ORIGINAL ARTICLE

α -lipoic acid protects testis and epididymis against linuron-induced oxidative toxicity in adult rats

P. Prathima¹ · K. Venkaiah¹ · T. Daveedu¹ · R. Pavani¹ · S. Sukeerthi¹ · M. Gopinath² · Sri Bhashaym Sainath¹

Received: 29 September 2019 / Revised: 20 November 2019 / Accepted: 10 December 2019 / Published online: 3 March 2020
© Korean Society of Toxicology 2020

Abstract

Linuron is well known for its antiandrogenic property. However, the effects of linuron on testicular and epididymal pro- and antioxidant status are not well defined. On the other hand, α -lipoic acid is well known as universal antioxidant. Therefore, the purpose of this study was twofold: firstly to investigate whether linuron exposure alters antioxidant status in the testis and epididymis of rats and if so, whether the supplementation of α -lipoic acid mitigates linuron-induced oxidative toxicity in rats. To address this question, α -lipoic acid at a dose of 70 mg/Kg body weight (three times a week) was administered to linuron exposed rats (10 or 50 mg/Kg body weight, every alternate day over a period of 60 days), and the selected reproductive endpoints were analyzed after 60 days. Respective controls were maintained in parallel. Linuron at selected doses reduced testicular daily sperm count, and epididymal sperm count, sperm motility, sperm viability, and number of tail coiled sperm, reduced activity levels of 3β - and 17β -hydroxysteroid dehydrogenases, decreased expression levels of StAR mRNA, inhibition of testosterone levels, and elevated levels of testicular cholesterol in rats over controls. Linuron intoxication deteriorated the structural integrity of testis and epididymis associated with reduced the reproductive performance over controls. Conversely, α -lipoic acid supplementation enhanced sperm quality and improved the testosterone synthesis pathway in linuron exposed rats over its respective control. Administration of α -lipoic acid restored inhibition of testicular and epididymal enzymatic (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase) and non-enzymatic (glutathione content), increased lipid peroxidation and protein carbonyl content produced by linuron in rats. α -lipoic acid supplementation inhibited the expression levels of testicular caspase-3 mRNA levels and also its activity in linuron treated rats. To summate, α -lipoic acid-induced protection of reproductive health in linuron treated rats could be attributed to its antioxidant, and steroidogenic properties.

Keywords Alpha lipoic acid · Linuron · Oxidative stress · Rat · Sperm · Testosterone

Introduction

Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) is a urea-based herbicide which is widely used to control the growth of broad leaf weeds and grasses in agricultural

crops [1, 2]. Due to its wide usage in agriculture, it enters into the surface water via agriculture runoff thereby escapes into the environment [3]. Linuron has been detected in food and drinking water [4] and according to the European Food Safety Authority ([1]), it has been classified as a carcinogen and toxic for reproduction under category 2 and 1B, respectively. This is particularly alarming and attained public attention. The anti-androgenic effects of linuron have been demonstrated in mammals and other vertebrates including fishes and amphibians [5–9]. Though considered as weak anti-androgen [10, 11], it can able to compete with androgens for androgen receptors (ARs) with a K_i of 100 μ M [12, 13]. It has been shown that linuron exposure adversely affect the androgen dependent activities such as reduction in anogenital distance, retention of nipples, suppressed testosterone levels, poor sperm quality and quantity and even resulted

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s43188-019-00036-y>) contains supplementary material, which is available to authorized users.

✉ Sri Bhashaym Sainath
drsainathvsu@gmail.com

¹ Department of Biotechnology, Vikrama Simhapuri University, Nellore, AP 524 320, India

² Department of Pharmacy, Ratnam Pharmacy College, Muthukur, Nellore, AP, India

in epididymal and testicular malformations [10, 11, 14–17]. Further, studies of Nam et al. [18] indicated that linuron negatively affects enzymatic antioxidants in the prostate gland of rats. In order to develop therapeutic strategies, it is considered important to gain insights into the mechanism of action by which linuron-mediated repro-toxicity. Though, several studies highlighted the adverse effects of linuron on androgen-dependent tissues [14, 15, 17], studies linking the probable effect of linuron on testicular and epididymal antioxidant status is poorly understood, especially in adults.

The therapeutic role of antioxidants against the chemical-induced testicular oxidative damage is widely acknowledged. Among a range of antioxidants, α -lipoic acid (LA; 1,2, dithiolane-3-pentanoic acid) is well recognized as universal antioxidant and also for its key role in the energy metabolism [19]. Based on its extraordinary antioxidant properties, several studies elaborated the beneficial effects of LA on male reproductive health against a broad spectrum of toxic insults [20–22] wherein oxidative stress is a part of the underlying etiology. In addition, anti-apoptotic properties against the arsenic [23] and the steroidogenic properties of LA against the 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin has been demonstrated [24].

Considering the above literature, the present study was aimed to address the following questions: a) to elucidate the alterations in reproductive endpoints with linuron exposure in adult rats and to investigate whether the linuron-induced alterations in the selected reproductive endpoints underlie impairment of pro- and anti-oxidant status, and b) if so, whether the supplementation of LA ameliorate linuron-induced suppressed male reproductive health in rats.

Materials and methods

Chemicals

The chemicals such as linuron and α -lipoic acid were obtained from Sigma chemicals Co. (St. Louis, MO, USA). The kits and other chemicals used in the current study were of reagent grade and obtained from Sigma Chemicals Co. (St. Louis, MO, USA), and Merck Laboratories (India). These chemicals were purchased from local suppliers.

Animals

Albino rats of Wistar strain were selected as animal models in this study. They were obtained from the Narayana Medical College, Nellore, AP, India. The rats were maintained in standard plastic cages (18"×10"×8") and provided the sterile paddy husk as the bedding material. The bedding material was changed twice a week. During the acclimatization period, the rats were maintained under controlled

laboratory conditions (temperature: 22–25 °C; light: 12 h light/dark cycle and the relative humidity: 50±5 °C) for about 2 weeks prior to the start of the experiment. After completion of acclimatization period, the selected experiments were designed and performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India [25] and approved by the Institutional Animal Ethical Committee (vide No. 1558/PO/a/11/CPCS EA/RIP/2015/03-10). During their acclimatization period, rats had free access to standard chow (NIN, Hyderabad, Telangana state, India) and water.

Experimental design

Wistar male rats (185±10 g; 90 days old) were randomly allocated to six groups (n=10 rats/group). Animals in group I served as controls and received corn oil (solvent used to dissolve linuron) in a volume of 2 ml. The corn oil was given on every alternative day over a period of 60 days. Rats in group II and III were exposed to linuron via gavage every alternate day at a final dose of 10 and 50 mg/Kg bodyweight (BW), respectively over a period of 60 days. Rats in group IV were intraperitoneally injected with α -lipoic acid three times a week over a period of 8 weeks at a dose of 70 mg/Kg body weight [26]. Whereas the animals in group V were exposed to linuron at a dose of 10 mg/Kg bodyweight via gavage every alternate day over a period of 60 days and intraperitoneally injected with α -lipoic acid three times a week over a period of 8 weeks at a dose of 70 mg/Kg body weight. Rats in group VI were exposed to linuron at a dose of 50 mg/Kg bodyweight via gavage every alternate day over a period of 60 days and intraperitoneally injected with α -lipoic acid three times a week over a period of 8 weeks at a dose of 70 mg/Kg body weight.

The dose selection of linuron was based on the previous studies [10, 18, 27] and the time line 60 days was to evaluate the probable effect of linuron on one complete cycle of spermatogenesis [28]. The selected doses of linuron were prepared in the corn oil and the animals received linuron at selected doses in a volume of 2 ml according their body weights. The required concentration of LA was prepared freshly by dissolving LA in saline at an alkaline pH (7.8). The test chemicals were prepared freshly and before their administration, they were adjusted according to the body weights of animals. Rats were analyzed for the selected reproductive endpoints after completion of the experimental period.

Fertility examinations

To evaluate the fertility efficacy, rats from control and experimental groups were cohabited with the normal cycling

females (90 days old: the female rats with proestrus stage were carefully monitored and kept for mating) of proven fertility in a one male to one female ratio. The mating period was restricted to 6 days. During the six day mating period, every morning the vaginal smear was collected and examined microscopically for the presence of sperm. The occurrence of sperm in the vaginal smear was considered as the day 1 of pregnancy/gestation day 1. The number of successful mating (conception days) was calculated on the presence of sperm in the vaginal plug in the morning. The mating- and fertility-indices were determined by the formula: [(number of sperm positive females/number of pairing) $\times$ 100] and [(number of pregnant rats/number of sperm positive females) $\times$ 100], respectively. Total number of pups delivered to respective females cohabited with the control and experimental rats were recorded. The body weights of pups were recorded. Pups were also analyzed for the androgen-dependent developmental landmarks such as age of testicular descent and anogenital distance.

