GONADOPROTECTIVE EFFECT OF ELLAGIC ACID IN CADMIUM-INDUCED TESTICULAR TOXICITY IN WISTAR ALBINO RATS

Dissertation submitted in partial fulfilment of the requirements for the degree of Master of Philosophy in Zoology

By

Bidanchi R. Momin

Registration No: MZU/M.Phil/394 of 26.05.2017

Under the Supervision of

Prof. G. Gurusubramanian

Department of Zoology

Department of Zoology
School of Life Sciences
Mizoram University
Aizawl-796004, Mizoram

2017

MIZORAM UNIVERSITY
DEPARTMENT OF ZOOLOGY

Post Box No.:190 **Gram: MZU Aizawl – 796004**
Prof. G. Gurusubramanian **Mobile: 9862399411**
Head **email: gurus64@yahoo.com**

CERTIFICATE

This is to certify that ***Gonadoprotective Effect of Ellagic Acid in Cadmium-Induced Testicular Toxicity in Wistar albino Rats*** written by **Bidanchi R. Momin bearing Reg. No.: MZU/M. Phil/394 of dt. 26.05.2017** has been written under my supervision.

She has fulfilled all the required norms laid down within the M. Phil. Regulations of Mizoram University. The dissertation is a result of her owned investigation. Neither the dissertation as a whole nor any part of it was submitted by any other University for any research degree.

(Prof. G. Gurusubramanian)

Supervisor and HOD

Department of Zoology

Mizoram University

Mizoram University

Aizawl, Mizoram

20th November, 2017

DECLARATION

I, Ms. **Bidanchi R. Momin**, bearing **Reg. No.: MZU/M. Phil/394 of dt. 26.05.2017**, hereby declare that the subject matter of this dissertation ***Gonadoprotective Effect of Ellagic Acid in Cadmium-Induced Testicular Toxicity in Wistar albino Rats*** is the record of work done by me, that the contents of this dissertation did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the dissertation has not been submitted by me for any other University or Institute.

This is being submitted to Mizoram University for the **Degree of Master of Philosophy in Zoology**.

Bidanchi R. Momin

(Candidate)

(Prof. G. Gurusubramanian)

(Head of the Department)

(Prof. G. Gurusubramanian)

(Supervisor)

ACKNOWLEDGEMENT

It is my great privilege and opportunity to thank all those who have inspired and helped me a lot during my course of dissertation/project work.

First and foremost, I thank the **Almighty God** for His abundance of blessings undeservingly bestowed upon me and for granting me with wisdom, knowledge, understanding and good health to undertake this project/dissertation without which it would have been impossible to complete it successfully.

It is indeed a genuine pleasure to express my debt of gratitude to my supervisor and guide, **Prof. G. Gurusubramanian**, Head of the Department of Zoology, Mizoram University, Aizawl, who gave me such an incredible and tremendous opportunity to do this astounding dissertation on the topic **"Protective effect of *Ellagic acid* against cadmium chloride-induced testicular toxicity in rats: A behavioural, biochemical and molecular approach"**. This has helped me a lot in learning and accomplishing a better quality in me especially for my future research purpose for which I am remarkably thankful to him. His worthwhile guidance, encouragement, support, timely help and supervision has helped me to complete this project in time.

I am also obliged to acknowledge my co-guide **Dr. Vikas Kumar Roy,** Assistant Professor, Department of Zoology, Mizoram University, for assisting and guiding me with my works. Also, I would like to thank **Prof. Ganesh Chandra Jagetia**, Professor of the Department of Zoology, Mizoram University, Aizawl, for allowing me to use his fluorescent microscope for my work.

I am immensely grateful to all my lab mates especially **Sanasam Sanjeev** and **Meesala Krishna Murthy** for their endless co-operations and suggestions during the course of this project and for willingly extending their helping hands despite their busy schedules.

Also, I would like to acknowledge the **D.B.T., Ministry of Science and Technology, Mizoram University, Mizoram** for rendering me the traineeship through 'DBT Twinning Programme for the NE and Bioinformatics Infrastructure Facility, Mizoram University for four months and helping me manage my modest requirements.

Last in sequence but not least in importance, I want to thank my parents, family members and friends for their aspiring, sensational and phenomenal support both morally and physically which they have provided me during my course of project work without which it would have been impossible to complete this project within a stipulated time.

Bidanchi R. Momin

Place: Mizoram University

Zoology Department

Date:

Mizoram University

Ex/MP/ZOOL

No. 10

PASSED

MIZORAM UNIVERSITY
AIZAWL

School of Life Sciences | **Department of Zoology**

Master of Philosophy First Semester Award-Sheet

The following is the assessment record of ***Bidanchi R. Momin,*** *Roll No.* ***Zoo/M. Phil /16/10,*** *Registration No.* ***7120 of 2014*** *in the M. Phil. First Semester Examination held in December,* ***2016***

SEMESTER EXAMINATION						Total Grade Point	Grade Point Average	Grade
LS-601		LS-602		LSZ-603(C)				
Research Methodology		Instrumentation: Tools and Techniques		Entomology and Toxicology				
Grade Point	Grade	Grade Point	Grade	Grade Point	Grade			
7.7	'O'	8.1	'O'	7.7	'O'	23.5	7.83	'O'

First Tabulator | Second Tabulator | Controller of Examinations

Date: 27 MAR 2017

EVALUATION : *The following indicates the corresponding grades of grade points :*

'O'	'A'	'B'	'C'
7.00-10.00	6.00-6.99	5.00-5.99	Below 5.00

The performance of the scholar shall be evaluated in the following grades : ***'O'*** *– Outstanding,* ***'A'*** *and* ***'B'*** *with grade point valuation in the 10 point scale, i.e., 7.00-10.00 corresponding to* ***'O'*** *grade, 6.00-6.99 corresponding to* ***'A'*** *grade, 5.00-5.99 corresponding to* ***'B'*** *grade. Those securing less than 5.00 points shall be graded as* ***'C'****. A scholar will be eligible for the award of M. Phil Degree if he/she secures at least a 'B' grade in each course.*

URKUND

Urkund Analysis Result

Analysed Document:	final thesis - Copy.docx (D33780895)
Submitted:	12/14/2017 12:41:00 PM
Submitted By:	MZUT207@mzu.edu.in
Significance:	6 %

Sources included in the report:

1220030021-S.pdf (D15892448)
Rahash-Ahmed-Reproductive Dysfunction-BioChemicla alterations in male rat.doc (D15042889)
Tuhina Das Thesis Physiology 250717.pdf (D29761384)
Bhabani Prasad Pakhira PhD BMLSM 201117.pdf (D33205286)

Instances where selected sources appear:

13

Table of Contents Page No.

LIST OF TABLES

LIST OF FIGURES

GONADOPROTECTIVE EFFECT OF ELLAGIC ACID IN CADMIUM-INDUCED TESTICULAR TOXICITY IN WISTAR ALBINO RATS

Dissertation submitted in partial fulfilment of the requirements for the degree of Master of Philosophy in Zoology

By

Bidanchi R. Momin

Registration No: MZU/M.Phil/394 of 26.05.2017

Under the Supervision of

Prof. G. Gurusubramanian

Department of Zoology

Department of Zoology
School of Life Sciences
Mizoram University
Aizawl-796004, Mizoram

2017

I. INTRODUCTION

Impotency in men is one of the most extensive disputed areas in association to medicine (Jamsai et al., 2011).Various determinants are accounted for such botheration viz., natural origin (toxic pollutants) or acquired (food and drugs). Cadmium (Cd) has been considered as one of the most noxious pollutants in the biosphere (Goyer et al., 2001). Exposure of Cd due to numerous industrial and environmental pollution effects has lead to a tremendous health issues conceiving a broad series of biochemical and physiological disintegration in humans and laboratory animals (Santos et al., 2004).

Cd can accumulate in the body for up to 40 years, thereby, causing dangerous human health complications in a comprehensive way (Wang et al., 2016). Cd causes reproductive competence by enhancing testicular dysfunction, malicious testicular deterioration, necrosis, seminiferous tubular damage and condensed androgen secretion in rats (Xu et al., 2005; Yari et al., 2010). The risk of Cd decay augmented due to its long biological half-life (17–30 years), ensuing the accumulation and relentless injurious possessions (Shukla et al., 2009).

Certainly, the International Agency for Research on Cancer has classified Cd as one of the known human mutagen in 1993 (IARC, 1993) and it has been ranked the 7th toxicant in the Priority List of Hazardous Substances of the Agency for Toxic Substances and Disease Registry (ATSDR, 2007). Experimentally, it has been identified that cadmium is detrimental to both the reproductive and immune systems furthermore, to its nephrotoxicity and hepatotoxicity (Ogawa et al., 2013). Also, recent studies have confirmed that acute $CdCl_2$ exposure causes a significant reproductive injury in male rats through diverse assets (Oguzturk et al., 2012).

1.1.CADMIUM TOXICITY IN TESTIS

Testicular injury is among the lethal effects of $CdCl_2$ exposure causing alarming ramifications viz., blood–testis barrier disruption, germ cell loss, testicular oedema, haemorrhage, necrosis, infertility and sterility in mammalians such as rodents (Deng et al., 2010). It has also been thought that the development of testicular tumors in rats is related to the chronic degenerative effects of cadmium in this tissue, resulting in loss of androgen production and a subsequent overstimulation of remnant testicular cells by the pituitary (Waalkes et al., 1997).

Cd negatively affects the reproductive functions and revelation to Cd toxicity has led to the impairment in testicular function, mechanisms of which causes damage to the vascular endothelium, leydig and sertoli cells, intercellular connections, the induction of oxidative

stress, impaired antioxidant defense mechanisms and the severity of the inflammatory response ultimately leading to a morphological and functional changes in inhibition of testosterone synthesis and spermatogenesis impairment (Goyer et al., 2004). Various demonstrations have been made that exposure to cadmium causes harmful effects in the testes and subsequent infertility in experimental animals (Fouad et al., 2015). Experimentally, it has been shown that disclosure to low dose of $CdCl_2$ induces no significant interruption in spermatogenesis, but, it does elevate the immunological microcircumstances in the testis, leading to increased susceptibility in testicular autoimmunity (Ogawa et al., 2013). Previously, some studies have shown that Cd causes infertility by impairment in spermatogenesis (Yari et al., 2016), seminiferous tubular atrophy, decreased pipe diameter, spermatogonial stem cells falling off the inner lining and reduced germ cell layers of disorderly arrangements in cadmium-treated rats hematoxylin and eosin stained testicular sections (Wang et al., 2016). Also, recently it has been proved that successful spermatogenesis and the attainment of optimal sperm function requires protection from increased levels of oxidative stress (Correia et al., 2017).

