ORIGINAL ARTICLE

L-carnitine-loaded nanoparticle ameliorates cypermethrin-induced reproductive toxicity in adult male rats

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ABSTRACT

The objective of this investigation was to find out whether L-carnitine-loaded nanoparticle (LCn) could reduce the reproductive toxicity of cypermethrin (CYP), the widely used insecticide in veterinary medicine in male rats. Twenty male Wistar rats that weighed between 210 and 240 g were split into four groups and treated daily for 2 months. The control group was given 0.9% normal saline solution daily. The second group received CYP (3.83 mg/kg b. w. p. o.) daily. The third group was administered with LCn and CYP (50 mg/kg b. wt. p. o. and 3.83 mg/kg b. wt. p. o., respectively) daily, whereas the fourth group received LCn alone (50 mg/kg b. wt. p. o.) daily. On day 60, all rats were sacrificed and samples were collected. CYP-treated animals exhibited inhibition of testicular anti-oxidative stress mechanisms, testicular steroidogenesis enzyme activity (3β-hydroxysteroid dehydrogenase [3β-HSD] and 17β-HSD), and downregulation of steroidogenic acute regulatory (StAR) gene expression. In addition, it decreased testosterone, follicle-stimulating hormone, and LH levels and had detrimental consequences for sperm quality. LCn attenuated CYP-induced reproductive toxicity via the alleviation of testicular oxidative stress status, improvement of steroidogenic enzyme activity, and upregulation of StAR gene expression, which are probably responsible for the concomitant improvement in testicular hormonal levels and improvement in sperm properties. Intriguingly, LCn treatment alone could enhance the functions of the male reproductive system.

Key words: Antioxidant activity, cypermethrin, hormones, L-carnitine nanoparticle, reproductive toxicity

INTRODUCTION

Environment-related toxicants, such as pesticides, have been linked to impaired sperm quality and male infertility.[1]

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Cypermethrin (CYP) is an extensively employed synthetic pyrethroid with multiple applications in many different fields, including farming, forestry, health care, and livestock care.[2] However, experiments on animals have demonstrated that CYP exposure is harmful to various organs, including the reproductive system.[3,4] Numerous antioxidant enzymes and free radical scavengers are present in testicular tissue that neutralize any potential damage. There is widespread agreement that free radical damage is the primary driver of declining sperm quality.[5] Researchers have found that CYP decreases the activity of antioxidative

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enzymes such as catalase (CAT) and glutathione peroxidase (GPx) while simultaneously elevating the amount of malondialdehyde (MDA).[6] In addition, the lipophilic nature of CYP allows it to bioaccumulate in membranes, resulting in the production of reactive oxygen species that have a detrimental effect on the structure of transport proteins.[7-9]

L-carnitine (LC) is an amino acid which is reported to have antioxidant properties.[10,11] Significant amounts of the LC are detectable in the epididymis, where it is indispensable for spermatogenesis, sperm maturation, and metabolism.[12] Due to its antioxidant properties, LC has a protective effect against the toxicity of various reprotoxic compounds.[13] Oral administration of LC increases sperm motility in men whose sperm have normal glutathione peroxidase phospholipid hydroperoxide levels.[14]

One limitation of the therapeutic use of LC is its lower oral bioavailability, which is attributed to its higher hydrophilicity, which prevents it from freely diffusing through the lipid membrane of the intestine epithelial cells.[15] Thus, one strategy to overcome this obstacle is to use a nanoparticle approach to deliver this compound to increase its bioavailability and cellular uptake. Using the present knowledge at hand, we imply that the potential ameliorative influences of LC loaded into nanoparticles (LCn) against the oxidative stress induced by CYP have not been thoroughly studied. Our objective in this investigation was to ascertain whether LCn may reduce the detrimental effects of CYP on rat testes by assessing some testicular oxidative stress markers, sex hormones, and androgenic enzyme alterations, as well as sperm quality.