After completion of the cohabitation period, male rats from control and experimental groups were fasted overnight, weighed, and euthanized via cervical dislocation. Animals were humanely sacrificed and the testes, epididymis, seminal vesicles, vas deferens and ventral prostate were collected immediately, weighed to the nearest milligram using an Electric balance (Shimadzu Model: BI-220H). Tissue somatic index (TSI) was determined using the following formula: [(weight of the tissue/weight of the rat) $\times$ 100].

Semen analysis

Testis was used to analyze the daily sperm production and epididymis was used to assess the sperm motility, number of viable, motile, and tail coiled sperm in control and experimental rats. Sperm suspension obtained by mincing the cauda part of epididymis in 2 ml of physiological saline (0.9 g NaCl in 100 ml autoclaved distilled water) at 37 °C. Sperm count and progressive sperm motility were determined according to Belsey et al. [29]. Trypan blue reagent (1%) was used to determine the viable sperm in the sperm suspension [30]. Sperm membrane integrity was analyzed by exposing the sperms to hypo-osmotic solution and observed for coiled tails under the microscope [31]. The basic principle behind the hypo-osmotic swelling test (HOS-T) is based on the fact that the tails of sperm with intact membrane coils and swell due to the influx of the fluid under hypo-osmotic stress, whereas the tails of sperm with membrane damage do not respond to the hypo-osmotic pressure [32]. The motility, viability and HOS-coiled sperms were expressed as a percentage of total sperm counted. The sperm count was expressed as millions/ml. Daily sperm count (DSP) from the testis was performed according to the method described by Robb et al. [33] and the units of DSP were expressed as

millions/g testis. Morphological changes in the sperms were identified according to the protocol described by Linder et al. [34]. One hundred sperms from each animal were evaluated and analyzed for the sperm abnormalities such as head defect, middle piece defect, tail defect and detached head. The morphological changes in the sperms were determined by light microscope (Model no. HV-12TR) and the percent of abnormality was calculated. To assess the sperm DNA damage, comet assay technique was performed [35]. For visualization of comets, the slides were stained with ethidium bromide. The formation of comets was visualized using fluorescence microscope (Model: CX43; Make: Olympus, Japan) and the resulting images were captured on a computer attached to the microscope. The captured images were analyzed using Image analysis software, CASP Lab. To avoid the exposure of sperm samples to light, all the experiments were performed in dark. All the experiments were performed in triplicate (100 cells were analyzed for comet formation per slide and a total of 3 slides per animal were prepared) for each treatment and were independently coded and scored. Accordingly, 300 cells were analyzed per animal for comet formation. Finally, the data was expressed as percent number of comets formed per 100 cells.

Testicular steroidogenesis

Cholesterol levels

Total cholesterol levels were determined according to the method described by Zlatkis et al. [36]. Briefly, the reaction mixture containing the 0.2 ml of testicular homogenate which was prepared in 5 ml of ferric chloride solution (0.05% of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in glacial acetic acid) and 3 ml of sulphuric acid were thoroughly mixed followed by incubation for 20 min. The incubation mixture without testicular homogenate was used as the blank. The absorbance was noted at 540 nm on a spectrophotometer (Make: Analytical Technologies; Model No: 2060). The cholesterol levels were expressed as mg/g testicular weight.

Steroidogenic marker enzyme activity levels

The activity levels of 3β hydroxysteroid dehydrogenase (3β HSD) (EC 1.1.1.51) and 17β hydroxysteroid dehydrogenase (17β HSD) (EC 1.1.1.64) were determined based on the protocol described by Bergmeyer [37]. Briefly, the testicular homogenate (5% w/v) prepared in ice cold Tris-HCl at pH 6.8 was centrifuged to separate the microsomal fraction. This was used as an enzyme source. A 2 ml reaction set up was prepared to analyze the activity levels of testicular 3β -HSD and 17β -HSD. The reaction mixture for 3β -HSD contains 0.08 μmol of dehydroepiandrosterone, 100 μmol of NAD and 0.1 M pyrophosphate buffer (pH 7.4) and the

reaction mixture for 17 β -HSD contains 0.08 μ mol of androstenedione, 100 μ mol of NADPH and 0.1 M pyrophosphate buffer (pH 7.4). To the reaction mixtures, 20 mg of enzyme source was added separately. The reaction mixtures without the substrate(s) were used as blank. The absorbance was measured at 340 nm on a spectrophotometer. The units for 3 β - and 17 β -HSDs were expressed as nmol of NAD converted to NADH/mg protein/min and nmol of NADPH converted to NADP/mg protein/min, respectively.

Serum testosterone levels

Circulating testosterone levels of the control and experimental rats were analyzed by using an enzyme linked immunosorbent assay (ELISA) kit [Diametra, Italy] purchased from the local suppliers. Briefly, blood was collected from the heart using a heparinized syringe from each animal before necropsy, followed by a centrifugation at 2000 *g* for 15 min. The serum was separated from the blood by overnight incubation at 4 °C and stored at –20 °C until all of the samples were collected. To avoid interassay variation, all the samples were run at the same time. The sensitivity of the test ranges between 0 to 16 ng/ml. The circulatory levels of testosterone were expressed as ng/ml.

Oxidative stress parameters

Tissues such as testis and cauda epididymis were selected to determine the oxidative stress markers. The selected tissues were homogenized in ice-cold phosphate buffer (pH 7.0) using mortar and pestle followed by centrifugation step at 10,000 *g* for 30 min at 4 °C. The enzymatic evaluations, protein carbonyl and lipid peroxidation contents were determined in the resultant supernatant.

Enzymatic antioxidants

Superoxide dismutase (SOD; EC: 1.15.1.1) activity was determined based on its ability to inhibit the auto oxidation of epinephrine at alkaline medium and the oxidation of epinephrine was followed in terms of production of adrenochrome, which exhibits absorption maximum at 480 nm [38]. The reaction mixture consists of 0.05 M carbonate buffer (pH 10.2), EDTA (4%), epinephrine (3×10^{-2} M) dissolved in 0.1 N HCl and the enzyme source. The SOD activity was calculated as Units/mg protein. The activity of catalase (CAT; EC: 1.11.1.6) was determined based on its ability to decompose hydrogen peroxide at 240 nm spectrophotometrically [39]. The reaction mixture of 1 ml contained 0.1 M phosphate buffer (pH: 7.4), 0.05 M hydrogen peroxide (H₂O₂) and the enzyme source. The CAT activity was represented as micromoles H₂O₂/mg protein/min. The activity levels of glutathione peroxidase (GPx; EC: 1.11.1.9)

and glutathione reductase (GR; EC: 1.1.8.7) were performed according to the methods described by Flohe and Gunzler [40] and Carlberg and Mannervik [41], respectively. GPx activity was determined via the system glutathione/NADPH glutathione reductase by the dismutation of *tert*-butylhydroperoxide at 340 nm. GR activity was determined through the oxidation rate of NADPH at 435 nm spectrophotometrically in a reaction medium containing 0.1 M NaH₂PO₄ (pH 7.0), including 1 mM EDTA, 0.1 mM oxidized glutathione (GSSG) and 0.1 mM NADPH.

Glutathione (GSH) assay (non-enzymatic antioxidant)

The GSH content in the testis and epididymal regions were determined based on the protocol described by Beutler [42]. Briefly, after tissue excision, the tissue acid extracts were obtained immediately by the addition of 12% trichloroacetic acid. (1:4 v/v) and then centrifuged. The resultant supernatant was used as the GSH source. The reaction mixture contains the following components: 0.25 mM Ellman's reagent (5,5'-dithio-bis-[2-nitrobenzoic acid prepared in 0.1 M NaH₂PO₄ (pH 8.0) and the GSH source. The formation of thiolate anion was measured at 412 nm on the spectrophotometer.

Lipid peroxidation (LPx) levels

The level of lipid peroxidation in the selected tissues was determined as per the method described by Ohkawa et al. [43] using thiobarbituric acid (TBA) reagent. The reaction mixture (2.5 ml) of LPx contains the tissue homogenates prepared in phosphate buffer (pH 7.2), 1.0 ml of TCA and 1.0 ml of TBA. The reaction mixture was thoroughly mixed followed by heating step in a boiling water bath for 20 min. After cooling, the tubes containing the reaction mixture were centrifuged at 1000 *g* for 10 min. The absorbance was then measured in the resultant supernatant at 535 nm using a spectrophotometer. The rate of LPx was expressed as μ moles of malondialdehyde formed/g wet wt. of tissue.