1.2.CADMIUM AND OXIDATIVE STRESS

Oxidative stress manifest in the aetiology of many diseases and male sterility is not an exception. Oxidative stress is one of the major threats for inducing germ cell apoptosis (Tremellen, 2008) and it is also the major harmful factors responsible for male reproductive function causing male sterility because of its harmful effects on the developing germ cells and sperm function [fig.1] (Aitken et al., 2011) . Reactive oxygen species (ROS) play a major role in spermatogenesis and reproduction. Conversely, firm physiological conditions which may be induced by inflammation, obesity or toxins aggravate the production of these species culminating in sperm DNA damage (Malik et al., 2017). The specific role of ROS in the activation of signal transduction pathways involved in defence mechanisms during Cadmium stress, still needs to be clarified. Therefore, in order to counteract the damaging ROS, aerobic cells are provided with extensive antioxidant defence mechanisms consisting mainly of antioxidant enzymes and small molecule antioxidant (Khojastehfar et al., 2014).

The oxidative stress that arises in cells exposed to cadmium weakens their antioxidant defense mechanisms, significantly increasing the lipid peroxidation level while decreasing the glutathione-SH-related proteins and changes antioxidant enzyme activity, activates proto-oncogenes leading to excessive production of protein products that stimulate cell proliferation in testis of Cd-treated rats. Experimentally, it has also been proved that Cd-treated rats had

significantly reduced sperm count, motility, sialic acid, luteinising hormone and testosterone relative to controls (Adaramoye et al., 2016). However, successful spermatogenesis and the achievement of optimal sperm function require protection from increased levels of oxidative stress (Correia et al., 2017). Some studies have also proved that oxidative stress also induces apoptosis in vitro (Angenard et al., 2010) and in vivo. Furthermore, Cd has been revealed to increase the expression of the proapoptotic member B cell- lymphoma (Bcl)-2-associated X protein (Bax) and of caspase- 3 and to reduce expression of the antiapoptotic Bcl-xL (Bu et al., 2011). Recently, it has also been shown that the immunohistochemical studies of the Cd-induced testicular tissue possessed positive immunostaining for the increased level of TNF-∝, but decreased number of proliferating cell nuclear antigen (PCNA) stained cells (Mohammed et al., 2017). The increased production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) allows the synthesis of cholesterol by activating the enzymes of cholesterol biosynthesis, including cholesterol ester hydrolase. Furthermore, LH and FSH production regulates the uptake of cholesterol esters (Alkhedaide et al., 2016).

Numerous approaches have been made therapeutically to tackle the toxic effects of Cd. Some of the plant species such as Moringa oleifera leaf extract (Mallya et al., 2017), Physalis peruviana L. (Mohamed et al., 2014) and antioxidants such as kolaviron and quercetin (Farombi et al., 2012) were found to be effective in reducing Cd-induced tissue damages, including the testes by rejuvenating spermatogenesis.

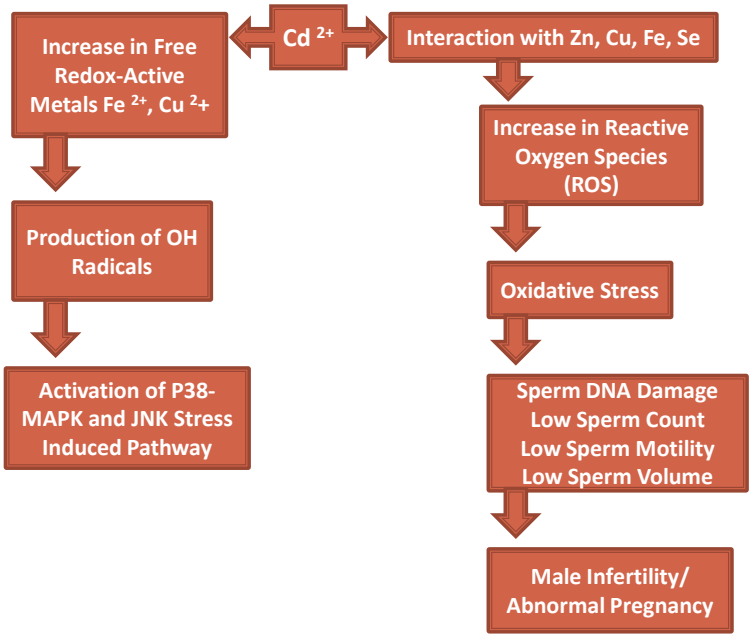


Fig. 1: The consequence and mechanism of induction of oxidative stress in male infertility.

1.3.Ellagic Acid a flavonoid

Ellagic acid, a member of flavonoids (EA; $C_{14}H_6O_8$; MW: 302.202; 3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1] benzopyran-5,10-dione) found in various berries and plants (Wang et al., 2017) has been in identification mostly due to its potential antioxidant activity (Syed et al., 2017), radical scavenging capacity, chemopreventive and antiapoptotic (Ceribasi et al., 2010; Turk et al., 2010) properties. It is believed that EA functions contradictory to the negative effects of oxidative stress by directly acting as an antioxidant or by activating／inducing cellular antioxidant enzyme systems (Atessahin et al., 2010). The no-observed-effect level (NOEL) is estimated to be 5% (3011 mg/kg b.w./day) for males and the no-observed-adverse-effect level (NOAEL) and NOEL in females will be estimated to be 5% (3254 mg/kg b.w./day) and <1.25% (778 mg/kg b.w./day), respectively (Tasaki et al., 2008).

1.4. Ellagic Acid (EA) an antiproliferating phytochemical

It is known that EA has been one of the most intoxicating flavonoid that is in wide use across countries. Many flourishing studies have been conducted to prove its potentiality in improving the grievances of some inefficient phytochemicals (Zhao et al., 2013; Farbood et al., 2015; Yousef et al., 2016). Investigations on the influential effect as an antiproliferating agent also has been made which elucidates that EA is a promising therapeutic to improve the cell proliferation by down-regulating the expression of PCNA (Syed et al., 2014; Syed et al., 2015; Wang et al., 2016; Cheng et al., 2017).

On the other hand, some studies are believed to have failed the antioxidant therapy in limiting cadmium induced testicular damage (Alkhedaide et al., 2016). Hence, the present study was designed to investigate the protective role of EA in cadmium induced testicular toxicity. However, to date, there is no available information on the impact of EA on testicular toxicity after Cd exposure in rats. Therefore, the aim of the present study was to investigate whether ellagic acid (EA) has protective effect on cadmium chloride ($CdCl_2$)-induced testicular and spermatozoal toxicity associated with the oxidative stress.

II. REVIEW OF LITERATURE

2.1. The Anticarcinogenic Influences Of Ellagic Acid On Cadmium-Induced Testis Injury

Cadmium is a commonly used chemical in agricultural and industrial operations and is released into the environment causing atmosphere, soil, and water pollution. It can accumulate in the body for up to 40 years, thereby, affecting the human health over an extensive period of time (Wang et al., 2016). There has been a rush in Cd pollution of the environment partially due to urbanization, eventually leading to the increase in Cd-related diseases because of the disclosure in human beings (Adaramoye et al., 2016). Phenolic phytochemicals such as ellagic acid (EA) are important components of fruits and vegetables and are partly conscientious for their beneficial health effects against oxidation-linked chronic diseases such as cancer and cardiovascular diseases (Atessahin et al., 2010). Ellagic acid (EA) has received particular attention because of its wide array of biological properties (Tasaki et al., 2008). Previous studies observed that EA significantly improved the damages in sperm parameters, oxidant/antioxidant balance and testicular apoptosis induced by chemotherapeutics such as cisplatin (Turk et al., 2008) and cyclophosphamide (Turk et al., 2010a).

Experimentally, it is known that cadmium is toxic to both the reproductive and immune systems, in addition to its nephrotoxicity and hepatotoxicity (Ogawa et al., 2013). Some investigations have shown that Cd can provoke oxidative stress and apoptosis of germ cells (Khojastehfar et al., 2014). It is determined that acute $CdCl_2$ exposure causes a significant reproductive damage via increased oxidative stress (increased TBARS levels and decreased SOD, CAT, GPx and GSH levels), histological alterations (necrosis, oedema etc.) and spermatological damage (decreased sperm motility and sperm concentration and increased abnormal sperm rate) in male rats (Oguzturk et al., 2012) . Several agents have been reported to mitigate Cd-induced toxicity in testis (Jahan et al., 2014). The formation of multinucleated giant cells in Cd-intoxicated rats suggests continuous deterioration of spermatogenic epithelium and appears to represent a distracted reaction to injury (Ponnusamy and Pari, 2011). Also, exposure to low dose of $CdCl_2$ induces no significant disturbance in spermatogenesis, but, it does change the immunological microcircumstances in the testis, resulting in increased susceptibility to testicular autoimmunity (Ogawa et al., 2013). So, in order to neutralize the damaging ROS, aerobic cells are provided with extensive antioxidant defence mechanisms consisting mainly of antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidase etc.) and small molecule antioxidant (e.g., glutathione, N-acetyl-

cysteine, vitamin E and vitamin C). In view of the above-mentioned, the expedition for safer and active agents that can alleviate cadmium-induced pathologies is required.

2.2. Antigenic Influences Of Cadmium And Ellagic Acid

Bax is a proapoptotic marker, which is increased in the early stages of intoxication and disease. Conversely, Ki-67 expression is a marker for cell proliferation. High expression of Ki-67 has been reported in cells during G2 and early M stages of cell growth. The resultant effect of the study confirmed that Bax marker was increased in the spermatogenic cells of the $CdCl_2$-treated group, while Ki67 was decreased (Alkhedaide et al., 2016). Experimental studies have also shown that, $CdCl_2$ intoxication increases the expression of pro-apoptotic proteins p53 and Bax, while reducing the expression of Bcl-2, an anti-apoptotic protein. CdCl2 induced concomitant increases in expression of P53 and Bax but led to decreased expression of Bcl-2, suggesting their important roles in testis damage, apoptotic cell death and decreased semen parameters elicited by $CdCl_2$ (Eleawa et al., 2014). The expression of PCNA, a nuclear protein mostly involved in the replication and in the DNA repair machinery was found to be associated with the cell proliferation process (Shivaji et al., 1992). Exposure of immunostaining in PCNA in the testis was considered as a proliferative marker for the efficiency of spermatogenesis, thereby, initiating the study of PCNA to quantitatively analyze the spermatogenesis status (Agarwal et al., 2003).

Hence, in the light of prospective antioxidant activity of flavonoids, we hypothesized that prophylactic treatment of EA may have protective effects against $CdCl_2$ induced on testicular toxicity by interfering with pathogenetic pathway and oxidative processes. Therefore the present study will be undertaken to investigate the protective role of EA against $CdCl_2$ induced deteriorated epididymal sperm characteristics, damaged oxidant/antioxidant balance and testicular apoptosis in rats.

