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Pleurotus tuber-regium mushrooms in the diet of rats ameliorates reproductive and testicular injury caused by carbon tetrachloride

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Abstract

Background: The incidence of male infertility arising from male sexual dysfunction is high especially in the sub saharan Africa. African foods may hold promise to reverse this trend. The aim of this study therefore is to evaluate the improvement of the reproductive and testicular injuries mediated by CCl_4 by the use of a wild edible mushroom, *P. tuber-regium*.

Methods: Sixty rats were divide into six groups. Group I received 3 mL/kg olive oil by intraperitoneal injections twice weekly. Group II received 3 mL/kg (30% in olive oil) injected twice weekly *i.p*, Groups III, IV and V received 100 mg, 200 mg and 500 mg wild edible *P. tuber-regium* (33.3% in feed) daily in addition to 3 mL/kg CCl_4 in oil injected twice weekly *i.p*. Group VI received 500 mg *P. tuber-regium* (33.3% in feed) daily. After 13 weeks, the animals were sacrificed and testes weighed.
Testicular counts and viability were evaluated. Serum levels of FSH, LH, testosterone, estrogen and prolactin were assayed. Malondialdehyde (MDA), ascorbic acid, α-tocopherol, superoxide dismutase (SOD), catalase, total glutathione and peroxidase were determined in testis homogenate. Also, histopathological examinations of the testes were performed.

Results: Administration of CCl_4 to rats significantly ($p < 0.01$) increased the relative testicular mass in treated group when compared to control group. Also, CCl_4 administration decreased significantly ($p < 0.01$) the levels of sperm motility, epididymal and testicular sperm count and viability ratio in the CCl_4 group when compared to the control group. Exposure to CCl_4 decreased significantly the levels of FSH, LH and testosterone when compared to the control while increasing the levels of estrogen, prolactin and MDA when compared to the control. The levels of ascorbic acid, α-tocopherol, SOD, catalase, total glutathione and peroxidase decreased significantly ($p < 0.01$) in treated groups when compared to control group. These changes were reversed by diets containing *Pleurotus tuber-regium* mushrooms in a dose-dependent manner. Photomicrographs also showed that *P. tuber-regium* prevented the edema, spermatogenic distortions and maturation arrest observed in the CCl_4 only group.

Conclusion: *P. tuber-regium* is effective in protecting the testes from the free radical injuries mediated by CCl_4.

Keywords: *P. Tuber-regium*, CCl_4, Mushroom, Sperm analysis, Hormones, Antioxidants, Histopathology

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Background

The global incidence of male sexual dysfunction (MSD) is alarming with estimates put at over 30% [1]. In sub Saharan Africa, there is lack of data on the true incidence of MSD but researchers believed that it is higher than 60% [2]. MSD is a syndrome with many diseases that usually but not always involve problems of sperm concentration, morphology and motility, hormonal imbalance that could result from low levels of testosterone, aging process, and drugs like antidepressants or environmental chemicals and cigarettes [3–5]. Carbon tetrachloride is a well-known industrial solvent that had been banned for its toxicological concerns and is known to be a toxicant to the liver, kidney, lungs, testis etc. [6]. Metabolism of carbon tetrachloride produces free radicals ($CCL_3^{\bullet}$ and $CCl_3O_2^{\bullet}$) that bind to poly unsaturated free acids (PUFA) of sperm membranes to generate alkoxy and peroxy radicals that can perturb signal transduction mechanisms and promote infertility [7]. These radicals are unstable; the pathological consequences in the testis of these very reactive agents may include reduced sperm counts, disrupted hormone levels, impaired enzyme activities, and necrosis [8]. Also, these radicals react with molecular structures of the body's defense system like the sulfhydryl groups of glutathione and protein thiols altering their structure and leading to loss of activity [9]. The mammalian body is endowed with protective mechanisms against the destructive effects of free radical damage [10]. Normal protective mechanisms may be overwhelmed in cases of excessive free radical production; cellular damage will ensue in these situations unless additional free radical scavenging capabilities are available through dietary constituents or other exogenous sources. Abundant evidence confirms the ability of antioxidants to treat or prevent CCl_4-induced testes injury [5, 7]. Several natural products, especially medicinal plants and mushrooms, have been shown to possess antioxidant properties [11, 12].

Mushrooms, such as *Pleurotus tuber-regium*, are often used as foods and as folk medicines [13]. Medicinal mushrooms are known to contain several phytoactive constituents which are mainly secondary metabolites like flavonoids, tannins, alkaloids, phenols etc. *Pleurotus tuber-regium* is an edible mushroom belonging to the family *Pleurotaceae.* In addition to its nutritive properties, it had been used as an anti-inflammatory, antioxidant, antiviral, antipyretic and hepatoprotective [14, 15].

The present study has evaluated the potential of *Pleurotus tuber-regium* to protect the reproductive functions of the testes from free radical injury mediated by carbon tetrachloride.

