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Effect of *Thymus vulgaris* leaf extract on cadmium-induced testicular toxicity in rats

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Abstract

Background: Cadmium (Cd) is a known metallohormone which mimics the action of steroid hormones with adverse effect on testicular function. It is highly toxic and a prevalent environmental contaminant with no conventional antidote. This study investigates the possible ameliorative effects of *Thymus vulgaris* extract on testicular toxicity induced by Cd in male rats.

Results: The testicular and epididymal weights, serum concentration of follicle stimulating hormone, luteinizing hormone, and testosterone were significantly ($p \leq 0.05$) lower in the cadmium-treated group compared to the control. Necrosis of germ cells of the seminiferous tubules was observed in the testicular tissues of the cadmium-treated group. Administration of extract showed mild but non-significant ($p \geq 0.05$) protective effect on the cadmium-induced decrease in sex hormones and sperm count as well as oxidative stress and histological changes.

Conclusion: *Thymus vulgaris* leaf extract had weak ameliorative effect on cadmium-induced testicular injury in rats but with promising antioxidant activity.

Keywords: Cadmium, Rat, Testis, *Thymus vulgaris*, Toxicity

Background

Cadmium (Cd) is a highly toxic environmental contaminant resulting from industrial activities. It is a heavy metal which is broadly utilized in industry but adversely affects animal and human health through occupational exposure, contaminated food and water or smoking (El-Demerdash et al. 2004; de Souza et al. 2010). This toxic metal enters and accumulates in different organs of the body in animals and man to cause severe tissue damage ranging from cellular degeneration, inflammation to cancers. Some of these organs include the kidney, liver, testicles, pancreas, thyroid, salivary glands, bone and brain (Thompson and Bannigan 2008; Ognjanović et al. 2010). However, the primary target organ for cadmium toxicity is the male reproductive organ. In the testes, a single non-carcinogenic dose of Cd is known to cause significant testicular atrophy and calcification following blood-testis barrier disruption, inflammation, germ cell loss and haemorrhage (Acharya et al. 2008; Ola-Mudathir et al. 2008; Deng et al. 2010). Previous reports have associated the toxic effects of Cd to its induction of oxidative stress and alteration in the antioxidant defence system in several tissues leading to a decrease in the activity of antioxidant enzymes and a change in cell membrane structure through lipid peroxidation (Bagchi et al. 1997; Zikic et al. 1998; Siu et al. 2009). This testicular oxidative stress and its associated cellular damage have been established as a major cause of severe male infertility due to Cd toxicity (Tremellen 2008; Turner and Lysiak 2008). Thus, heavy metal poisonings like Cd toxicity are suspected to be one of the major reasons for the recent declining fertility associated with reduced sperm count and testicular function in men in developed countries (Siu et al. 2009), as animals and humans that inhabit industrial areas where Cd is used for manufacturing certain products are

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vulnerable to accidental Cd exposure. However, modern research has shown that phytogenic compounds or bioactive compounds of plant origin are rich in antioxidants, such as flavonoids, phenols, terpenes, polysaccharides, saponins, alkaloids, vitamins, and trace elements (Miliauskas et al. 2004; Gouthamchandra et al. 2010; Chaves et al. 2020). These antioxidants directly or indirectly exert their effects on the body's antioxidant system by eliminating excessive free radicals and thus protecting the body (Chaves et al. 2020). *Thymus vulgaris* L. (Lamiaceae) is an indigenous perennial herb in Africa, Asia, central and southern Europe, that is known to be rich in essential oils and phenolic substances (WHO 1999). In folk medicine, it is widely used for the treatment of diseases such as gastroenteric and bronchopulmonary disorders and as an anthelmintic (Rustaiyan et al. 2000). It is also known to have immunomodulatory, anti-inflammatory, antioxidant and free radical scavenging effects (Vigo et al. 2004; El-Nekeety et al. 2011). It contains potent antioxidants such as carvacrol, linalool and thymol (Satyal et al. 2016). Therefore, this study was designed to evaluate the effect of *Thymus vulgaris* extract (TVE) on cadmium-induced testicular toxicity using male albino rats as they are genetically similar to human.