III. OBJECTIVES

To evaluate the protective effect of ellagic acid against cadmium - induced testicular toxicity in rats with respect to:-

- Oxidative stress (MDA) and antioxidant enzymes (SOD, GST, GSH) concentrations.
- Sperm quality and serum hormone (LH, FSH, estradiol and testosterone) analyses.
- Histopathological analysis and DNA damage detection.
- Assessment of modulation of steroidogenic, apoptosis, cell proliferation and cytoprotection activities by immunohistochemical, Western blotting and RT-PCR.

IV. MATERIALS AND METHODS

4.1. CHEMICALS AND DRUGS

Cadmium Chloride (GLS pharma Ltd., Hyderabad) was purchased from a local drug store, whereas Ellagic acid >98.0% purity was obtained from Tokyo Chemical Industry (TCI) Co., Ltd., Ethanol, disodium phosphate (Na_2HPO_4), Hydrogen peroxide (H_2O_2), dipotassium hydrogen phosphate (K_2HPO_4), Potassium di-hydrogen phosphate (KH_2PO_4) and carboxymethyl cellulose were procured from MERC, India Ltd., Mumbai, whereas 1-chloro-2,4 dinitrobenzene (CDNB), phenazinemethosulfate, nitrobluetetrazolium, sodium pyruvate, cumenehydroperoxide, thiobarbituric acid, -5'-dithiobis [2-nitrobenzoic acid](DTNB), 5-thionitrobenzoic acid, tert-butyl-hydroperoxide and nicotinamide adenine dinucleotide (NADH) were supplied by the Sigma Aldrich Chemical Company, Kolkata, India, etc.

4.2. Glasswares, Equipment, Other Materials Required

The following glasswares listed below were used during experimentation were test tubes, conical flasks, beakers, centrifuge and micro-centrifuge tubes, petridishes, measuring cylinders, pipette tips etc. The following equipments were used through the study: Spectrophotometer, Electronic balance, Micropipettes, Centrifuge, Vortex machine, Homogenizer, Water bath, Refrigerators, Flat spin, Thermometer, etc. Other materials required are scissors, dissecting box and tray, scalpel, forceps, syringes, cotton etc.

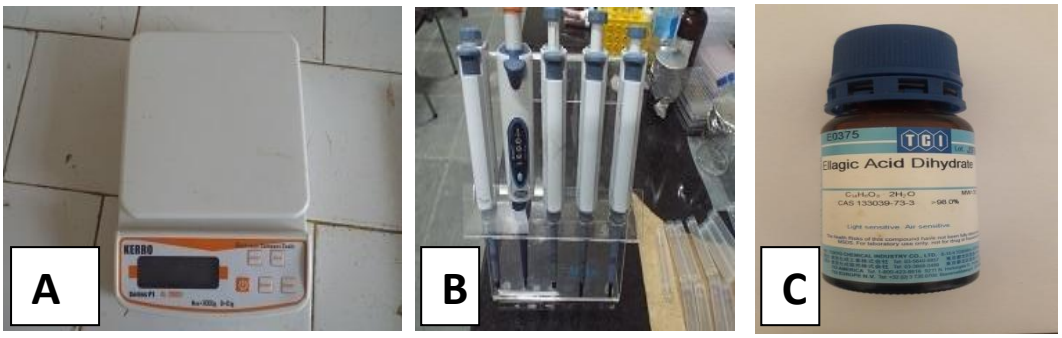


Fig.2: (A) Electronic weighing balance, (B) Micropipettes and (C) Ellagic Acid drug.

4.3. Animals and experimental design

The animal care and handling was done according to the guidelines approved by the Animal Ethics Committee of Mizoram University, Aizawl, India (MZUIAEC 17-18/1). Usually, three months old male Wistar rats (150 - 180 g) was procured and maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and 12 h of light and dark cycle,

respectively. The animals had free access to the standard food and water. The animals were divided into four groups where six animals each were housed in a polypropylene cage containing wood powder (procured locally) as bedding throughout the experiment:

Group I: Control where the animals were given any treatment.

Group II: Cadmium Chloride alone (5 mg/kg b.wt of cadmium chloride in distilled water for 30 and 60 consecutive days) (Alkhedaide et al., 2016).

Group III: Ellagic acid alone (2 mg/kg b.wt. of ellagic acid in alkaline solution) (Atessahin et al., 2010).

Group IV: $CdCl_2$+Ellagic acid (5 mg/kg b.wt. of cadmium first and then 2 mg/kg b.wt. of ellagic acid administration).

The animals from all groups were sacrificed after 60 days $CdCl_2$ and ellagic acid administration.

4.4. Water consumption

Preweighed food was provided in standard stainless steel hoppers. The amount of food remaining, including any on the bottom of the cages was recorded. Intake was calculated as the weight (in grams) of food provided less that recovered for working weeks. Water consumed was also monitored twice a week during the baseline and during working weeks by measuring the initial and final volume of water.

4.5. Body weight Gain/decrease of animals

The animals were weighed twice a week and changes in weight of rats were noted individually, to note the differences in between the control and experimental samples.

The organs weight: On the day after the end of treatment, animals were weighed and sacrificed by decapitation. The liver, right kidney, right testis, epididymis, vas deferens, ventral prostate, and seminal vesicle (without the coagulating gland, full and empty of secretion) were removed and their weights were determined.

4.6. Organ weight Index

Relative organs weight was calculated as:

Organ Weight/Body Weight×100

4.7. Rectal temperature

The effect of the treatment doses of all groups on rectal temperature were assessed twice a week throughout the study. Rectal temperatures were measured using a digital thermometer with beeper, inserted 1 cm into the rectum and the temperature is allowed to stabilise for 1 min approximately.

4.8. Experiment 1: Biochemical analysis of Oxidative stress and Enzymatic Antioxidant status.

4.8.1. Tissue samples

After blood collection, all rats were rapidly sacrificed and the liver and kidney were immediately excised, weighed and blotted dry. A known weight of each of liver and kidney were stored at -20^{o}C for biochemical analysis. The specimens of each organ (kidney, liver and testis) were homogenized with tissue homogenizer individually to make 10% of lipid peroxidation, superoxide dismutase (SOD) activity and reduced glutathione (GSH). The homogenates were prepared for analysis by centrifugation at 18000 rpm (4^{o}C) for 30 minutes and the supernatant was kept for biochemical analysis.

4.8.2. Lipid Peroxidation (LOO)

The lipid peroxidation assay is based upon the formation of a red adduct (absorption maximum 532 nm) between TBA and malondialdehyde (MDA), a colourless end product of lipid peroxide decomposition (Janero, 1990). Lipid peroxidation was measured according to standard protocol (Satoh et al., 1978). The tissue homogenate was precipitated by the addition of trichloroacetic acid (15%) followed by the addition of 0.5 ml of thiobarbituric acid (0.8%). The mixture was incubated at 95°C for 25 minutes. The mixture was allowed to cool to room temperature and centrifuged at 8000 rpm. The supernatant was collected and the absorbance was read at 540 *nm* against the blank in a UV-VIS spectrophotometer. The concentration of thiobarbituric acid reactive substance was read from the standard calibration curve, which

was plotted using 1, 1, 3,3tetraethoxypropane. The lipid peroxidation is represented as nM TBARS/mg protein.

4.8.3. Glutathione (GSH)

The general thiol reagent, 5-5'-dithiobis DTNB (Ellman's Reagent) reacts with GSH to form the chromophore, 5-thionitrobenzoic acid (TNB) and GS-TNB which can be detected at 412 nm. The GS-TNB is subsequently reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TNB molecule and recycling the GSH; thus amplifying the response. Any oxidized GSH (GSSG) initially present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly reduced to GSH.

The glutathione (GSH) contents were determined by the method of Moron et al., (1979). Briefly, the cell homogenate was mixed with 25% TCA to precipitate proteins, centrifuged and the supernatant was collected. The supernatant was mixed with 0.2M sodium phosphate buffer (pH 8) and 0.06 mM DTNB. The solution was incubated for 10 minutes at room temperature. The absorbance of the samples was read against the blank at 412 nm with a double beam UV-VIS spectrophotometer and the GSH concentration was calculated from the standard curve.

4.8.4. Glutathione-S-Transferase (GST)

Glutathione-S-transferase catalyses the reaction of CDNB with the –SH group of glutathione. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance at 340 nm. The rate of increase is directly proportional to the GST activity of the sample.

Glutathione-S-transferase (GST) was estimated by the method of Habig and Pabst (1974). Briefly, the liver homogenate was mixed with 0.1 M potassium phosphate buffer, CDNB and 10 mM GSH, and incubated for 10 minute at 37□C. The absorbance was read against the blank at 340 nm using a double beam UV-VIS spectrophotometer.

4.8.5. Superoxide Dismutase (SOD)

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazan. The colour formed at the end of the reaction can be extracted into butanol and measured at 560 nm.

Briefly, the cell homogenate was mixed with reagent consisting of phenazene-methosulphate, nitroblue tetrazolium and NADH, and incubated for 90 seconds at 30°C and the reaction was stopped by adding acetic acid and n-butanol. Blank was prepared by adding the reagent without the sample and incubated for 90 seconds at 30°C and the reaction was stopped adding acetic acid and n-butanol. The absorbance of sample was measured against the blank at 560 nm in a UV-VIS spectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme and the sample as follows:

Activity= Sample – Blank/Sample X 100

4.9. Experiment 2: Sperm Anaysis and Serum Hormone Assay.

4.9.1. Sperm analysis

4.9.1a. Sperm count and motility assay

The epididymal sperm suspension was prepared in 1 ml of phosphate buffered saline (PBS) at pH 7.2. An aliquot from the suspension (1 ml) was diluted in a ratio of 1:40 with PBS. A sample of the diluted suspension was charged into a hemocytometer. The total sperm count in eight squares (except the central erythrocyte area) of one mm^2 each was determined and multiplied by 5×10^4 to get the total count (Bairy et al., 2010). Then the same eight squares were also examined for motile sperms and the percentage of motile sperms were recorded (Narayana 2008).

4.9.1b. **Determination of daily sperm production and testicular sperm number**

To evaluate the daily sperm production in testes, spermatozoa were counted as per the standard protocol of Robb et al. (1978). Briefly, after the testes have being removed and weighed, they were homogenised for 3 min in 25 ml of physiological saline containing 0.05% (v/v) Triton X-100. Then, 5.5 µl of sample aliquots was placed on the haemocytometer and counted twice at 100X magnification microscope to determine the average number of spermatids per sample. These values were used to obtain the total number of spermatids per testis, and this number was then divided by the testes weight to give spermatids per gram of testes. Developing spermatids spend 4.61 days in rats. Thus, the values for the number of spermatids per testis were divided by 4.61 to obtain daily sperm production. Motile sperm cells were counted and the percentage was calculated.