Methods

Collection of fungi

Fresh fruiting bodies of *P.tuber-regium* were collected from a forest close to University of Nigeria, Nsukka. They were identified by a taxonomist at the International Center for Ethnomedicine and Drug Development (INTERCEDD) Nsukka, Nigeria. The mushrooms were air dried, pulverized and stored in air-tight containers until use. The voucher specimen for the same is conserved under the reference number INTERCEDD/050.

Experimental Animals

Male *Sprague Dawley* rats with average weight of 170–180 g were obtained from the Animal House of University of Port Harcourt, Rivers State Nigeria. The animals were maintained under standard laboratory conditions at ambient temperature of 25 °C ± 15%, with darkness cycle of 12 h. They were allowed access to standard commercial pellet diet and water *ad libitum*. All the experimental studies conformed to the guidance for care and use of animals in experimental studies of the University of Port Harcourt Ethical protocols.

Acute toxicity test

Various doses of *P. tuber-regium* were administered to male *Sprague Dawley* rats *per oral* (50–5000 mg kg^{-1} b.w). The animals were observed for gross behavior, neural and autonomic toxicity as described on OECD guidelines [16]. There was no mortality or toxic signs recorded in this period even up to 5000 mg/kg dose.

Experimental Design

The animals were divided into six (6) groups ($n = 10$). They were treated as follows;

Group I Control (received olive oil 3 mL/kg i.p. twice weekly for 13 weeks in addition to feed and water).

Group II Carbon tetrachloride treated (received 3 mL/kg i.p. of 30% CCl_4 in olive oil twice weekly for 13 weeks) [5] modified.

Group III Rats were treated with 3 mL/kg i.p. of 30% CCl_4 in olive oil twice weekly in addition to 100 mg/kg BW of *Pleurotus tuber-regium* (33.3% in feed) for 13 weeks [17].

Group IV Rats were treated with 3 mL/kg i.p. of 30% CCl_4 in olive oil twice weekly in addition to 200 mg/kg BW of *Pleurotus tuber-regium* (33.3% in feed) for 13 weeks.

Group V Rats were treated with 3 mL/kg i.p. of 30% CCl_4 in olive oil twice weekly in addition to 500 mg/kg BW of *Pleurotus tuber-regium* (33.3% in feed) for 13 weeks.

Group VI Rats received olive oil 3 mL/kg i.p. twice weekly in addition to 500 mg/kg BW of *Pleurotus tuber-regium* (33.3% in feed) for 13 weeks.

Measurement of organ weights

At the end of the 13th week post treatment, the animals were sacrificed under ether anesthesia and the right testes removed, carefully trimmed of fats and rinsed with ice cold saline before being weighed using a digital weighing balance. The relative organ weights were determined as follows;

Relative organ weight (ROW) = Organ weight/Animal weight x 100.

Part of the testes were homogenized in phosphate buffered saline for lipid peroxidation and antioxidant studies while part were preserved in 10% formalin for histological evaluations.

Collection of Blood samples

At the end of the 13th week post treatment, blood samples were withdrawn from the medial canthus of the eye using micro hematocrit tubes into a clean test tube [18]. The blood was allowed to coagulate for 30–60 min, then centrifuged at 1800 x g relative centrifugation force (RCF) for 30 min to effect separation of serum from blood cells. The serum was stored below 0 °C in 1.5 mL tubes until used for hormonal analyses.

Sperm analysis

Sperm motility was determined by macerating the epididymis in mortar and pestle and mixing it with 1 ml of phosphate-buffered saline (37 °C) in order to release the sperm cells, and a drop of the suspension was quickly placed on a glass microscope slide, covered with a glass slip and examined using a light microscope at × 40 magnification. At least 200 sperm cells were counted and the number of motile sperm cells reported as a percentage of the total cells [19]. Sperm viability was assessed microscopically from smears that were prepared by mixing equal volumes of eosin-nigrosin stain with epididymal suspensions, incubation for 30 s, distributing the stained suspensions on glass slides, and drying in air. After 30 s, a drop of the mixture was placed on a glass slide and spread out to make a smear, and this was allowed to air-dry. The smear was examined under x100 oil immersion using a light microscope. Live sperm were left unstained whereas dead sperm stained pink or red. At least 100 spermatozoa were evaluated and the sperm vitality recorded as Live to Dead sperm ratio [20]. The caudal epididymal sperm reserves and testicular counts were determined using the standard hemocytometric method [19].

Lipid peroxidation and antioxidant assays

At the end of the 13th week post treatment, one gram of testis tissue was homogenized in 9 ml ice cold Phosphate buffered saline (pH 7.4) to make 10% homogenates. The homogenates were centrifuged at 1800 x g RCF for 15 min at 4 °C and the supernatants were used to determine the levels of malondialdehyde (MDA), ascorbic acid, alpha tocopherol, and total glutathione as well as superoxide dismutase, catalase, and peroxidase enzyme activities. Lipid peroxidation was quantified as malondialdehyde [21]. Ascorbic acid was assayed colorimetrically using the 2,4-dinitrophenylhydrazine method [22]. Alpha tocopherol was analysed colorimetrically with 2, 4, 6 – tripridyl - s – triazin and $FeCl_3$ after extracting with absolute ethanol and xylene as described by Martinek [23]. Superoxide dismutase (SOD), catalase, total glutathione and peroxidase were assessed using a commercial kit (Biovision, Mountain View, CA, USA) obtained from a local representative and assayed according to the manufacturer's protocol.