Methods

Chemicals

Cadmium chloride ($CdCl_2$—99%) was obtained from Sigma Aldrich Chemicals Co. (St. Louis, Mo, USA). All chemicals and reagents utilized were obtained from commercial suppliers.

Extraction of plant material

The leaves of *T. vulgaris* were purchased from Ogige market in Nsukka and identified by Mr. A. Ozioko, a botanist at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, and the voucher specimen was deposited at the University of Nigeria Herbarium museum, with herbarium number UNNH648. The plant material was dried under shade for 10 days and ground to powder using a grinding machine. The powdered material was soaked in 70% methanol for 3 days after which it was filtered through Whatman paper (No. 1) and concentrated using a rotary evaporator (Daud et al. 2017). The *T. vulgaris* extract (TVE) obtained was stored at 4 °C and dissolved in 2% tween 80 in distilled water prior to administration (Onoja et al. 2020).

Acute toxicity (LD50) study

Acute toxicity (LD50) of the TVE extract was determined according to the Organization of Economic Cooperation and Development (OECD) guideline 423 (OECD 2003). Adult male rats were administered 625, 1250, 2500, and 5000 mg/kg doses of the extract orally and observed for clinical signs of toxicity and mortality. No mortality or signs of toxicity were observed in animals administered 5000 mg/kg of the extract. Hence, the median lethal dose (LD50) was considered to be greater than 5000 mg/kg body weight in rats.

Animals

Thirty two healthy male albino rats about 10–12 weeks old, weighing between 160 and 180 g were obtained from the Experimental Animal Unit of the Zoological Garden, University of Nigeria, Nsukka. They were housed in standard metal cages with wood shavings as bedding in the Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka and housed at a temperature of 25 ± 4 °C and relative humidity of $65 \pm 5\%$ with an alternating 12 h light and dark cycle. The animals had free access to food and water daily, and were acclimatized for one week before the commencement of the study.

Experimental design

The experiment was conducted in compliance with the National Institutes of Health guidelines on the care and use of laboratory animals (National Research Council 2011) as approved by the Faculty of Veterinary Medicine, Institutional Animal Care and Use Committee (IACUC), University of Nigeria, Nsukka (FVM No.20/20/11/7). The rats were randomly (randomized controlled trial) divided into four groups of eight rats each. Group A was administered 0.5 ml of 2% tween 80 in distilled water orally and a single subcutaneous dose of phosphate buffered saline (PBS); Group B was administered a single subcutaneous (SC) dose of cadmium at 3 mg/kg in PBS only; Group C was administered 500 mg/kg TVE in 2% tween 80 in distilled water orally, daily for 3 weeks while Group D was administered single (SC) dose of cadmium (SC) at 3 mg/kg + 500 mg/kg TVE in 2% tween 80 in distilled water orally, daily for 3 weeks. The group allocation and treatment were concealed from the laboratory technologist and pathologist involved in sample analysis and interpretation. The effective dose of Cd and the extract used and the duration of study were based on previous reports (Ponnusamy and Pari 2011; Onoja et al. 2020).

Sample collection

At the end of the experiment, the rats were fasted overnight and 2 mL of blood was collected via the retro-orbital plexus into plain sample bottles after euthanasia by intraperitoneal injection of 90 mg/kg body weight ketamine hydrochloride and 5 mg/kg body weight xylazine (Zarei and Shahrooz 2019). Blood in the plain sample bottles was allowed to clot, centrifuged (3000 rpm

for 10 min) and serum was collected for hormonal assay. Thereafter, the rats were dissected and the epididymis was collected for sperm count while the testes were harvested for antioxidant enzyme activity, lipid peroxidation assay and histopathology.

Spermatogenic activity

The testicular and epididymal weights were determined using a sensitive Mettler weighing balance (manufactured by Mettler Toledo, Switzerland) while the epididymal sperm count was assessed using the standard haemocytometric method (Obembe and Ige 2016).