4.9.1c. Sperm Abnormality

The technique of Wyrobek et al. (1984) was adopted for sperm abnormality. To evaluate the sperm abnormalities, the sperm suspension was stained with eosin, dropped on slides to determine the motility, abnormality (using Olympus microscope by objective (40×). Abnormality was classified in head and tail. Abnormal sperm cells were counted and the percentage was calculated. To assess motility, the sperms were classified as motile sperm (M) and non-motile (NM).

4.9.2. ELISA for Serum LH, FSH and testosterone

Follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were studied in serum by ELISA. The samples were analyzed in duplicate and the manufacturers' protocols were carefully followed. The mean absorbances were obtained and correlated with those from standard curves. The data were expressed in IU/L for FSH and LH and nmol/L for testosterone.

4.10. Experiment 3: Histological and Sperm DNA damage analysis.

4.10.1. Histological studies

The testes tissues were removed and fixed in bouin's fluid. The tissues were then dehydrated in different grades of alcohol, embedded in paraffin wax. Sections (5 μM) were then cut using a microtome and processed for general histological staining using hematoxylin and eosin stain (Sigma-Aldrich), based on previously stated protocols (Bancroft and Gamble, 2002). The sections were examined under a light microscope. All tubular sections in one section of the testicular biopsy are evaluated systematically and each given a score from 1-10 according to the listed criteria (Johnsen, 1969).

4.10.2. Morphometric evaluation of seminiferous tubules

From each section, seminiferous tubule diameter and area (essentially from circular tubular cross sections) was determined using pre-calibrated measuring eyepiece. The sections were then examined at X400 and X1000 magnifications and measurements were made using the software (NIS-Elements, Basic Research, Tokyo, Japan). About 20 sections of seminiferous tubules that were round or nearly round were chosen randomly and measured for each group. The tubular diameter was measure at X400 magnification. The diameter of

the seminiferous tubule was measured across the minor and maor axes and the mean diameter was obtained.

4.10.3. DNA damage detection- Acridine orange stain

Sperms with normal DNA fluorescence inhibit green colour and those with abnormal DNA fluorescence red or yellow. It is important to score DNA normality immediately after staining (Tejeda et al., 1984).

4.11. Experiment 4: Immunohistochemical and Western blot analysis.

4.11.1 Immunohistochemistry

For immunohistochemistry detection, testes sections (5 μm) were deparaffinized in xylene and dehydrated in various ascending concentrations of ethanol. The endogenous peroxidase activity was blocked with 3%/ 0.03% H_2O_2 in absolute methanol for 15 min. Blocking of non-specific binding will be conducted using normal horse serum (Sigma-Aldrich) for 20 min at room temperature. Subsequently, the testicular sections were incubated with the following primary antibodies overnight: 1) Anti-B-cell lymphoma 2-associated X protein (**Bax**), 2) Anti-**Bcl-2** (B-cell lymphoma 2) and 3) anti-**PCNA** at dilutions of 1:1000 for each antibody in phosphate-buffered saline (PBS). After removal of the unbound primary antibodies by rinsing with PBS, slides were incubated with a specific dilution of biotinylated antigoat secondary antibody. Bound antibodies were detected with avidin-biotinylated peroxidase complex ABC-kit Vectastain, and the chromogen 3,3′-diaminobenzidine tetrachloride (DAB) was used as substrate. After appropriate washing in PBS, slides were counterstained with hematoxylin and images were viewed microscopically and captured. All sections were incubated under the same conditions with the same concentration of antibodies and at the same time; so, the immunostaining was comparable among the different experimental groups.

4.11.2. Western blotting

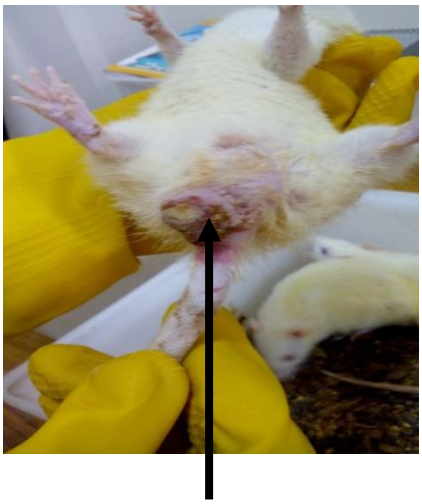
The protein extraction from the testis homogenate was conducted following the method of Cifuentes et al. (Cifuentes et al., 2005) and western blot analysis was performed as previously described (Roy VK., 2011). In brief, a 20% homogenate (w/v) of adipose tissue was made in suspension buffer containing 0.1 mol l^{-1} NaCl, 0.01 mol l^{-1} Tris-HCl (pH 7.6), 0.001 mol l^{-1} EDTA (pH 8.0) and 10 μg ml^{-1} phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 5000 ***g***, 4°C for 15 min; the supernatant was extracted with an

equal volume of chloroform and the aqueous phase was recovered. Equal amounts of proteins (40 μg) as determined by Folin's method were used for 10% SDS-PAGE, whereas a 15% gel was used for PCNA and Bcl2 western blotting. Thereafter, proteins were transferred electrophoretically to a PVDF membrane (Millipore India Pvt. Ltd, Bangalore, Karnataka, India) overnight at 4°C. Membranes were blocked for 60 min with Tris-buffered saline [TBS; Tris 50 mmol l^{-1} (pH 7.5), NaCl 150 mmol l^{-1}, 0.02% Tween 20] containing 5% fat-free dry milk. The membranes were further incubated with rabbit anti-human insulin receptor-β antibody (at a dilution of 1:1000) and mouse monoclonal anti-human GLUT4 antibody (at a dilution of 1:4000), rabbit polyclonal GLUT8 (at a dilution 1:2000), rabbit anti-p44/42 MAPK (at a dilution of 1:1000) and rabbit polyclonal anti-human leptin for 60 min in blocking solution. Immunoreactive bands were revealed by incubating the membranes with biotinylated secondary antibody for 30 min followed by three washings with PBS (0.2 mol l^{-1}, pH 7.4) for 10 min each. After washing, the blots were incubated with avidin–peroxidase conjugate (at a dilution of 1:2000; Vector Laboratories) for 30 min. Finally, the blot was washed three times with PBS and developed with an enhanced chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA, USA). Similarly, a blot was developed for β-actin (Santa Cruz) at a dilution of 1:1000 as a loading control. Immunoreactive bands were later quantified using ImageJ software (Image J 1.36, NIH, Bethesda, MD, USA). Validation of GLUT4, GLUT8 and IR for use in bat tissue is described earlier (Srivastava, 2008) (Clark et al., 1994).

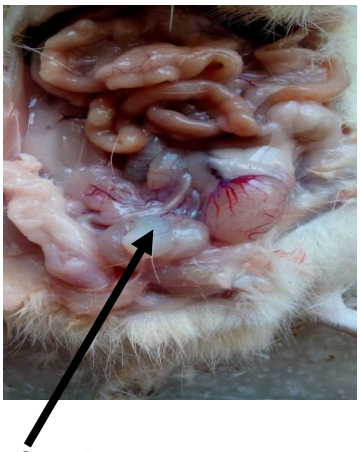
4.11. Statistical analysis

All values were presented as mean ± S.E.M. Differences were considered to be significant at $p < 0.05$. One-way ANOVA and post hoc Tukey-high significant differrence (HSD) test will be used to determine differences between groups. The SPSS / PC Program will be used for the statistical analysis.

V. RESULTS



Impaired Rectum



Disruption of Right Testis

Fig. 3: (A) Impairment of rectum after oral treatment of $CdCl_2$ and (B) After 60 days of $CdCl_2$ treatment it is observed that the left side of the testis was extremely disrupted.

The effect of drug on tissue was observed by the impairment in the rectum and the disruption of one testis as we can see in the Fig. 3 above. The results of all the sperm and biochemical parameters studied are represented as mean ± standard error (SEM). The bar graph of the sperm parameters (Fig. 5), hormone assay (Fig. and biochemical results are shown in the figures. Testicular histopathologic and immunohistochemical views and DNA damage of sperms are also shown in the figures.

5.1. Water Consumption

The treated groups did not show any significant changes in water retention capacity when compared to the control group.

Table 1: The initial and final volume of the water consumed (ml) by the Wistar albino rats during the treatment process.

Treatment groups	**Control**	**Cd**	**Cd + EA**	**EA**	**$F_{3,19}$**	**p value**
Initial (ml)	274.71 ± 23.39	245.33 ± 24.88	244.67 ± 22.78	265.33 ± 20.56	0.4240	NS
Final (ml)	238.23 ± 25.45	205.33 ± 21.64	192.00 ± 17.19	212.00 ± 23.55	0.7681	NS

Values are expressed as mean ± standard error mean (n = 5/treatment). The water consumption capacity was calculated as initial volume - final volume. NS - Not significant, Cd-Cadmium and EA-Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

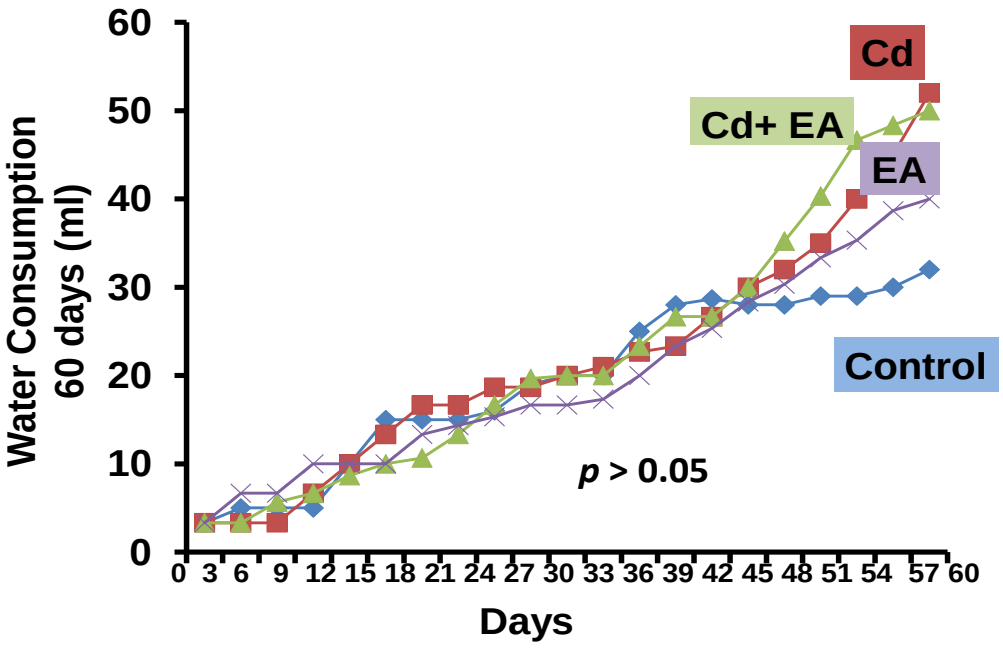


Fig. 4: Effect of treatment with EA on the water consumption of rats showing the lowest consumption of water per ml.