Serum analysis of hormones

Serum analysis of follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, estrogen and prolactin were estimated using commercial kits (Biocheck Inc, 323 Vintage Park Dr, Foster City, CA 94404) obtained through a local representative and assayed according to the manufacturer's protocol.

Histological studies

Portions of the testis from all animals were sliced and dehydrated with a range of concentrations (70%, 80%, 90% and absolute) of alcohol and then cleared with xylene (twice) before embedding in paraffin. The embedded tissue blocks were section with Shandon AS 325 rotary microtomes into approximately 5 μm thick section and slides were prepared with the sections. The tissues were stained with Ehrlich's haematoxylin and eosin blue.

Statistical analysis

All statistical analyses were done on GraphPad Prism version 5.02 (www.graphpad.com/scientific-software/prism). Data were expressed as mean ± standard deviation (SD). ANOVA test was used to analyze the difference among various treatments with least significance difference (LSD) at 0.05 followed by Bonferroni's posttest.

Results

Effect of *P. tuber-regium* on the average kidney weight of CCl_4 treated animals

Figure 1 shows the effect of *P. tuber-regium* treatment on testes of animals administered CCl_4 for 13 weeks. Administration of CCl_4 to the rats resulted in a significant ($p < 0.05$) increase in relative weight of the testis 0.92 ± 0.15 in the control groups when

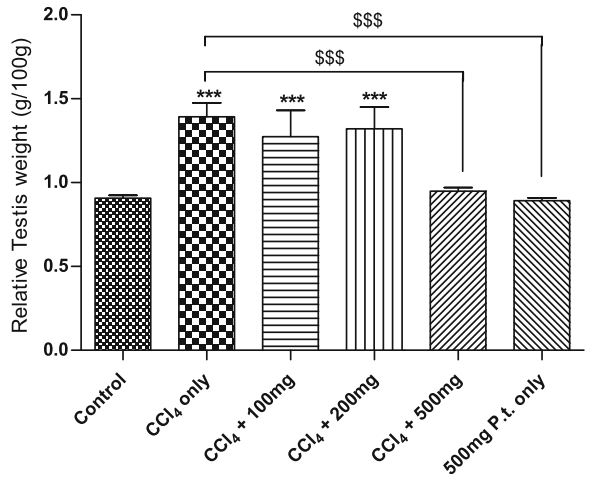

Fig. 1 Effect of *P. tuber-regium* on relative weight of testes of animals treated with CCl_4 for 13 weeks ($n = 10$). ***: significantly different from control at $p < 0.001$. $$$: significantly different from CCl_4 only at $p < 0.001$

compared to the relative weight 1.2 ± 0.24 of the treated group 1.2 ± 0.24. *P. tuber-regium* in the diet resulted in a dose-dependent reversal of the increases in testicular weight caused by CCl_4 treatments.

Effect of *P. tuber-regium* on sperm analysis

Administration of CCl_4 to rats significantly ($p < 0.05$) decreased the levels of motile sperms (74.0 ± 1.50%), epididymal reserve (46.0 ± 2.10 x 10^6), testicular count (449 ± 37 x 10^6) and viability (46.33 ± 4.62) in the control group when compared to the CCl_4 only treatment group values (1.70 ± 1.1%, 10.0 ± 2.1 x 10^6, 180 ± 13.0 x 10^6 and 6.23 ± 0.30 respectively). Treatment with *P. tuber-regium* restored the levels of these biomarkers to different degrees when compared to the control group. However, treatment with *P. tuber-regium* alone did not alter the levels of these markers significantly ($p < 0.05$) when compared to the control group (Fig. 2).

Effect of *P. tuber-regium* on reproductive hormones

The effects of *P. tuber-regium* on follicle stimulating hormone, luteinizing hormone, testosterone, estrogen and prolactin are shown in Fig. 3. 13 weeks