Hormonal assay

Enzyme-linked immuno-absorbent assay (ELISA) kit was used for the quantitative determination of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentration according to the manufacturer's protocols.

Lipid peroxidation and antioxidant assay

Lipid peroxidation biomarker, malondyaldehyde (MDA) was measured by spectrophotometric method as described previously (Ohkawa et al. 1979), while catalase (CAT) and superoxide dismutase (SOD) activity was estimated according to standard methods (Nishikimi et al. 1972; Hadwan 2018).

Histopathological evaluation

Testes from the different groups were dehydrated in graded concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. Five-micrometer thick sections were cut, mounted on glass slides, and stained with haematoxylin and eosin for light microscopy (Bancroft and Gamble 2008).

Statistical analysis

Statistical analyses of data were carried out by one way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) version 22.0. The mean differences were considered significant at $p \leq 0.05$.

Results

Body, testicular and epididymal weights

Table 1 shows the body weights, testicular and epididymal weights of rats. Compared with control, there was no significant difference in body weight between the $CdCl_2$-treated rats and control group. It was observed that $CdCl_2$-treated rats showed a significant decrease in testes and epididymal weights when compared with the control group. However, the testis and epididymal weights of rats administered $CdCl_2$ and treated with TVE were not significantly ($p \geq 0.05$) different compared to the control.

Epididymal sperm counts

The epididymal sperm counts were significantly ($p \leq 0.05$) lower in the $CdCl_2$-treated group compared to the control (Fig. 1). Although, the $CdCl_2 + TVE$ group showed an increase in sperm count, this was not significantly ($p \geq 0.05$) different from the $CdCl_2$-treated group.

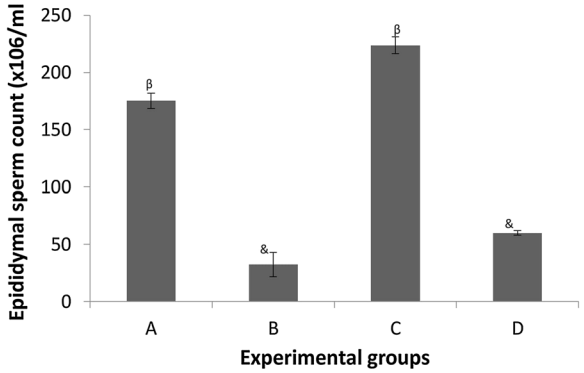

Fig. 1 A graph showing the mean epididymal sperm counts of rats from the experimental Group A (control), Group B (3 mg/kg $CdCl_2$), Group C (500 mg/kg TVE) and Group D (3 mg/kg $CdCl_2$ + 500 mg/kg TVE). The values are expressed as mean ± S.E.M ($n = 8$). The level of significance was set at $p \leq 0.05$. Bars with different superscripts are significantly different

Table 1 Effects of *Thymus vulgaris* extract on body weight, testicular and epididymal weights of rats

Parameters	A (Control)	B ($CdCl_2$)	C (TVE only)	D ($CdCl_2$ + TVE)
Body weight (g)	193.50 ± 6.68^a	185.60 ± 9.97^a	191.50 ± 8.88^a	189.50 ± 5.41^a
Testicular weight (g)	2.83 ± 0.12^a	1.57 ± 0.47^b	3.83 ± 0.39^a	1.77 ± 0.09^b
Testicular/BW ratio (%)	1.45 ± 0.01^a	0.83 ± 0.03^b	1.98 ± 0.08^a	0.92 ± 0.08^b
Epididymal weight (g)	0.87 ± 0.19^a	0.53 ± 0.03^b	1.63 ± 0.38^a	0.65 ± 0.13^b
Epididymal/BW ratio (%)	0.42 ± 0.15^a	0.25 ± 0.05^b	0.83 ± 0.14^a	0.32 ± 0.03^b