5.2. Body weight and organ weight

The oral treatment of rats with 5 mg/kg cadmium caused a decreased in body weight outgrowth and organ weights rate when compared to control. Table 2 shows the changes in body weights and organ weights after receiving different treatments. The body weight of rats in the cadmium treated group was significantly reduced as compared to the control group ($p<0.05$). Though not statistically significant, there was a dose dependent increase in the body weight of rats which received cadmium and ellagic acid at 5 and 2 mg/kg. The weight of the testes in the toxic group (cadmium, 5 mg/kg) was significantly reduced compared to the control group ($p<0.001$) and there was a significant increase in the weights of the testes in the groups received ellagic acid (2 mg/kg) as compared to the toxic group.

Table 2: Effects of EA treatment for 60 days on body weight (g), absolute (g) and relative (g/100 g) weights of reproductive organs of $CdCl_2$ induced testicular toxicity in male rats.

Parameters	**Experimental group**				**$F_{3,19}$ value**	***p* value**
	Control	**$CdCl_2$**	**$CdCl_2$+EA**	**EA**		
Initial body weight (g)	117.70 ± 2.35a	117.77 ± 0.57a	149.23 ± 1.11b	143.07 ± 3.19c	160.04	<0.0001
Final body weight (g)	167.10 ± 4.10a	110.00 ± 0.97b	180.73 ± 1.80c	171.57 ± 2.30a,c	156.56	<0.0001
Liver (g)	8.64 ± 0.10a	5.37 ± 0.21b	6.56 ± 0.21c	7.69 ± 0.38a	33.05	<0.0001
Liver (g/100 g)	5.18 ± 0.11a	4.89 ± 0.10a	3.62 ± 0.15b	4.49 ± 0.12a	30.53	<0.0001
Kidney (g)	0.88 ± 0.02a	0.49 ± 0.01b	0.52 ± 0.05c	0.64 ± 0.01d	38.94	<0.0001
Kidney (g/100g)	0.52 ± 0.01a	0.45 ± 0.02b	0.29 ± 0.01c	0.38 ± 0.01d	43.85	<0.0001
Testis (g)	0.99 ± 0.01a	0.90 ± 0.01b	0.93 ± 0.01b	0.98 ± 0.01a	13.75	<0.0001
Testis (g/100 g)	0.59 ± 0.04a	0.82 ± 0.02b	0.51 ± 0.01a	0.57 ± 0.07a	10.32	0.0005
Cauda epididymis (g)	0.24 ± 0.00a	0.14 ± 0.03b	0.11 ± 0.01b	0.17 ± 0.00b	12.20	0.0002
Cauda epididymis (g/100g)	0.14 ± 0.02a	0.12 ± 0.01a	0.07 ± 0.01b	0.09 ± 0.01a,b	5.81	0.0070
Caput epididymis (g)	0.19 ± 0.00a	0.17 ± 0.00b	0.13 ± 0.01c	0.21 ± 0.00a	44.33	<0.0001
Caput epididymis (g/100g)	0.11 ± 0.00a	0.16 ± 0.01b	0.08 ± 0.00c	0.12 ± 0.00d	2823.10	<0.0001
Prostate (g)	0.19 ± 0.01a	0.15 ± 0.00a	0.09 ± 0.00b	0.18 ± 0.02a	13.59	<0.0001
Prostate (g/100 g)	0.11 ± 0.01a	0.13 ± 0.00b	0.47 ± 0.00c	0.10 ± 0.00a	830.71	<0.0001
Seminal vesicle (g)	0.36 ± 0.00a	0.26 ± 0.00b	0.23 ± 0.01b	0.32 ± 0.00c	60.46	<0.0001
Seminal vesicle (g/100 g)	0.21 ± 0.01a	0.24 ± 0.00a	0.12 ± 0.00b	0.11 ± 0.00b	71.03	<0.0001
Vas deferens(g)	0.17 ± 0.00a	0.12 ± 0.01b	0.09 ± 0.00c	0.15 ± 0.00a	40.22	<0.0001
Vas deferens (g/100 g)	0.09± 0.00a	0.11 ± 0.00a	0.05 ± 0.01b	0.09 ± 0.00a	31.05	<0.0001

Values are expressed as mean ± standard error mean (n = 5/treatment). The relative organ weight was calculated as {[organ weight/body weight] × 100}. NS - Not significant, Cd- Cadmium, EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

5.3. Rectal Temperature

Elevation in Cadmium (Cd) treated group (101.90°F) was significantly observed due to the stress culminated by the Cd. A drop in the EA treated group by 93.47 °F was found which may be due to the radical scavenging capacity of the EA. A significantly slight elevation in the control group by 94.3°F was seen when compared to that of the co-treated group (94.20°F).

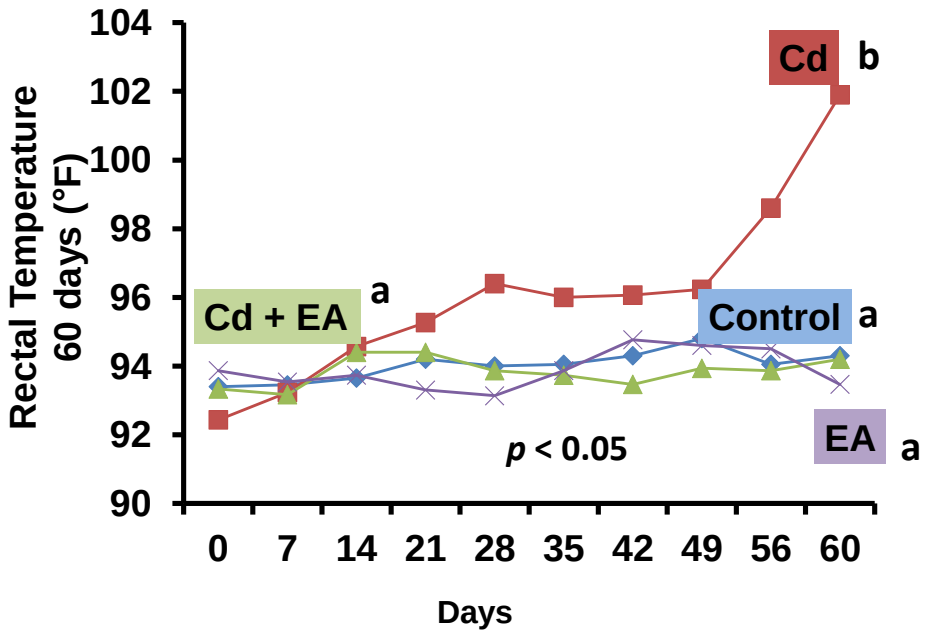


Fig.5: Graph showing the drastic increase in Cadmium (Cd) treated group due to the stress culminated by the Cd. A drop in the Ellagic Acid (EA) treated group line graph was found which may be due to the radical scavenging capacity of the EA.

5.4. Biochemical Assay

5.4.1. Lipid Peroxidation (LOO)

$CdCl_2$ administration elevated LOO, whereas treatment of rat with FA significantly reduced lipid peroxidation when compared to $CdCl_2$ treatment alone (Table 3 and figure 6).

Table 3: LOO (nmol of MDA/mg protein) contents of a drug dose.

Control	Cd	Cd + EA	EA
15.83 ± 0.13a	54.88 ± 0.40b	23.99 ± 0.53c	19.31 ± 0.22d

Values are expressed as mean ± standard error mean (n = 5/treatment). Cd- Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

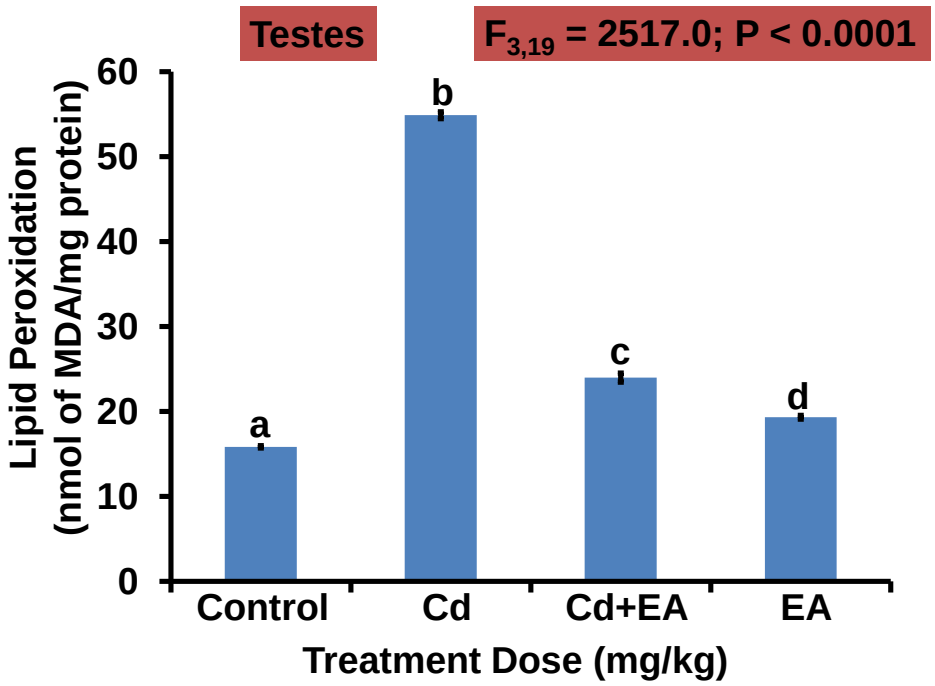


Fig. 6: Effect of EA treatment on the Cadmium-induced alteration in the LOO contents in the rat testis. Tissue of intoxicated male rats values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at p<0.05.

5.4.2. Gluthathione (GSH)

The $CdCl_2$ administration decreased the glutathione content, whereas combination of EA and $CdCl_2$ increased it (Table 4 and figure 7).

Table 4: GSH (nmol/mg protein) contents of a drug dose.