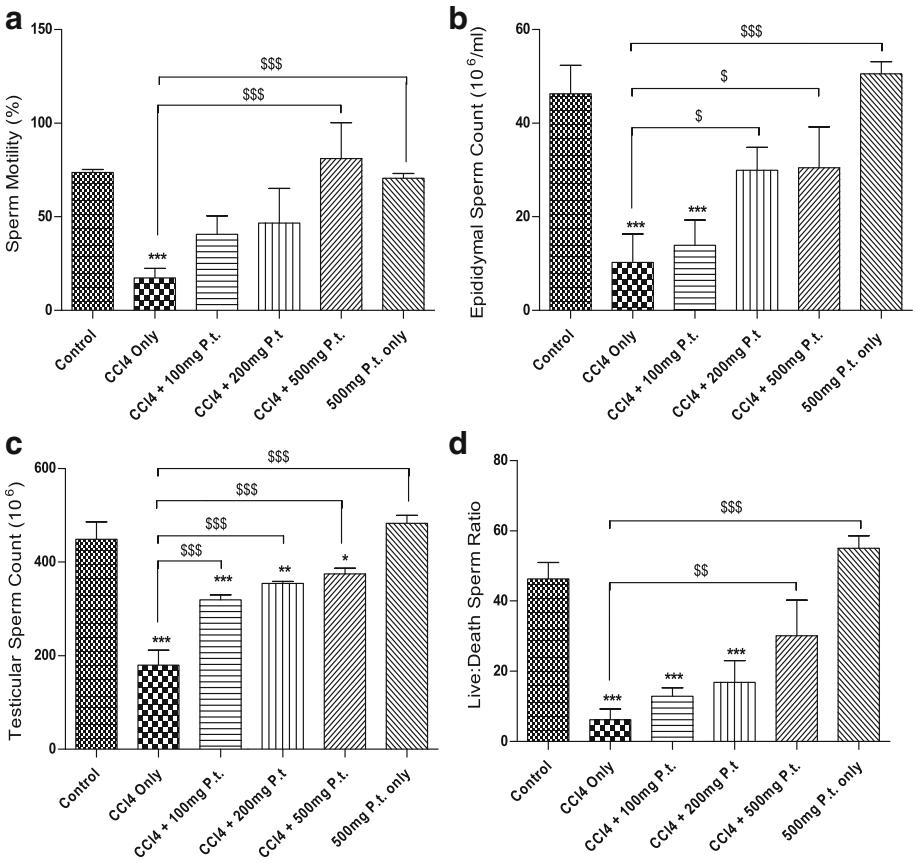

Fig. 2 Effect of *P. tuber-regium* treatment on sperm parameters of animals treated with CCl_4 for 13 weeks ($n = 10$). ***, * significantly different from control at $p < 0.001$and $p < 0.05$. $$$, $$, $ significantly different from CCl_4 only at $P < 0.001$