MATERIALS AND METHODS

Chemicals

Sigma Aldrich (USA) was the supplier of the CYP, and LCN (47 nm) was obtained from NANOSHEL (USA). Analytical-grade chemicals were used in this study.

Experimental design

Twenty adult male Wistar rats, whose weights ranged from 210 to 240 g, were acclimated for 2 weeks and provided with free food and water. The temperature in the housing was kept at 25°C, and light and dark cycles of 14 and 10 h were utilized. Humane care and management guidelines for laboratory animals were followed, and all animals used in the experiment were handled humanely, which was approved by the University of Al-Qadisiyah (IRAS0822019). Rats were assigned into four experimental groups (five rats per group) and treated as described in the following:

In the control group, 0.9% normal saline was given to the rats through a gastric drencher for 60 days.

The CYP group: They were administered 3.83 mg/kg b. wt. p. o. of CYP daily.[8]

The LCn + CYP group: Administered with LCn as a pretreatment at a dose of 50 mg/kg b. w. p. o. daily, and then 30–60 min later, CYP was administered at a dose of 3.83 mg/kg b. w. p. o.[16]

The LCn group: Administered with LCn by a gastric drencher at a dose of 50 mg/kg b. w. p. o.

The rats utilized in the study were euthanized by inhalation of ether and their testicles were removed. One testicle was utilized to measure lipid peroxidation, enzymatic antioxidants, steroidogenic enzymes, and the level of gene expression. Another testicle was used to test the sperm parameters.

Estimation of testicular oxidative stress status

An ice-cold buffer (0.1 M phosphate buffer + 150 mM KCl, pH 7.4) was used with a tissue homogenizer to create a 10% homogenate of the testis. A portion of the homogenate was tested for lipid peroxidation using spectrophotometry at 530 nm to estimate the amount of MDA produced.[17] In order to measure the antioxidative enzyme activity, the remaining homogenate was centrifuged at 10,000 ×g at 4°C for 10 min and analyzed colorimetrically. To get an estimate of the CAT activity, GR activity, and GPx activity, absorbance was measured at 570 nm, 340 nm, and 420 nm, respectively.[18-20]

Steroid hormones assay

The serum levels of testosterone hormone (TH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were analyzed utilizing rat-specific ELISA kits that were purchased from Qayee-Bio Life Science®, China, and the assays were performed based on the competitive binding principle.

Assessment of steroidogenic enzymes

About 100 mg of testicle tissue was washed and homogenized in phosphate buffered saline (PBS); the sample was then kept at − 20°C overnight. Further, the sample was centrifuged at 5000 ×g for 5 min at 4°C. Thereafter, 3β-hydroxysteroid dehydrogenase (3β-HSD) and the 17β-HSD were measured in the supernatant utilizing rat-specific ELISA kits (MyBioSource, USA).

Quantification of steroidogenic acute regulatory gene expression

RNA Isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction

The homogenate was directly suspended in TRIzol reagent, followed by the extraction of mRNA. The mRNA was reverse transcribed into cDNA using qScript cDNA SuperMix (Quantabio) using 2 μg of total RNA.

The transcripts were amplified with primers detailed in Table 1. Real-time quantitative polymerase chain reaction amplification was done utilizing PowerUp™ SYBRTM Green Master Mix (Thermo Fisher Scientific), and data were analyzed with an Applied Biosystems and software version 3.1 (Thermo Fisher Scientific, MA, USA). Steroidogenic acute regulatory (StAR) gene expression levels were measured using the comparative Ct (ΔΔCt) approach to determine the relative quantitative expression of the StAR gene relative to the control (untreated sample) and then normalized to the β-actin reference gene.