Protein carbonyl content

Protein carbonyl content in the tissues was determined according to the method developed by Levine et al. [44]. This is one of the most common and reliable methods to analyze the protein carbonyl content in the tissues based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form a 2,4-dinitrophenylhydrazone. Briefly, the testis or cauda epididymis was homogenized in 10 volumes of 1.15% KCl, tris-HCl (10 mM, pH 7.4) at 4 °C. The homogenates were centrifuged at 10,000 rpm for 15 min and the resultant supernatant was used to determine

the protein carbonyl content. The units for protein carbonyls were expressed as nmol carbonyl/mg protein.

Estimation of proteins

The protein content in the testis and different regions of epididymis were determined according to method described by Lowry et al. [45]. The unknown protein concentrations in the samples were determined using bovine serum albumin as the standard.

Testicular caspase-3 and steroidogenic acute regulatory proteins (StAR) mRNA levels

The expression of steroidogenic acute regulatory proteins and caspase-3 mRNA levels was determined using semi-quantitative PCR. Total RNA was isolated using the Trizol plus purification system (Invitrogen, Carlsbad, USA) and the procedure to isolate the total RNA was strictly adhered to the manufacturer's instructions. The purity of RNA was analyzed spectrophotometrically and by agarose gel electrophoresis. The first strand cDNA synthesis was performed as per the manufacturer's instructions of iscript™ cDNA synthesis kit (Biorad, India) using 1 µg of total RNA. The first strand cDNA was used to determine the expression levels of cholesterol transport proteins, steroidogenic acute regulatory proteins (StAR), oxidative stress marker, caspase-3 and house-keeping gene or internal standard, glyceraldehydes phosphate dehydrogenase (GAPDH) via semi-quantitative PCR (Applied Biosystems, SimpliAmp™, Thermal cycler). The reaction mixture contains 10 µl of Phusion mixture (Thermo Scientific), 1 µl of forward primer (FP), 1 µl of reverse primer (RP), 2 µl of cDNA and 6 µl of nuclease free water. The PCR cycle conditions were as follows: step 1 includes 1 cycle of 95 °C, for 30 s followed by 30 cycles of 95 °C for 5 s, 55 °C for 15 s (step 2) and final step includes 1 cycle of 72 °C for 10 min. The primers [StAR (NM_031558; FP: 5'-TTGGGCATACTCAACAACCA-3'; RP: 5'-ATGACA CCGCTTGCTCAG-3'); caspase-3 (NM_012922; FP: 5'-TACCCTGAAATGGGCTTGTGT-3'; RP: 5'-GTTAAC ACGAGTGAGGATGTG-3'); and GAPDH (NM017008; FP: 5'-AGACAGCCGCATCTTCTTGT-3'; RP: 5'-CTT GCCGTGGGTAGAGTCAT-3')] used in this study were based on our previous studies [18]. The expression levels of voltage dependent. The amplified products were run on 1.8% agarose gels in TAE buffer and the relative intensities of the StAR and caspase-3 bands were normalized against the corresponding GAPDH band intensities. GAPDH was selected as the house keeping gene in this study.

Caspase-3 activity

Caspase-3 activity in the testis was determined using the method described by Cid et al. [46]. Testis was homogenized in the homogenization buffer containing HEPES buffer, 100 mM NaCl, 10 mM DTT, 1 mM, EDTA, 0.1% CHAPS and 10% glycerol. The testicular homogenate was incubated for 3 h at 4 °C followed by centrifugation at 10,000 g for 10 min. The reaction buffer comprises of 50 µM fluorogenic substrate, 7-amino-4- methylcoumarin (AMC) and 100 µg of protein. Kinetic analysis was performed to measure the fluorescence intensity for 2 h using a fluorimeter (Hitachi) at 380 nm (excitation) and 460 nm (emission). The units for caspase-3 activity was measured as pmole of AMC liberated per minute per 100 µg protein.

Histology of testis

Histological sections of selected tissues (testis and cauda epididymis) were prepared according to the protocol described by Brancraft and Stevens [47]. Briefly, the testis and cauda part of epididymis from each rat of control and experimental groups was individually fixed in the Bouin's solution for 24 h followed by dehydration steps in ascending alcoholic series. After dehydration step, the samples were cleaned in xylol and embedded in the paraffin wax for section cutting and staining procedures. The sectioned specimens were stained with hemotoxylin and eosin Y and analyzed for histological studies using Hovers microscope (Model no. HV-12TR).

Statistical analysis

Results were shown as mean ± SD for each group. Statistical analysis was performed using one-way analysis of variance (ANOVA) using post hoc Tukey test. All statistical tests were performed by using Statistical Package for Social Sciences version 16.0 (SPSS Inc, Chertsey, UK). The differences in the values between the groups were considered significant at $p < 0.05$.

Results

No abnormal signs of behaviour such as head flicking, scratching, biting, circling, licking, and lethargic movements were noticed in male rats exposed to linuron. No mortality was found in this study from control and experimental rats and none of the animals was excluded from the control and experimental groups. No changes in food and

water intake were noticed in control and experimental rats (data not shown).

Effect of α -lipoic acid on reproductive performance in linuron exposed adult rats

The fertility ability of rats was shown in Table 1. Significant changes were noticed in the number of conception days in linuron exposed rats, LA plus linuron treated rats over controls. The rats in linuron exposed groups took more number of days to impregnate the female rats. The mating index was decreased in rats cohabited with linuron exposed rats (9 out of 16 rats and 7 out of 16 rats showed copulatory plugs when cohabited with 10 mg/Kg BW linuron and 50 mg/Kg BW linuron treated rats, respectively). On the other hand, LA plus linuron treated rats showed a reduction in the number of days to impregnate the female rats over their respective controls. The mating indices in female rats cohabited with LA plus linuron treated rats was found be increased as compared to linuron exposed rats (12 out of 16 rats and 10 out of 16 rats showed copulatory plugs when cohabited with LA plus linuron at 10 mg/Kg BW and LA plus linuron at 50 mg/Kg BW treated rats, respectively). No changes were noticed in the fertility indices of experimental rats over controls. However, the number of litters delivered to rats cohabited with linuron exposed rats at a dose of 10 mg/Kg BW and 50 mg/Kg BW were decreased by 55.55% and 59.82%, respectively as compared to controls. Whereas, the number of litters delivered to rats cohabited with LA plus linuron at 10 mg/Kg BW and LA plus linuron at 50 mg/Kg BW were increased by 61.54% and 58.98%, respectively as compared to their respective controls. No significant changes were noticed in the body weights of litters delivered to rats cohabited with either controls or experimental rats. However, the age at

testicular descent in male pups exposed to linuron via paternal route were significantly enhanced over controls, while the age at testicular descent was significantly decreased in male pups exposed to LA plus linuron via paternal route as compared to their respective controls.

Effect of α -lipoic acid on body and organ weights in linuron exposed adult rats

No significant changes in the body weights were noticed in control and experimental groups (Table 2). Linuron showed marked effects on the weights of testis in rats. Linuron intoxication at 50 mg/Kg BW caused a significant increase in the weights of testis, while linuron at 10 mg/Kg BW significantly reduced the weights of testis in rats over controls. The photomicrographs of testis in linuron treated rats at 50 mg/Kg BW were shown in the Fig. 1. LA supplementation ameliorated the weights of testis and accessory sex organs in linuron exposed rats as compared to respective controls (Table 2). No changes were observed in LA alone treated rats as compared to controls (Table 2).

Effect of α -lipoic acid on selected sperm variables in linuron exposed adult rats

The data showing sperm variables were represented in Table 3. The selected sperm variables such as testicular daily sperm count and epididymal sperm variables such as sperm count, sperm motility, sperm viability, and number of tail coiled sperm were significantly reduced in linuron exposed rats at selected doses over controls. Significant increase in the sperm head abnormalities such as pointed head, rod shaped head and headless sperm were noticed in linuron exposed rats as compared to controls (Table 3, Fig. 2).

