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EXPERIMENTAL STUDY

Effects of tramadol administration on male reproductive toxicity in Wistar rats

The role of oxidative stress, mitochondrial dysfunction, apoptosis-related gene expression, and nuclear factor kappa B signalling

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ABSTRACT

AIM: The present study investigated the role of redox balance, inflammation, mitochondrial dysfunction, and apoptosis in Tramadol (Tra)-induced testicular toxicity.

METHOD: Twenty-four male Wistar rats were randomly divided into either the control group or the groups receiving different doses of Tra (25, 50, and 75 mg/kg/day, i.p.) for 21 successive days. Testicular tissues were collected for oxidative stress, mitochondrial function, sperm assays and histopathological evaluation. Real-time polymerase chain reaction was performed to evaluate the markers of inflammation and apoptosis.

RESULTS: Tra caused a significant reduction in the sperm count, motility and morphology, while it caused a marked increase in oxidative stress parameters. In addition, Tra induced testicular mitochondrial dysfunction due to the collapse of mitochondrial membrane potential and mitochondrial swelling. It also led to the significant inhibition of anti-apoptotic Bcl-2 expression, besides a significant increase in pro-apoptotic Bax expression. There was a significant increase in the level of tumour necrosis factor-α, interlukin-1β and nuclear factor kappa B. Histopathological degenerative changes were observed in the testis after Tra exposure.

CONCLUSIONS: The present results suggest that Tra exposure may lead to reproductive toxicity due to the loss of the antioxidant defence system, mitochondrial dysfunction, and activation of inflammatory and apoptotic pathways *(Tab. 4, Fig. 5, Ref. 63)*. Text in PDF *www.elis.sk.*

KEY WORDS: tramadol, oxidative stress, apoptosis, inflammation; mitochondria, testis.

Introduction

Tramadol (Tra) is a centrally acting opioid analgesic and a synthetic analogue of codeine (1). IMS Health reported 43.6 million and 41.0 million Tra prescriptions in the United States in 2016 and 2017, respectively (2). Tra consumption is increasing worldwide due to two major reasons. Firstly, despite the availability of many new analgesic drugs, opioids remain the first choice for the treatment and management of moderate to severe pain. Tra has fewer side effects than other opioids, as it binds to μ-opioid receptors and inhibits the neuronal reuptake of norepinephrine and serotonin (3). Secondly, addiction is one of the most common health and social problems worldwide, and Tra is frequently abused in many countries around the world, especially by men (4).

In 2016, the National Survey on Drug Use and Health (NSDUH) reported that 1.6 million people over the age of 12 years used Tra for non-medical reasons in the United States (2). In previous reports, a wide range of side effects has been attributed to Tra consumption, including histopathological and biochemical damage to the liver, kidneys, testis (4–6) and brain of rats (7). According to previous studies, several mechanisms are involved in Tra-induced toxicity, such as oxidative stress (5, 8) and cell death (9).

Today, infertility is recognized as a common social problem, and nearly 40 % of infertility problems are reported in men (10). Male infertility is mostly caused by a reduced level of sexual hormones, low sperm count, abnormal sperm morphology, and lack of sperm motility (11). Some studies suggested that long-term administration of Tra could damage the sperm cell membrane, decrease the level of testosterone in the serum (4), and induce apoptosis in interstitial cells of testis in rats (12).

Mitochondria are the major source of reactive oxygen species (ROS) and adenosine triphosphate (ATP) under physiological

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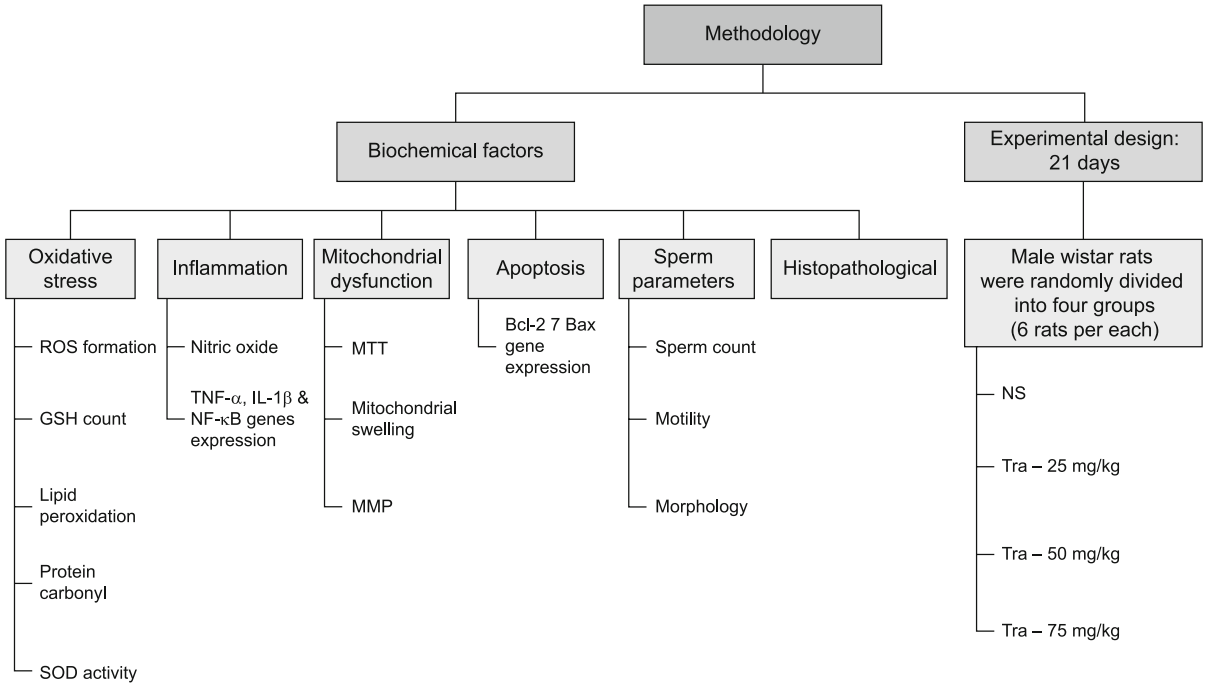

Fig. 1. The experimental study method is illustrated.

conditions (13). Any disturbances in the mitochondrial electron transport chain can lead to an excess ROS production. Oxidative stress is a condition, which results from the imbalance between ROS production and deficient ROS detoxification This condition is known to play a crucial role in pathological cell signalling during apoptosis and necrosis (14). The mechanism of ROS-mediated apoptosis involves mitochondrial permeability transition (MPT) pore opening and release of cytochrome C from mitochondria as an important indicator of cell apoptosis (15).