Sperm parameter analyses

Sperm count

In 5 mL of physiological saline, the epididymis was chopped using micro-scissors and shaken for 10 min. Following the inoculation, the sample was diluted in a ratio of 1:100 with 5 g $NaHCO_3$, 1 mL of formalin (35%), and 25 mg of eosin/100 mL of water. Using a hemocytometer, the total number of sperm was calculated. After diluting the sperm solution, approximately 10 µL was placed in each counting chamber of the hemocytometer. A light microscope at a magnification of ×400 was utilized to calculate the sperms that had settled over this time.[22]

Sperm motility

Sperms were collected and diluted with 2.9% Tris buffer solution on a slide. Instantaneously, the percentage of motility was optically assessed at ×400. The mean was utilized to determine the final motility score.[23]

Sperm morphology and viability

The sample of sperm suspension was diluted with formaldehyde, and then 10 µl of the sample was stained with eosin and nigrosin.[24] Using the microscope's ×40 objectives, both stained and unstained sperm cells were counted, and their averages were calculated. The percentage of sperm viability was determined by counting the living and dead cells in five separate fields for each sample, where eosin stains only dead sperm, turning them a dark pink color, while living sperm remain white.

Statistical analysis

For this study, we employed one-way analysis of variance (ANOVA) and LSD to analyze normally distributed data and find statistically significant differences using GraphPad Prism, version 9.5.0; $P \leq 0.05$ was considered statistically significant.

RESULTS

Effect of L-carnitine nanoparticle administration against cypermethrin-induced testicular oxidative stress in rats

The levels of MDA in the testis and the activity of testicular CAT, GR, and GPx are shown in Figure 1. The MDA levels of the CYP groups were significantly ($P \leq 0.05$) greater than those of the controls. In the LCn + CYP group, LCn remarkably ($P \leq 0.05$) minimized the increase in MDA. LCn treatment independently considerably ($P \leq 0.05$) reduced the MDA level relative to the control group, indicating a reduction in lipid peroxidation. On the other hand, the CAT, GR, and GPx activities were reduced considerably ($P \leq 0.05$) following CYP treatment relative to the control group. Comparing the LCn + CYP group to the positive control (CYP) group showed that CAT, GR, and GPx activities in testes were markedly ($P \leq 0.05$) improved in the LCn + CYP group. CAT, GR, and GPx activities were all significantly ($P \leq 0.05$) enhanced by administration of LCn alone relative to the control group [Figure 1].

The effect of L-carnitine nanoparticles against cypermethrin-induced inhibition of steroid hormones levels

CYP significantly ($P \leq 0.05$) decreased TH, FSH, and LH levels in rats. Treatment with LCn significantly ($P \leq 0.05$) improved the level of these reproductive hormones in the LCn + CYP group in contrast to the CYP-treated group. Conversely, rats treated with LCn alone demonstrated significant ($P \leq 0.05$) increases in TH, FSH, and LH relative to the negative control group [Table 2].

The values indicate the mean ± standard deviation [SD], $n = 5$. At the $P \leq 0.05$ level, the statistical significance among groups is indicated by different letters arranged vertically.

The effect of L-carnitine nanoparticles against cypermethrin-induced inhibition of steroidogenic enzyme activity

In the CYP-treated group, both 3β-HSD and 17β-HSD

Table 1: Primer sequence that is used in real-time polymerase chain reaction[21]

Gene	Sense primer	Antisense primer
StAR	5′-GGGCATACTCA ACAACCAG-3′	5′-ACCTCCAGTC GGAACACC-3′
β-actin	5′-TCGTGCGTGACATT AAAGAG-3′	5′-ATTGCCGATAGT GATGACCT-3′

StAR: Steroidogenic acute regulatory

Table 2: The effect of L-carnitine nanoparticles on reproductive hormone changes induced by cypermethrin in rats

Groups	TH (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)
Control	3.12±0.05[a]	0.121±0.004[a]	0.098±0.001[a]
CYP	2.21±0.04[b]	0.078±0.003[b]	0.068±0.001[b]
LCn + CYP	2.71±0.06[c]	0.095±0.002[c]	0.086±0.002[c]
LCn	3.61±0.04[d]	0.162±0.005[d]	0.151±0.003[d]