Control	Cd	Cd + EA	EA
5.39 ± 0.00a	3.59 ± 0.02b	4.63 ± 0.01c	4.27 ± 0.01d

Values are expressed as mean ± standard error mean (n = 5/treatment). Cd- Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

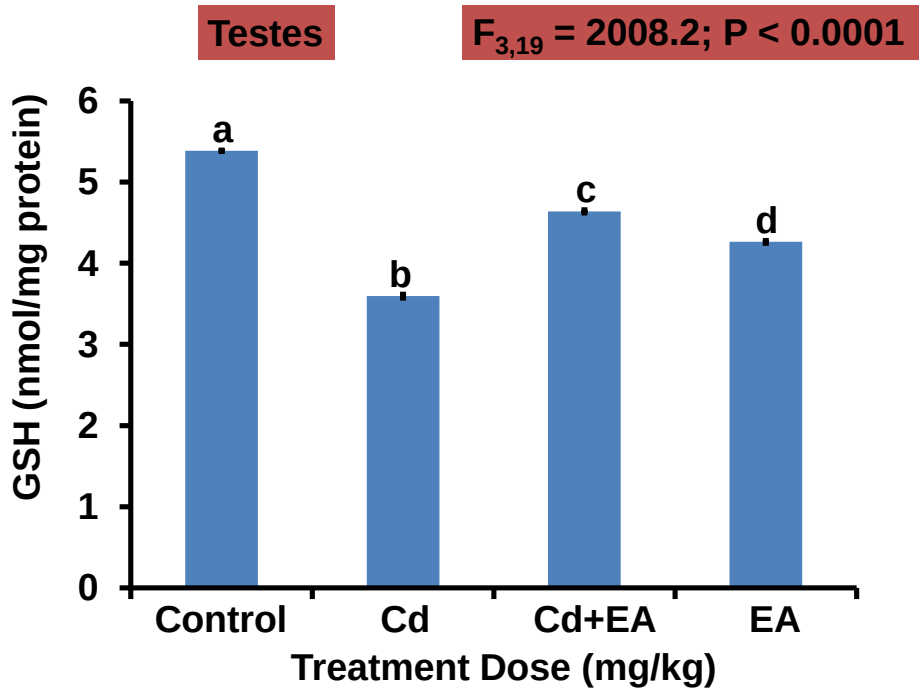


Fig. 7: Effect of treatment with EA on GSH antioxidant and cadmium contents in testis. Tissue of intoxicated male rats values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at p<0.0001.

5.4.3. Gluthatione-S-Transferase (GST)

The $CdCl_2$ administration decreased the GST content significantly, whereas combination of EA and $CdCl_2$ increased it (Table 5 and figure 8).

Table 5: GST (IU/L of protein) contents of a drug dose.

Control	Cd	Cd + EA	EA
10.93 ± 0.024a	7.81 ± 0.023b	10.59 ± 0.026 c	8.16 ± 0.024d

Values are expressed as mean ± standard error mean (n = 5/treatment). Cd- Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

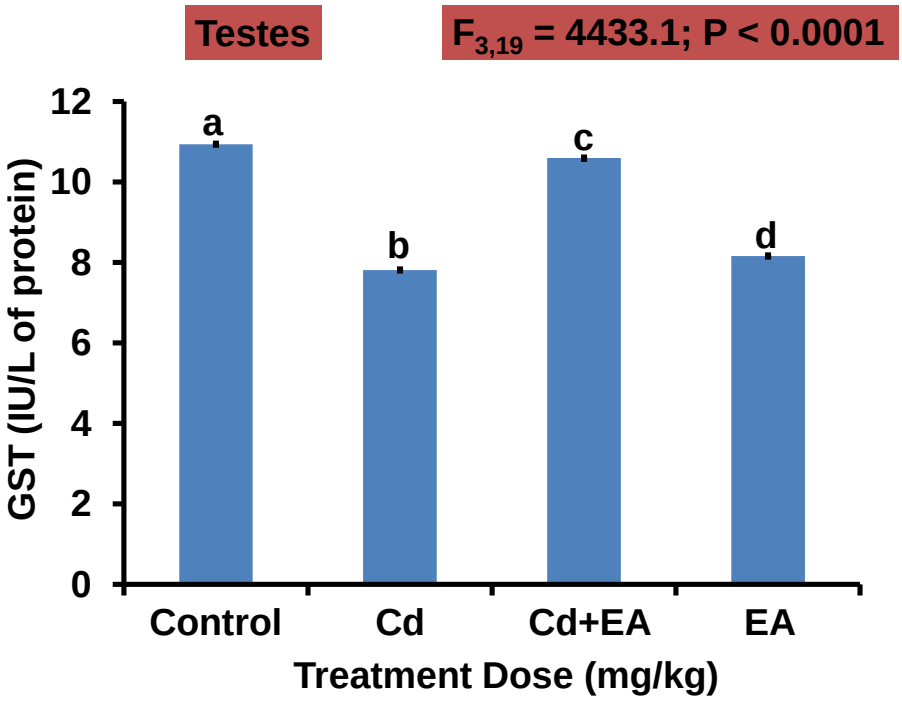


Fig.8: Effect of EA treatment on the Cadmium-induced alteration in the GST contents in the rat testis. Tissue of intoxicated male rats values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at p<0.0001.

5.4.4. Superoxide Dismutase (SOD)

The superoxide dismutase activity showed significant change after EA administration (Table 6 and Figure 5e).

Table 6: SOD (U/mg protein) contents of a drug dose.

Control	Cd	Cd + EA	EA
42.02 ± 0.00a	40.64 ± 0.01b	42.46 ± 0.00c	43.28 ± 0.00c

Values are expressed as mean ± standard error mean (n = 5/treatment). Cd- Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

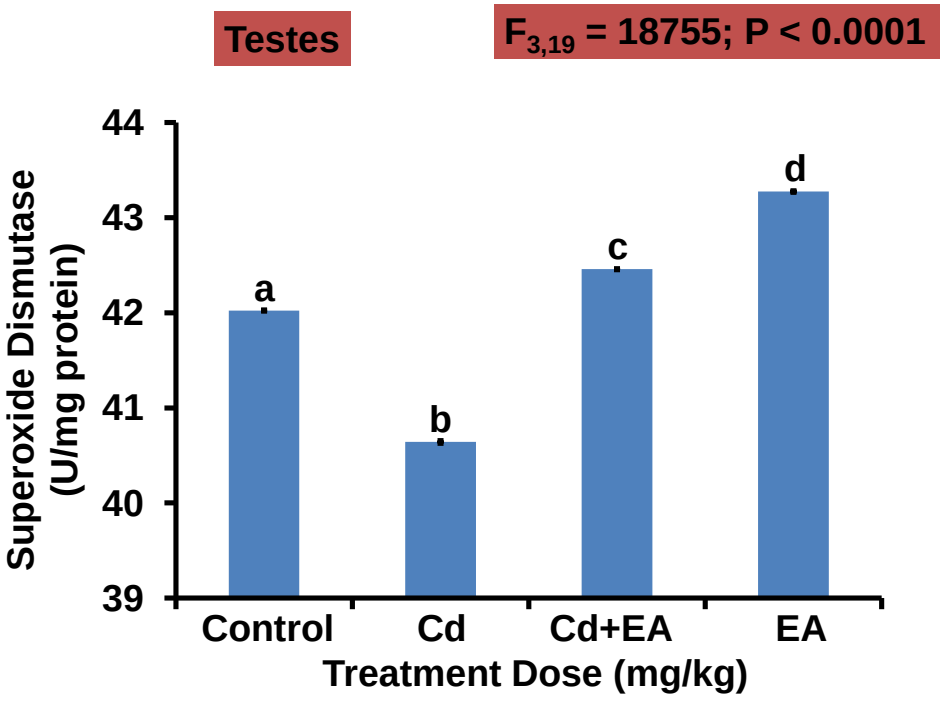


Fig.9: Effect of EA treatment on the Cadmium-induced alteration in the SOD contents in the rat testis. Tissue of intoxicated male rats values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at $p<0.0001$.

5.5. Sperm analysis

5.5.1. Sperm motility and Daily Sperm Production

As shown in the figure 10 and table 7 below, Cd-treated rats showed significant decrease in sperm motility and epididymal sperm concentration along with significant elevation in the percentages of total abnormalities in spermatozoa compared with the control groups. However, co-administration of EA to Cd-treated rats significantly prevented Cd-mediated decrease in sperm motility and epididymal sperm concentration and renovated the level of sperm abnormality to near control. The daily sperm production (DSP) was not significantly different in cadmium-treated rats and in rats co-treated with EA when compared with the control rats.

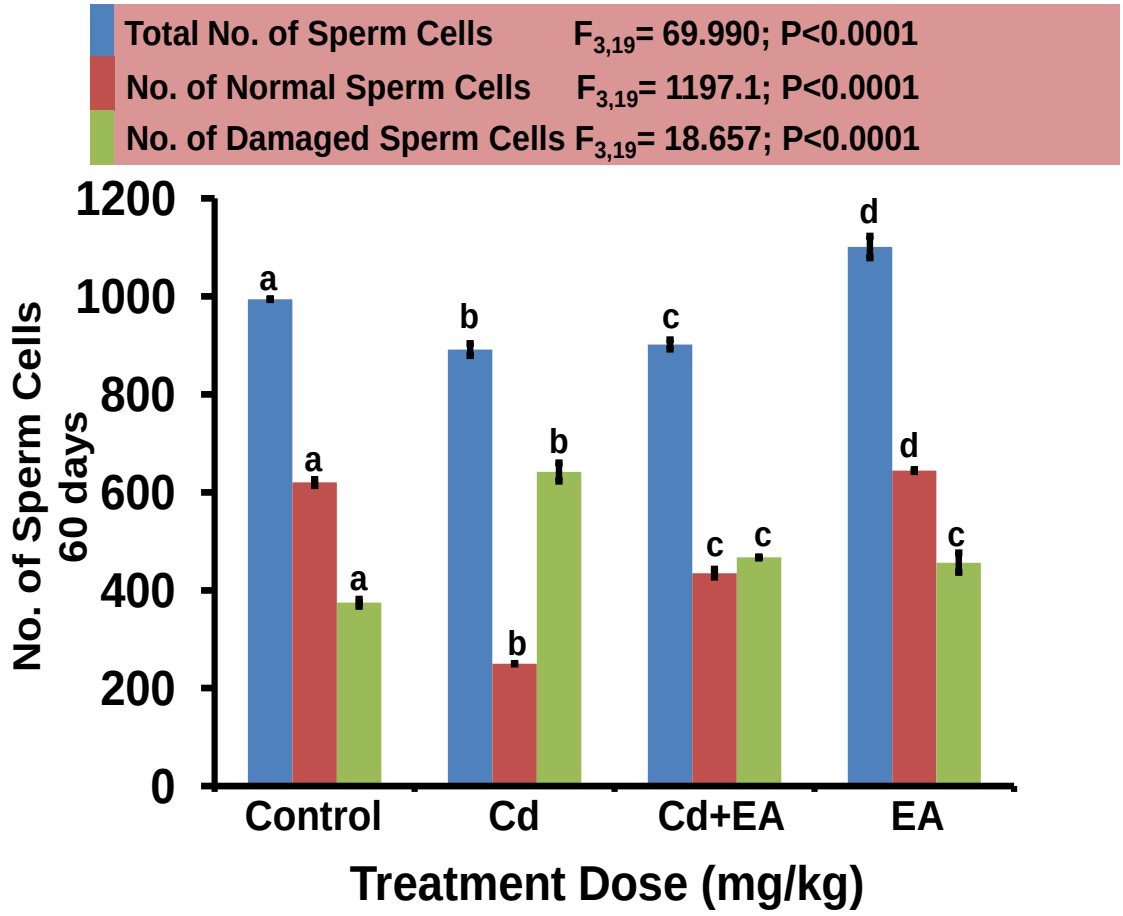


Fig.10: A bar graph showing the highest number of dead sperm cells in Cd treated groups significantly when compared to the other groups.

