



Protective effect of carvacrol on ketamine induced testicular damage in mouse model of schizophrenia

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ABSTRACT

Introduction: Ketamine is applied to induce symptoms of schizophrenia in animal models. Besides the nervous system, ketamine also affects male lower genitourinary tracts. The present study evaluated the effects of carvacrol on antioxidant enzymes and examined the histopathologic changes in the testes of ketamine induced schizophrenic mice.

Methods: A total of 48 male mice were treated with 25 mg/kg ketamine or saline for a period of 14 days. Between the 8th and 14th days, the animals received carvacrol (25 and 50 mg/kg) or saline. At the end of the experiment, blood samples were taken to measure luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone; the testes were also collected for biochemical and histopathological evaluations.

Results: The results indicated that induction of schizophrenia by ketamine led to an oxidative stress by increasing malondialdehyde (MDA) level ($P < 0.05$). Treatment with 50 mg/kg carvacrol resulted in significant decrease in oxidative injury by decreasing MDA level and increasing antioxidant enzymes ($P < 0.05$). Testosterone, FSH and LH levels showed no significant difference between treatment groups and control groups except for testosterone which increased in mice treated with 50 mg/kg carvacrol ($P < 0.05$). Administration of carvacrol reduced the deleterious histopathologic changes caused by ketamine.

Conclusion: The present study showed that ketamine causes oxidative stress and damage in testicular tissues and co-administration with carvacrol prevents the harmful effects of ketamine.

Implication for health policy/practice/research/medical education:

The results of the present study show that oxidative stress exerted on testicular tissues by ketamine is effectively prevented by carvacrol. Hence co-administration of carvacrol with ketamine is recommended.

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Introduction

Schizophrenia is a chronic and debilitating mental disease from which about 1% of the world's population suffer (1). Ketamine, an anesthetic drug in veterinary and medical surgery (antagonist of the glutamatergic *N*-methyl-d-aspartate [NMDA] receptors) is applied to induce some symptoms of schizophrenia in animal models. Ketamine can cause hallucinatory effects in human as well, so it is used as a common recreational drug among abusers (2, 3). At sufficiently high doses, subjects may experience a state of dissociation called the "K-hole", supposed to mimic the phenomenology of schizophrenia (4). Besides the toxicity to the nervous system, ketamine can affect the lower urinary tract, causing dysuria, polyuria, hematuria, nocturia, and decreased sperm motility and quality (5,6).

The results of several studies suggest that patients with schizophrenia, especially men, have lower fertility rates compared to healthy subjects (7).

There are evidences suggesting that reactive oxygen species (ROS) are involved in membranous pathologies of central nervous system and may have an important role in neuropsychiatric disorders like schizophrenia (8). Interestingly, excess amounts of free oxygen radicals also can induce production of abnormal sperms and result in infertility (9).

Carvacrol (CAR), [2-Methyl-5-(1-methylethyl)], a monoterpene phenol found in many essential oils of the family Labiatae, such as *Origanum*, *Satureja*, *Thymbra*, *Thymus*, and *Coridothymus* species (10), possesses free radical scavenging activity. Some authors demonstrated its

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protective effect on the nervous system and testis (11,12). The US Food and Drug Administration (FDA) approved carvacrol to be used in food processing as an additive (13). The aim of the present study was to investigate the effects of ketamine on biochemical parameters of oxidative stress damage and histopathological alterations in testicular tissues of mice and also to evaluate the effects of co-administration of carvacrol as a potent and safe radical scavenger on antioxidant enzyme activities and on the levels of lipid peroxidation in the testicular tissues of schizophrenic mice. To our knowledge, this is the first study investigating the biochemical and histopathological effects of carvacrol on schizophrenic mice testes.

Material and Methods

Animals and experimental design

Forty-eight adult male Swiss albino mice (25-35 g) aging about 2 months were purchased from the Pasteur Institute of Iran (North research center, Amol, Iran) and were randomly allocated into 6 groups. Animals were allowed to adapt to the new condition for a week. They were housed in groups of three or four in temperature-controlled rooms (24°C) with constant humidity (55 ± 10%) and 12-hour light/dark cycle prior to undergoing experimental protocols. They accessed commercial rodent chow (Pars, Tehran, Iran) and water ad libitum. All experiments were performed at the same time of the day.

Chemicals

Carvacrol, C₁₀H₁₄O with a molecular weight of 150.22, was purchased from Sigma (Sigma-Aldrich, Germany). According to Sigma, the degree of purity was >97%. Tween 80 (Sigma, USA), ketamine (Alfasan, Woerden, Holland) and all the other chemicals were analytical grade and commercially available. Carvacrol was emulsified with 0.2% Tween 80 and dissolved in distilled water.