The statistical significance ($P \leq 0.05$) among groups is depicted by different letters ([a,b,c,d]) arranged vertically. The values indicate the mean±SD, $n=5$. At the $P \leq 0.05$ significance level, the statistical significance among groups is indicated by different letters arranged vertically. LCn: L-carnitine nanoparticles, CYP: Cypermethrin, TH: Testosterone hormone, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, SD: Standard deviation

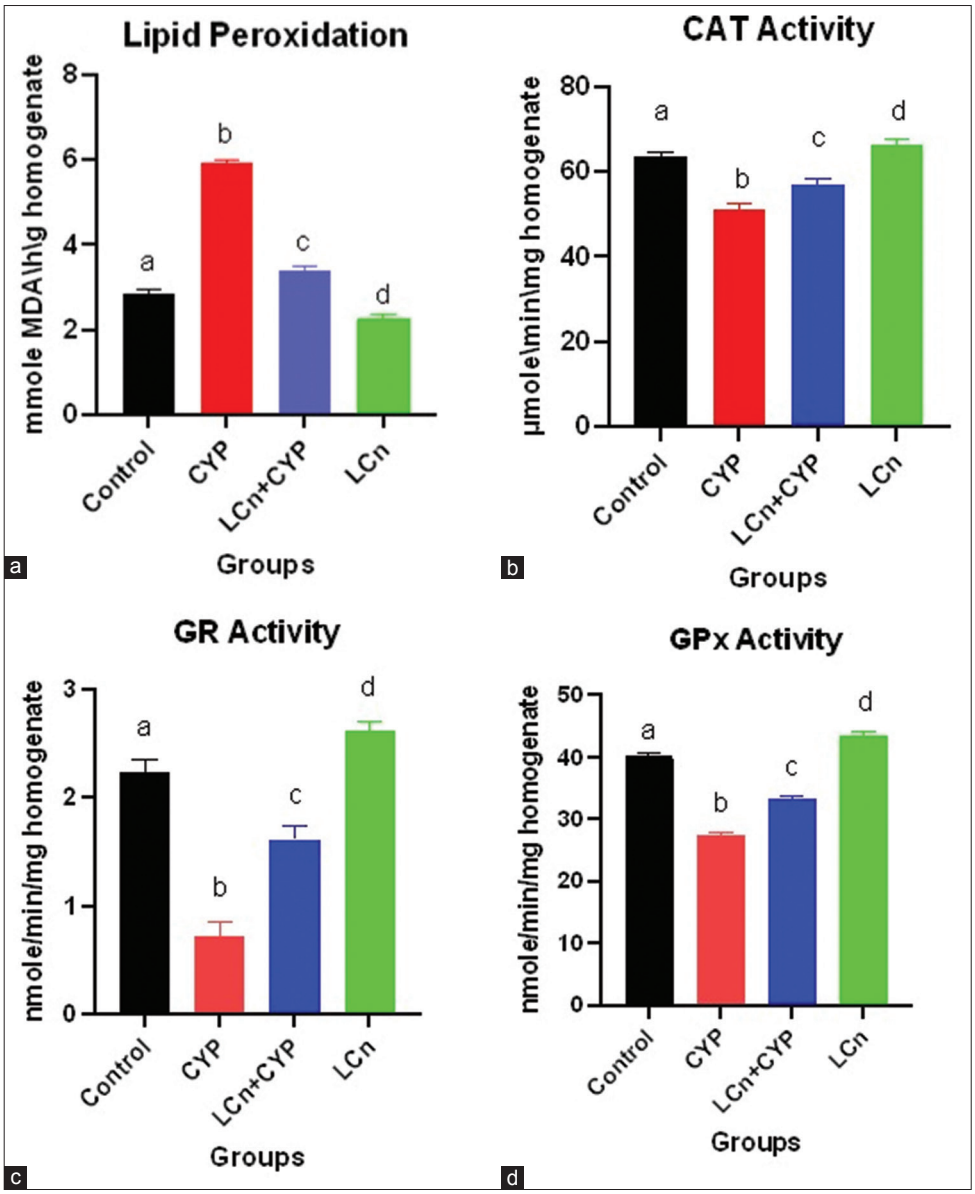

Figure 1: The influence of LCn on oxidative stress caused by CYP in testicular tissues. Oxidative stress parameters in rat testicular tissue included (a) Lipid peroxidation, (b) CAT, (c) GR activity, and (d) Glutathione GPx activity. The figures represent the mean ± SD, $n = 5$. LCn: L-carnitine nanoparticles, CYP: Cypermethrin, CAT: Catalase, SD: Standard deviation

activity were decreased significantly ($P \leq 0.05$) relative to the control group. LCn treatment significantly ($P \leq 0.05$) counteracted the decline in 3β-HSD and 17β-HSD activity induced by CYP treatment. Interestingly, following treatment with LCn alone, the steroidogenic enzyme activity was significantly ($P \leq 0.05$) improved relative to the control group [Table 3].

Table 3: The effect of L-carnitine nanoparticles against cypermethrin-induced inhibition of some testicular androgenic key enzymes

Groups	17-ß HSD (ng/mL)	3-ß HSD (pg/mL)
Control	1.01±0.14[a]	0.84±0.18[a]
CYP	0.49±0.11[b]	0.33±0.11[b]
LCn + CYP	0.78±0.13[c]	0.58±0.13[c]
LCn	1.23±0.11[d]	1.12±0.12[d]

The statistical significance ($P \leq 0.05$) among groups is depicted by different letters ([a,b,c,d]) arranged vertically. LCn: L-carnitine nanoparticles, CYP: Cypermethrin, HSD: Hydroxysteroid dehydrogenase

L-carnitine nanoparticle treatment enhances steroidogenic acute regulatory gene expression