Table 1 Effect of α -lipoic acid (LA) on reproductive performance and androgen-dependent developmental landmarks in F1 pups of controls and linuron-treated rats

Parameters	Controls	Linuron		Linuron + LA	
		10 mg/Kg	50 mg/Kg	10 mg/Kg	50 mg/Kg
Number of female rats	10	10	10	10	10
Number of male rats	10	10	10	10	10
Conception time (days)	1.2 ^a ± 0.68	3.8 ^b ± 0.42	4.91 ^c ± 0.31	2.3 ^d ± 0.21	2.5 ^e ± 0.31
Mating index (%)	100 (10/10)	70 (7/10)	60 (6/10)	80 (8/10)	80 (8/10)
Fertility index (%)	100 (10/10)	100 (7/7)	100 (6/6)	100 (8/8)	90 (7/8)
No. of. live pups/rat	11.7 ^a ± 1.2	5.2 ^b ± 0.22	4.7 ^b ± 0.35	7.2 ^c ± 0.38	6.9 ^c ± 0.36
Body weights of pups# (Postnatal day: PND 1)	5.04 ^a ± 0.28	4.97 ^a ± 0.34	4.9 ^a ± 0.40	5.1 ^a ± 0.21	5.07 ^a ± 0.33
Anogenital distance# (cm) on PND 1	0.37 ^a ± 0.028	0.36 ^a ± 0.038	0.37 ^a ± 0.041	0.35 ^a ± 0.057	0.36 ^a ± 0.052
Testicular descent# (PND)	24.9 ^a ± 1.10	32.1 ^b ± 0.99	33.9 ^c ± 1.72	26.6 ^d ± 0.69	30.3 ^e ± 1.41

Data are expressed as mean ± SD

^{a-c}Different superscripts in the same row indicate a significant difference ($p < 0.05$)

#n = 10

Table 2 Effect of α -lipoic acid (LA) on body weights (g) and tissue somatic indices (W/W%) in linuron-exposed rats

Parameters	Controls	LA	Linuron exposed		Linuron exposed + LA	
			10 mg/Kg	50 mg/Kg	10 mg/Kg	50 mg/Kg
Body weight	260.72 ^a ± 10.28	260.82 ^a ± 13.42	263.84 ^a ± 12.67	259.42 ^a ± 10.67	264.71 ^a ± 14.27	265.21 ^a ± 14.81
Testis	0.889 ^a ± 0.034	0.924 ^a ± 0.023	0.519 ^b ± 0.017	1.129 ^c ± 0.018	0.759 ^d ± 0.024	0.969 ^e ± 0.038
Epididymis	1.178 ^a ± 0.047	1.185 ^a ± 0.073	1.115 ^a ± 0.040	0.772 ^b ± 0.034	1.139 ^a ± 0.058	0.912 ^c ± 0.081
Seminal vesicles	0.396 ^a ± 0.0048	0.395 ^a ± 0.0052	0.345 ^b ± 0.0031	0.343 ^b ± 0.0028	0.375 ^c ± 0.0024	0.384 ^d ± 0.0047
Ventral Prostate	0.188 ^a ± 0.0019	0.192 ^a ± 0.0027	0.124 ^b ± 0.0092	0.116 ^c ± 0.0038	0.144 ^d ± 0.0041	0.138 ^d ± 0.0051
Vas deferens	0.142 ^a ± 0.0021	0.146 ^a ± 0.0059	0.144 ^a ± 0.0036	0.083 ^b ± 0.0041	0.147 ^a ± 0.0038	0.106 ^c ± 0.0071

Data are expressed as mean ± SD of 10 individuals

^{a–e}Different superscripts in the same row indicate a significant difference ($p < 0.05$)

Fig. 1 Photomicrographs of testis in control (A), linuron treated rats at 50 mg/Kg body weights (B) and α -lipoic acid supplemented linuron treated rats at 50 mg/Kg body weights (C)

Whereas, LA supplementation improved linuron induced deteriorated sperm quality and quantity in rats. No changes were observed in selected sperm variables in LA alone injected male rats as compared to the controls (Table 3). The results of comet assay indicated that linuron exposure at both doses negatively affect the integrity of sperm DNA in rats. Conversely, LA supplementation reduced the sperm DNA injury in linuron exposed rats (Figs. 3 and 4). The photomicrographs of sperm head abnormalities (Fig. 2).

Effect of α -lipoic acid on testicular steroidogenesis in linuron exposed adult male rats

Significant elevation in the testicular cholesterol levels (Fig. 5) accompanied by a significant reduction in the serum testosterone levels were observed in linuron treated rats (Fig. 6). The activity levels of 3β - and 17β -HSD in the testis of linuron exposed rats were significantly reduced over controls (Table 4). RT-PCR studies indicated that the expression levels of steroidogenic acute regulatory protein mRNA was decreased in linuron treated rats over controls (Fig. 7A, B). Co-treatment with LA on the other hand, showed reversal effects on the selected testicular steroidogenic variables in rats exposed to linuron as compared to their respective controls (Table 4). No changes were observed in the steroidogenic machinery of testis in LA alone injected rats as compared to controls (Table 4).

Effect of α -lipoic acid on testicular and epididymal oxidative stress parameters in linuron exposed adult rats

The changes in the selected oxidative stress variables in the testis and cauda epididymis were shown in Table 5. In linuron exposed rats at a dose of 10 mg/Kg BW, the activity

Table 3 Effect of α -lipoic acid (LA) on sperm parameters in controls and linuron exposed rats

Parameters	Controls	LA	Linuron exposed		Linuron exposed + LA	
			10 mg/Kg	50 mg/Kg	10 mg/Kg	50 mg/Kg
Testicular daily sperm count (10^6 /g testis)	29.82 ^a ± 2.98	28.28 ^a ± 4.92	19.67 ^b ± 1.43	16.27 ^c ± 1.28	24.41 ^d ± 1.08	20.12 ^b ± 1.09
Sperm count (10^6 /ml)	72.81 ^a ± 4.68	73.12 ^a ± 4.09	47.01 ^b ± 2.38	42.71 ^c ± 2.48	59.21 ^d ± 2.41	53.78 ^e ± 1.21
Sperm viability (%)	69.27 ^a ± 3.21	70.12 ^a ± 3.39	45.09 ^b ± 2.42	36.12 ^c ± 1.48	54.27 ^d ± 2.68	49.27 ^e ± 1.24
Sperm motility (%)	65.42 ^a ± 2.82	68.42 ^b ± 2.19	40.38 ^c ± 1.27	34.61 ^d ± 1.29	48.62 ^e ± 3.17	44.87 ^f ± 1.08
HOS-tail coiled test (%)	63.09 ^a ± 2.61	64.12 ^a ± 2.79	38.19 ^b ± 1.42	30.72 ^c ± 2.38	45.71 ^d ± 2.91	40.21 ^b ± 1.11
Sperm head abnormalities (%)	2.5 ^a ± 0.52	2.35 ^a ± 0.35	25.72 ^b ± 2.72	34.89 ^c ± 2.34	9.65 ^d ± 3.27	13.28 ^d ± 3.09

Data are expressed as mean ± SD of 10 individuals

^{a–f}Different superscripts in the same row indicate a significant difference ($p < 0.05$)

Fig. 2 Sperm head morphology in control rat (normal sperm with hook: **A**) and loss of characteristic hook shape in the sperm head of linuron treated rats (**B–F**) and restoration of sperm head morphology in α -lipoic acid supplemented linuron treated rats (**G**)

Fig. 3 Sperm without comet tail (control: **A**), sperm showing a tail as a mobilized DNA fragments due to damage of DNA (linuron treatment: **B**, arrows indicate comets) and restoration of sperm DNA damage in α -lipoic acid supplemented linuron exposed rats (**C**)

Fig. 4 Changes in sperm DNA damage (%) of control and experimental rats (comet assay)

levels of testicular and epididymal SOD, CAT, GPx and GR were significantly reduced as compared to controls. Whereas, the GSH content were unchanged in the testis and significantly reduced in the cauda epididymis of linuron

Fig. 5 Changes in the testicular cholesterol levels of linuron treated rats supplemented with or without α lipoic acid (LA). Bar graphs indicate mean $\pm$ SD of 10 individual rats. Bars with different letters indicate a significant difference ($p < 0.05$)

treated rats at 10 mg/Kg BW over controls. Linuron intoxication at 50 mg/Kg BW showed a reduction in the activity

Fig. 6 Changes in the circulatory levels of testosterone in linuron treated rats supplemented with or without α lipoic acid (LA). Bar graphs indicate mean $\pm$ SD of 10 individual rats. Bars with different letters indicate a significant difference ($p < 0.05$)

levels of selected enzymatic and non-enzymatic antioxidants in the testis and cauda epididymis of rats over controls. The lipid peroxidation levels and protein carbonyl content in the selected tissues of linuron at either 10 or 50 mg/Kg BW were elevated as compared to controls. Co-treatment with LA showed an enhancement in the enzymatic and non-enzymatic antioxidants with a concomitant decrease in the lipid peroxidation levels and protein carbonyl content in the selected tissues of linuron treated rats over their respective controls. No significant changes were noticed in the oxidative stress parameters in the testis and cauda epididymis of LA alone injected rats over controls. RT-PCR studies indicated that the expression of testicular caspase-3 mRNA levels (Fig. 7C) was increased in linuron treated rats at selected doses over controls, while administration of LA substantially reduced the expression levels of caspase-3 mRNA in the testis of linuron exposed rats at 10 or 50 mg/Kg BW over their respective controls. Caspase-3 activity was noticed in linuron exposed rats (Fig. 8). However, it is pertinent to mention that caspase-3 activity was not detected in the controls, lipoic acid alone supplemented rats, lipoic acid plus linuron treated rats in the conditions used.