Table 7: Effect of treatment of $CdCl_2$ for 60 days on sperm analysis in adult male Wistar albino rat.

Parameters	Experimental group				$F_{3,19}$ value	*p* value
	Control	$CdCl_2$	$CdCl_2$+EA	EA		
Testis						
Sperm number ($\times 10^6$)	125.12 ± 9.12	67.50 ± 5.00	83.25 ± 4.25	97.36 ± 12.36	8.569	NS
Sperm number ($\times 10^6$/g)	12.63 ± 0.96	7.50 ± 1.12	8.95 ± 4.45	9.93 ± 1.55	0.7672	NS
Daily sperm production ($\times 10^6$ testis/day)	20.51 ± 1.49	11.06 ± 0.82	13.647 ± 0.69	15.97 ± 2.02	8.569	NS
Daily sperm production relative to testis weight ($\times 10^6$ testis/day/g)	2.08 ± 0.02a	1.22 ± 0.00b	1.46 ± 0.01c	1.62 ± 0.05d	175.42	<0.0001
Cauda epididymis						
Sperm number ($\times 10^6$)	51.25 ± 7.86	31.84 ± 2.26	42.71 ± 0.48	47.45 ± 2.16	5.855	NS
Sperm number ($\times 10^6$/g)	5.18 ± 1.33	3.537 ± 0.70	4.59 ± 1.00	4.84 ± 1.12	0.4497	NS
Caput epididymis						
Sperm number ($\times 10^6$)	21.60 ± 1.29	13.19 ± 2.08	14.65 ± 3.63	15.05 ± 0.44	3.968	NS
Sperm number ($\times 10^6$/g)	2.19 ± 0.03a	1.465 ± 0.08b	1.57 ± 0.05b	1.53 ± 0.02b	43.483	<0.0001
Sperm motility (%)	62.34 ± 1.55a	28.04 ± 1.19b	48.22 ± 0.90c	58.53 ± 2.18a	100.68	<0.0001

Values are expressed as mean ± standard error mean (n = 5/treatment). NS - Not significant. Cd-Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

5.5.2. Sperm Abnormality

Control rats showed about 21.02% of total abnormal sperms with 13.69% of sperm head abnormalities and 7.33% of tail abnormalities. The rats exposed to $CdCl_2$ showed teratozoospermia i.e., a greater degree of sperm abnormalities when compared to the normal control group ($p<0.0001$). The head abnormalities accounted for about 26.61% whereas tail abnormalities were about 16.02%. A decrease in the sperm abnormalities was observed in all the three groups treated with different doses of ellagic acid compared to the toxic group ($p<0.001$). The rats which received ellagic acid, percentage of head abnormalities were reduced to 13.20% respectively and percentage of tail abnormalities to 4% respectively.

Table 8: Effects of Cadmium chloride on sperm head and tail abnormalities (%).

Parameters	Experimental group				$F_{5,19}$ value	*p* value
Sperm morphology	Control	$CdCl_2$	$CdCl_2$+EA	EA		
Normal	57.90 ± 1.37a	14.67 ± 0.09b	31.95 ± 1.21c	65.60 ± 0.89d	533.36	<0.0001
Head abnormalities						
Amorphous	4.15 ± 0.52a	3.63 ± 0.65a	6.77 ± 0.41c	4.00 ± 0.15a	9.367	0.0008
Banana head	5.37 ± 0.12a	14.82 ± 0.51b	6.77 ± 0.21a	4.40 ± 0.41a	185.74	<0.0001
Detached head	4.15 ± 0.54a	8.16 ± 0.65b	11.61 ± 0.35c	4.80 ± 0.35a	49.255	<0.0001
Total	13.69 ± 1.02a	26.61 ± 1.52b	25.15 ± 1.02b	13.20 ± 0.84a	40.855	<0.0001
Tail abnormalities						
Bent tail	5.13 ± 0.39a	13.91 ± 0.52b	5.25 ± 0.54a	2.40 ± 0.54a	99.484	<0.00011
Broken tail	2.20 ± 0.21a	2.11 ± 0.19a	3.59 ± 0.22b	1.60 ± 0.15c	19.216	<0.0001
Total	7.33 ± 0.92a	16.02 ± 0.14b	8.84 ± 0.98a	4.00 ± 0.65c	45.783	<0.0001

Values are expressed as mean ± standard error mean (n = 5/treatment). The relative organ weight was calculated as {[organ weight/body weight] × 100}. NS - Not significant, Cd- Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

5.6. Serum Assay

5.6.1. Serum Testosterone levels

Testosterone levels in all groups were gradually elevated. Cadmium showed a significant decrease of testosterone levels when compared with the control group(Table 9 and Fig. 11). Results suggest that Leydig cell regeneration could be retarded by cadmium.

Table 9: Testosterone content of a drug dose.

Control	Cd	Cd + EA	EA
9.75 ± 0.42a	1.26 ± 0.53b	2.93 ± 0.35b	7.39 ± 0.58c

Values are expressed as mean ± standard error mean (n = 5/treatment). Cd- Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

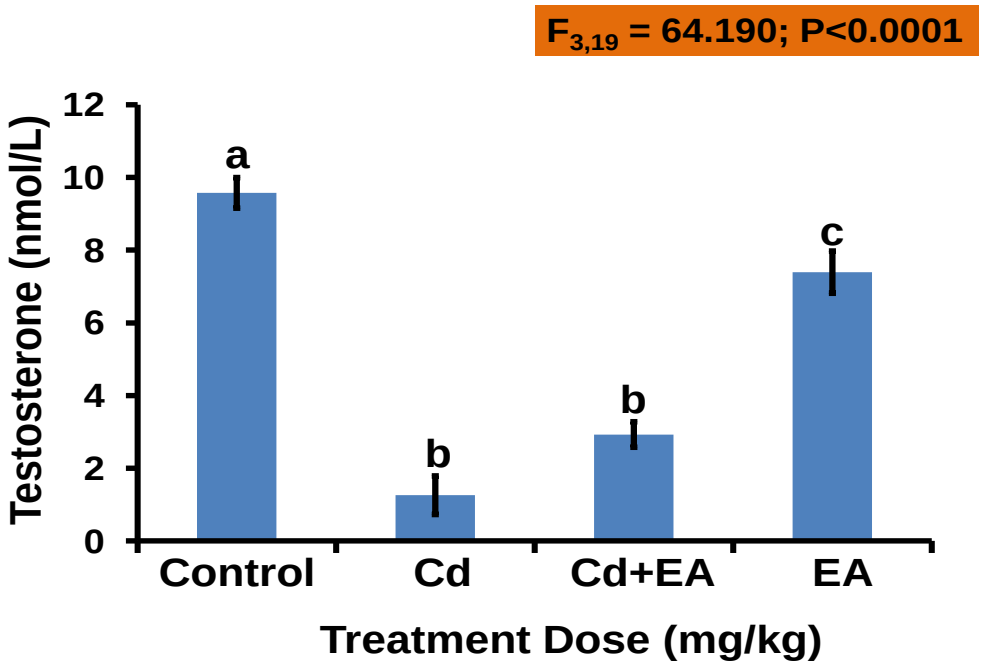


Fig.11: Effect of treatment with EA on testosterone and cadmium content in testis. Tissue of intoxicated male rats values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at $p<0.05$.

5.6.2. Serum LH and FSH levels

Serum FSH significantly elevated, whereas combination of EA and $CdCl_2$ decreased it (Table 10 and Fig. 12) while the LH content significantly decreased after $CdCl_2$ exposure whereas treatment of rat with EA significantly increased LH when compared to $CdCl_2$ treatment alone (Table 10 and Fig. 13).

Table 10: FSH and LH content of a drug dose at different days.

Parameters	Control	Cd	Cd + EA	EA
FSH (IU/L)	0.33 ± 0.19a	3.55 ± 0.15b	2.32 ± 0.23c	1.23 ± 0.38a
LH (IU/L)	5.34 ± 0.04a	2.26 ± 0.03b	0.39 ± 0.02c	4.23 ± 0.05 d

Values are expressed as mean ± standard error mean (n = 5/treatment). Cd- Cadmium and EA- Ellagic. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

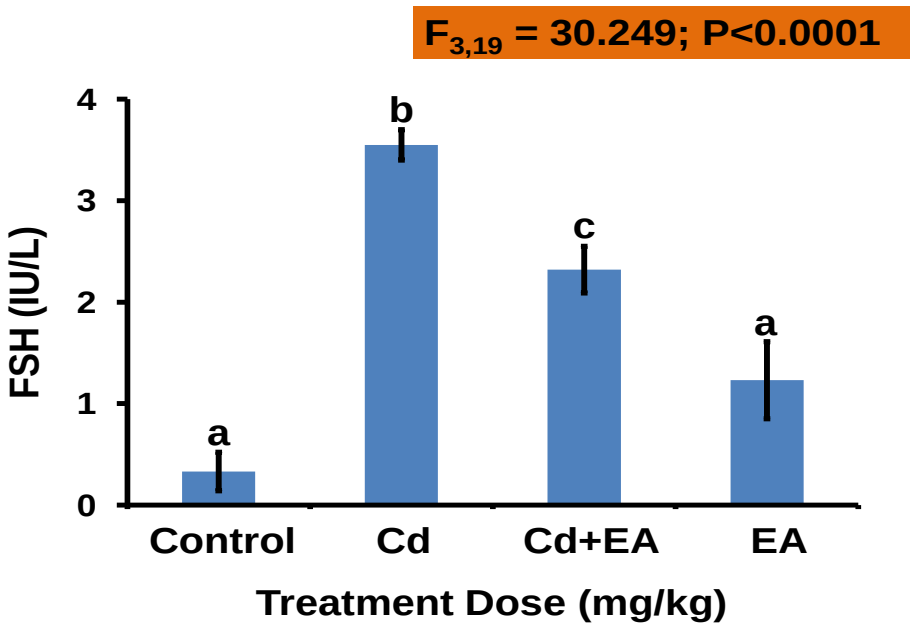


Fig.12: Effect of treatment with EA on FSH and cadmium content in testis. Tissue of intoxicated male rats values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at p<0.05.