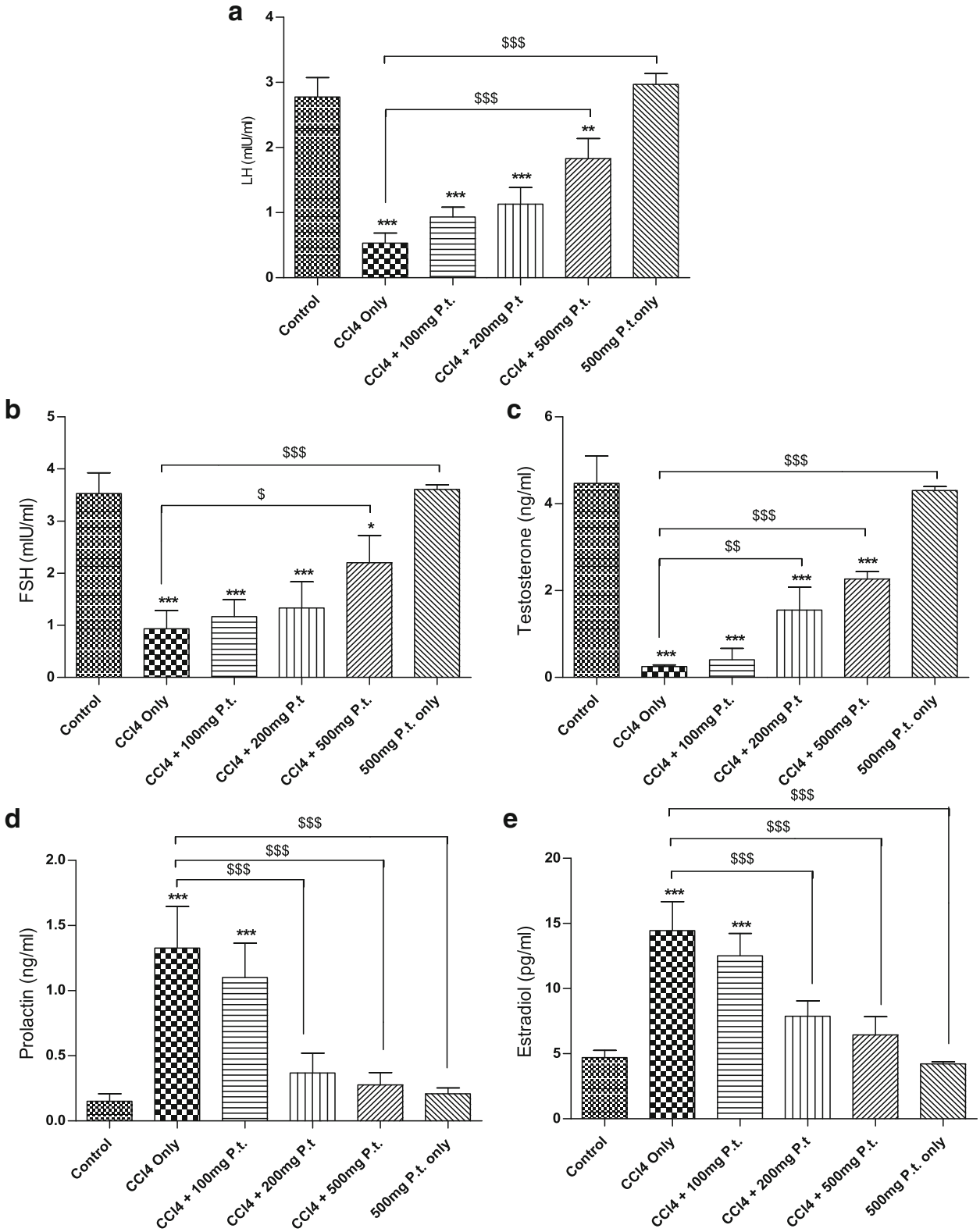

Fig. 3 Effect of *P. tuber-regium* treatment on hormonal profile of animals treated with CCl_4 for 13 week ($n = 10$). ***, ** significantly different from control at $p < 0.001$, $p < 0.01$ probability levels. $$$, $$, $ significantly different from CCl_4 only at $p < 0.001$, $p < 0.01$, $p < 0.05$ probability levels

administration of CCl_4 significantly decreased ($p < 0.05$) the hormonal levels of FSH, LH and testosterone (0.93 ± 0.04 mIU/mL, 0.53 ± 0.02 mIU/mL and 0.25 ± 0.04 ng/mL) when compared to the control group (3.53 ± 0.40 mIU/mL, 2.78 ± 0.30 mIU/mL and 4.48 ± 0.60 ng/mL respectively). Alterations of these hormones were significantly reversed ($p < 0.05$) by the treatment with P. tuber-regium at different doses in a dose dependent manner. However, there is no significant difference between the negative control treated and the control. Also, administration of CCl_4 increased significantly ($p < 0.05$) the levels of estrogen and prolactin from 4.70 ± 0.56 pg/mL and 0.15 ± 0.06 ng/mL in control group to 14.5 ± 2.2 pg/mL and 1.33 ± 0.12 ng/mL in CCl_4 only group respectively. These alterations were reversed with *P. tuber-regium* treatment.

Effect of *P. tuber-regium* on lipid peroxidation and antioxidant vitamins

The effects of *P. tuber-regium* on the levels of malondialdehyde, ascorbic acid, and alpha tocopherol in the testis are shown in Fig. 4. Administration of CCl_4 significantly ($p < 0.05$) increased the level of MDA in the testes from 0.68 ± 0.08 μmol/L in control group to 1.60 ± 0.30 μmol/L in the treated group. Ascorbic acid and alpha tocopherol levels decreased from 0.65 ± 0.04 mg/dL and 3.50 ± 0.03 μg/mL in the control group to 0.31 ± 0.03 mg/dL and 0.69 ± 0.01 μg/mL respectively. *P. tuber-regium* administration decreased the levels of MDA and increased the levels of ascorbic acid and alpha tocopherol significantly in a dose dependent manner in the groups that took this mushroom. Also, the levels of MDA, ascorbic acid and α-tocopherol in the negative control treated is comparable to control.

Effect of *P. tuber-regium* on antioxidant enzymes

The effect of *P. tuber-regium* treatment on the antioxidant enzymes in the testis is shown in Fig. 5. There was a significant ($p < 0.001$) decrease on all the antioxidant enzymes assayed; SOD decreased from 10.30 ± 0.40 U/mL in control group to 2.28 ± 0.03 U/mL in the treated group, catalase from 93.90 ± 4.0 mU/mL to 31.40 ± 1.83 mU/mL, total glutathione from 1.52 ± 0.07 μg/mL to 0.47 ± 0.04 μg/mL and peroxidase from 11.20 ± 0.40 mU/mL to 4.57 ± 0.30 mU/mL respectively. Co-administration of CCl_4 with *P.tuber-regium* resulted in improvement of the aforementioned antioxidant enzymes in a dose dependent manner. However, treatment of *P. tuber-regium* to negative control produced no significant difference in antioxidants levels when compared to the control.