According to animal studies, the expression of pro-apoptotic Bax and caspase-3 increases due to Tra exposure in rats, whereas anti-apoptotic Bcl-2 expression decreases in the cerebral cortex and lung tissues (16). On the other hand, uncontrolled production of free radicals can induce inflammatory responses (14). Also, free radicals can act as inflammatory effectors by, for example, activating the transcription factor, nuclear factor-kappa B (NF-ƙB), which in turn leads to the transcription of genes involved in the synthesis of inflammatory cytokines (17, 18).

In many previous studies, sperm mitochondria have been introduced as one of the main sources of ATP formation, which is necessary for sperm motility (19). Therefore, the activity of sperm mitochondria seems to be closely related to sperm parameters, such as: sperm concentration, motility, function (14, 20), and there is a correlation between mitochondrial dysfunction and testicular injury. However, the exact effects of Tra on mitochondria, NF-ƙB signalling pathway, expression of anti-apoptotic and pro-apoptotic genes in testicular tissues, and reproductive function have not been investigated so far. Therefore, in the present study, we aimed to highlight the mechanisms of oxidative stress, mitochondrial dysfunction, apoptosis, and NF-ƙB signalling pathway as possible underlying mechanisms of Tra-induced testicular toxicity after 21 consecutive days of Tra administration in male rats.

Materials and methods

Animals

Male Wistar rats, weighing 200–250 g, were purchased from the Laboratory Animals Research Centre of Mazandaran University of Medical Sciences, Sari, Iran. All experimental procedures were conducted according to ethical standards and protocols, approved by the Committee of Animal Experimentation of Mazandaran University of Medical Sciences, Sari, Iran. We tried to minimize the number of animals in this study and performed anaesthesia induction for painful experimental procedures. The rats were kept in the animal house in a 12:12 hour light-dark cycle at constant temperature (22 ± 2 °C) with free access to water and food (21).

Drugs and treatments

Tra (Sigma Aldrich, St. Louis, MO, USA) was dissolved in normal saline and administered intraperitoneally (i.p.) at three different doses (25, 50, and 75 mg/1 kg body weight). All reagents and chemicals used were of analytical grade. Tra doses were determined based on previous studies respectively and modified during pre-tests (22). All of the solutions were prepared freshly at the beginning of each test day. The animals were randomly divided into the four groups, each group consisting of six rats (n = 24), including: group 1: A Vehicle control group receiving normal

saline (NS, i.p.), group 2: Tra (25 mg/kg, i.p.), group 3: Tra (50 mg/kg, i.p.) and group 4: Tra (75 mg/kg, i.p.).

The doses of Tra were determined based on the previous studies and modified during pre-tests. The study was carried out over 21 days (three weeks). Twenty-four hours after the final administration, the rats were anesthetized by ketamine (80 mg/kg) and xylazine (5 mg/kg). Next, testicular tissue of rats was separated, minced, and homogenized with a glass handheld homogenizer. Some parts of the tissue were used for mitochondrial isolation, using a differential centrifugation technique, which was confirmed by the measurement of succinate dehydrogenase (23). The flowchart of the experimental study design is shown in the Figure 1. Three animals from each group were used for the histopathological assay and gene expression evaluation. Also, three rats from each group were sacrificed for other tests, including apoptosis, oxidative stress, and mitochondrial function assessments. The testicular tissue, which was intended for real-time polymerase chain reaction (RT-PCR), was stored in RNA protector solution (Cib Zist Fan Co., Iran) at –80 °C. In addition, the tissue collected for the histopathological study was kept in formalin (10 % w/v) at room temperature.

Measurement of total protein

Protein content was determined in testis tissues with the Bradford method. Bovine serum albumin was used as a standard, homogenate samples mixed with Coomassie blue and after 10 min, absorbance was determined at 595 nm by spectrophotometer (24).

Markers of oxidative stress

Assessment of reactive oxygen species (ROS)

The ROS level measurement was performed using the dichlorodihydro-fluorescein diacetate (DCFH-DA) as an indicator. Shortly, PBS buffer (pH 7.4) was used to dilute the testis homogenate to 1:20 (v/v). Then, 190 µl of homogenate and 10 µl of 1 mM DCFH-DA were mixed together and incubated for 30 min at 37 °C. The conversion of DCFH-DA to 2′,7′-dichlorofluorescein was measured with Shimadzu RF5000U fluorescence spectrophotometer (Agilent Technologies, USA) at excitation/emission wavelength of 485/520 nm, respectively. The results were expressed as fluorescent intensity per 1mg protein (25) .

Evaluation of lipid peroxidation (LPO)

The indicator that was utilized for LPO determination is thiobarbituric acid (TBA) and consequently malondialdehyde (MDA) production. Briefly, determination of the supernatant absorbance at 532 nm on the ELISA reader (Tecan, Rainbow Thermo, Austria) is the method used to evaluate the amount of MDA formation. The standard is tetramethoxypropane and findings were expressed as micromolar (µM)/mg protein (26).

Determination of reduced intracellular glutathione (GSH)

Reduced glutathione content in testis tissue homogenates was measured by the dithio-bis (2-nitrobenzoic acid) (DTNB) as an indicator and yellow colour developed was read at 412 nm on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). A standard curve was drawn using different specified concentrations of GSH solution. With the help of this standard curve, the GSH content was calculated and expressed as nanomolar protein (27) .

