IJPSR (2019), Volume 10, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 21 October 2018; received in revised form, 25 January 2019; accepted, 31 January 2019; published 01 July 2019

RECOVERY OF DIMINISHED SPERMATOGENESIS BY RESVERATROL AGAINST THE PYRETHROID, LAMBDA CYHALOTHRIN-INDUCED REPRO-TOXICITY IN ALBINO RATS

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Keywords:

Lambda-cyhalothrin, Resveratrol, Rats, Spermatogenesis, Oxidative stress

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ABSTRACT: Lambda-cyhalothrin [-cyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate] is one of the type II synthetic broad-spectrum pyrethroids used to protect the crops against insects. The aim of the present study was to evaluate the protective effect of resveratrol on male reproductive health in lambda-cyhalothrin exposed rats. Exposure of male rats to lambda-cyhalothrin resulted in a significant reduction in the reproductive organ weights accompanied by a reduction in the testicular daily sperm count and epididymal sperm count, sperm motility, and sperm viability. Further, a significant decrease in the activity levels of superoxide dismutase and catalase with a significant increase in the levels of lipid peroxidation were observed in the testis of lambda-cyhalothrin administered rats over the controls. Moreover, the integrity of testicular architecture was deteriorated in lambda-cyhalothrin exposed rats. Conversely, supplementation of resveratrol enhanced the activity levels of testicular enzymatic antioxidants and inhibited lipid peroxidation levels in lambda-cyhalothrin exposed rats as compared to its respective controls. Significant increase in the selected epididymal sperm variables accompanied by the restoration of testicular architecture was recorded in resveratrol plus lambda-cyhalothrin treated rats over lambda-cyhalothrin exposed rats. On the other hand, no changes were observed in the selected reproductive endpoints in resveratrol administered rats over controls. To conclude, resveratrol could plausibly inhibit lambda cyhalothrin-induced testicular oxidative stress and improves the sperm quality and quantity in rats.

INTRODUCTION: Male infertility is a serious ongoing problem all over the world. Several studies claimed that the exposure of humans to a range of environmental contaminants, including pyrethroids causes several male reproductive disorders [1]. Pyrethroids are used to protect the crops against insects.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(7).3474-81

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(7).3474-81

Type II pyrethroids belong to the broad-spectrum potent organic insecticides which contain cyhalothrin rings and widely used in agricultural, veterinary and household applications [2]. Lambda-cyhalothrin (LCT), is one of the synthetic type II pyrethroid insecticides extensively used to control pests in food crops, non-food crops and against to kill disease vectors such as insect, ticks and flies [3].

Due to its wide usage, LCT has been detected in vegetables and fruits [4], milk and blood of dairy cows [5] as well as in cattle meat [6]. Although LCT exhibits low mammalian toxicity, several studies indicated that LCT exposure in mammals might cause genotoxicity, neurotoxicity, and mutagenicity

[7- 10]. Nowadays, there is compelling evidence that human beings have an increased risk of exposure to LCT [11]. About reproduction, it has been shown that the exposure of male mice to LCT adversely affects the testicular architecture as indicated by Leydig cell degeneration and sperm morphology, quality, and quantity [12]. Studies of Yousef [13] reported that exposure of male rabbits to LCT negatively affects reproductive organ weights, diminished serum testosterone levels accompanied by enhanced testicular oxidative stress. These studies, at least in part, claim that the LCT induced male reproductive toxicity underpins reduced testosterone levels and induction of oxidative stress.

Antioxidants are widely used to protect the cellular systems against a range of environmental pollutants, including testicular toxicants. Resveratrol (RES) (trans-3,4,5-trihidroksi-stilben), is a phenolic dietary phytoalexin with antioxidant properties which is found in various plants and different foods, especially grapes [14]. The therapeutic potential of resveratrol is well acknowledged [15, 16]. Previously, the role of resveratrol against the testicular oxidative damage induced by several testicular toxicants including cypermethrin, pharmaceutical agents, cisplatin and also during obese conditions has been reported [17-22]. However, its probable effects on male reproduction in LCT-exposed rats is not well defined. Given this, the present study was aimed to investigate the possible protective role of resveratrol against lambda cyhalothrin-induced testicular toxicity in adult Wistar rats.

