ± 2°C, 12 hours of light and 12 hours of darkness. Access to water and regular food for mice in the study will be unrestricted.

The total number of mice were 48. Rats in this study were divided into control and experimental groups. The mice kept in standard cages in Animal House School of Medicine were used. 6 rats were placed in each cage. Mice were divided into two groups. The experimental group was divided into eight groups.

Control group receive saline, experimental group1 received 20mg/kg of morphine, the experimental group 2 got 10mg/kg curcumin + 20mg/kg of morphine, experimental group 3 received 30 mg/kg curcumin + 20 mg/kg of morphone, experimental group 4 got 60 mg/kg curcumin + 20mg/kg of morphine, the experimental group 5 received 10mg/kg curcumin, experimental group 6 had 30mg/kg curcumin, experimental group 7 had 60mg/kg curcumin, for the saline group as the control group, normal saline solution (0.9%) of Razi serum company was used. For the experimental groups, curcumin initially weighed based on the concentration listed and then dissolved in a solution of absolute ethanol (C2H5OH), and the desired volumes and doses of the study was reached by solution of normal saline 9.0% (Hughes et al., 2004; Taepongsorat et al., 2008).

After 28 days of intraperitoneal injection, the rats anesthetized in the supine position, incision in the cardio, bleed from the left ventricle was done immediately by 5ml syringes to measure hormone levels available in blood serum. The blood was incubated for half an hour, then centrifuged at around 2500 rpm for 30 minutes. Serum was pick up and kept at -20°C until blood sample measurement. Using ELISA method, serum testosterone levels were determined by specific kits. Used kit was prepared from Monobind and Immunotech company.

Sperm sample preparation
The testicles and epididymis in the inferior quadrant of abdomens were removed by an incision and the left testis and epididymis were removed immediately. After anesthetizing the rats and blood sampling of the cardio, the left epididymis immediately removed and the tissues separated under stereo microscope. The tail of epididymis was separated and transferred to a petri dish containing DMEM/F12 (including CO2) by 5ml and divided into smaller pieces in order to move sperms into the culture medium and incubate for 7 minutes at 37°C.

Evaluation of sperm motility
Sperm motility of each sample was evaluated by a light microscope at 40x10 magnification. About 50ml of growth medium containing semen liquid was taken and poured on glass slides which have already been cleaned and dried by alcohol and covered with coverslip and viewed under a microscope. Sperm motility performed at 4°C and based on WHO procedures. Sperm counting was done via cell counter and about 100 sperm per sample were counted.

Sperm motility grading such that, zero: do not move, Class 1: move forward, Class 2: rapid move forward Class 3: progressive move. Counting was repeated in all experimental groups and the control group.
Evaluation of Viability

The most common way to determine cell survival and identification of live sperm from dead sperm, is cell counting using eosin staining based on dye uptake by the dead cells membrane and disposal by the membrane viable cells. To determine the effect of each concentration lonely, about 20uL of medium containing semen liquid was taken from each Petri dish after a given time, then mixed with equal volume of eosin stain solution (20 ml of each). After 2-5 minutes, some of this mixture was poured onto Neubauer slide.

Live sperms remained transparent and dead sperms becomes pink colored. Prepared glass slides were investigated at 40x magnification. 100 spermatozoa (including transparent and colored sperms) were counted in different microscopic fields of view. Finally, the survival rate was calculated by the following formula:

\[
\text{Cell viability (%) = } \frac{\text{No of viable cells (unstained cells)}}{\text{Total no of cells (stained and unstained)}} \times 100
\]

Sperm Morphology

“Feathering” is a way to prepare the non-diluted semen liquid. In this method, the semen liquid is taken by Pasteur pipette and spilled on a slide, then semen drops spreads along the back edge of the angled slide and is pulled forward over the flat slide to form the smear. In smear, both sides of the glass slide must be washed and dried with alcohol primarily, then put a label on that as specification using a diamond pen and about 5-10uL of semen liquid was taken and transferred on slides. For quantitative and qualitative assessment of sperm morphology, involves detection of abnormal sperms from normal ones.