Values are Mean ± S.E.M, $n = 8$; BW, body weight; g, gram; $CdCl_2$, Cadmium chloride; TVE, *Thymus vulgaris* extract. Mean values bearing different superscripts in the same row differ significantly at $p \leq 0.05$

Table 2 Serum concentration of reproductive hormones of the experimental groups of rats

Groups	FSH (ng/dl)	LH(ng/dl)	Testosterone(ng/dl)
A (Control)	2.77 ± 0.15^{a}	0.35 ± 0.05^{a}	5.47 ± 0.48^{a}
B ($CdCl_2$)	0.70 ± 0.21^{b}	0.19 ± 0.02^{b}	2.13 ± 0.18^{b}
C (TVE only)	3.03 ± 0.20^{a}	0.34 ± 0.03^{a}	4.77 ± 0.43^{a}
D ($CdCl_2$ + TVE)	1.03 ± 0.29^{b}	0.16 ± 0.03^{b}	2.77 ± 0.20^{b}

Values are Mean ± S.E.M, $n = 8$; ng/dl, nanogram per decilitre; TVE, *Thymus vulgaris* extract; $CdCl_2$, Cadmium chloride; LH, luteinizing hormone; FSH, follicle stimulating hormone. Mean values bearing different superscripts in the same column differ significantly at $p \leq 0.05$

Table 3 Effect of *Thymus vulgaris* extract testicular oxidative stress marker and antioxidant enzyme activity of the experimental groups of rats

Groups	MDA(nmol/g tissue)	SOD ((U/g tissue)	CAT(U/g tissue)
A (Control)	36.40 ± 1.04^{a}	41.80 ± 1.28^{a}	34.57 ± 1.04^{a}
B ($CdCl_2$)	103.70 ± 3.42^{b}	16.87 ± 1.01^{b}	18.80 ± 0.81^{b}
C (TVE only)	27.17 ± 6.18^{a}	42.80 ± 2.87^{a}	44.00 ± 2.60^{c}
D ($CdCl_2$ + TVE)	88.50 ± 5.39^{c}	22.30 ± 0.91^{b}	24.80 ± 0.78^{d}

Values are Mean ± S.E.M, $n = 8$; nmol/g, nanomoles per gram; U/g, units per gram; TVE, *Thymus vulgaris* extract; $CdCl_2$, Cadmium chloride; MDA, malondyaldehyde; CAT, catalase; SOD, superoxide dismutase. Mean values bearing different superscripts in the same column differ significantly at $p \leq 0.05$

Serum testosterone, LH and FSH

The hormonal profile in the experimental groups showed a significantly ($p \leq 0.05$) lower serum FSH, LH and testosterone in $CdCl_2$-treated rats when compared to the control (Table 2). However, the serum FSH, LH and testosterone levels in the rats administered $CdCl_2$ and treated with TVE were comparable to the group administered $CdCl_2$ only.

Testicular oxidative stress and antioxidant markers

The administration of Cd to rats in group B led to a significant ($p \leq 0.05$) decrease in SOD and CAT activity with increased MDA concentration in the testis compared to the control group A and TVE treated group C. However, the co-administration of Cd and TVE in group D increased activity of CAT and SOD with a significant decrease in the MDA level in testicular tissues when compared to group B (Cd only) as shown in Table 3.

Histopathology

Histopathological evaluation (Fig. 2) revealed normal seminiferous tubular epithelium with spermatogenic cell lines and lumen filled with numerous spermatids in the control group. The $CdCl_2$-treated group showed severe testicular damage which included degeneration, coagulative necrosis of seminiferous tubular epithelium with increased interstitial space, mononuclear cells infiltration and Sertoli cells-only pattern of tubules, while rats administered Cd and treated with TVE had mild restoration in testicular architecture.