Measurement of protein carbonyl (PrC)

The protein carbonyl level was evaluated by the spectrophotometric method based on guanidine hydrochloride. The carbonyl content was determined by reading the absorbance at 365 nm wavelength. Briefly, samples were extracted in 500 µL of 20 % (w/v) TCA. Then, Samples were placed at 4 °C for 15 min. The precipitates were treated with 500 µL of 0.2 % 2, 4-dinitrophenylhydrazine (DNPH) and 500 µL of 2 mol l^{-1} HCl for the control group, and samples were incubated at room temperature for 1 h with vortexing at 5 min intervals. Then proteins were precipitated by adding 55 µL of 100 % TCA. The microtubes were centrifuged and washed three times with 1000 µL of the ethanol-ethyl acetate mixture. The microtubes were dissolved in 200 µL of 6 mol l^{-1} guanidine hydrochloride. The carbonyl content was determined by reading the absorbance at 365 nm wavelength (28).

Estimation of superoxide dismutase activity (SOD)

The testis homogenate was centrifuged (4°C, 4000 rpm) for 10 min. Then, its supernatant was collected and used to determine the activity of SOD in tissue with commercial kits (ZellBio GmbH assay kits, Ulm, Germany) following the manufacturers' protocols (29).

Measurement of NO level as an inflammation marker

Nitrite is an indicator for the nitric oxide (NO) production. The accumulation of nitrite in the testis tissue was measured with Greiss reagent using rat specific ELISA kit (Cib Zist Fan Co., Iran). In this method, Sulfanilic acid was quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt was then coupled to N-(1-naphthyl) ethylenediamine, forming an azo dye that could be spectrophotometrically quantified based on its absorbance at 548 nm by Perkin Elmer Lambda 20 spectrophotometer. The concentration of nitrite in the supernatant was determined from the sodium nitrite standard curve (30).

Mitochondrial function assay

Isolation of mitochondria

The tissues were homogenised and mitochondria were isolated by differential centrifugation. Briefly, the homogenates were centrifuged at 1000×g for 8 min at 4 °C. Supernatants were collected in fresh Eppendorf and then centrifuged at 10,000×g for 10 min at 4 °C. Pellets, thus, obtained were resuspended in isolation buffer and spun again at 12,300×g for 10 min at 4 °C. The resulting supernatants were transferred and top off with isolation buffer with EGTA (215 mM mannitol, 75 mM sucrose, 0.1 % BSA, 20 mM HEPES, 1.0 µM EGTA, and pH is adjusted to 7.4 with KOH) and again spun at 12,300×g for 10 min at 4 °C. Pellets containing pure mitochondria were resuspended in isolation buffer. All the procedures were performed on ice throughout the protocol (31).

Determination of mitochondrial function

The determination of mitochondrial toxicity was done with the use of a dye called tetrazolium salt (MTT). This yellow indicator is reduced to purple formazan by mitochondrial succinate dehydrogenase. The crystals of formazan were dissolved in dimethyl sulfox-

Tab. 1. Special primers for Bcl-2, Bax, TNF-α, IL-1β, and NF-ƙB genes and GAPDH as a housekeeping gene used for quantitative RT-PCR.

Gene	Forward primers	Reverse primers
GAPDH	5′CCCCCAATGTATCCGTTGTG3′	5′TAGCCCAGGATGCCCTTTAGT3′
NF-ƙB	5′AGCACCAAGACCGAAGCAA3′	5′TCTCCCGTAACCGCGTAGTC3′
TNF-α	5′AGCCCTGGTATGAGCCCATGTA3′	5′CCGGACTCCGTGATGTCTAAGT3′
IL-1β	5′GGAAGGCAGTGTCACTCATTGTG3′	5′GGTCCTCATCCTGGAAGCTCC3′
Bcl-2	5′ACTTCTCTCGTCGCTACCGTCGC3′	5′AGAGCGATGTTGTCCACCAGGG3′
Bax	5′CCAGGACGCATCCACCAAGAAG3′	5′CCCAGTTGAAGTTGCCGTCTGC3′

Tab. 2. Program of Real-Time PCR.

Cycle	Cycle Point
Hold	Hold at 95 °C, 15 min 0 s
Cycling (40 repeats)	Step 1: Hold at 95 °C, 15 s Step 2: Hold at 60 °C, 30 s Step 3: Hold at 72 °C, 15 s, acquiring to Cycling A([Green] [1] [1])
Melt	Ramp from 72 °C to 95 °C Hold for 90 s on the 1st step Hold for 5 s on next steps, Melt A([Green] [1] [1])

ide and its absorbance was evaluated with an ELISA reader (Tecan, Rainbow Thermo, Austria) at the wavelength of 570 nm (32).

Quantification of the mitochondrial membrane potential (MMP)

To evaluate MMP, uptake of cationic fluorescence probe rhodamine123 by mitochondria was evaluated. The rhodamine123 fluorescence was followed up utilizing Schimadzou RF-5000U fluorescence spectrophotometer at the excitation and emission wavelengths of 490 nm and 535 nm, respectively (33).

Assessment of mitochondrial swelling

To examinate mitochondrial swelling, changes in light scattering in isolated mitochondria was measured. The monitoring was done at 540 nm (30°C) with an ELISA reader (Tecan, Rainbow Thermo, Austria) (34).

Evaluation of inflammatory and apoptosis-associated genes expressions by real-time polymerase chain reaction (RT-PCR)

For total RNA extraction, 100 mg from each sample were used. The samples were digested in a microtube by Hybrid-R™ total RNA isolation kit (Seoul, South Korea) due to the manufacturer's instructions. Evaluation of extracted RNA integrity was done by certain qualitative and quantitative methods. For quantitative measuring, 3 µl total RNA was mixed with 97 µl diluted water and absorption was measured at 260 and 280 nm using a spectrophotometer (Agilent Technologies, USA). For qualification testing, electrophoresis over Agarose gel was utilized. The cDNA was synthesized by Thermo Scientific ReverAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Wilmington, USA). Real-time PCR was performed for all the samples using specific primers (Tab. 1) of Bcl-2, Bax, tumour necrosis factor-alpha (TNF-α), interlukin-1β (IL-1β) and NF-ƙB genes, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene by Corbett machine (Rotor-Gene 6000). Table 2 shows the real-time PCR program for Bcl-2, Bax, TNF-α, IL-1β, NF-ƙB and GAPDH genes. Gene expression analysis was carried out using the $2^{-\Delta\Delta CT}$ method (35).