Assessment of Seminiferous pipes diameter

Testicular tissue samples in a serial tissue cut were prepared from each rat. 5 cutting from each sample were stained and studied. Control samples considered as normal and changes caused by morphine and curcumin were evaluated compared to controls.

Curcumin effects on sperm parameters in rats
Roshankhah et al.,

Statistical analysis

Data was entered and analyzed using SPSS software (version 20.0). Quantitative data were compared using one-way ANOVA and Tukey test considering significant (P<0.05).

RESULTS

Assessment of testicles weight changes

Results of the left testicle weight between two groups showed a significant decrease in testicular weight in the groups receiving morphine compared to the control group. In the group receiving morphine + curcumin at a dose of 60mg/kg, significant increase in testicular weight was observed compared to group receiving morphine alone. Also increasing the weight of testes in groups receiving Curcumin with doses of 30 and 60mg/kg was observed compared to morphine receivers that the increase was significant. There was a significant decrease in testicular weight in the groups receiving morphine compared to the control group. In the group receiving morphine + curcumin at a dose of 60mg/kg significant increase in testicular weight was observed compared to group receiving morphine alone. Also increases in weight of testes in groups receiving curcumin at doses of 30 and 60 mg/kg was observed compared with the group receiving just morphine which the increase was significant (Figure 1).

Evaluation of sperm parameters

The results of counting the number of sperm in study groups showed a significant reduction in groups receiving morphine as well as groups receiving morphine + curcumin at doses of 30, 50 and 60
Curcumin effects on sperm parameters in rats
Roshankhah et al.,

compared to control group. Also in groups receiving curcumin with doses of 10, 30 and 60mg/kg significant increase in the number of sperm observed compared to morphine alone. There was a significant reduction in groups receiving morphine as well as groups receiving curcumin with doses of 10, 30 and 60 compared to control group. Due to the reductions in the number of sperm in group receiving doses of 30, 60 and 10mg/kg of curcumin + mor-

Figure 2: Comparison of changes in sperm count in the studied groups

Figure 3: Effect of different concentrations of curcumin and morphine on the viability of sperm in the study groups

phine rather to the control group, this reduction was statistically significant. Furthermore, in the groups receiving curcumin + morphine at the doses of 10,30 and 60mg/kg significant increase in the number of sperm was observed compared to morphine alone (Figure 2).

Investigation of the viability of sperm in the study groups (Viability)
The results of the viability of sperm in the study groups showed a significant reduction in the groups receiving morphine compared to the control group. Despite the increase in viability of sperm in groups receiving curcumin at doses of 10, 30 and 60mg/kg compared with the control group, this increase was not significant. There was a significant reduction between morphine receivers and groups receiving morphine + curcumin with 10, 30 and 60 milligrams per kilogram doses with the control group (Figure 4).

Figure 4: Effect of different concentrations of morphine and curcumin on the sperm normal morphology in study groups
doses of 10, 30 and 60mg per kilogram compared with the control group, this increase was not significant. There was a significant reduction between morphine receivers and groups receiving morphine + Curcumin with 10, 30 and 60 milligrams per kilogram doses with the control group (Figure 4).
Sperm Motility (Motility)
Sperm motility assessments in study groups in four steps shows a significant increase between the groups receiving morphine and morphine + curcumin with doses of 10 and 30mg/kg in the control group at absence of sperm motility. As well as significant increase in the groups receiving Curcumin with doses of 10, 30 and 60mg/kg, there is a for-

Figure 5: Effect of different concentrations of morphine and curcumin on sperm motility in the study groups

Figure 6: Effect of different concentrations of morphine and curcumin on the Seminiferous pipe diameter in study groups

Curcumin effects on sperm parameters in rats
Roshankhah et al.,
ward movement compared with the control group. Moreover, a significant decrease in groups receiving morphine and groups receiving morphine + curcumin with doses of 10, 30 and 60mg/kg, there was a progressive fast forward movement compared to the control group. Also, in groups receiving morphine + Curcumin with doses of 10, 30 and 60 milligrams per kilogram significant increase in sperm motility and progressive motility fast forward observed compared to the groups receiving morphine alone (Figure 5).