Effect of α -lipoic acid on testicular and epididymal architecture in linuron-exposed adult rats

The testicular and epididymal histology was depicted in Fig. 9. In control and LA alone supplemented rats, the testicular architecture shows intact epithelial basement membrane with lumen occupied by spermatozoa (Fig. 9a, b). Whereas, linuron at a dose of 10 or 50 mg/Kg BW showed disrupted testicular architecture with lumen devoid of sperm in rats (Fig. 9c, d). Further, the diameter of lumen in the seminiferous tubules was increased in rats exposed to linuron at a dose of 50 mg/Kg BW. LA supplementation repaired the ruptured epithelial membrane with lumen occupied by sperm in linuron treated rats at selected doses, suggesting restoration of testicular organization (Fig. 9e, f). The epididymal architecture in control and LA supplemented rats showed intact pseudostratified epithelium with stereocilia and lumen occupied by sperm (Fig. 9g, h). Linuron intoxication at either 10 or 50 mg/Kg BW caused abnormal stratified layer as indicated by ruptured the pseudostratified epithelium membrane with lumen with few sperm (Fig. 9i, j). On the other hand, LA supplementation sustained the integrity of pseudostratified layer accompanied by sperm in the lumen of epididymis in linuron treated rats (Fig. 9k, l).

Discussion

The findings of this study indicated that linuron-induced suppression of male reproduction in rats occurs via inhibition of testosterone synthesis and testicular and epididymal oxidative toxicity. On the other hand, α -lipoic acid ameliorated suppressed male reproductive health in rats by promoting testicular spermatogenesis, steroidogenesis and neutralizing the excess generation of free radicals through stimulation of enzymatic and non-enzymatic antioxidants in linuron treated rats.

Numerous studies indicated that the testicular toxicants underlie oxidative damage to interfere with male reproductive tract functions [48]. Several researchers used different indicators to determine oxidative stress to exemplify

Table 4 Effect of α -lipoic acid (LA) on testicular steroidogenesis in controls and linuron exposed rats

Parameters	Controls	LA	Linuron exposed		Linuron exposed + LA	
			10 mg/Kg	50 mg/Kg	10 mg/Kg	50 mg/Kg
3 β -HSD ^W	0.026 ^a $\pm$ 0.001	0.027 ^a $\pm$ 0.0021	0.014 ^b $\pm$ 0.0011	0.011 ^c $\pm$ 0.0021	0.020 ^d $\pm$ 0.0011	0.017 ^e $\pm$ 0.0012
17 β -HSD ^Y	0.018 ^a $\pm$ 0.001	0.019 ^b $\pm$ 0.0003	0.009 ^c $\pm$ 0.0002	0.008 ^d $\pm$ 0.0003	0.013 ^e $\pm$ 0.0002	0.011 ^f $\pm$ 0.0001

Data are expressed as mean $\pm$ SD of 10 individuals

^{a-f}Different superscripts in the same row indicate a significant difference ($p < 0.05$)

^WUnits for 3 β -hydroxysteroid dehydrogenase (HSD) = μ moles of NAD converted to NADH/mg protein/min;

^YUnits for 17 β -hydroxysteroid dehydrogenase (HSD) = μ moles of NADPH converted to NADP/mg protein/min

Fig. 7 Determination of the expression levels of StAR mRNA levels and caspase-3 mRNA levels normalized against GAPDH band intensities in linuron treated rats supplemented with or without α lipoic acid (LA). The band intensities obtained from four testicular samples per group were used for normalization procedures. **A** Gel images of PCR products of GAPDH, StAR and Caspase-3; **B** Changes in testicular StAR mRNA expression levels of linuron treated rats supple-

mented with or without LA. Bars represent mean $\pm$ S.D. of four individual samples. Bars with same letters did not differ from each other at $p < 0.05$; and **C** Changes in testicular Caspase mRNA expression levels of linuron treated rats supplemented with or without LA. Bars represent mean $\pm$ S.D. of four individual samples. Bars with same letters did not differ from each other at $p < 0.05$

Table 5 Effect of α -lipoic acid (LA) on oxidative stress parameters in the testis and epididymis of controls and linuron-exposed rats

Tissue	Groups Parameters	Controls	LA	Linuron		Linuron $\pm$ LA	
				10 mg/Kg	50 mg/Kg	10 mg/Kg	50 mg/Kg
Testis	SOD	17.28 ^a $\pm$ 1.41	16.42 ^a $\pm$ 1.54	10.07 ^b $\pm$ 1.32	8.57 ^b $\pm$ 1.08	13.08 ^d $\pm$ 1.97	13.58 ^d $\pm$ 1.11
	CAT	11.48 ^a $\pm$ 1.08	12.38 ^a $\pm$ 1.52	7.75 ^b $\pm$ 0.93	5.67 ^c $\pm$ 0.76	10.66 ^d $\pm$ 0.34	9.65 ^d $\pm$ 0.47
	GR	4.012 ^a $\pm$ 0.075	4.11 ^b $\pm$ 0.092	3.82 ^c $\pm$ 0.021	1.86 ^d $\pm$ 0.045	3.72 ^e $\pm$ 0.063	2.64 ^f $\pm$ 0.077
	GPx	3.12 ^a $\pm$ 0.026	3.09 ^a $\pm$ 0.083	2.97 ^b $\pm$ 0.033	0.98 ^c $\pm$ 0.067	3.05 ^a $\pm$ 0.052	2.39 ^d $\pm$ 0.034
	GSH	14.54 ^a $\pm$ 1.72	15.63 ^a $\pm$ 2.27	14.28 ^a $\pm$ 1.72	7.28 ^b $\pm$ 1.24	15.72 ^a $\pm$ 1.35	11.28 ^c $\pm$ 1.23
	LPx	11.48 ^a $\pm$ 2.55	10.67 ^a $\pm$ 1.78	20.21 ^b $\pm$ 3.65	27.12 ^c $\pm$ 2.64	16.98 ^d $\pm$ 1.21	19.08 ^d $\pm$ 2.13
Cauda epididymis	PC's	2.04 ^a $\pm$ 0.28	2.73 ^b $\pm$ 0.11	5.63 ^c $\pm$ 0.26	6.01 ^c $\pm$ 0.48	3.21 ^d $\pm$ 0.34	3.48 ^d $\pm$ 0.17
	SOD	19.18 ^a $\pm$ 2.02	20.27 ^a $\pm$ 1.18	8.71 ^b $\pm$ 0.64	9.27 ^{c,b} $\pm$ 1.02	16.42 ^d $\pm$ 1.24	13.18 ^e $\pm$ 0.47
	CAT	7.23 ^a $\pm$ 0.26	8.01 ^b $\pm$ 0.88	4.27 ^c $\pm$ 0.17	3.18 ^d $\pm$ 0.23	6.21 ^e $\pm$ 0.19	5.98 ^e $\pm$ 0.21
	GR	5.07 ^a $\pm$ 0.025	4.97 ^b $\pm$ 0.064	3.28 ^c $\pm$ 0.042	2.53 ^d $\pm$ 0.061	4.01 ^e $\pm$ 0.038	3.56 ^f $\pm$ 0.011
	GPx	3.25 ^a $\pm$ 0.063	3.29 ^a $\pm$ 0.041	2.32 ^b $\pm$ 0.085	1.34 ^c $\pm$ 0.091	2.76 ^d $\pm$ 0.021	2.83 ^d $\pm$ 0.038
	GSH	10.18 ^a $\pm$ 0.79	12.31 ^b $\pm$ 0.28	5.72 ^c $\pm$ 0.39	4.49 ^d $\pm$ 0.27	8.65 ^e $\pm$ 0.17	7.27 ^f $\pm$ 0.12
	LPx	12.34 ^a $\pm$ 1.07	13.18 ^a $\pm$ 1.36	26.21 ^b $\pm$ 2.47	27.18 ^b $\pm$ 3.68	18.07 ^c $\pm$ 1.03	20.98 ^d $\pm$ 1.39
	PC's	1.98 ^a $\pm$ 0.27	2.01 ^a $\pm$ 0.39	5.99 ^b $\pm$ 0.25	6.08 ^b $\pm$ 0.47	3.08 ^c $\pm$ 0.17	3.12 ^c $\pm$ 0.53