Sperm examination

Quantification of sperm count

Semen samples were prepared from the caudal epididymis separated from the testis and placed in a Petri dish. Epididymal spermatozoa were obtained by mincing the epididymis with scissors. Diluted sperm suspension was placed in physiological saline and incubated at 32 °C for 10 min. The epididymal sperm count was determined by hemocytometer. 5mLof diluted sperm was placed on the central square of Neubauer hemocytometer slide. After counting the sperms by a microscope at ×40 magnification in five squares, their numbers were expressed as one million in one mL of sample size (36).

Assessment of sperm motility

Sperm motility was measured according to the World Health Organization (WHO) guidelines. The count was evaluated twice for each sample by a microscope at ×40 magnifications and the means were announced. At least 200 sperm per each animal were examined. Sperm motility was reported as a per cent of motile sperm of total sperm calculated in each replicated (36).

Determination of sperm morphology

For determined morphological defects, sperm smears were prepared on clean and grease-free slides and allowed to dry in air overnight. The slides were stained with 1 % Eosin-Y/5 % nigrosin. The specimens were examined under microscope at ×100 magnifications for morphological abnormalities such as: amorphous, hookless, bicephalic, coiled or abnormal tails. At least 200 sperm per each animal were examined (36).

Histopathological assay

After the animals were anesthetized, testis tissues were immediately removed and washed with cold normal saline and samples

Tab. 3. Effect of Tramadol on biomarkers of oxidative stress and NO level in the testis tissue.

Groups	ROS formation (Fluorescence intensity)	LPO (µM)	GSH (µM)	PrC (mM)	SOD activity (U/ml)	NO (nmol/ml)
NS	104.19±5.18	11.984±1.11	329.326±10.05	0.178±0.02	18.412±0.12	22.465±0.98
Tra (25 mg/kg)	169.615±6.46^{ns}	14.067±1.58^{ns}	324.07±11.67^{ns}	0.186±0.03^{ns}	17.849±0.58^{ns}	28.115±2.22*
Tra (50 mg/kg)	251.743±6.76***	27.982±1.63****	309.615±4.95**	0.255±0.03**	7.599±0.16****	77.218±4.96****
Tra (75 mg/kg)	314.176±6.5***	29.407±1.66****	303.52±5.05***	0.266±0.03***	4.735±0.36****	106.053±3****

Data expressed as Mean ± SD and analysed by ANOVA followed by Tukey test. n = 3. NS (normal saline, control), Tra (Tramadol) and ns (non-significant). ns p> 0.05, when compared to control group. ** p < 0.01, when compared to control group. *** and **** p < 0.001, when compared to control group.

were fixed in 10% (w/v) formalin solution for 24 hours. After processing and embedding in paraffin using a standard protocol, five-micron tissue sections stained with haematoxylin and eosin (H&E) for evaluation of testis damage. Sample sections were measured using ×400 magnification for determination of the degree of testis by a histologist that was blinded to the control group. An Olympus light microscope (Olympus, Tokyo, Japan) was used for histological evaluation (4).

Statistical analysis

Results are presented as the mean ± SD. All statistical analyses were done using the SPSS software, version 14, one-way ANOVA and Kruskal-Wallis tests, followed by Tukey test. Average, standard, minimum, and maximum deviations were calculated for each group of data. $p < 0.05$ was considered statistically significant. For gene expression analysis, Bcl-2, Bax, TNF-α, IL-1β and NF-ƙB genes expression in relation to GAPDH as a reference gene were measured by Ct variations and $2^{-\Delta\Delta cT}$ (Ct test- Ct reference) formula.

Results

Parameters of oxidative stress

Reactive oxygen species level in testis

In order to determine possible oxidative damage, induced by 3 weeks' administration of Tra, the level of ROS was evaluated in testis tissue. As shown in Table 3, the mean values of ROS were non-significant ($p > 0.05$) at a low dose of Tra (25 mg/kg) group, when compared to the control group. Administration 50 and 75 mg/kg of Tra showed a significant ($p < 0.001$) increment in testis tissue, when compared to the control group.

Levels of LPO, GSH and PrC in testis

The measured data of LPO, GSH and PrC in the testis tissue are summarized in the Table 3. These data showed a remarkable change in comparison with the data of the control group. The intracellular GSH concentration, MDA level and PrC content were non-significant ($p > 0.05$) at low doses of Tra in comparison to the control group. While 3 weeks' exposure to 50 mg/kg of Tra caused a significant increase in MDA level as well as PrC content ($p < 0.001$ and $p < 0.01$, respectively) and significantly ($p < 0.01$) decreased in GSH concentration in testis tissue as in comparison with the control group. Interestingly, at 75 mg/kg of Tra, there was a significant ($p < 0.001$) decrease in GSH level and increase in LPO as well as PrC content in tissue, when compared to the control group.

Superoxide dismutase activity in testis

The activities of antioxidant enzyme (SOD) in the testis of the experimental animals is shown in the Table 3. Three weeks' administration of Tra at doses 50 and 75 mg/kg, significantly ($P<0.001$) decreased the SOD activities compared to the control rats, whereas chronic exposure to 25 mg/kg of Tra resulted in non-significant ($P>0.05$) reduction of SOD activities compared to the control group.