To further investigate the role of LCn in protecting male rats from CYP-induced reproductive toxicity. As shown in Figure 2, CYP significantly ($P \leq 0.05$) inhibited testicular StAR expression to approximately 60% of the control group's level. However, this CYP-induced suppression was significantly ($P \leq 0.05$) counteracted in the presence of LCn. However, it was not fully restored to the level of the control group. Intriguingly, the LCn treatment

significantly ($P \leq 0.05$) increased the expression of the StAR gene relative to the control group [Figure 2].

L-carnitine nanoparticle treatment improves sperm parameters

Upon exposure to CYP, there were a substantial reduction ($P \leq 0.05$) in sperm parameters, including their motility, count, and viability, and an increase ($P \leq 0.05$) in sperm morphological deformity. The outcomes demonstrate that the LCn group significantly ($P \leq 0.05$) restored sperm parameters in the CYP + LCn treated group. LCn administration in rats significantly ($P \leq 0.05$) enhanced motility, viability, and morphological features relative to control animals; however, it did not increase sperm count in comparison to the control group [Figure 3].

DISCUSSION

This study focused on LCn and presented positive data regarding it as a potential strategy for mitigating the harmful effects of CYP-induced testicular toxicity in rats. In addition, this study has shown that LCn alone can enhance testicular functions.

The safety of CYP is increasingly a source of concern because it may have off-target effects on humans, animals, and the environment as a result of its extensive application.[2,25] CYP is one of the most potent and commonly employed insecticides, with a high reproductive toxicity.[4] Peroxidative damage is contemplated as the leading cause of markedly reduced testicular activity underlying the pathological effects of a wide variety of circumstances, such as toxic compound exposure, making the antioxidant defense mechanisms critically important.[26]