MATERIALS AND METHODS:

Procurement and Maintenance of Experimental Animals: Adult male Wistar rats 170 to 180 g (70 days old) were procured from an authorized vendor (M/s. Raghavendra Enterprises, Bangalore, India). They were housed (four per cage) in polypropylene cages (18″ × 10″ × 8″) lined with sterile paddy husk, under standard laboratory conditions (temperature 30 ± 2 °C; light and dark 12:12 h) at the Animal Facility, Sri Padmavati Mahila Visvavidyalayam, Tirupati. The rats were fed on standard pellet chow (purchased from HLL Animal Feed, Bangalore, India) *ad libitum*. The experiments were carried out by guidelines of the Committee for Control and Supervision of Experiments on Animals, Government of India [23]. The experiments were also reviewed and approved by the Institutional Animal Ethical Committee resolution at Sri Padmavathi Mahila Visvavidyalayam, Tirupati ((Reg. No. 1677/PO/Re/ S/2012 CPCSEA dated 21-12-2015).

Test Chemicals: The test chemicalLambda-cyhalothrin was purchased from a local pesticide store in Tirupati, A.P. Resveratrol (>99% purity) was obtained from TCI Chemicals, Pvt. Ltd., Chennai, India. The other chemicals used in this study are of analytical grade and were obtained from Merck and HIMEDIA (Bangalore, India).

Experimental Design: Rats were randomly divided into four groups with every six animals. Rats in the first group served as controls and did not receive any treatment. Rats in group 2 and 3 were administered orally with lambda-cyhalothrin at a dose of 10.83 mg/body weight ($1/7^{th}$ of LD_{50} value of lambda-cyhalothrin) and resveratrol (20 mg/kg body weight/day) in 30% DMSO, respectively for 55 days. Rats in group 4 received the same experimental regimen like that of rats in group 2 and 3 through oral for 55 days.

Necropsy and Determination of Tissue Somatic Indices: The rats were fasted overnight, weighed and killed by cervical dislocation on the 56^{th} day, and the tissues such as testes, epididymis, seminal vesicles, prostate and vas deferens were dissected out immediately, cleared from adhering tissues and weighed to nearest milligram by using a Shimadzu electronic balance. Tissue somatic indices (TSI) were calculated by using the following formula:

TSI = [weight of the tissue (g) / Body weight of the animal (g)] × 100

Testes were used for determination of daily sperm production, biochemical studies and histopathology and cauda epididymis was used for sperm analysis.

Sperm Analysis: Epididymal sperm suspension was obtained by mining cauda epididymis in 2.0 ml of physiological saline at 37 °C. A 10 ml of epididymal fluid was placed in Neubauer Chamber as described by Belsey *et al*., [24] and total sperms counted. Sperm count was expressed as a million/ml. The number of motile and non-motile sperms was determined according to the method described by Belsey *et al*. [24] The whole process was

performed within 5 min following their isolation from cauda epididymis. Non-motile sperms were counted first next followed by motile sperms.

Sperm motility was expressed as a percentage of the total sperm counted. Sperm viability was determined by the method of Talbot and Chacon [25] using 1% trypan blue. Briefly, mixed one drop of epididymal sperm suspension with a drop of 1% trypan blue solution on a microscope slide and incubated at 37 °C for 15 min. After incubation, the slides were observed under a microscope, unstained spermatozoa were taken as viable and stained were counted as dead. Sperm viability was expressed as a percentage of the total sperm counted.