Treatment protocol

The animals received 25 mg/kg ketamine or saline intraperitoneally (i.p.) for 14 days (14). Between the 8th and 14th days, the animals received carvacrol (25 and 50 mg/kg, i.p.) or saline. This research design was led to 6 experimental groups, as follows:

- 1) saline plus saline (as control group); 2) ketamine plus saline (called ketamine group); 3) saline plus carvacrol 25 mg/kg (called carvacrol 25 group); 4) saline plus carvacrol 50 mg/kg (called carvacrol 50 group); 5) ketamine plus carvacrol 25 mg/kg (called ketamine + carvacrol 25 group); and 6) ketamine plus carvacrol 50 mg/kg (called ketamine + carvacrol 50).

Biochemical analysis

At the end of the experiment, blood samples were collected from mice for measuring luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone; sera were separated by centrifuging the samples at 4000 rpm for 10 minutes. Levels of serum testosterone, LH and FSH were assayed using automated chemiluminescence

immunoassay systems (ADVIA Centaur, Bayer Vital, Fernwald, Germany).

Histopathological analysis

After excising the testicular tissues of the euthanized mice, sections of the testis were fixed in 10% formalin and were embedded in paraffin wax. Sections were then cut at a 5-µm thickness, deparaffinized and stained with hematoxylin and eosin (H&E) according to the standard methods. For histopathological evaluation, eight sections from each testicular tissue were examined by a pathologist who was blinded to the study groups.

Tissue preparation

The testicular tissue was collected and rinsed with ice-cold saline and stored at -80°C. Before biochemical analysis, the tissue from testis was homogenized at 4°C after adding pre-cooled 0.9% saline in a ratio of 1:9. When testicular tissue was disrupted; the homogenate was centrifuged at 3000 × g for 10 minutes at 4°C. The supernatant was used for biochemical measurements. Protein content of the tissue homogenate was determined using a colorimetric method of Lowry with bovine serum albumin as the standard (14).

Determination of malondialdehyde

The formation of thiobarbituric acid in organ samples was assessed to measure the lipid peroxidation according to an original method (15). Briefly, the supernatant of the tissue homogenate was mixed with 20% trichloroacetic acid and the mixture was centrifuged. Then, thiobarbituric acid was added to the supernatant and heated. The absorbance of the supernatant was measured at 532 nm.

Catalase

A 10% tissue homogenate was prepared in 2 mL of potassium phosphate buffer (pH 7.4). This homogenate was centrifuged at 3000 rpm for 15 minutes. Catalase activity was measured in the supernatant obtained after centrifugation. 2.95 mL of 19 mM H₂O₂ was poured in the cuvette. To it, 0.05 mL of cytosolic supernatant was added and the change in absorbance at 240 nm was recorded at 1 minute intervals for 3 minutes, knowing that the presence of catalase decomposes H₂O₂ leading to a decrease in absorbance (16).

Total antioxidant power

The total antioxidant capacity was determined by the ferric reducing antioxidant power (FRAP). Briefly, the stocks' solutions included 300 mM acetate buffer 10 mM TPTZ (2, 4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM FeCl₃.6H₂O solution. The fresh working solution (FRAP reagent) was prepared by mixing acetate buffer, TPTZ solution, and FeCl₃.6H₂O solutions. The samples and deionized water were mixed with 3 mL of the FRAP reagent and allowed to react for 5 minutes in the dark. The changes in absorbance at 593 nm were considered to be related to the total reducing power of antioxidants of the

tissues (17).

Statistical analysis

Values are presented as mean \pm SEM. The statistical differences between groups were applied by one-way analysis of variance (ANOVA), followed by Duncan's test when appropriate. A *P* value of 0.05 was considered significant.

Results

Histopathological results

In the control group, seminiferous tubules were surrounded by dense interstitial tissue. These tubules showed round or oval structures. Different stages of spermatogenesis were identified; Spermatogonial cells, spermatocytes, spermatids (different steps) and spermatozoa and also Sertoli cells were identified. Mature spermatozoa occupied most of the central luminal portion of the seminiferous tubules. Interstitial tissue was filled with Leydig cells, blood vessels, fibroblasts and collagen fibers (Figure 1A). Compared to the control group, there were significant pathological changes in the testicular tissues in the ketamine 25 group. In this group, most seminiferous tubules showed significant derangement and morphological changes of different cell lines. Affected seminiferous tubules lost