Assessment of the Seminiferous Pipes Diameter in study groups
The results of seminiferous pipe diameter evaluation in study groups showed a significant decrease in groups receiving morphine in comparison of control group. Also there was a significant reduce in groups receiving morphine + curcumin at of doses 10 and 30mg/kg compared to the control group. Other groups received a dose of curcumin alone, despite the increase in diameter of seminiferous pipes compared to the control group, the difference was not significant. There was a significant reduction in groups receiving morphine rather to the control group. There was also a significant reduce in morphine + curcumin receiving groups with doses of 10, 30 mg/kg compared with the control group (Figure 6).

The results of measuring testosterone levels of serum in the study groups
The results of the testosterone levels in the study groups showed that there was a significant decrease in the groups receiving morphine and groups receiving morphine + curcumin with doses of 10,30, and 60mg/kg compared to the control group. There was also a significant increase in groups receiving curcumin by doses of 30 and 60mg/kg compared with the control group (Figure 7).

Discussions
Results of studying the left testis weight in two groups showed a significant decrease in testicular weight between the groups receiving morphine compared to the control group. Also, increases in weight of testes in groups receiving curcumin with doses of 30 and 60mg/kg significant increase was observed compared to the control group. The observed increase in testicular weight in groups receiving curcumin could possibly happen due to development of seminiferous tubules or increase the number of cells such as spermatozoon in the testi-
cles (Taepongsorat et al., 2008). Testis weight significantly depends on of spermatogenesis cell density in different steps. Farombi et al study showed that administration of curcumin significantly decreases body weight and testicular weight in the groups receiving di-n-butylphthalate (Ilbey et al., 2009). Tousson et al, study shows that curcumin injection increases in the epididymis and testis weight in groups receiving cisplatin (Tousson et al., 2014). Results of this study are consistent with the results of this study.

As morphine has ability to generate free radicals and reactive oxygen species in the cells, it can be argued that the generation of free radicals in testes cells which are very sensitive, causes termination and testicular weight loss (Khaki et al., 2009a). Other studies have also shown that ROS generation reduces the quality and quantity of semen liquid and by increasing the cell permeability causes the sperm viability loss. It can also be concluded that one of the possible reasons of testicular atrophy are unknown factors which interfere in the process of spermatogenesis, thereby reducing the number of sexual cells causes testis weight loss (Bu et al., 2011). Thomas study shows that a daily intake of methadone for 5-10 days caused a significant reduction in the weight of sexual organs in mice (Lakhman et al., 1989) which is consistent with the results of this study. Morphine causes reducing in release of LH and LHRH in adult rats of both sexes (Miller and Gibson, 1994).

Considering that the weight of testes in rats receiving morphine declined sharply, it may indicate a direct effect of morphine on testis and thus, effects on steroidogenic activity and reduce the performance of it. Morphine inhibits the release of norepinephrine from the hypothalamus in female mice. On the other hand, several studies indicated that increasing density of receptors (u) in hypothalamus and the forebrain hypothalamus of the female mice that were in contact with morphine during pregnancy. Thus, it may reduce the amount of norepinephrine and recycling it and consequently reduce the hormone LH due to (u) receptors in the hypothalamus the increased density in these rats (Khaki et al., 2009b; Bu et al., 2011).

Considering conducted studies, we can conclude that morphine consumption increases oxidative stress and subsequently, increase generation of ROS which stops the cell cycle, and increases in apoptotic process that cause reduction in daily generation of sperm hence reduce the total number of sperms (Kaushal and Bansal, 2007). Morphine causes oxidative stress through increase lipid peroxidation and by changes in intracellular glutathione levels. Since the sperm plasma membrane is rich in polyunsaturated fatty acids on one hand, and has a weak antioxidant system on the other hand, therefore very lipid peroxidation is very sensitive by ROS (Baumber et al., 2000).

Antioxidants have an important defensive factor against oxidative stress induced by free radicals. Curcumin has antioxidant effects due to the unique conjugate structure including 2-methoxyphenol and enol dike tone. Special structure of curcumin has the ability to trap free radicals through its antioxidant chain. Due to curcumin antioxidant properties, it can increase the mobility and viability of sperm (Sneha., 2014). Chan and Wu study shows that curcumin has protective effects against methylglyoxal inducing oxidative damage to DNA and human mononuclear cells (Chan and Wu, 2006). Sneha et al., study also showed that curcumin significantly improves the motility and viability of sperm in a dose-dependent against toxicity induced by diethanolamine in human spermatozoa cells on in vitro (Sneha., 2014).