Data are expressed as mean $\pm$ SD of 10 individual rats

SOD Superoxide dismutase (Units/mg protein), CAT Catalase (μ moles of H_2O_2 decomposed/mg protein/min), GSH Reduced glutathione (μ mol of thiourea/mg protein/h), LPx Lipid peroxidation (μ mol of malondialdehyde formed/g tissue), PC Protein carbonyl content (nmol carbonyl/mg protein), GR Glutathione reductase (μ mol of NADPH oxidized/mg protein/min), GPx Glutathione peroxidase (μ mol of NADPH oxidized/mg protein/min)

^{a-f}Different superscripts in the same row indicate a significant difference ($p < 0.05$)

Fig. 8 Changes in the caspase-3 activity in linuron treated rats. Bar graphs indicate mean $\pm$ SD of 6 individual rats. *Significantly different from that of control at $p < 0.05$. For evaluation of P, students t test was conducted. For linuron treated rats at 50 mg/Kg body weight, linuron treated rats at 10 mg/Kg body weights were served as controls

the chemical-induced toxicity. In this study, we evaluated lipid peroxidation levels and protein carbonyl content as markers of oxidative stress in the cells. This is because, free radicals generate the lipid peroxidation process in cells

and malondialdehyde levels is one of the final products of peroxidation of polyunsaturated fatty acids in cells. Thus, an increase in malondialdehyde levels reflects overwhelming generation of free radicals. Excess carbonyl content is an indicator of protein oxidation. In the present study, we found a significant increase in the lipid peroxidation levels and protein carbonyl content with a concomitant decrease in the activity levels of enzymatic (SOD, CAT, GPx and GR) antioxidants in the testis and cauda epididymis might be an indication of failure of counterattack mechanism(s) against the oxidative damage induced by linuron in rats. Our results corroborates with the studies of Nam et al. [18] where these authors suggested that the exposure of rats to linuron at 50 or 100 mg/Kg BW impairs the cellular antioxidant system of prostate gland. Thus, linuron-induced oxidative stress leads to peroxidation of lipid rich sources such as plasma membrane of testis, epididymis and sperm, thereby interferes with their functions. Testis performs two important functions: spermatogenesis and steroidogenesis. A significant reduction in the testicular daily sperm count and circulatory levels of testosterone accompanied by increased testicular lipid peroxidation levels and protein carbonyl content might suggest that linuron-induced oxidative toxicity at the level of Sertoli- and Leydig-cells.

Biosynthesis of testosterone in the Leydig cells is critically regulated by a cascade of enzymes, 3β - and 17β -HSDs,

Upper panel: Testis; Lower panel: Cauda epididymis

Fig. 9 Photomicroscopic pictures of the transverse sections of testes (upper panel) and epididymis (lower panel) in control and experimental rats. Testis from control and α -lipoic acid (LA) injected rats, shows compactly arranged seminiferous tubules with complete spermatogenesis. Testis from linuron exposed rats at 10 mg/Kg body weight, shows ruptured epithelium with lumen devoid of sperm. Testis from linuron exposed rats at 50 mg/Kg body weight, shows enlarged seminiferous tubules with lumen devoid of sperm. Testis from LA treated linuron exposed rats at both 10 and 50 mg/Kg body weight, shows intact epithelium as basement membrane with amelioration of spermatogenesis showing sperm in the lumen of seminiferous tubules. Cauda epididymis from control and LA injected rats show intact tubular architecture with lumen occupied by spermatozoa. Cauda epididymis from linuron exposed rats show disrupted membrane with lumen devoid and/or filled with few spermatozoa. Cauda epididymis from LA treated linuron exposed rats show restoration of tubular architecture and lumen filled with spermatozoa. Scale bar = 50 μ m. Double head arrows dilation of seminiferous tubules; *ruptured membrane; arrow head: lumen devoid of spermatozoa

ration of spermatogenesis showing sperm in the lumen of seminiferous tubules. Cauda epididymis from control and LA injected rats show intact tubular architecture with lumen occupied by spermatozoa. Cauda epididymis from linuron exposed rats show disrupted membrane with lumen devoid and/or filled with few spermatozoa. Cauda epididymis from LA treated linuron exposed rats show restoration of tubular architecture and lumen filled with spermatozoa. Scale bar = 50 μ m. Double head arrows dilation of seminiferous tubules; *ruptured membrane; arrow head: lumen devoid of spermatozoa

which play a key role in the conversion of pregnenolone to effectual hormones and a rate limiting step operated at the level of StAR protein, which acts as a channelling proteins for cholesterol from outer to inner mitochondrial membrane [49]. A significant reduction in the activity levels of 3β - and 17β -HSDs and expression levels of StAR mRNA associated with accumulation of cholesterol in the testis of linuron treated rats might suggest that the hydroxysteroid dehydrogenases and StAR proteins could be susceptible targets of linuron-induced toxicity. These events subsequently lead to reduced steroidogenesis thereby diminished serum testosterone levels [50, 51]. Previously, studies of Bai et al. [14] and Ding et al. [15] suggested that prenatal exposure to linuron at least in part interfere with the steroidogenic cascade of enzymes and proteins thereby profoundly affect the testosterone levels in F1 generation males. Testosterone is crucial for normal spermatogenesis and epididymal functions [52]. Therefore, disturbances in the testicular daily sperm count and epididymal sperm variables such as sperm count, motile sperm, viable sperm, and tail coiled sperm testis associated with reduced circulatory levels of testosterone in linuron treated rats might support this notion [51]. It is well known that sperm attains maturity and fertilizing ability in the epididymal region during its transit time [53]. Therefore, the deterioration of sperm quality might be attributed to the disorganization of epididymis as indicated by disruption of stratified layer with a significant increase in the % sperm head abnormalities (head less, rod shape, and pointed shape) associated with reduced sperm motility and number of tail coiled sperm (HOS-test) could be linked to the acceleration of sperm transit time in the epididymis of linuron treated rats [54]. Moreover, significant increase in the % sperm DNA damage in linuron-intoxicated rats as evidenced by comet assay technique could reflect genotoxic effects at the level of male gametes [36].

A reduction in the weights of seminal vesicles, epididymis, vas deferens, and ventral prostate in linuron exposed rats could be linked to the inadequate bioavailability of testosterone [10]. On the other hand, a transient increase in the weights of testis in rats treated with linuron at 50 mg/Kg BW [55]; McIntyre et al. [11], while a significant reduction in the weights of testis in rats exposed to linuron at 10 mg/Kg BW. The reduction in the weights of testis in 10 mg/Kg linuron treated rats were linked to the testicular atrophy, while a transient increase in the testicular weights in 50 mg/Kg linuron treated rats could be due to dilation of seminiferous tubules. Previously, it has been shown that the rats exposed to short term exposure to 200 or 300 mg/Kg of atrazine over a period of 15 or 7 days, respectively showed a transient increase in the weights of testis over the rats exposed to atrazine at 200 mg/Kg over a period of 40 days [51]. Further, a transient in the testicular weights might be linked to the estrogenic ability of the toxicants that disrupt

estrogen-mediated re-absorption of luminal fluid in the efferent tubules thereby leads to testicular swelling followed by atrophy [56]. Experiments of Spirhanzlova et al. [57] using short-term based whole genome bioassays demonstrated that linuron can able to disrupt androgen, estrogen and thyroid signalling in vertebrate systems. Molecular docking analysis performed in this study also showed interactions between the linuron and rat estrogen receptor (supplementary material: Table 6). Conversely, the body weights were comparable between the control and experimental rats, suggesting that the overt condition of linuron treated rats were apparently normal. In the current study, we did not found any abnormal signs of behaviour and mortality in linuron treated rats suggesting that the selected doses of linuron were nontoxic. Moreover, the selected doses of linuron in our study were well below the oral LD₅₀ dose levels of linuron in rats (i.e. 2600 mg/Kg: EPA, [58]).

The ability of male to sire an offspring is directly linked to the sperm fertilizing ability [59]. The deterioration of sperm quality and quantity associated with a reduction in the number of live pups per rat cohabited with linuron treated male rats might reflect compromised sperm fertility, which in turn might be linked to abnormal sperm number and function. Our findings indicated that linuron exposure resulted in spermatotoxicity as evidenced by sperm DNA damage (Comet assay) reduced motility, reduced viability, reduced sperm membrane integrity, increased sperm head abnormalities and also decreased sperm number. Thus, the impaired sperm cannot able to reach the zona pellucida of ova; thereby reduction in fertility efficacy of linuron exposed adult rats. On the other hand, significant increase in the number of copulation trials in linuron treated male rats could reflect intact sexual desire.