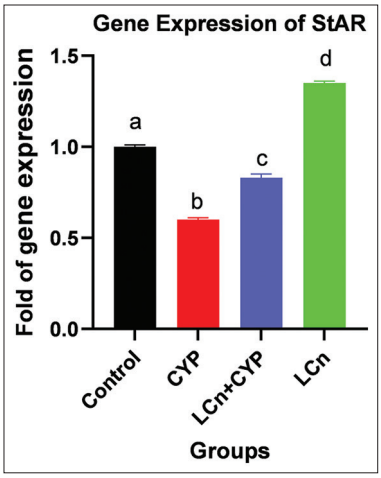

Figure 2: Effect of CYP and/or LCn treatment on StAR gene expression. Figures reflect a mean ± SD; $n = 3$. The use of different letters denotes a statistically significant difference at $P \leq 0.05$ based on a one-way ANOVA followed by the Tukey *post hoc* test. LCn: L-carnitine nanoparticles, CYP: Cypermethrin, StAR: Steroidogenic acute regulatory, SD: Standard deviation

L-carnitine nanoparticles reduce testicular oxidative stress

When CYP accumulates in the testicles, it generates reactive oxygen species (ROS) and causes them to deteriorate, which elevates free radical production and weakens the antioxidant defenses of the body. In this study, CYP exposure induces a marked increase in MDA, a characteristic marker of lipid peroxidation that indicates the excessive formation of free radicals.[27] In this experiment, the treatment with CYP decreased CAT activity. This could be due to hydrogen peroxide buildup in the testicles, where an increase in hydrogen peroxide levels significantly decreases CAT function;[28] it is also possible that CYP could affect the CAT enzyme structure. Furthermore, CYP treatment caused a reduction in GR activity. This might have occurred due to the probable detrimental effect of CYP on the GR enzyme structure. The GPx enzyme is another antioxidant enzyme that has the ability to degrade hydrogen peroxide and other organic hydroperoxides by neutralizing H_2O_2 and lipid peroxides.[29] In this experiment, CYP treatment inhibited GPx activity, which was consistent with the previous report showing a decline in GPx activity following pyrethroid exposure.[30] Damage to membrane and organelle structures occurs when free radicals are not neutralized. LC is reported to have antioxidant effects,[31] as shown in this study. Our results showed that rats that were given LCn before CYP had more CAT and GR activity and less MDA in their testes compared to rats administered with CYP. By boosting the antioxidant defense, LCn may reduce the damage caused by CYP accumulation, resulting in a decreased MDA level and increased activity of the antioxidant enzymes CAT, GR, and GPx.

Positive effect of L-carnitine nanoparticles on steroid hormones

In this analysis, the serum levels of TH, LH, and FSH were reduced in CYP-exposed rats, but they improved after treatment with LCn. Pesticide exposure has been linked previously to reduced levels of pituitary gonadotrophins (LH and FSH) and TH secretions.[32] When TH, LH, and FSH levels fall, it is reasonable to assume that CYP is also impacted further along the hypothalamic–pituitary axis. As LH stimulates TH synthesis by Leydig cells, low LH levels may also result in low TH. The lack of TH, LH, and FSH decreases spermatogenesis and results in insufficient amounts of healthy sperm and ultimately decreased fertility.[33] The results of this investigation provided further corroboration that CYP has negative effects on fertility. The pesticide CYP can also lower LH, FSH, and TH levels, possibly by increasing oxidative stress and interfering with antioxidant enzyme synthesis and activity. Therefore, LCn functions as an antioxidant by reducing oxidative stress via blocking the production of free radicals and boosting antioxidant activity. Thus, oxidative stress is decreased, and the negative consequences of CYP on testicular tissue are mitigated, leading to increased concentrations of sex