Daily sperm production was determined in the testis of rats using the method of Blazak *et al*. [26] Briefly; decapsulated testes were homogenized in 50 mL of normal saline (0.9% NaCl) solution containing Triton X-100 (0.01%) using a mortar and pestle. The homogenate is allowed to settle for 1 min then filtered and the filtrate was used for count number of released sperm heads in an improved Neubauer-type hemocytometer. The number of sperm produced per gram tissue of testis per day was calculated [27].

Oxidative Stress Parameters:

Superoxide Dismutase (SOD): The activity of superoxide dismutase was determined by the method of Mishra and Fridovich [28]. Briefly, 0.2 ml of testes homogenate was added to 2.0 ml of carbonate buffer (0.05 M at pH 10.2) and 0.3 ml of freshly prepared epinephrine (30 mM). Immediately changes in absorbance were measured at 420 nm every 10 s interval for 3 min in a spectrophotometer. The activity of SOD was expressed in units per min per mg protein.

Catalase (CAT): The activity of catalase was assayed using the method described by Claiborne [29]. Briefly, to 50 µL of an enzyme, 2.40 mL of 50 mM phosphate buffer (pH 7.0) and 10 µL of 19 mM hydrogen peroxide were added and allowed for a reaction. The decrease in absorbance was measured immediately at 240 nm, against a blank at the 10s interval for 3 min in the spectrophotometer. The activity of CAT was expressed in µM of H_2O_2 consumed/min/mg protein.

Lipid Peroxidation: The levels of lipid peroxidation in testes was measured by a malonaldehyde, a breakdown product was estimated by using thiobarbituric acid (TBA) reagent method described by Hiroshi et al. [30]. Briefly, 10% of tissue homogenization was prepared with 1.15% potassium chloride solution. To 2.5 ml of the homogenate, 0.5 of saline (0.9% sodium chloride), 1.0 ml of 20% (w/v) trichloroacetic acid (TCA) were added, centrifuged at 4000 X g at 4°C for 20 min and collect the supernatant. Mixed the 1.0 ml of supernatant with 0.25 ml of TBA-TCA-HCl reagent (Thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) then the mixture was incubated in a water bath at 95 °C for 30 min.

After incubation, 1 ml of n-butanol was added to samples, mixed thoroughly, and the aliquots were centrifuged at 4000 X g at 4 °C for 15 min. Transferred the organic layers into a clear tube, and the absorbance was measured at 532 nm against a blank and constructed the standard curve using a known amount of malondialdehyde at 532 nm in a spectrophotometer. The rate of lipid peroxidation was expressed as µ moles of malondialdehyde formed/gram wet weight of tissue.

Protein Estimation: The protein content in the testis was estimated by the method of Lowry *et al*. [31] using bovine serum albumin as the standard.

Histological Evaluation of Testes: Testes were collected from both control and experimental rats, cleared off adhering tissues, and fixed separately in Bouin's solution for 24 h. The fixed specimens were dehydrated in ascending series of alcohol and embedded in paraffin blocks after clearing in xylol. 6 µm thick sections were made from fixed testis blocks and then stained with hematoxylin-eosin (H&E), and observed under Olympus phase contrast microscope (Model no: BX41TF) [32].

Statistical Analysis of Data: All data are presented as mean ± standard deviation. Values of $p<0.05$ were considered statistically significant. The differences between control and experimental groups were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc

test. Statistical analysis was performed using the SPSS software for Windows, version 16.0.

RESULTS: No mortality was observed in rats from both control and experimental groups, and none of the rats were excluded from this study. No clinical signs of toxicity, such as loss of fur, salivation, urination, and lethargic activity were noticed in control and experimental rats.

Body Weights and Tissue Indices: The body weights and reproductive tissue indices of control and experimental rats are presented in **Table 1**. No significant changes were observed in the body weights of controls and experimental rats **Table 1**. However, a significant ($p<0.05$) decrease in the indices of the testis and accessory sex organs such as epididymis, seminal vesicles, vas deferens, and prostate gland were observed in LCT administered rats when compared to the control rats. On the other hand, administration of resveratrol significantly ($p<0.05$) increased the weights of reproductive organ weights in LCT exposed rats. Whereas, no changes were observed in the reproductive organ weights of resveratrol exposed rats as compared to the controls **Table 1**.