Discussion

Human exposure to $CdCl_2$ usually occurs through occupational contact in factories and consumptions of contaminated food and drinking water (El-Demerdash et al. 2004; de Souza et al. 2010). Cd can cause blood-testis barrier disruption, germ cell loss, testicular oedema, haemorrhage and necrosis leading to impaired reproductive physiology and irreversible infertility (Zikic et al.1998; Takiguchi and Yoshihara 2006; Blanco et al. 2007; Acharya et al. 2008; Deng et al. 2010; Oguzturk et al. 2012). As seen in the present study, the testicular and epididymal weights were decreased in the $CdCl_2$-treated group compared to the control. It is well known that the weight of the testis depends on the mass of undifferentiated spermatogenic cells (Ponnusamy and Pari 2011) and can also serve as the primary indicator of a possible alteration in androgen status (Biswas et al. 2001). This was observed in the serum FSH, LH and testosterone levels in $CdCl_2$-treated group which showed a substantial decrease when compared to the control. Cd exerts its known toxic effects on organs of the body like the testis through its induction of oxidative stress (Dzobo and Naik 2013; Kumar et al. 2019).

In experimental models, Cd exposure is known to affect testis weight and induce pathology leading to reduced sperm count which adversely affects male fertility (Biswas et al. 2001; Yang et al. 2006). This was also confirmed by the severe necrosis of the seminiferous tubules and interstitial inflammation of the testis, as seen in the $CdCl_2$-group. This study also showed that administration of TVE did not elicit meaningful amelioration of cadmium-induced testicular damage. However, the consistent tendencies for increase in testicular and epididymal weights, epididymal sperm counts, serum FSH and testosterone, and mild reduction in the severity of interstitial inflammation in the group that was administered $CdCl_2$ and treated with TVE is promising. Hence, it can be stated that at higher doses and/or longer duration of study, TVE can reverse or ameliorate the testicular toxicity induced by Cd which is in contrast with previous studies (El-Newary et al. 2017; Onoja et al. 2020) where TVE was shown to protect against hepatotoxicity induced by Cd. The discrepancies were partly, attributed to differences in sensitivity of the organs to TVE. However, the antioxidant enzyme activity in the testes of rats administered $CdCl_2$ and treated with TVE increased but was relatively lower compared to those reported in the