Kobayashi et al study in 2001 showed that continuous decline in the number of alive and active sperm cells are related to increasing the amount of ROS. Because it increases the amount of malondialdehyde enzymes, therefore dysregulation and disorders in cell membrane (Kobayashi et al., 2001). Since oxidative stress, lipid peroxidations and change in morphine induced membrane properties caused the death of germ cells in different phases of growth and reduce the number of sperms, hydrogen peroxide is able to make sperm immobile (Venkatesh et al., 2009). Antioxidants protect sperm from damage and improve sperm quality (Khaki et al., 2009a). Fetouh et al study showed that curcu-
Curcumin effects on sperm parameters in rats
Roshankhah et al.,

Curcumin has protective effects against infertility by improving sperm production, motility and sperm count in male pigs exposed with gentamicin. Also Abarikwu and collegues study indicated that curcumin significantly improves sperm production in the testes against toxicity induced by Gallic acid (Fetouh and Azab, 2014).

Sperm mobility and sperm viability also are considered as the most important parameters to assess the ability of fertilization and sperm membrane integrity. Mammalian sperm membranes contain large amounts of polyunsaturated fatty acids, which is sensitive to lipid peroxidation obtained from oxidative stress which causes rapid loss of intracellular ATP and thereby, reducing sperm viability and motility (Bansal and Bilaspuri, 2010). Thus, it is possible that changes in motion design and reduce sperm viability in mice affected by morphine induced by the ability of pollutants to induce oxidative stress by lipid peroxidation in sperm membrane (Pena et al., 2003).

Studies show that there is a significant relationship between the generation of active oxygen species and disorders in sperm morphology. Researchers believe that in terms of increased levels of free radicals, loss of epithelial cells results in damage of sertoli cells and cytoplasmic bridges collapse as well as reducing the number of sperm and sperm malformation (Aziz et al., 2004).

The results of this study show that a significant decrease in the average inner diameter of seminiferous tubules observed in group receiving morphine, compared to the control group. This feature arises when the cells of seminiferous tubule in groups receiving morphine quickly differentiated from groups morphine + curcumin and released from the walls of the tubes, which cause a slight increase in internal pipes diameter. According to Wing et al., study, phytoestrogens bind to estrogen receptors in the testes and through mechanisms such as increased epithelial layer, the seminiferous tubular diameter and increase the diameter of the lumen in the steps of spermatogenesis, will stimulate this process (Wing and Christensen, 1982).

Mikolajczyk et al study showed that morphine consumption during pregnancy decrease LH and serum testosterone level in the male offspring (Sokolowska-Mikolajczyk et al., 2005), which is consistent with the results of this study. Study on heroin addicts showed that declining testosterone levels in these men accompany with lower levels of LH and FSH compared to normal subjects (Kalra and Kalra, 1984). Lamfon. (2007) study showed that morphine affects testicular function and structure, as well inhibit axis of hypothalamic-pituitary-gonadal. Histological study also showed that morphine causes termination and damage in spermatogenic cell which is consistent with results of the presented study (Lamfon., 2007). Sakr and Badawy (2013) study indicates that curcumin can cause a significant increase in testosterone serum levels and improve sperm number in rats exposed to monosodium glutamate.

CONCLUSION
Curcumin administration as a powerful antioxidant on animals with normal spermatogenesis as well in groups receiving morphine, especially in high doses, which can be effective on fertility. The results also indicated that curcumin possible antioxidant properties on sperm parameters, has protective effect against free radicals and is effective in improving sperm parameters. Hence, curcumin can have positive effects on reproductive parameters at high doses.

COMPETING INTERESTS
The authors declare that they have no competing interests.

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Curcumin effects on sperm parameters in rats
Roshankhah et al.,


Curcumin effects on sperm parameters in rats
Roshankhah et al.,