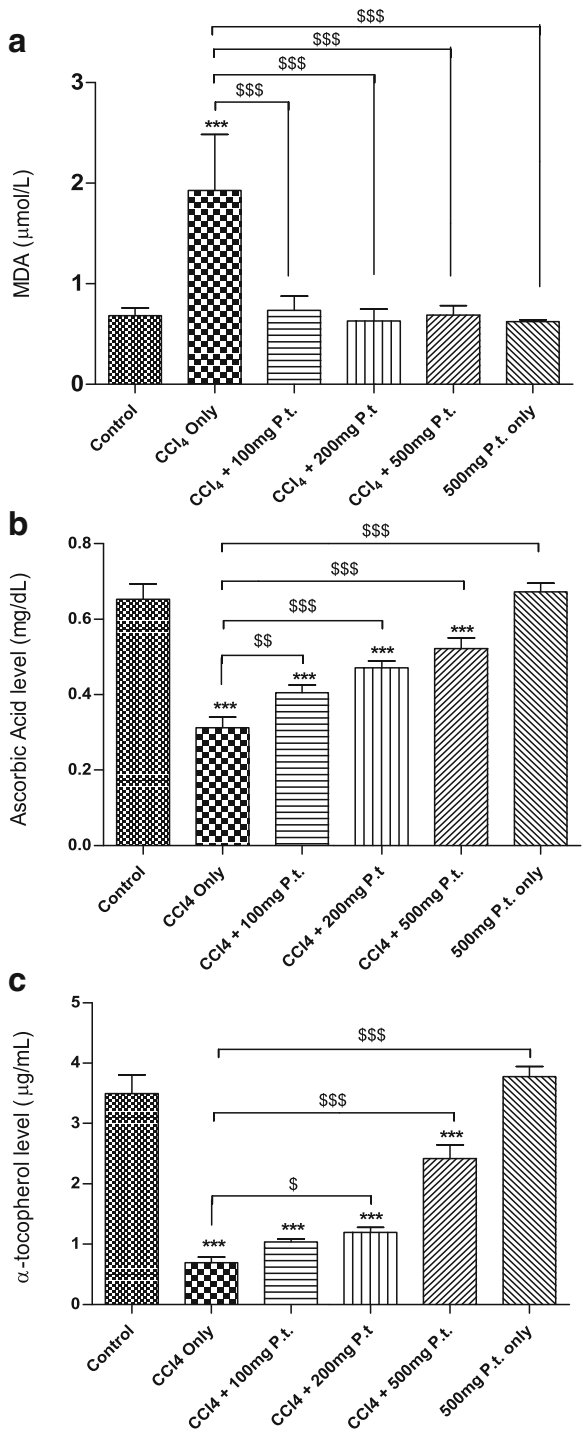

Fig. 4 Effect of *P. tuber-regium* on lipid peroxidation and antioxidant vitamins of animals treatment with CCl_4 for 13 weeks ($n = 10$). *** Significantly different from control at $p < 0.001$. $$$, $$, $ significantly different from CCl_4 only at $P < 0.001$, $P < 0.01$, $P < 0.05$

Histological studies of the Testes

The histoarchitecture of the testes at different treatments are presented in Fig. 6. The histological studies of the testes showed well preserved testicular architecture