their typical spherical shapes and were wrinkled with an irregular basement membrane, which indicated tubular atrophy, however, some tubules were preserved. The spermatogenic cells showed degeneration and/or necrosis. The most commonly affected cells were spermatocytes and spermatids, however, some spermatogonial cells were also affected. Sloughed germ cells filled the lumens of tubules. Some multinucleated giant cell and also cytoplasmic mass were formed within the lumen. Some tubules showed severe epithelial atrophy and were only covered by Sertoli cells and spermatogonia and an arrest of spermatogenesis process was seen. These tubules had distended lumens compared to normal tubules. Some Sertoli cells showed vacuolization and swelling and necrotic cell debris were seen in the cytoplasm of some of them. In the interstitial tissue and under the tunica albuginea, vascular congestion was observed and in some areas reduced number of Leydig cells was present (Figure 1B and C).

In the carvacrol 25 group, there were no significant pathological changes in the testicular tissues compared to the control group. In detail, normal spermatogenic cells, Sertoli cells and well-delineated tubular basement membrane were seen. The interstitial tissue and Leydig cells were intact (Figure 1D). In the carvacrol 50 group, histopathological evaluation showed normal structures

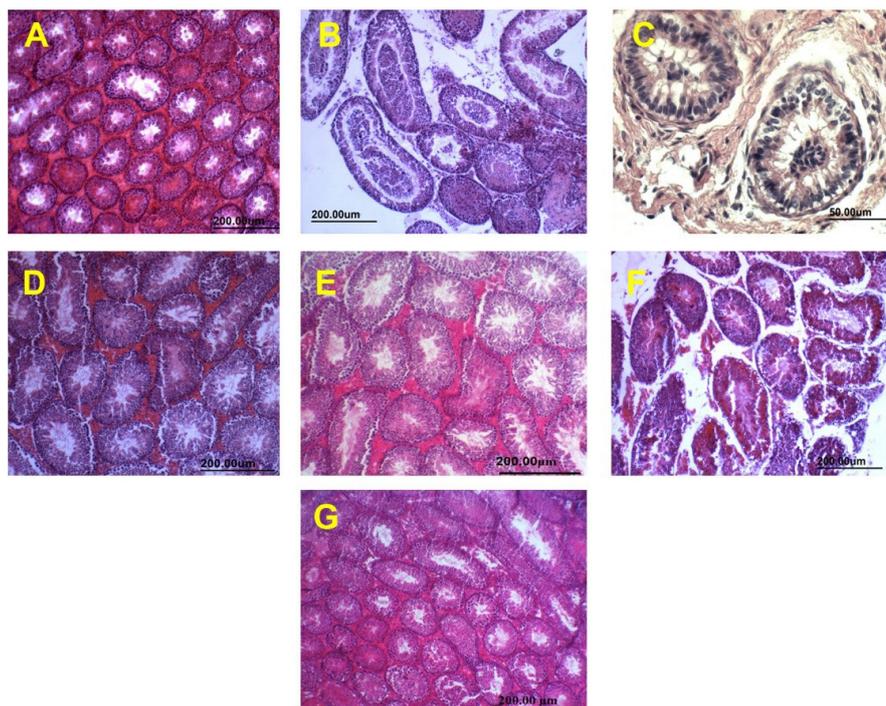


Figure 1. (A) Testicular sections of control group. Note to the normal spermatogenesis and the normal cell arrangement in the seminiferous tubules. The interstitial spaces also appear normal. (B and C) Photograph of testes treated with ketamine. B: Note to the damaged seminiferous tubules. The seminiferous tubular epithelium is irregular and degenerated. Damaged tubules containing degenerated spermatogenic cell. Lumen is full of cellular and spermatogenic debris. C: Note the degeneration of spermatogenic cells and the separation of spermatogenic cells from the basement membrane in some tubules in this group, with giant cell formation within lumen. Degeneration of interstitial tissue, interstitial edema and atrophy of Leydig cell also occurred. (D) Testes of mice in carvacrol 25 group. Seminiferous tubules more or less are similar to that of control. (E) Note to the normal aspect of the seminiferous tubules, without microscopic alterations in structure in carvacrol 50. (F) Pathologic changes in seminiferous tubules had partly ameliorated and restoration of spermatogenic cells in most seminiferous tubules is seen. However mild vacuoles still exist within cells in ketamine + carvacrol 25 (G) Testes of the mice in ketamine + carvacrol 50 group, showing nearly normal mature active seminiferous tubules with complete spermatogenic series. H&E.

similar to the control and carvacrol 25 groups (Figure 1E). In ketamine + carvacrol 25 group, pathologic changes in seminiferous tubules were partly ameliorated, but the number of spermatozoa within the lumen was yet less than the control group. Vacuolization of the seminiferous epithelium was also seen (Figure 1F). In ketamine + carvacrol 50 group, histopathological changes were reduced remarkably. Spermatogenic cells within these tubules had normal stratification and relatively in most of the tubules all stages of spermatogenesis were normal, however slight degeneration (vacuolization and some sloughed germ cells) was observed in a few tubules (Figure 1G).