In our experiments, LA significantly ameliorated oxidative stress and reproductive indicators. This is indicated by a decrease in the levels of lipid peroxidation levels and protein carbonyl content with normalized enzymatic and non-enzymatic antioxidants and protection of reproductive endpoints in the LA plus linuron treated rats as compared to controls. LA shielding effects on the testis and cauda epididymis against linuron-induced oxidative toxicity might be responsible for their structural and functional integrity in LA + linuron treated rats. Previously, several studies elaborated antioxidant effects of LA against a range of testicular toxicants [21, 60, 61]. The normalization of antioxidants in linuron exposed rats by LA is linked to direct and indirect effects. Directly, LA and its redox couple dihydrolipoic acid can able to quench the excess free radicals in both aqueous and lipid layers and indirectly protects the cellular systems via boosting the endogenous antioxidants. The present results support this notion [20, 23, 62]. SOD and CAT are believed to be first line of defence enzymes in the cellular systems to negate the excess generation of free radicals. A significant

reduction in the activity levels of SOD, and CAT levels in the testis and epididymis of linuron treated rats might reflect improper dismutation of superoxides, and accumulation of hydrogen peroxides [64]. LA supplementation via its scavenging activity mitigates superoxide radicals [63] and also enhanced cellular NADPH levels (Shila et al. 2003) might seem to be responsible for offset of linuron-induced decline in the activities of testicular and epididymal SOD and CAT. Linuron treatment significantly inhibited GPx in the testis and epididymis of rats, suggesting excess accumulation of hydrogen peroxides. LA supplementation appears to enhance the GPx activity levels in the selected tissues via stimulation of intracellular de novo synthesis of GSH, a substrate for GPx and also via enhancement of GR activity in linuron intoxicated rats. These events could probably mitigate the testicular and epididymal oxidative damage in LA plus linuron treated rats. Caspase-3 is classically known as an executioner caspase where its stimulation marks the point of no return in the apoptotic process [65]. In the present study, the enhanced expression levels of testicular caspase-3 mRNA accompanied by caspase-3 activity could reflect acceleration of apoptotic caspase-3 dependent cascade in linuron treated rats over controls. On the other hand, LA inhibited the expression levels of caspase-3 mRNA levels, might reflect LA-induced antiapoptotic property [66].

Linuron-induced inhibition of testicular steroidogenesis could be recovered by the supplementation of LA in rats as evidenced by the amelioration of serum testosterone levels, restoration of the activity levels of 3 β - and 17 β -HSDs and expression of StAR mRNA levels in the testis and reduction of testicular cholesterol levels in LA + linuron treated rats. These events could be linked to the steroidogenic effects of LA or protective effects at the level of Leydig cells and/or both. Enhanced activity levels of 3 β - and 17 β -HSDs in the testis of rats subjected to LA and pesticide mixture (zineb, carbamate, dimethoate, dithiophosphate and glyphosate) [67]. The recovery of sperm in the lumen of testis and epididymis accompanied by promotion of testicular DSP and selected epididymal sperm endpoints might be linked to the protective effects of LA at the level of Sertoli cells and adequate supply of androgens in LA + linuron treated rats [60].

The observation that the age of testicular descent which was significantly increased in the F1 generation male rats delivered to rats cohabited with linuron treated rats could possibly suggest paternal mediated developmental toxicity. On the other hand, we found a reduction in the age of testicular descent in F1 male rats delivered to rats cohabited with LA + linuron treated rats might possibly suggest protective effects of LA against linuron-mediated developmental toxicity. Based on our results, we hypothesize that the restoration of sperm DNA integrity by LA against the linuron toxicity in male rats could be one of the possible explanations for this observation.

To summate, inhibition of testosterone levels and provoking oxidative damage might be potential mechanisms of linuron-induced suppressed male fertility in rats. However, our results also indicated that the linuron-induced suppressed male reproductive health was reversible as evidenced by restoration of male fertility in LA plus linuron treated rats. These protective effects of LA could be due to its steroidogenic, and antioxidant properties in linuron treated rats.

Acknowledgements We thank the Head, Dept. of Biotechnology, VSU, Nellore, AP, India for providing laboratory space and allowed us to utilize the equipments purchased under DST-FIST programme, New Delhi. We thank the Head, Department of Genetics, Narayana Medical College, Nellore for providing animals and the Dr. M. Gobinath, Ratnam Pharmacy College, Muthurkur for providing the animal house facilities. Our special thanks to the Head, Department of Marine Biology for allowing us to utilize ELISA Microplate Reader.

Funding This research work was not supported by any funding agency.

Compliance with ethical standards

Conflict of interest Nothing to disclose.

References

1. EFSA (European Food Safety Authority) (2016) Conclusion on the peer review of the pesticide risk assessment of the active substance linuron. EFSA J 14:4518
2. Gupta PK (2018) Herbicides and fungicides. In: Veterinary toxicology, basic and clinical principle, 3rd edn. pp 553–567
3. Gatidou G, Thomaidis NS, Stasinakis AS, Lekkas TD (2007) Simultaneous determination of the endocrine disrupting compounds nonylphenol, nonylphenol ethoxylates, triclosan and bisphenol A in wastewater and sewage sludge by gas chromatography–mass spectrometry. J Chromatogr A 1138:32–41
4. Pest management regulatory agency (2012) Proposed re-evaluation decision PRVD2012-02 linuron. Tecnical report prepared for pest management regulatory agency. Health Canada: Ottawa Ontario Canada
5. Kojima H, Katsura E, Takeuchi S, Niiyama K, Kobayashi K (2004) Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. Environ Health Perspect 112:524–531
6. Orton F, Lutz I, Kloas W, Routledge EJ (2009) Endocrine disrupting effects of herbicides and pentachlorophenol: in vitro and in vivo evidence. Environ Sci Technol 43:2144–2150
7. Freyberger A, Witters H, Weimer M, Ahr HJ (2010) Screening for (anti) androgenic properties using a standard operation protocol based on the human stably transfected androgen sensitive PALM cell line first steps towards validation. Reprod Toxicol 30:9–17
8. Wilson VS, Lambright CR, Furr JR, Howdeshell KL, Earl Gray L (2009) The herbicide linuron reduces testosterone production from the fetal rat testis during both in utero and in vitro exposures. Toxicol Lett 186:73–77
9. Uren TM, Perry MH, Santos EM (2015) The herbicide linuron inhibits cholesterol biosynthesis and induces cellular stress responses in brown trout. Environ Sci Technol 49:3110–3118