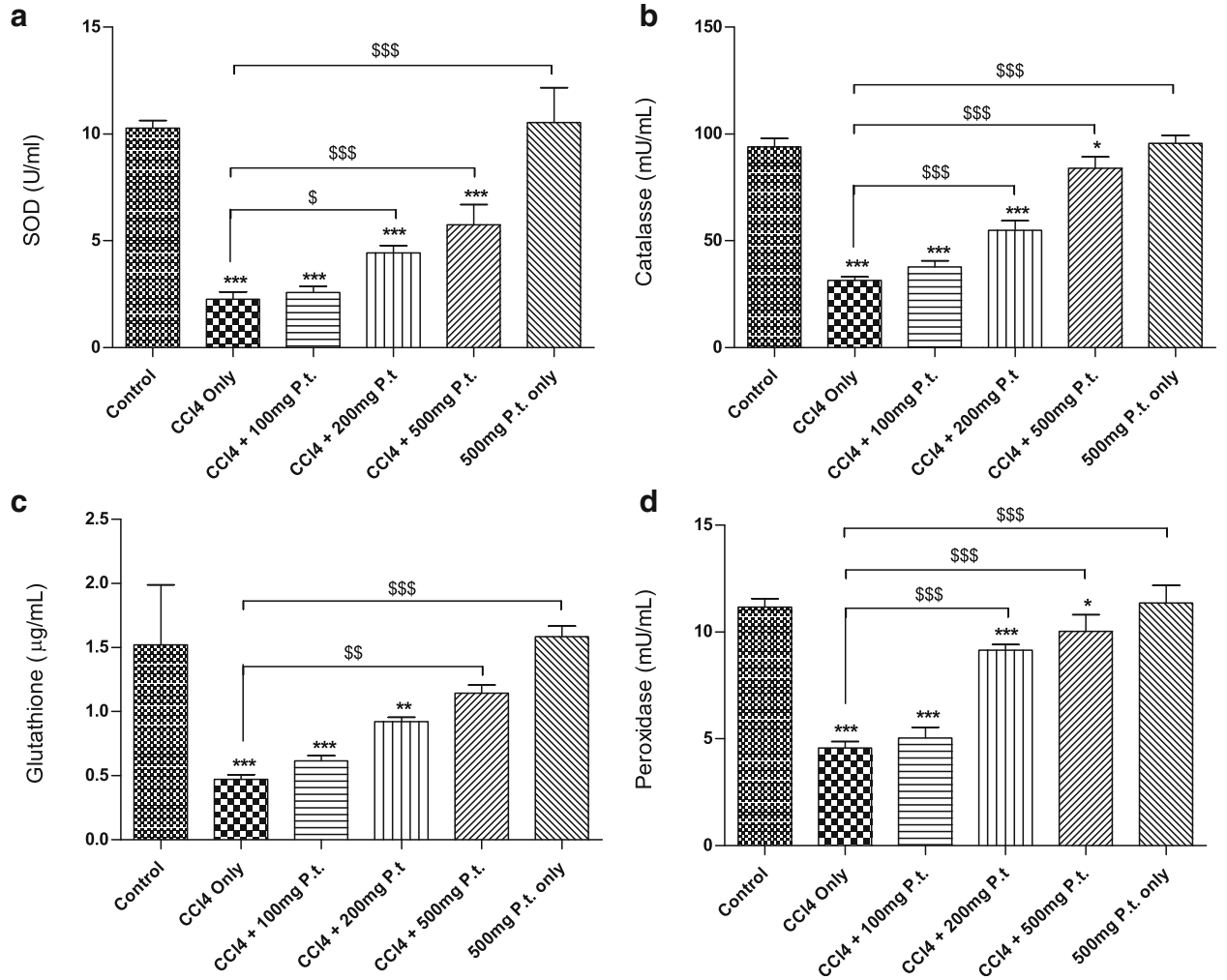

Fig. 5 Effect of *P. tuber-regium* on antioxidant enzymes of animals treated with CCl_4 for 13 weeks ($n = 10$). ***, **, * significantly different from control at $p < 0.001$, $p < 0.01$, and $p < 0.05$ probability level. $$$, $$, $ significantly different from CCl_4 only group at $p < 0.001$, $p < 0.01$, $P < 0.05$ probability level

(Fig. 6a). Administration of CCl_4 resulted in scanty spermatogenic cells, maturation arrest (MA) and edema (Fig. 6b). Treatment with *P. tuber–regium* improved these observed pathologies in a dose dependent manner (Fig. 6c, d and e). Micrographs of *P. tuber-regium* only group shows well preserved architecture which is comparable to control group (Fig. 6f).

Discussion

Oxidative stress resulting from increased generation of free radicals or depletion of antioxidant defense systems is well known to produce toxic effects in animal models [4, 6]. Carbon tetrachloride, a toxic industrial solvent and environmental contaminant, exerts its destructive effects by the generation of free radicals. [24]. CCl_4 is bio transformed by phase 1 metabolism of the P-450 system to generate trichloromethyl radical ($CCl_3^{\bullet}$) and peroxy trichloromethyl radicals ($CCl_3OO_2^{\bullet}$) [25]. These free radicals have the ability to bind poly unsaturated fatty acids (PUFA) abundant in sperm cell membranes to generate alkoxy ($R^{\bullet}$) and peroxy radicals ($ROO^{\bullet}$) that in turn will generate lipid peroxides that are very reactive with potential to alter enzymes and cause necrosis [5, 26]. The resultant effects of these free radicals is the depletion of the antioxidant capacity *in vivo*, caused by decreasing the levels of strategic antioxidant enzymes and vitamins, leading ultimately to oxidative stress [27].

In this study, we tested the hypothesis that *P. tuber-regium* will protect and reduce lipid peroxidation in the testes of CCl_4 intoxicated *Sprague Dawley* rats. *P. tuber-regium* possesses active phytochemicals that, when present in the diet of rats, can supply exogenous antioxidant capacity to ameliorate oxidative stress [13]. Oxidative stress appears to be a major contributor in male infertility