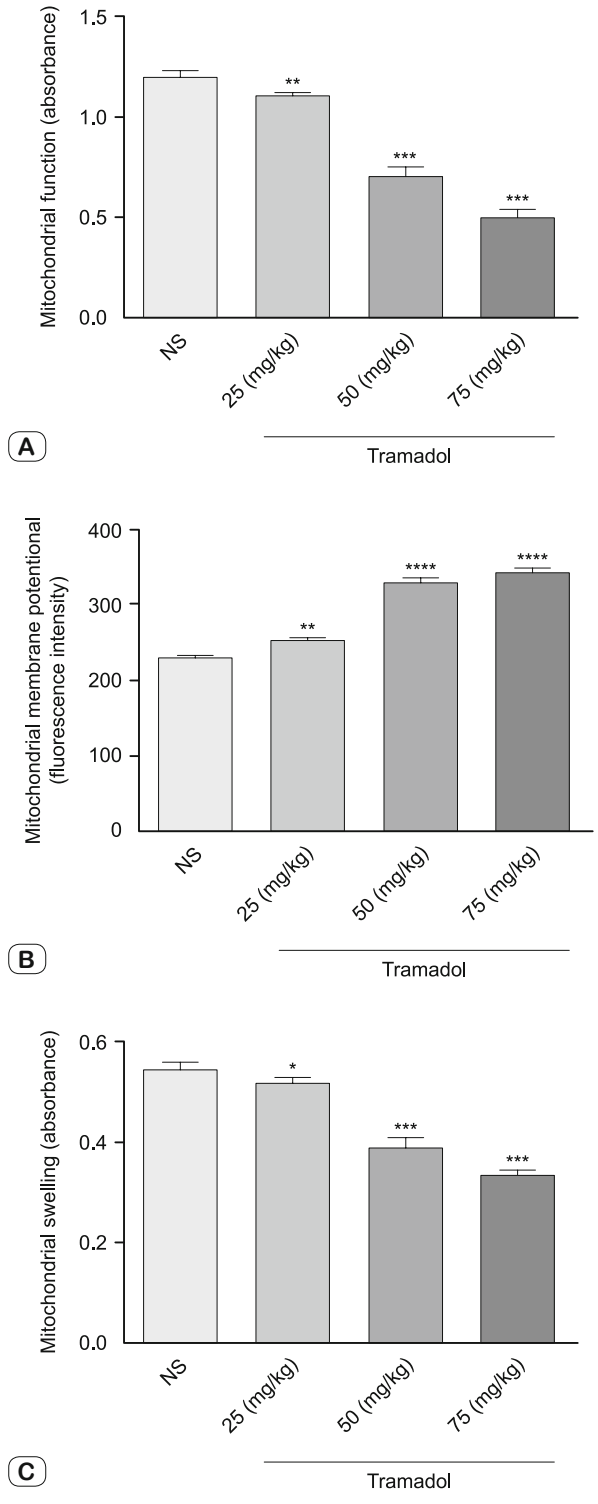

Fig. 2. Effect of Tramadol on (A) mitochondrial function, (B) mitochondrial membrane potential and (C) mitochondrial swelling in testis-isolated mitochondria of Wistar rat. Data expressed as Mean ± SD and analysed by ANOVA followed by Tukey test. n = 3. NS (normal saline, control). * $p < 0.05$, when compared to control group. ** $p < 0.01$, when compared to control group. * and **** $p < 0.001$, when compared to control group.**

Nitric oxide concentration in testis

The results of NO evaluation in the testis, 3 weeks' exposure to Tra showed a significant ($p < 0.001$) increase in NO level at the middle and high doses (50 and 75 mg/kg), while low doses of Tra (25 mg/kg) showed a significant ($p < 0.05$) increase in NO level, compared to the control group (Tab. 3).

Mitochondrial function assays

Mitochondrial function

As shown in the Figure 2A, 3 weeks' administration of Tra in 50 and 75 mg/kg, markedly ($p < 0.001$) decreased mitochondrial function in comparison with the control group in sperm mitochondria. Also, a low dose of Tra (25 mg/kg) significantly ($p < 0.01$) increased Tra-induced sperm mitochondrial toxicity, when compared to the control group.

Mitochondrial membrane potential

As was shown in Figure 2B, increased MMP collapse as an electrochemical potential in Tra-treated middle and high doses rats (50 and 75 mg/kg) were observed as a consequence of mitochondrial dysfunction, when compared to the control group ($p < 0.001$). Also, a low dose of Tra (25 mg/kg) significantly ($p < 0.05$) incremented MMP collapse in sperm mitochondrial membrane in comparison with the control group.

Mitochondrial swelling

Testis mitochondrial swelling was significantly ($p < 0.001$) elevated in animals treated with Tra for 3 weeks in 50 and 75 mg/kg doses in comparison with the control group. Further, lower dose of Tra (25 mg/kg) caused a significant ($p < 0.05$) increase in testis mitochondrial swelling in comparison with the control group (Fig. 2C).

Bcl-2 and Bax genes expressions in testis

According to the RT-PCR analysis, the expression level of anti-apoptotic Bcl-2 gene in the Tra groups (50 and 75 mg/kg) were markedly ($p < 0.001$) decreased, when compared to the control group. While the expression of these genes was non-significant ($p > 0.05$) at a low dose of Tra (25 mg/kg) group, when compared to the control group. Besides, the gene expression of pro-apoptotic Bax was significantly ($p < 0.001$) upregulated in the testis tissue of Tra groups (50 and 75 mg/kg) in comparison with the normal group. Whereas lower dose of Tra (25 mg/kg) caused a non-significant ($p > 0.05$) gene expression of Bax in comparison with the control group (Fig. 3).

TNF-α and IL-1β genes expressions in testis

Using the RT-PCR analysis, the expression of TNF-α and IL-1β genes as pro-inflammatory cytokines in the 50 and 75 mg/kg doses of Tra was significantly ($p < 0.001$) upregulated, when compared to the control group. Additionally, a low dose of Tra (25 mg/kg) produced a significant ($p < 0.05$) genes expression of TNF-α and IL-1β in comparison with the control group (Fig. 4A–4B).

NF-ƙB gene expressions in testis

When compared to control group, the gene expression of NF-ƙB as an inflammation associated complex in Tra-treated groups