10. Lambright C, Ostby J, Bobseine K, Wilson V, Hotchkiss AK, Mann PC, Gray LE (2000) Cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats. *Toxicol Sci* 56:389–399
11. McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, Foster PM (2000) Effects of in utero exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD) BR rat. *Toxicol Appl Pharmacol* 167:87–99
12. Waller CL, Uma BW, Gray LE Jr, Kelce WR (1996) Three dimensional quantitative structure-activity relationships for androgen receptor ligands. *Toxicol Appl Pharmacol* 137:219–227
13. Bauer ER, Meyer HH, Stahschmidt-Allner P, Sauerwein H (1998) Application of an androgen receptor assay for the characterisation of the androgenic or antiandrogenic activity of various phenylurea herbicides and their derivatives. *Analyst* 123:2485–2487
14. Bai J, Han H, Wang F, Ding H, Hu X, Hu B, Li H, Zheng W, Li Y (2017) Maternal linuron exposure alters testicular development in male offspring rats at the whole genome level. *Toxicology* 389:13–20
15. Ding HW, Zheng W, Han H, Hu X, Hu B, Wang F, Su L, Li H, Li Y (2017) Reproductive toxicity of linuron following gestational exposure in rats and underlying mechanisms. *Toxicol Lett* 266:49–55
16. Hotchkiss AK, Parks-Saldutti LG, Ostby JS, Lambright C, Furr J, Vandenbergh JG, Gray LE (2004) A mixture of the antiandrogens linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biol Reprod* 71:1852–1861
17. Santos SM, Videira RA, Fernandes MA, Santos MS, Moreno AJ, Vicente JA, Jurado AS (2014) Toxicity of the herbicide linuron as assessed by bacterial and mitochondrial model systems. *Toxicol In Vitro* 28:932–939
18. Nam RK, Hang W, Siminovich K, Shlien A, Kattan MW, Klotz LH, Trachtenberg J, Lu Y, Zhang J, Yu C, Toi A, Loblaw DA, Venkateswaran V, Stanimirovic A, Sugar L, Malkin D, Seth A, Narod SA (2011) New variants at 10q26 and 15q21 are associated with aggressive prostate cancer in a genome-wide association study from a prostate biopsy screening cohort. *Cancer Biol Ther* 12:997–1004
19. Biliska A, Wodek L (2005) Lipoic acid – the drug of the future? *Pharmacol Rep* 57:570–577
20. El-Bishbishy HA, Aly HA, El-Shafey M (2014) Lipoic acid mitigates bisphenol A induced testicular mitochondrial toxicity in rats. *Environ Occup Health* 29:875–887
21. Gules O, Eren U (2016) Protective role of alpha lipoic acid against polychlorobiphenyl (Aroclor 1254)-induced testicular toxicity in rats. *Rom J Morph Embryol* 57:451–459
22. Prathima P, Venkaiah K, Pavani R, Daveedu T, Munikumar M, Gobinath M, Valli M, Sainath SB (2017) α -lipoic acid inhibits oxidative stress in testis and attenuates testicular toxicity in rats exposed to carbimazole during embryonic period. *Toxicol Rep* 4:373–381
23. Prathima P, Pavani R, Sukeerthi S, Sainath SB (2018) α -Lipoic acid inhibits testicular and epididymal oxidative damage and improves fertility efficacy in arsenic-intoxicated rats. *J Biochem Mol Toxicol*. <https://doi.org/10.1002/jbt.22016>
24. Koga T, Ishida T, Takeda T, Ishii Y, Uchi H, Tsukimori K, Yamamoto M, Himeno M, Furue M, Yamada H (2012) Restoration of dioxin-induced damage to fetal steroidogenesis and gonadotropin formation by maternal co-treatment with α -lipoic acid. *PLoS ONE* 7:e40322
25. CPCSEA (2003) Committee for the Purpose of control and supervision on experiments on animals PCSEA guidelines for laboratory animal facility. *Indian J Pharmacol* 35:257–274
26. Dixit S, Dhar P, Mehra RD (2015) Alpha lipoic acid (ALA) modulates expression of apoptosis associated proteins in hippocampus of rats exposed during postnatal period to sodium arsenite (NaAsO₂). *Toxicol Rep* 2:78–87
27. O'Connor TG, Heron J, Golding J, Beveridge M, Glover V (2002) Maternal antenatal anxiety and children's behavioural emotional problems at 4 years. *Br J Psychiatry* 180:502–508
28. Hayes W, Kruger CL (2014) Hayes' principles and methods of toxicology, 6th edn. CRC Press, Taylor and Francis Group, Boca Raton, Florida
29. Belsey MA, Moghissi KS, Eliasson R, Paulsen CA, Gallegos AJ, Prasad MR (1980) Laboratory manual for the examination of human semen and semen cervical mucus interaction WHO 1211 Geneva-27 Switzerland
30. Talbot P, Chacon RS (1981) A triple-stain technique for evaluating normal acrosome reactions of human sperm. *J Exp Zool* 215:201–208
31. Jeyendran RS, Van der Ven HH, Zaneveld LJD (1992) The hypoosmotic swelling test: an update. *Arch Androl* 29:105–116
32. Ramu S, Jeyendran RS (2013) The hypo-osmotic swelling test for evaluation of sperm membrane integrity. *Methods Mol Biol* 927:21–25
33. Robb GW, Amann RP, Killian GJ (1978) Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *J Reprod Fertil* 54:103–107
34. Linder RE, Strader LF, Slott VL, Suarez JD (1992) Endpoints of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants. *Reprod Toxicol* 6:491–505
35. Miranda-Spooner M, Paccola CC, Neves FM, de Oliva SU, Miraglia SM (2016) Late reproductive analysis in rat male offspring exposed to nicotine during pregnancy and lactation. *Andrology* 4:218–231
36. Zlatkis A, Zak B, Boyle AJ (1953) A new method for the direct determination of serum cholesterol. *J Lab Clin Med* 41:486–492
37. Bergmeyer HU (1974) Beta-hydroxysteroid dehydrogenase. In: Bergmeyer HU (ed.) *Methods Enzy Anal* Academic Press, New York, pp 447–489
38. Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 247:3170–3175
39. Chance B, Maehly AC (1955) Assay of catalase and peroxidases. *Methods Enzymol* 2:764–775
40. Flohe L, Gunzler WA (1984) Assays of glutathione peroxidase. *Methods Enzymol* 105:114–120
41. Carlberg I, Mannervik B (1985) Glutathione reductase assay. *Methods Enzymol* 113:484–495
42. Beutler E (1975) The preparation of red cells for assay. In: Beutler E (ed) *Red cell metabolism: a manual of biochemical methods*. Grune and Straton Company, New York, pp 8–18
43. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95:351–358
44. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186:464–478
45. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275
46. Cid C, Alvarez-Cermenon JC, Regidor I, Plaza J, Salinas M, Alcazar A (2003) Caspase inhibitors protect against neuronal apoptosis induced by cerebrospinal fluid from multiple sclerosis patients. *J Neuroimmunol* 136:119–124
47. Brancraft JD, Stevens A (1982) Theory and practice of histological techniques, 2nd edn. Churchill Livingstone, New York
48. Turner TT, Lysiak JJ (2008) Oxidative stress: a common factor in testicular dysfunction. *J Androl* 29:488–498

49. Stocco DM, Clark BJ (2000) Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. *Biochem Pharmacol* 51:197–205
50. Clair E, Mesnage R, Traver C, Seralini GE (2012) A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro and testosterone decrease at lower levels. *Toxicol In Vitro* 26:269–279
51. Victor-Costa AB, Bandeira SM, Oliveira AG, Mahecha GA, Oliveira CA (2010) Changes in testicular morphology and steroidogenesis in adult rats exposed to atrazine. *Reprod Toxicol* 29:323–331
52. Knez J (2013) Endocrine-disrupting chemicals and male reproductive health. *Reprod Biomed Online* 26:440–448
53. Mehrpour O, Karrari P, Zamani N, Tsatsakis AM, Abdollahi M (2014) Occupational exposure to pesticides and consequences on male semen and fertility: a review. *Toxicol Lett* 230:146–156
54. Espinoza Navarro O, Bustos Obregon E (2014) Effect of Malathion on cellularity and sperm differentiation in testis and epididymis of adult rats. *Int J Morphol* 32:119–124
55. Nakai M, Hess RA, Moore BJ, Guttroff RF, Strader LF, Linder RE (1992) Acute and long-term effects of a single dose of the fungicide carbendazim (methyl-2-benzimidazole carbamate) on the male reproductive system in the rat. *J Androl* 13:507–518
56. Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL (1997) Estrogen receptor (alpha and beta) expression in the excurrent ducts of the adult male rat reproductive tract. *J Androl* 18:602–611
57. Spirhanzlova P, De Groef B, Nicholson FE, Grommen SVH, Marras G, Sébillot A, Demeneix BA, Pallud-Mothré S, Lemkine GF, Tindall AJ, Du Pasquier D (2017) Using short-term bioassays to evaluate the endocrine disrupting capacity of the pesticides linuron and fenoxycarb. *Comp Biochem Physiol C Toxicol Pharmacol* 200:52–58
58. U S EPA (1995) Reregistration Eligibility Decision (RED): Linuron. US Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs US Government Printing Office, Washington DC, EPA 738-R-95-003
59. Pratap Reddy K, Girish BP, Sreenivasula Reddy P (2014) Reproductive and paternal mediated developmental toxicity of Benzo(a) Pyrene in adult male Wistar rats. *Toxicol Res (Camb)*. <https://doi.org/10.1039/c4tx00121d>
60. Hamdy BG, Nabil T, Saad NN, Abdelwahab M, Mohamed L (2016) Protective role of alpha lipoic acid against the deleterious effects of both natural and artificial sweetener (Sucrose and Aspartame) in albino rats. *Alex J Vet Sci* 49:105–115
61. Lebda M, Gad S, Gaafar H (2014) Effects of lipoic acid on acrylamide induced testicular damage. *Mater Soc Med* 26:208–212
62. Holmquist L, Stuchbury G, Berbaum K, Muscat S, Young S, Hager K, Engel J, Munch G (2007) Lipoic acid as a novel treatment for alzheimer's disease and related dementias. *Pharmacol Ther* 113:154–164
63. Biewenga GP, Haenen GR, Bast A (1997) The pharmacology of the antioxidant lipoic acid. *Gen Pharmacol* 29:315–331
64. Shila S, Kokilavani V, Subathra M, Panneerselvam C (2005) Brain regional responses in antioxidant system to alpha-lipoic acid in arsenic intoxicated rat. *Toxicology* 210:25–36
65. McIlwain DR, Berger T, Mak TW (2013) Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol* 5:a008656
66. Santos PS, Campelo LM, Freitas CM, Saldanha GB, Freitas RM (2011) Lipoic acid effects on glutamate and taurine concentrations in rat hippocampus after pilocarpine-induced seizures. *Arq Neuropsiquiatr* 69:360–364
67. Astiz M, de Alaniz MJT, Marra CA (2012) The oxidative damage and inflammation caused by pesticides are reverted by lipoic acid in rat brain. *Neurochem Int* 61:1231–1241