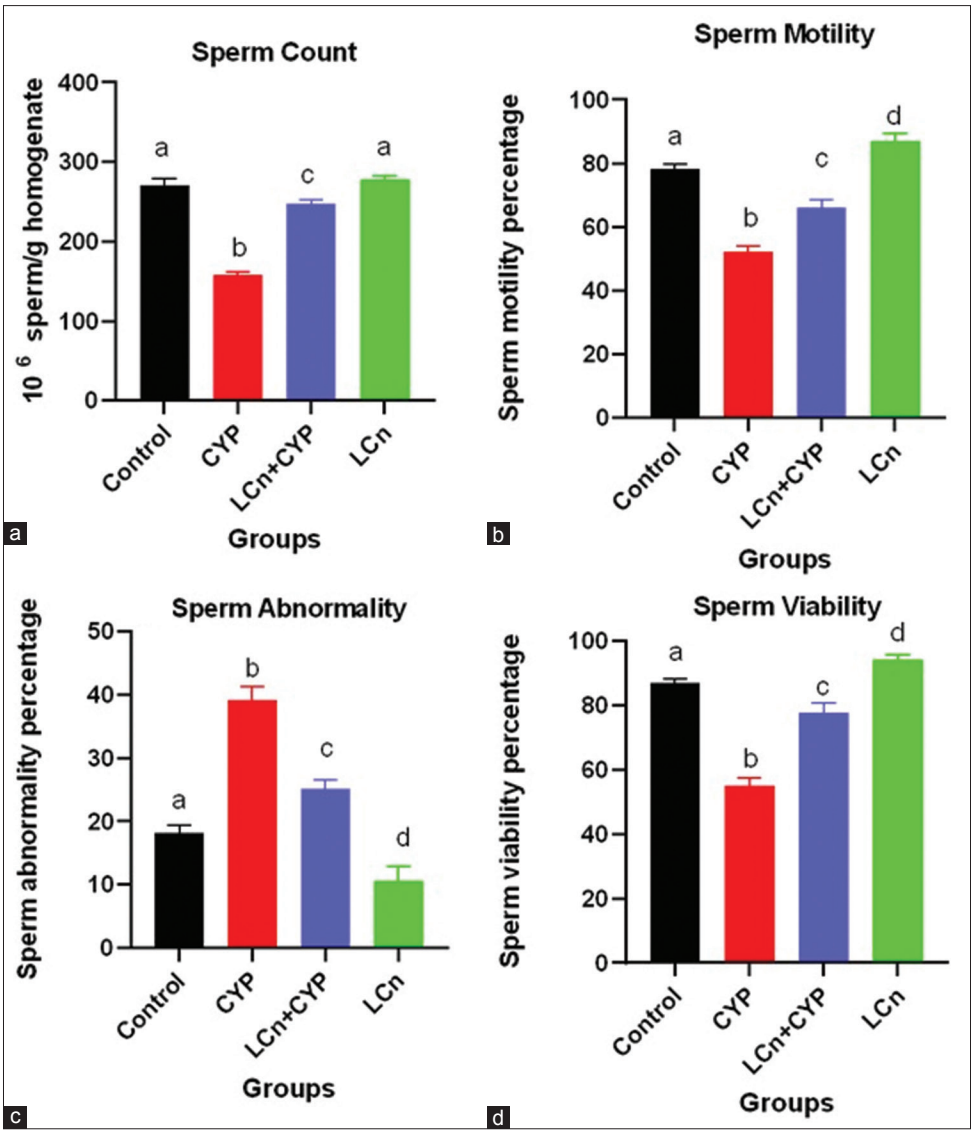

Figure 3: Effect of CYP and/or LCn on sperm parameters. Sperm parameters included (a) Sperm count, (b) Sperm motility, (c) Sperm abnormality, and d: Sperm viability. The figures represent the mean ± SD of normally distributed data. n = 5. Different letters represent the statistically significant differences ($P \leq 0.05$) among groups. CYP = Cypermethrin, and LCn = L-carnitine-loaded nanoparticles

hormones in the blood. On the other hand, aromatase converts testosterone to estrogen, hence playing a crucial role in the steroidogenesis process.[34] Interestingly, LCn was found to reduce the mRNA expression of aromatase in mice.[35] Therefore, increasing the levels of TH in the blood is possible by interfering of LCn with this aromatase activity.