Biochemical results

According to the results illustrated in Table 1 the testicular malondialdehyde (MDA) levels in the ketamine group were significantly higher than those of the control group ($P < 0.01$). In the groups which received 25 and 50 mg/kg carvacrol, no significant difference was found in the testicular MDA levels compared to the control group ($P > 0.05$). In the animals receiving 50 mg/kg carvacrol plus ketamine (ketamine+ carvacrol 50 group), the testicular MDA levels were revealed to be closer to the control group and showed significant difference in comparison to ketamine group ($P < 0.01$). Lower levels of total antioxidant power (TAP) were obtained in the ketamine (schizophrenic) group compared to the control group but the difference was not statistically significant. Moreover, the ketamine + carvacrol 50 and ketamine + carvacrol 25 showed a significantly higher TAP level compared to the ketamine group ($P < 0.05$). There was no significant difference in CAT levels in comparison between the

ketamine group and the control mice ($P > 0.05$), while the carvacrol 50, the ketamine + carvacrol 25 and ketamine + carvacrol 50 presented higher levels of CAT compared to the control group ($P < 0.05$).

An increase was seen in the testosterone levels of the group receiving 50 mg/kg carvacrol and animals treated with ketamine + carvacrol 50 ($P < 0.05$). There was no significant difference in LH and FSH levels between the treated groups (Table 2) ($P > 0.05$).

Discussion

In the present study, both biochemical and histopathological examinations indicated that ketamine induced a marked reproductive toxicity through induction of oxidative stress which was prevented by co-administration of carvacrol. Even though effects of oxidative stress on testicular tissues were demonstrated in several studies (18-22), research on the effects of schizophrenia on oxidant levels and the expression of antioxidant enzymes in testicular tissues is sparse in literature. Both the male and female genital systems are rich in enzymatic and non-enzymatic antioxidants. Imbalance in oxidant/anti-oxidant status could cause molecular and genetic defects leading to infertility (23). ROS can reduce male fertility by damaging the sperms' cellular membrane. These incidents can reduce sperm functionality through impairment of metabolism, motility, acrosomal reactivity, fusogenic capacity, and damage to the DNA of sperm (6,24,25). In some studies, it was shown that the antioxidants levels in the blood were correlated with spermatoc counts and motility (5,26,27). In the present study, an increase in lipid peroxidation (MDA) and reduction in TAP in the testes of schizophrenic group (following ketamine administration at a dose of 25 mg/

Table 1. Determination of malondialdehyde, catalase and total antioxidant power in different treatment groups

Groups	MDA (nmol/mg protein)	CAT (U/mg protein)	TAP (mmol/mg protein)
Control	15.50 ± 0.19	33.19 ± 3.64	255.4 ± 18.08
Ketamine	27.5 ± 3.28 ^a	34.83 ± 7.75	239.6 ± 11.40
Carvacrol 25	21.58 ± 0.58	38.92 ± 4.02	260 ± 11.40
Carvacrol 50	19.65 ± 0.92	54.14 ± 4.10 ^c	276 ± 59.80
Ketamine + Carvacrol 25	24.17 ± 1.64	54.41 ± 4.95 ^c	359.2 ± 57.39 ^b
Ketamine + Carvacrol 50	18.22 ± 1.41 ^b	51.60 ± 3.38 ^c	392 ± 34.40 ^b

Data are presented as Mean ± SEM.

^a Significantly different from control group as $P < 0.01$; ^b Significantly different from ketamine group as $P < 0.05$; ^c Significantly different from control group as $P < 0.05$.

Table 2. Determination of testosterone, FSH and LH in different treatment groups

Groups	Testosterone (ng/mL)	FSH (ng/mL)	LH (ng/mL)
Control	2.25 ± 0.06	0.28 ± 0.02	0.27 ± 0.04
Ketamine	2.05 ± 0.05	0.27 ± 0.05	0.26 ± 0.03
Carvacrol 25	2.45 ± 0.03	0.22 ± 0.04	0.26 ± 0.02
Carvacrol 50	3.00 ± 0.09 ^a	0.23 ± 0.02	0.25 ± 0.02
Ketamine + Carvacrol 25	2.47 ± 0.05	0.27 ± 0.04	0.28 ± 0.06
Ketamine + Carvacrol 50	3.03 ± 0.06 ^a	0.25 ± 0.04	0.25 ± 0.03

Data are presented as Mean ± SEM.