Spermatology: Significant decrease in the testicular daily sperm production and epididymal sperm variables such as sperm count, sperm motility, and sperm viability was observed in rats exposed to LCT as compared to controls. Conversely, oral administration of resveratrol showed an increase in the selected sperm endpoints in LCT exposed rats **Table 2**. No significant ($p<0.05$) changes were observed in the sperm parameters of resveratrol administered rats as compared to control rats **Table 2**.

TABLE 1: CHANGES IN THE BODY WEIGHTS (g) AND ORGAN INDICES (w/w %) OF LAMBDA CYHALOTHRIN (LCT) TREATED RATS SUPPLEMENTED WITH OR WITHOUT RESVERATROL (RES)

Parameter	Control	LCT	RES	LCT + RES
Body weight	$268.92^{a} \pm 18.49$	$263.86^{a} \pm 21.32$ (-1.88)	$272.14^{a} \pm 21.09$ (1.19)	$269.95^{a} \pm 18.03$ (0.38)
Testes	$1.08^{a} \pm 0.05$	$0.56^{b} \pm 0.03$ (-48.14)	$1.08^{a} \pm 0.04$ (0)	$0.91^{c} \pm 0.365$ (-15.74)
Epidydimis	$0.98^{a} \pm 0.08$	$0.61^{b} \pm 0.06$ (-37.75)	$1.16^{a} \pm 0.14$ (18.36)	$0.88^{c} \pm 0.07$ (-10.20)
Vas deference	$0.08^{a} \pm 0.02$	$0.06^{b} \pm 0.01$ (-25)	$0.11^{a} \pm 0.03$ (37.5)	$0.09^{c} \pm 0.02$ (12.5)
Prostate gland	$0.18^{a} \pm 0.02$	$0.12^{b} \pm 0.02$ (-33.33)	$0.22^{a} \pm 0.03$ (22.22)	$0.16^{c} \pm 0.04$ (-11.11)
Seminal vesicle	$0.55^{a} \pm 0.07$	$0.37^{b} \pm 0.04$ (-32.72)	$0.58^{a} \pm 0.06$ (5.45)	$0.46^{c} \pm 0.10$ (-16.36)

Values are mean ± S.D. of 6 individuals. Values in parenthesis are percent change from that of control. For calculation of % change for RES and LCT groups, rats in control group served as controls. Mean values with different superscripts in a row differ significantly at $p<0.05$.

TABLE 2: CHANGES IN THE TESTICULAR DAILY SPERM PRODUCTION (DSP), AND EPIDIDYMAL SPERM QUANTITY AND QUALITY OF LAMBDA CYHALOTHRIN (LCT) RATS SUPPLEMENTED WITH OR WITHOUT RESVERATROL (RES)

Parameter	Control	LCT	RES	LCT + RES
DSP (millions/g testis)	$22.69^{a} \pm 1.99$	$15.84^{b} \pm 0.40$ (-30.18)	$24.06^{b} \pm 0.84$ (6.03)	$20.11^{c} \pm 0.98$ (-11.37)
Sperm count (millions/mL)	$67.52^{a} \pm 3.40$	$46.12^{b} \pm 2.39$ (-36.69)	$69.54^{b} \pm 4.21$ (2.99)	$56.12^{c} \pm 2.12$ (-16.88)
Sperm motility (%)	$69.82^{a} \pm 2.30$	$40.23^{b} \pm 2.65$ (-42.38)	$68.23^{a} \pm 3.12$ (-2.27)	$51.95^{c} \pm 2.42$ (-25.59)
Sperm viability (%)	$70.45^{a} \pm 2.35$	$44.97^{b} \pm 3.24$ (-36.16)	$68.62^{a} \pm 2.54$ (-2.59)	$53.23^{c} \pm 2.16$ (-24.44)

Values are mean ± S.D. of 6 individuals. Values in parenthesis are percent change from that of control. For calculation of % change for RES and LCT groups, rats in control group served as controls. Mean values with different superscripts in a row differ significantly at $p<0.05$.