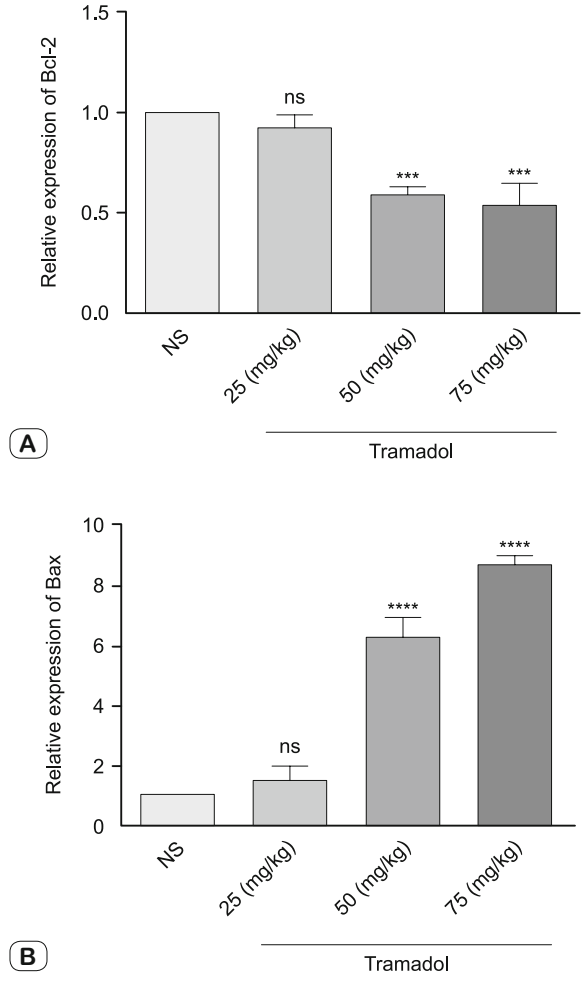

Fig. 3. Effect of Tramadol on (A) Bcl-2 and (B) Bax gene expression in testis of Wistar rat. GAPDH was served as an internal standard. Experiments were repeated three times, and similar results were obtained. Data expressed as Mean ± SD and analysed by ANOVA followed by Tukey test. n = 3. NS (normal saline, control). ns $p > 0.05$, when compared to control group. * and **** $p < 0.001$, when compared to control group.**

with middle and high doses (50 and 75 mg/kg) was remarkably enhanced in testis tissue ($p < 0.001$). Moreover, a low dose of Tra (25 mg/kg) was shown to non-significantly ($p > 0.05$) affect gene expression of a pro-inflammatory mediator, NF-ƙB, compared to the control group (Fig. 4C).

Sperm count, motility and morphology

Sperm count, motility and normal morphology were analysed for all the groups. As shown in the Table 4, the mean of epididymal sperm count, motility and normal morphology of sperm cells decreased significantly ($p < 0.001$) in Tra (50 and 75 mg/kg) treated groups, when compared to the control group. However, count, motility and normal morphology of sperm cells were non-significant ($p > 0.05$) in the lowest dose of Tra (25 mg/kg) group in comparison with the control group after 21 consecutive days.

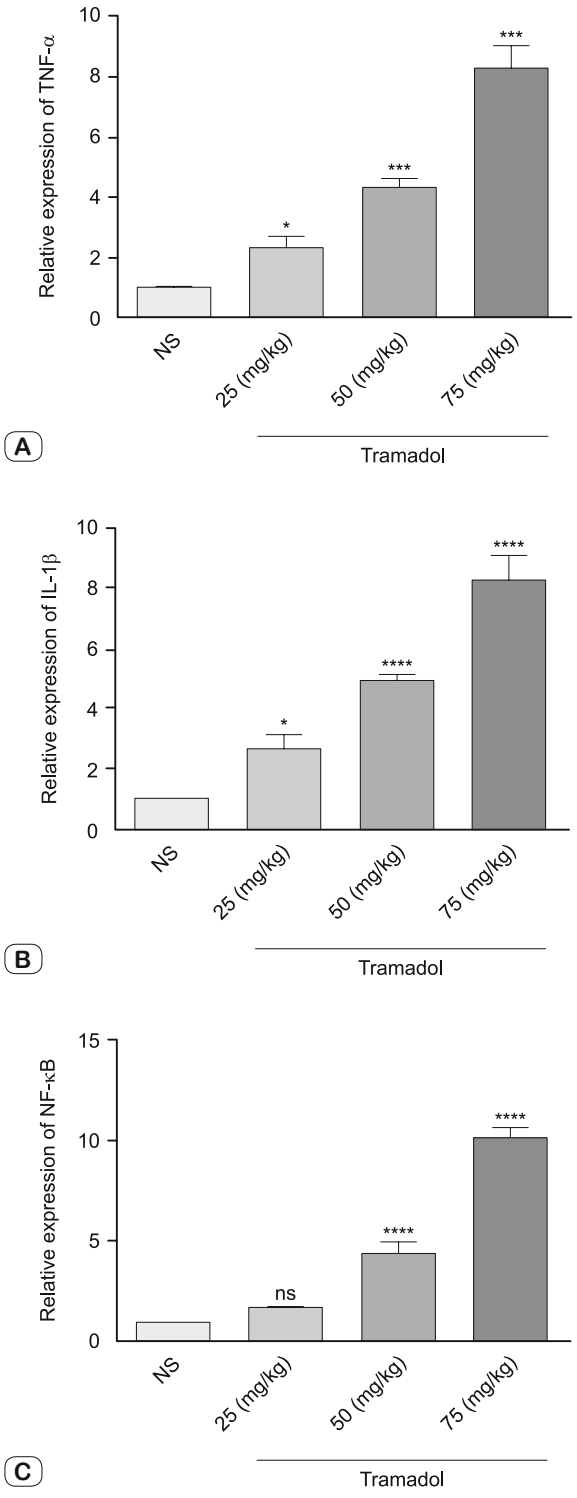

Fig. 4. Effect of Tramadol on (A) TNF-α , (B) IL-1β and (C) NF-ƙB gene expression in testis of Wistar rat. GAPDH was served as an internal standard. Experiments were repeated three times, and similar results were obtained. Data expressed as Mean ± SD and analysed by ANOVA followed by Tukey test. n = 3. NS (normal saline, control). ns p > 0.05, when compared to control group. * p < 0.05, when compared to control group. * and **** p < 0.001, when compared to control group.**

Histopathological examination

The photomicrographs of testis in all the groups are presented in the Figure 5. Histopathological analysis of the testis tissues of the control group showed no abnormality. Whereas, 3 weeks' administration of Tra caused testis damage and the increase in its dose led to an increase in histological changes. In testis tissue, the diameter of the lumen of the rat seminiferous tubule showed normal morphological appearance in the control group. After treatment with 25 mg/kg of Tra, a reduction of seminiferous epithelial thickness were observed (black arrow). Nevertheless, middle and higher doses of Tra (50 and 75 mg/kg) caused several alterations, such as increase of the diameter of the lumen of the rat seminiferous tubule (white arrow), reduction seminiferous epithelial thickness and oedema (Red arrow), when compared to the control group.