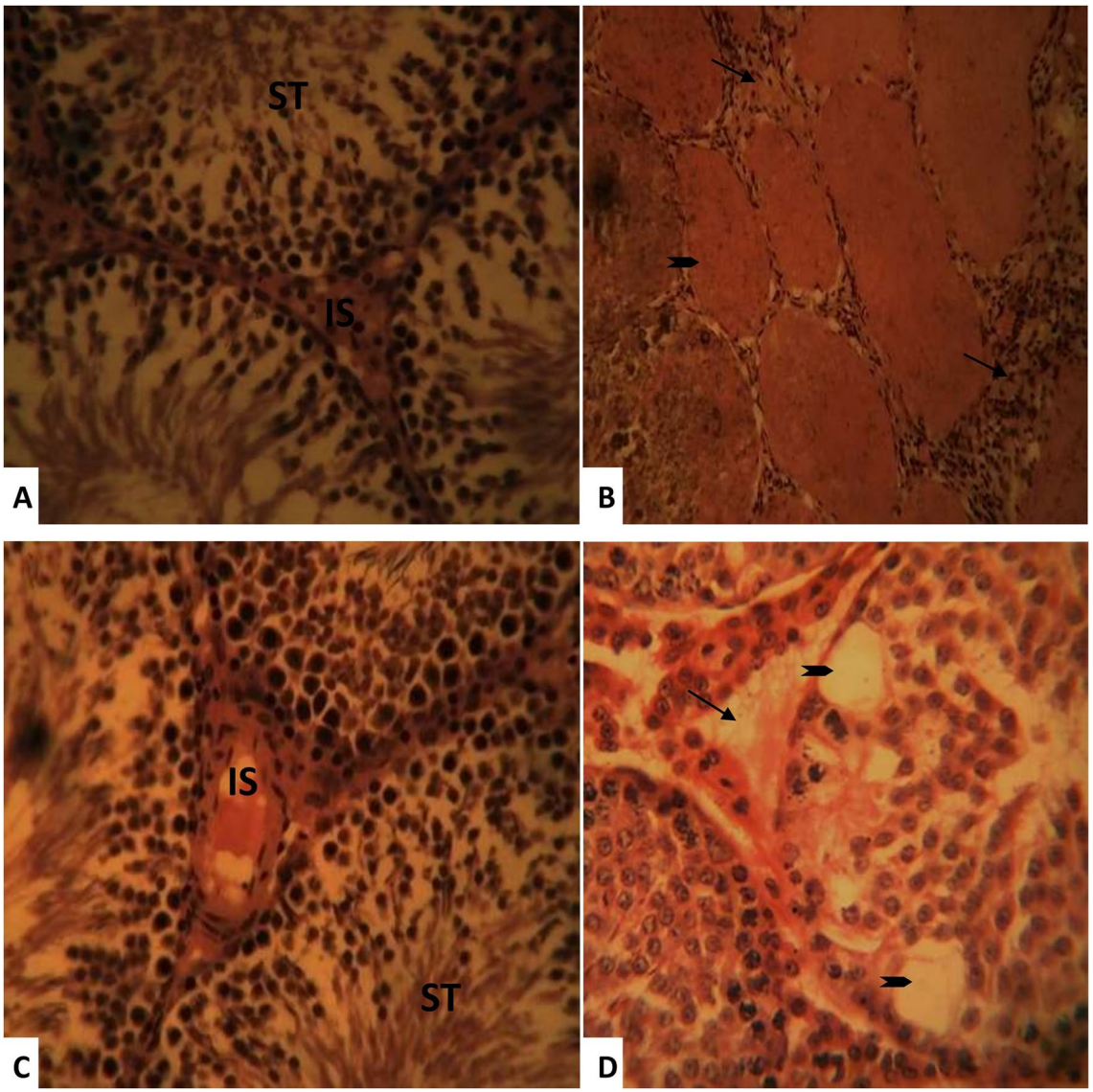

Fig. 2 Photomicrograph of the testis from Group A (control) rats showing normal seminiferous tubules (ST) and interstitial space (IS). Group B (3 mg/kg $CdCl_2$) rats showing severe necrosis of seminiferous tubules (arrowheads) and interstitial inflammation (thin arrows); Group C (500 mg/kg TVE) rats showing normal seminiferous tubules (ST) and interstitial space (IS) and Group D (3 mg/kg $CdCl_2$ + 500 mg/kg TVE) rats showing a preserved testicular histoarchitecture with mild vacuolation and necrosis of seminiferous tubules (arrowheads) and interstitial oedema (thin arrows). H & E, 400 ×

aforementioned studies. This may further account for the observed low potency of TVE on cadmium-induced testicular injury.

Conclusion

This study shows that *Thymus vulgaris* leaf extract has weak ameliorative effect on cadmium- induced testicular damage in rats and also high antioxidant activity.

Abbreviations

ARRIVE: Animal Research Reporting of In Vivo Experiments; TVE: *Thymus vulgaris* Extract; Cd: Cadmium; SC: Subcutaneous; PBS: Phosphate buffered saline; $CdCl_2$: Cadmium chloride; LD50: Lethal dose 50; LH: Luteinizing hormone; FSH: Follicle stimulating hormone; MDA: Malondialdehyde; CAT: Catalase; SOD: Superoxide dismutase; BW: Body weight.

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Licence/permission

Not applicable.

Authors' contributions
RIO and CUC were involved in research conceptualization, supervision and writing of the original draft. WO was involved in research conceptualization, interpretation of toxicological data and proof reading of the manuscript. EUU, DCA and BIE were involved in data collection and analysis. All authors read and approved the final manuscript.

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Availability of data and materials
All data analysed and generated in this study are mentioned in this article.

Declarations

Ethics approval and consent to participate
This study was approved by the Faculty of Veterinary Medicine Institutional Animal Care and Use Committee University of Nigeria, Nsukka (FVM2020117) in compliance with ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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