^a Significantly different from ketamine group as $P < 0.05$.

kg for 2 weeks) clearly demonstrated the failure in the antioxidant defense system. These results were compatible with Ozyurt et al who induced schizophrenia in rats by dizocilpine (MK-801) and showed a significant oxidative stress by increasing superoxide dismutase in the testes (18). They also reported an increase in tissue MDA and protein carbonyl levels. Parlaktas et al evaluated the effects of melatonin on oxidative stress and histopathologic changes in testis of MK-801-induced psychotic rat model. They demonstrated that MDA, protein carbonyl and NO levels were increased in testicular tissues of rats and treatment with melatonin led to a significant decrease in oxidative injury (28). Data obtained in the present study showed an increase in MDA level in testes of mice treated with ketamine and co-administration of carvacrol led to a significant decrease in MDA levels.

Absalan et al reported that a decrease in membrane integrity might be a result of ROS function induced by ketamine. CAT, which prevents ROS damage, has been found in both human testis and spermatozoa (6). An increase in free radical formation leads to compensatory mechanisms characterized by higher activity of the free radical scavenging enzymes such as CAT (29). Various researches have indicated lower, normal or elevated catalase activity in schizophrenic human patients' sera (30,31). However, in the present study CAT activity in the testes of mice receiving ketamine (schizophrenic model) did not change significantly ($P > 0.05$).

Lower testosterone, LH and FSH levels have been reported in patients with schizophrenia (32-34), although recent surveys detected that the serum testosterone levels showed no difference between schizophrenic and healthy subjects (35,36). This is in concordance with the results obtained from the present study which showed no significant changes in testosterone, LH and FSH in schizophrenic mice model.

Carvacrol possesses significant antioxidant properties. Potent antimutagenic and antioxidant effects of carvacrol have been demonstrated by *in vitro* (37,38) and *in vivo* studies (39). Haeri et al showed that administration of essential oil of *Satureja khuzestanica* (SKEO) (225 mg/kg) that contains carvacrol to male rats increases FSH and testosterone, and improves the weights of testes, epididymides, and accessory sex glands (40). Histopathological analysis also showed that in male rats treated with SKEO (150, 225 mg/kg), the number of spermatogonium, spermatids, spermatozooids and Leydig cells increased and the Sertoli cells underwent hypertrophy. These findings were compatible with our study; however we showed protective effects of carvacrol especially at a dose of 50 mg/kg in ketamine induced model of schizophrenia.

Satureja montana extract, containing carvacrol as a major component (79.75%), resulted in an increase in testosterone levels in rats. However, it led to no significant alteration in serum LH and FSH levels (41). Similar pattern of alterations in FSH, LH and testosterone levels in rats that received Iranian *S. khuzestanica* essential oil, mainly

containing carvacrol (93.9%), was reported by Rezvanfar et al (42). The evidences presented in our study are in accordance with aforementioned studies; we illustrated an increase in the testosterone level in the carvacrol 50 and ketamine + carvacrol 50 groups, with no changes in LH and FSH levels.

Daggulli et al (12) demonstrated that carvacrol decreased MDA levels in serum, and testicular tissue which was elevated through methotrexate therapy. In line with the mentioned study, in the present study, carvacrol 50 mg/kg along with ketamine caused a decrease of lipid peroxidation in the testis. So carvacrol may protect germ cells against oxidative stress and apoptosis.

Conclusion

Although the precise mechanism of oxidative stress as a result of ketamine treatment has not been clarified in the present study, the results of histologic and biochemical analysis support that an oxidative injury occurred in testicular tissues of mice. Our results suggest that oxidative stress exerted on testicular tissues by ketamine was reversed by carvacrol. Further experimental and clinical studies are needed to fully understand the mechanism of action of carvacrol.

Authors' contributions

All authors made substantial contributions to conception and design, and/or acquisition of data, and substantially to the writing of the manuscript. All authors read the final version and confirmed the manuscript publication.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Research protocol was in accordance with Pasteur Institute of Iran laboratory animals' guide and was accepted by the ethics committee of this institute (Ethical No: 930314). All efforts were made to minimize the animals' suffering and to reduce the number of the animals used.

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