Discussion

The results of the present study showed that Tra exposure of adult male rats for 21 consecutive days caused sperm dysfunctions via disturbing the sperm count, motility, and morphology. Moreover, histopathological findings indicated metabolic changes, haemorrhage, and cell damage. The increased level of oxidative stress, apoptosis, mitochondrial dysfunction, and triggering of the inflammatory pathway seems to be important in the pathogenesis of testicular injury in response to Tra exposure.

Oxidative stress is considered an important factor in the induction and progression of Tra-related reproductive toxicity (37). Our findings revealed that ROS generation significantly increased in testicular tissues following Tra exposure, which is in agreement with the previous studies (38, 39). ROS are extremely reactive molecules, produced under physiological and pathological conditions. Excess production of ROS, such as: superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide, causes drastic changes, which can destroy lipids, proteins, nucleic acids, and other cellular compounds, resulting in cellular death (40). Additionally, the increased level of free radicals in the testis causes a wide range of pathophysiological events, including infertility (41).

In the present study, Tra exposure (50 and 75 mg/kg) led to a significant increase in the testicular level of LPO, compared to the control group. The present findings are in agreement with the results reported by Abdel-Latif Ibrahim et al (42) and Ghoneim et al (12), which showed that LPO significantly increased after Tra exposure in some tissues. Some studies reported that an increase of oxidants in the testis led to an increase in LPO and decreased the activity of antioxidant enzymes. Lipid peroxidation results from the free radical attack to lipid membranes, and testis is considered a susceptible organ to lipid peroxidation because of its high lipid composition (unsaturated fatty acids) and high oxygen consumption (43, 44). Therefore, there is a close relationship between lipid peroxidation and reproductive toxicity.

Enzymatic and non-enzymatic antioxidants act as major defence systems against free radicals, removing them from biological systems. GSH is a major non-enzymatic tripeptide, and its sulfhydryl group (-SH) can directly interact with ROS or act as a cofactor of ROS-detoxifying enzymes (45). In the present study,

Tab. 4. Effect of Tramadol on sperm parameters.

Groups	Total sperm count (10^6/mL)	% Sperm motility	% Abnormal sperm morphology
NS	69.65±3.6	87.9±4.18	32.35±1.59
Tra (25 mg/kg)	64.5±7.63^{ns}	80.375±1.57^{ns}	34.15±0.77^{ns}
Tra (50 mg/kg)	42.63±2.93****	57.775±1.2****	54.55±0.8****
Tra(75 mg/kg)	40.28±6.8****	44.575±0.87****	61.275±0.62****

Data expressed as Mean ± SD and analysed by ANOVA followed by Tukey test. n = 3. NS (normal saline, control), Tra (Tramadol) and ns(non-significant). ns: p>0.05, when compared to control group. **** p < 0.001, when compared to control group.

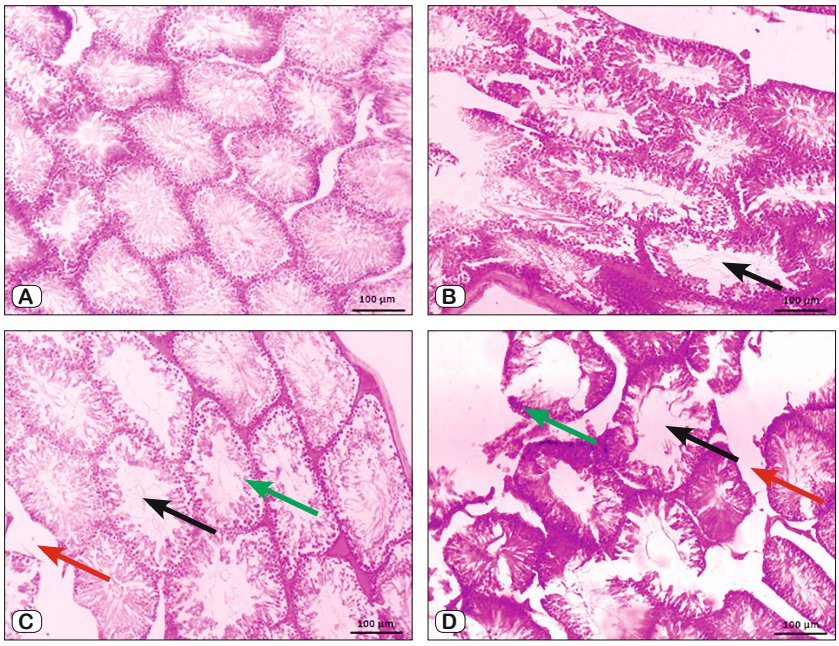

Fig. 5. Photomicrographs showed the effect of Tramadol on the histological architecture of Wistar rat testis. (A) Normal saline (control group); (B, C and D): Tramadol (25, 50 and 75 mg/ kg, respectively). Green arrow: Reduced seminiferous epithelial thickness; Black arrow: Increased of the diameter of the lumen of the rat seminiferous tubule; Red arrow: Oedema. H&E staining (Mag: ×400). (Scale bar: 100 µm).

Tra doses of 50 and 75 mg/kg significantly decreased the intracellular GSH content, compared to the control group. In agreement with our study, Ghoneim et al (12) and Sheweita et al (46) reported that Tra exposure decreased the GSH storage in the testis, cortex, liver and kidney tissues. Depletion of GSH in the testis can cause severe deficiency in the antioxidant defence system, leading to the further elevation of LPO. This condition leads to tissue necrosis or apoptosis via oxidation of thiol groups in mitochondrial membrane proteins (47).

On the other hand, SOD is an essential antioxidant enzyme against ROS production (46). The present findings indicated a significant reduction in the activity of SOD in Tra-exposed rats, which is consistent with some previous studies (37, 42). The observed reduction of antioxidant enzyme activity in the testis of Tra-exposed rats suggests changes in the oxidant defence system due to the suppression of ROS scavenging. On the other hand, a marked increase was observed in the PrC level of testicular tissues after the administration of different doses of Tra. In fact, PrC is introduced into protein oxidation, produced during LPO with ROS production. The higher level of PrC in the testis of rats reflects the high rate of oxidative stress (48). Therefore, there is a correlation between protein oxidation and male infertility due to the negative impact of oxidative stress on redox regulation of sperm function, and consequently, reproductive capacity (14).