L-carnitine nanoparticle effect on steroidogenic enzyme activity

A marked drop in TH after exposure to CYP that has been reported in this experiment could also be attributed to the direct action of pyrethroids on hormonal production pathways in the testicles. The steroidogenic enzymes 3β-HSD and 17β-HSD were both shown to be diminished in CYP-exposed animals. This decrease in 3β-HSD and 17β-HSD might have occurred due to testicular cell injury, as there was a remarkable rise in oxidative stress in the testicular tissue, or it could be the result of CYP's interference with the transcriptional regulation of the 3β-HSD and 17β-HSD genes. A drop in the levels of 3-HSD and 17-HSD in the testicles caused by CYP was reduced by the pretreatment with LCn. In addition, LCn treatment alone improved the level of these steroidogenic enzymes. This can be attributed to the potential of the LCn to act as an antioxidant as well as a positive regulator of testicular androgenesis.[36]

L-carnitine nanoparticles upregulate steroidogenic acute regulatory expression in testes

Our results showed that CYP treatment reduced testicular StAR gene expression in rats. LCn improved StAR expression in CYP-treated rats. LCn treatment has the ability to improve the expression of StAR compared to control animals. These unique findings might be explained by the

anti-oxidant properties of LCn that can positively affect the expression of this gene. Since pyrethroids are lipophilic, they tend to bioaccumulate in membranes, where they generate ROS.[7] The mitochondrial function is impaired, and the StAR protein is suppressed due to the increased production of ROS.[37] One of the StAR protein functions is to shuttle cholesterol from the cytoplasm to the matrix of the mitochondria.[38] One limitation in the production of TH is the transfer of cholesterol.[38] It has been suggested that pyrethroids inhibit the production of the StAR protein.[39] Thus, in the current experiment, reduced TH levels were observed after exposure to CYP, which might have been mediated by the suppression of StAR protein expression. Intriguingly, it has been observed that LC increases TH levels via upregulating the testicular StAR in male rats.[21] This could also explain why the level of TH increased after LCn was used in this experiment.

L-carnitine nanoparticles enhance sperm parameters in rats

This study demonstrated that treatment with CYP negatively affects sperm quality and dramatically increases sperm abnormalities. There are several possible mechanisms for aberrant sperm production in the testes after CYP exposure. One way is that the buildup of CYP in the testicles could have harmed the Sertoli cells directly, resulting in impaired spermatogenesis and fewer sperm. Second, a decrement in serum TH was observed in the current experiment. Third, increasing levels of ROS have been linked to lower sperm counts.[40,41] Fourth, any change in gonadotropin levels may have an impact on spermatogenesis.[42] Our results indicated that CYP exposure could cause a reduction in FSH and LH levels that might be accountable for the sperm impairment observed in this study. LCn considerably alleviated the drop in the quality of sperm caused by CYP treatment. The current study showed that LCn improved sperm quality, which was in line with earlier research on LC showing that LC regulates Sertoli cell functions and significantly boosts the release of pyruvate and lactate, which are necessary for energy production and sperm cell development in the testis.[43] The improvement of sperm quality by LCn in this part of the experiment is consistent with the antioxidant action of LCn and its beneficial effect on sex hormones demonstrated in this study.

CONCLUSION

It could be concluded that our study exhibited the effectiveness of LCn in protecting male rats against CYP-induced testicular toxicity that may be mediated, at least in part, by its apparent anti-oxidant characteristics, enhancement of steroidogenic enzyme activities, and stimulation of StAR gene expression. All of these components are crucial for the simultaneous improvement of testicular hormone levels, which is necessary for the improvement of sperm quality. An important limitation of this experiment is the lack of a conclusive determination of how LCn affects males' ability to reproduce. More research is needed to determine whether LCn can improve infertile males' fertility and procreative abilities.

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Conflicts of interest

There are no conflicts of interest.

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