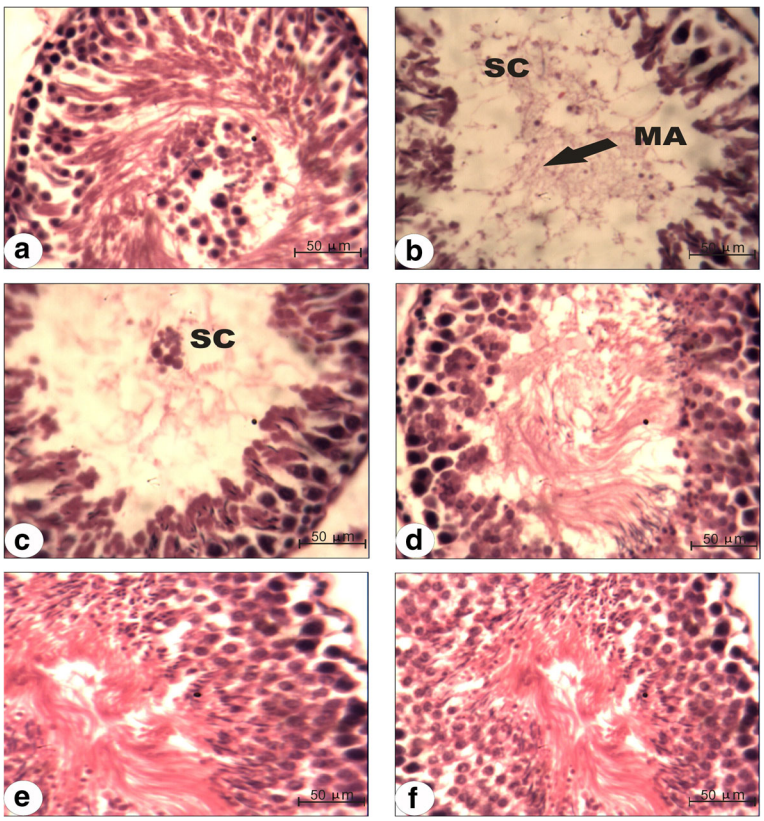

Fig. 6 Photomicrograph showing histopathological changes in the testes of rats (magnification H & E X 400). **a** Control testis showing normal histology. **b** CCl_4 only group showing scanty spermatogenic cells and maturation arrest (MA) (**c**) CCl_4 + 100 mg P.t. group showing seminiferous tubules without full population of spermatozoa (**d**) CCl_4 + 200 mg P.t. group showing seminiferous tubules without full population of spermatozoa. **e** CCl_4 + 500 mg P.t. group showing normal testis. **f** 500 mg P.t. only group with normal histology

and development of effective antioxidants to combat it will be a giant stride in the prevention and management of male sexual dysfunction [28, 29]. Administration of CCl_4 increased significantly both the absolute and relative organ weights of the testes. This increase may be due to increase in lipid peroxidation induced by the highly reactive metabolites of CCl_4 that may possibly activate the inflammatory pathway leading to edema [5, 30]. CCl_4 metabolites react with poly unsaturated fatty acids and form covalent adducts with lipids and proteins that lead to lipid peroxide formation and destruction of cell membranes and consequently testis injury [31]. Amelioration of this effect was seen in the groups that were treated with *P. tuber-regium* suggesting its abilities to mop up free radicals generated by CCl_4 [32]. The protective effects of *P. tuber-regium* against oxidative stress in rat testis that we observed in this study are consistent with those reported for medicinal plants with known antioxidant properties, such as *Launaea procumbens* [5].

CCl_4 induced significant reduction in the sperm motility (%), count and vitality when compared to the control group. Theses alterations in the sperm parameters were significantly restored to different degree in the groups of animals treated with *P. tuber-regium*. Other reports on the intoxication of animals with CCl_4 recorded similar reductions [26, 33]. Alterations in sperm parameters may result from low levels of reproductive hormones secondary to the effect of CCl_4 in the gonadal-pituitary axis or direct toxic effects to the sperm cells [4].

Effects of CCl_4 administration on FSH, LH and testosterone showed significant decrease in the CCl_4 group compared to the control group. Reductions in the FSH and LH may be due to the distortions in the hypothalamic-pituitary axis leading to testicular dysfunction as indicated by the results of sperm analysis [26, 34]. The decrease in testosterone level may be as a result of low levels of FSH and LH since its activity is under their direct influence. Also, decrease in testosterone level could be due to direct toxicity of CCl_4 on the sertoli cells [35]. The levels of estrogen and prolactin increased significantly in the CCl_4 only treatment group when compared with those of the control group. In the male reproductive system, prolactin and estrogens

promote infertility in males by antagonizing the effects of testosterone [36]. These observed hormonal alterations were reversed by the treatment with *P. tuber-regium*.

Antioxidant enzymes may have a major role in preventing male infertility due to oxidative stress since CCl_4 and reactive oxygen species are associated with impaired antioxidant enzyme status which determine in part the body's antioxidant capacity [26]. Administration of CCl_4 decreased the levels of ascorbic acid, α-tocopherol, SOD, catalase, total glutathione and peroxidase while increasing the level of lipid peroxidation as measured by MDA. Reduction in the levels of antioxidant enzymes and vitamins is inversely related to the MDA suggesting the involvement of oxidative stress in the levels of these enzymes. Decreased glutathione levels may be due toxic effects of CCl_4 on NADP (H) enzyme which is the limiting factor in its production [37]. Catalase is known to convert peroxides especially hydrogen peroxide to water and in the circumstance of its lipid peroxides, this enzyme will be consumed leading to its low levels [38]. Treatment with *P. tuber-regium* improved the levels of the antioxidant enzymes and vitamins further leading credence to the antioxidant properties of this mushroom. Plants with polyphenolics like flavonoids, phenolic compounds, glucans are known to be effective in antioxidation. This they do by scavenging hydroxyl and superoxide radicals, chelate metal ions or exert synergistic effects in conjunction with other antioxidant metabolites [7].

Histological studies revealed that CCl_4 administration caused degeneration of the testicular structure and germ cells with scanty spermatogenic cells, maturation arrest, and edema. The histological changes in the testis caused by CCl_4 were largely reversed by the presence of *P. tuber-regium* in the diet. This is in agreement with other researchers that used CCl_4 model of oxidative stress [26] and points to the involvement of oxidative stress in male infertility and the possible potential benefits natural products like mushrooms may play in the management of this ailment.

Conclusion

P. tuber-regium may be useful in the management of male reproductive hormonal dysfunction possibly secondary to oxidative stress.

Authors' contributions

KO: Carried out the bench work analysed data and write up, OO: Designed the study, analysed data and write up and IS: Design. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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