The process of inflammation involves different signal transduction pathways, including NF-ƙB. NF-ƙB is a complex of transcriptional activator proteins, found in the cytoplasm of normal cells in an inactive state, dimers with the inhibitory kappa B (IKB) subunit proteins (49). Oxidative stress can activate NF-ƙB via phosphorylation of IKB by IKB kinases (18). Free NF-ƙB binds to the corresponding DNA sequence of target genes, including TNF-α, IL-1β, and other genes, associated with an increased ROS generation (17, 50). In the present study, based on RT-PCR assay, moderate and high doses of Tra induced inflammatory reactions by increasing the gene expression of NF-ƙB, TNF-α, and IL-1β, which reflects inflammatory responses in testicular tissues. Generally, inflammatory cytokines, such as TNF-α and IL-1β, are produced by activated macrophages and trigger the apoptosis cascade during apoptosis (51).

Our results revealed that testicular levels of NO were elevated in Tra-treated rats, compared to the control group. In consistence with our results, Ahmed et al. found that Tra administration could increase the testicular levels of NO and LPO, improve the expression of endothelial NO synthase, and decrease the activity of antioxidant enzymes significantly, compared to the control group. They suggested that Tra-induced testicular dysfunction in adult male rats might be due to the overproduction of NO and oxidative stress (38). Moreover, there is a relationship between lipid peroxidation and endogenous inflammation, which is significantly associated with sperm damage by decreasing the level of sperm motility and mitochondrial function and increasing morphological sperm abnormalities (14, 20); these changes are similar to our observations of sperm parameters.

Mitochondria are not only the main source of cellular ROS, but are also highly susceptible to free radical attack (52). In this study, inhibition of testicular mitochondrial viability and increase of MMP collapse and mitochondrial swelling were reported after Tra exposure. In fact, oxidation of thiol groups in mitochondrial membrane proteins could cause conformational changes in the pore complex, leading to MPT pores opening. The opening of pores resulted in the collapse of MMP and mitochondrial swelling

(53). On the other hand, opening of MPT pores led to the release of mitochondrial cytochrome C and activated the mitochondrial-mediated pathway of apoptosis (41).

Apoptosis refers to programmed cell death and occurs in the testis to remove damaged cells via physiological processes (14). According to the study by Chen et al (54), there is a significant correlation between apoptosis and male infertility; therefore, dysfunction of testicular mitochondria can be a symptom of male infertility (14, 55). Various families of proteins are involved in the regulation of apoptosis. Bcl2 is the most important family that can regulate apoptosis(56). This family can indirectly regulate caspase activity to control apoptosis and regulate it via heterodimerization of Bcl-2 with Bax (a pro-apoptotic member), resulting in the prevention of mitochondrial changes during apoptosis (42). Therefore, dysregulation of anti-apoptotic Bcl2 and pro-apoptotic Bax proteins may prevent or initiate the development of cellular apoptosis (57, 58).

The level of apoptosis in the testis was evaluated via RT-PCR assay. Bax gene expression decreased due to a marked increase in Bcl2 gene expression in Tra-treated rats, compared to the control group, which is in agreement with the previous studies (9, 59). Therefore, this finding is consistent with the study by Awadalla et al (16), which indicated the significant upregulation of Bax, besides downregulation of Bcl-2 in the rat cortex and lung; Tra-induced damage occurred in these tissues through the cell apoptosis pathway. Consequently, Tra-induced ROS formation not only leads to LPO and GSH depletion, but it can also damage the mitochondrial membrane integrity and opening of MPT pores and lead to apoptosis (12, 52).

In the current study, moderate and high doses of Tra caused a significant decrease in sperm count and motility, while increasing the percentage of abnormal sperms, compared to the control group. This finding is in line with the result of the previous study, which attributed this effect to the disruption of spermatogenic cell maturation and confirmed the crucial role of NO overproduction and oxidative stress in the development of Tra-induced testicular dysfunction in male rats (37). Generally, the structural and functional integrity of sperm membranes is essential to the motility and viability of sperms (55). The sperm membranes are extremely rich in PUFAs; therefore, they are suitable for LPO (39).

Arabi et al (60) reported a significant correlation between MDA concentration and percentage of viable sperms. Overall, the high level of LPO may induce an excessive ROS generation. Oxidative stress may exert adverse effects on sperm function via alteration of mitochondrial function and induce apoptosis, as well as a dramatic loss in the fertilizing potential of sperms (61). Some studies reported that the loss of MMP may lead to a decreased sperm motility (20). Furthermore, motile sperms are important in infertility capacity, which is directly dependent on mitochondrial function (55). Analysis of oxidative stress status, mitochondrial function, and apoptosis in testicular tissues of male rats indicate the pathogenic molecular mechanism of the adverse effects of Tra on sperm parameters. Histopathological analysis of the testis supports the biochemical and molecular findings. We found severe irregular changes in the testicular tissues of Tra groups, compared to the control group; these findings are in line with previous studies (4, 62, 63). In general, the present findings support Tra-induced toxicity in the testicular tissue.

In conclusion, the present results reveal that Tra exposure for 21 consecutive days exerts negative effects on testicular function in adult male rats. Increased oxidative stress, mitochondrial dysfunction, and inflammation may play significant roles in the induction of pro-apoptotic Bax gene expression and inhibit anti-apoptotic Bcl-2 gene expression, leading to cell death in the testis of male Wistar rats. Consequently, these pathways may be majorly responsible for the reproductive toxicity induced by Tra exposure.

Learning points

- Tramadol (Tra) increases oxidative stress by suppressing antioxidant system in rat testis.
- Tra induces inflammatory responses through NF-κB pathway.
- Tra triggered mitochondrial-mediated apoptosis in rat testicular tissue
- Sperm count, motility, morphology and testis organ histology were affected by Tra.

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