SOD and CAT Activity Levels: The activity levels of SOD and catalase were significantly ($p<0.05$) decreased in the microsomal fraction of testis of LCT exposed rats as compared to control rats **Table 3**. Significant ($p<0.05$) increase was observed in the activity levels of SOD and CAT in resveratrol plus LCT treated rats as compared to its respective controls **Table 3**. No changes in the activity levels of SOD and CAT were recorded in resveratrol alone supplemented rats as compared to controls.

Lipid Peroxidation: The lipid peroxidation level was significantly ($p<0.05$) increased in the testis of rats administered with LCT as compared to control rats **Table 3**. A significant ($p<0.05$) reduction in the lipid peroxidation level was observed in rats administered with resveratrol and LCT as compared to its respective controls. Whereas no significant changes were observed in the lipid peroxidation levels of resveratrol alone treated rats as compared to controls **Table 3**.

TABLE 3: CHANGES IN THE OXIDATIVE STRESS PARAMETERS OF LAMBDA CYHALOTHRIN (LCT) RATS SUPPLEMENTED WITH OR WITHOUT RESVERATROL (RES)

Parameter	Control	LCT	RES	LCT + RES
Superoxide dismutase	$9.24^{a} \pm 1.03$	$4.59^{b} \pm 0.58$	$10.05^{a} \pm 1.09$	$7.12^{c} \pm 0.95$
(n moles pyrogallol oxidized/mg protein/min)		(-50.32)	(8.76)	(-22.94)
Catalase	$28.51^{a} \pm 2.08$	$15.87^{b} \pm 3.60$	$30.21^{a} \pm 2.01$	$22.66^{c} \pm 2.49$
(n moles of H_2O_2 metabolized/mg protein/min		(-44.33)	(5.96)	(-20.51)
Lipid peroxidation	$12.84^{a} \pm 0.92$	$25.11^{b} \pm 1.62$	$13.89^{a} \pm 1.10$	$17.20^{c} \pm 1.21$
(µ moles of malondialdehyde/g tissue)		(95.56)	(8.17)	(33.95)

Values are mean ± S.D. of 6 individuals. Values in parenthesis are percent change from that of control. For calculation of % change for RES and LCT groups, rats in control group served as controls. Mean values with different superscripts in a row differ significantly at $p<0.05$.

Histological Changes: In control rats, the histological examination of testicular sections showed a clear basement membrane with lumen completely occupied by spermatozoa. In LCT exposed rats, disruption of testicular architecture with lumen devoid of sperm was noticed. On the other hand, the architecture of testis was restored in resveratrol plus LCT treated rats as evidenced by the recovery of damaged seminiferous tubules with lumen occupied by spermatozoa accompanied by intact interstitium with good Leydig cells **Fig. 1**.

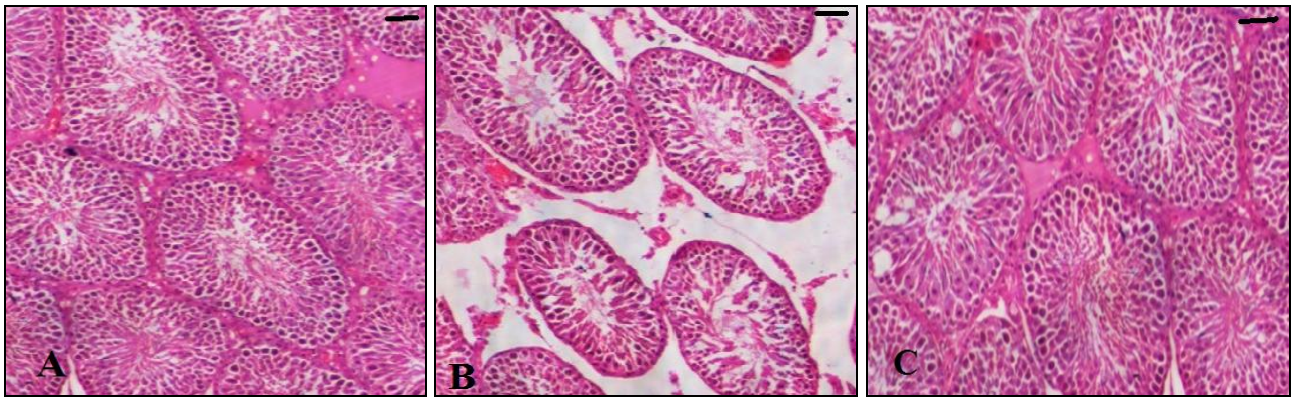

FIG. 1: PHOTOGRAPHS TESTICULAR HISTOLOGY OF CONTROL RATS (A) AND LCT (B), LCT + RESVERATROL (C) SCALE BAR = 25 µm

DISCUSSION: The role of testosterone in the regulation of structural and functional integrity of reproductive organs is well acknowledged. In this study, a reduction in the weights of testis and epididymis could be correlated to the inadequate supply of androgens in LCT exposed rats. The present results are in agreement with earlier studies where treatment with LCT reduced the weight of testes in rats [12, 33]. It is also known that the testicular weight is largely dependent on the mass of the differentiated spermatogenic cells. Thus, the significant reduction in the weight of testes in LCT-exposed rats could be attributed to a decrease in the number of spermatogenic cells and sperms.

Accordingly, we found a disrupted testicular architecture with lumen almost devoid of sperm in LCT-exposed rats. The results are in agreement with the studies of Al-Sarar *et al.* [12] Testicular spermatogenesis, and epididymal sperm maturation events are considered as crucial determinants for male fertility. In the present study, we found a reduction in the daily sperm production, and epididymal sperm variables such as sperm count, sperm motility and sperm viability in LCT-exposed rats might reflect diminished sperm quality and quantity.

Previous studies also reported the treatment with LCT and other pyrethroids decreased the spermatogenic cells leads to decreased the weights of testis [20, 33, 34, 35]. The functional integrity of testis and epididymis require an adequate supply of testosterone. Previously, it has been shown that LCT deteriorates the testicular Leydig cells testosterone biosynthesis [36]. These authors further demonstrated that LCT acts not only at the level of testis but also influence pituitary luteinizing hormone production, thereby testosterone synthesis.

At the molecular level, it has been shown that LCT negatively targets the expression level of 3β-hydroxysteroid dehydrogenase mRNA, an enzyme involved in the catalysis of cholesterol to testosterone and steroidogenic acute regulatory proteins mRNA, a protein involved in the transport of cholesterol from outer to inner mitochondrial membrane [36]. Previous studies reported that the exposure to pyrethroids significantly reduced the serum levels of testosterone, and it leads to decreased spermatogenesis [12, 37]. Thus, a significant reduction in the testicular daily sperm count and epididymal sperm endpoints accompanied by a significant reduction in the reproductive organ weights in cypermethrin exposed rats might support this notion.

Several studies claimed that oxidative stress is one of the main reasons for the testicular toxicant-induced repro-toxicity. We found a significant reduction in the activities of testicular SOD and catalase with a concomitant increase in the levels of lipid peroxidation in testes of LCT-exposed rats, suggesting oxidative imbalance. Under normal circumstances, equilibrium is maintained between pro- and anti-oxidants in cellular systems. SOD is the primary defense enzyme against oxidative stress [38] and plays a crucial role in dismutation of superoxide anions, and catalase neutralizes hydrogen peroxides to molecular oxygen and water [39]. A significant reduction in the activity levels of testicular SOD and CAT could reflect improper dismutation of superoxide anions and the inability to eliminate H_2O_2 in LCT-exposed rats. MDA being a useful indicator of oxidative damage into cells and tissues [40] is probably like the end product and an important molecular marker of lipid peroxidation.

The enhanced MDA levels with a reduction in the selected enzymatic antioxidants could reflect a failure of the antioxidant defense system to negate the testicular oxidative damage induced by free radicals in LCT-exposed rats. Being rich in PUFA, the sperm plasma membrane is susceptible to free radical attack. Thus, testicular oxidative stress environment could adversely affect sperm quality and quantity [41]. Many studies have suggested that in rats, LCT, and other pyrethroids-induced oxidative stress results in increased lipid peroxidation products and decreased antioxidant enzymes in the testes and epididymis and causes reproductive toxicity [20, 33].

Administration of resveratrol, a natural polyphenol ameliorated those above LCT-induced repro-toxic effects in rats. We found a significant increase in the weights of reproductive organs accompanied by an increase in the testicular daily sperm production and selected epididymal sperm endpoints in resveratrol plus LCT treated rats, suggesting the protective effects of resveratrol against the LCT at the level of testicular Sertoli- and Leydig cells. Previously, the protective effect of resveratrol directly on Leydig cell functions [20] and on spermatogenesis [42] (Jiang *et al.*, 2008) has been reported. In this study, we found a significant increase in the testicular daily sperm count and selected epididymal sperm endpoints supports this notion.

In general, SOD renders its activity *via* removal of superoxides and CAT neutralizes the toxic effect of hydrogen peroxide in cellular systems. A significant decrease in the activity levels of SOD and CAT with a significant increase in the lipid peroxidation levels could reflect testicular oxidative stress in rats treated with LCT. One of the interesting findings reveals that resveratrol ameliorated LCT-induced oxidative stress in the testis of rats as indicated by a significant increase in the activity levels of SOD and CAT with a decrease in the lipid peroxidation levels. This might be attributed to the dismutation of superoxides by SOD and removal of hydrogen peroxides by CAT in the testis of LCT treated rats supplemented with resveratrol.

Previously, the antioxidant potential of resveratrol has been elaborated against the testicular and epididymal oxidative damage in experimental animals [20, 21, 43, 44]. The improvement in the testicular antioxidant status and enhanced testicular steroidogenesis could probably restore testicular architecture in resveratrol plus LCT treated rats. Also, resveratrol treatment has been shown to improve the epididymal sperm parameters, and testicular antioxidant defense system in humans and animal models [45, 46, 47].

CONCLUSION: To conclude, LCT-induced male reproductive toxicity could be attributed to the

testicular oxidative stress, and resveratrol with its antioxidant properties ameliorated testicular oxidative stress and restored the testicular and epididymal functions.

ACKNOWLEDGEMENT: The authors are thankful to the Head, Department of Biotechnology, Tirupati for providing the laboratory and animal facilities. Authors are highly thankful to the Department of Biotechnology, New Delhi for financial support in the form of DBT-In house project for M.Sc., sanctioned to one of the author, Ms. Pravallika, M.Sc., Authors are also thankful to DST-CURIE, Central Instrumentation Facility, Sri Padmavati Mahila Visvavidyalayam (Women's University) Tirupati, for providing permission to utilize equipments to complete analysis of selected parameters.

CONFLICT OF INTEREST: The authors declare that there are no conflicts of interest concerning the authorship or publication of this article.

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How to cite this article:
Pravallika M, Vasudha K, Archana D, Sainath SB and Kishori B: Recovery of diminished spermatogenesis by resveratrol against the pyrethroid, lambda cyhalothrin-induced repro-toxicity in albino rats. Int J Pharm Sci & Res 2019; 10(7): 3474-81. doi: 10.13040/IJPSR. 0975-8232.10(7).3474-81.