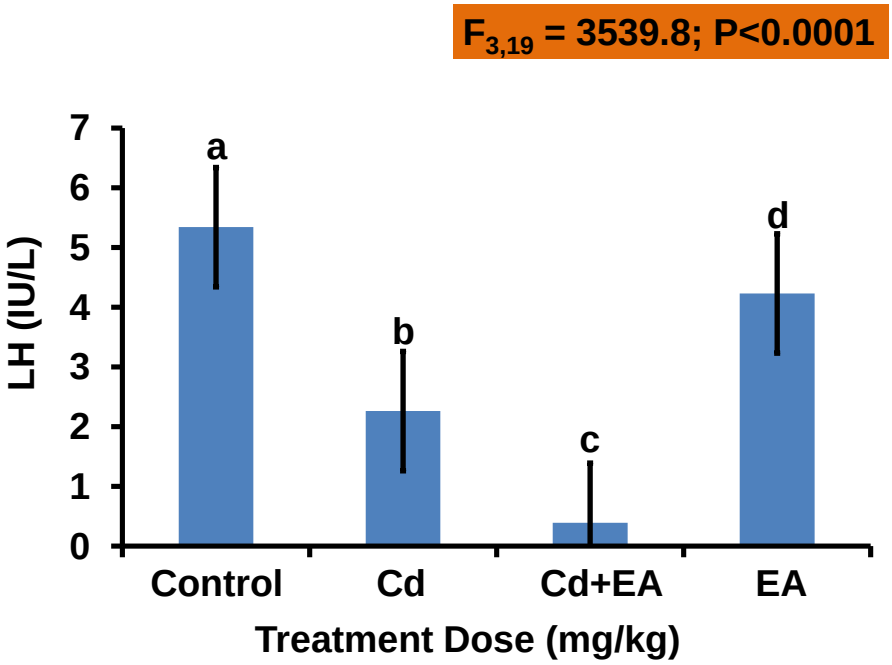


Fig. 13: Effect of treatment with EA on LH and cadmium content in testis. Tissue of intoxicated male rats values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at $p<0.05$.

5.7. Histopathology

The histological appearance of the testicular tissues of the control group was normal in appearance [Fig. 14 (A)]. Cd induced histopathological variations in the testis such as necrosis, germ cell degeneration, desquamation, edema and congestion in addition to degeneration and atrophy of seminiferous tubules [Fig. 14 (B)]. It was observed that there was a marked decrease in necrotic and degenerative changes in germinal cells of rats that received EA along with $CdCl_2$ [Fig. 14 (C)]. A reduction in Johnsen's scoring was observed in the $CdCl_2$ exposed group compared to the normal animals ($p<0.0001$; Table 11). An improvement in the Johnsen's score (Table 11, Fig. 15) and Mean Seminiferous Tubule Diameter (Table 12, Fig. 16) was noted in the groups which received ellagic acid at 2 mg/kg, thus showing its protective action.

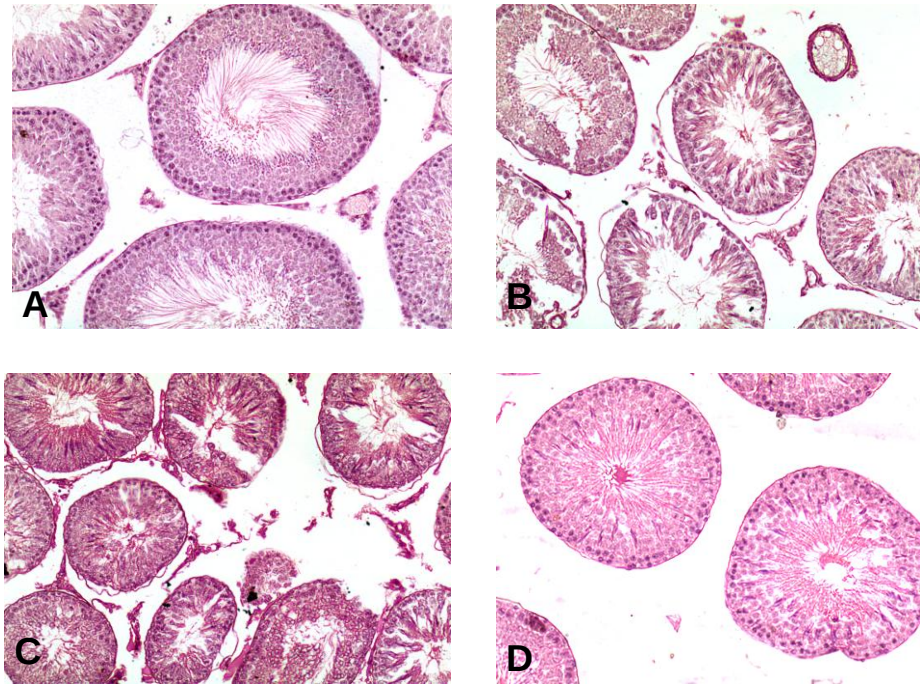


Fig. 14: Photomicrographs of the 60 days treated testes; magnification, x10. (A) In the control group, numerous seminiferous tubules, spermatogonia, spermatocytes and spermatozoa were detected. Interstitial connective tissue and Leydig cells were observed [hematoxylin & eosin (H&E)]. (B) In the cadmium chloride ($CdCl_2$) group, edema and sloughing of spermatogonia were detected (H&E). (C) In the co-treated group (EA and $CdCl_2$), the seminiferous tubules were characterized by congestion and slight edema (H&E). (D) The ellagic acid (EA) group activated spermatocytes and spermatozoa were detected (H&E).

Table 11: Johnsen's Scoring (JTBS) and Morphometric Seminiferous Tubules diameter (MSTD).

Parameters	Control	Cd	Cd + EA	EA	$F_{3,19}$	p value
Johnsen's Scoring	9.30 ± 0.30a	4.70 ± 0.86b	8.40 ± 0.29a	8.90 ± 0.31a	13.472	<0.0001
MSTD (µm)	273.50 ± 3.29a	203.00 ± 1.16b	256.33 ± 5.34c	269.50 ± 2.35a	91.819	<0.0001

Values are expressed as mean ± standard error mean (n = 5/treatment). Cd- Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

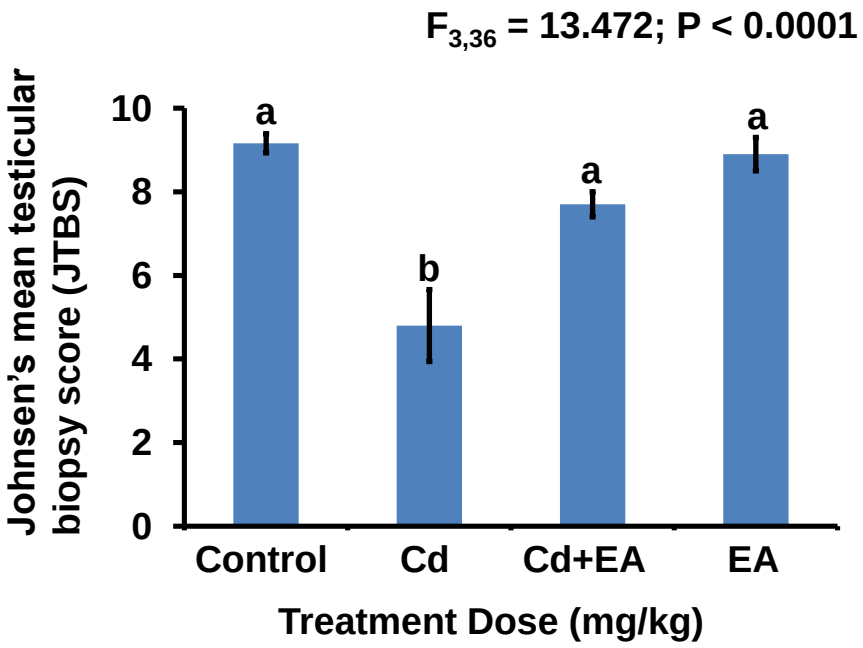


Fig. 15: Effect of Cd exposure on the testes significantly reduced the Johnsen's score and the Co-treatment with EA significantly increased its score. Values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at p<0.0001.

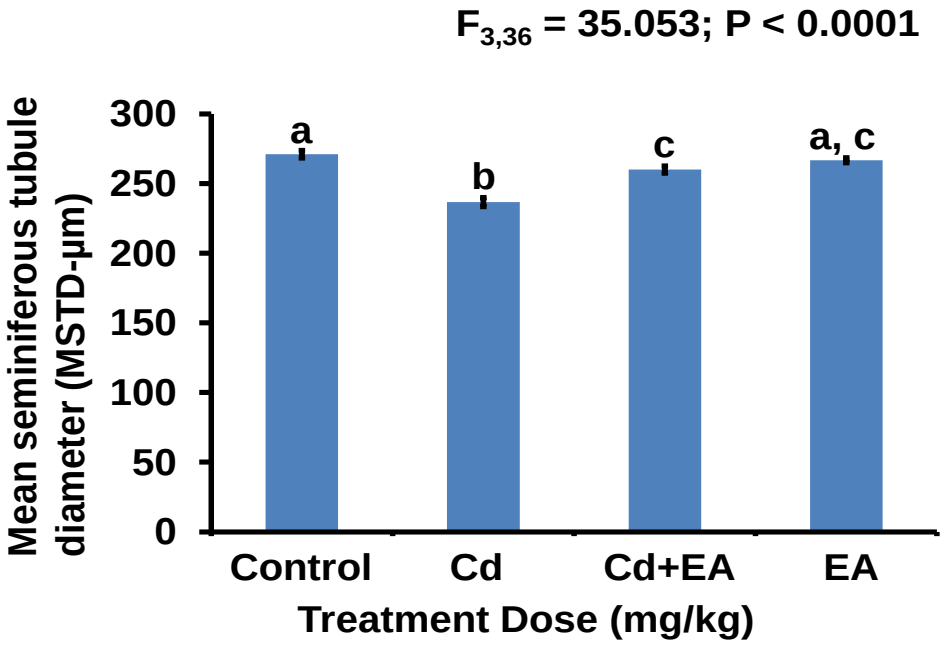


Fig. 16: The exposure of Cd on the testes significantly reduced the seminiferous tubule diameters and the Co-treatment with EA slightly improves it. The values are expressed as mean ± SEM. Columns followed by the different alphabetical letters are significantly different at p<0.0001.

5.8. DNA damage detection

The exposure of Cd to the sperms causes the abnormal DNA exhibiting more number of fluorescence red or yellow colour [Fig. 17 (B)] under fluorescence microscope as we can see in the figure. On the other hand, the control group showed normal DNA more exhibiting green colour [Fig. 17 (A)]. The co-treated group [Fig. 17 (C)] showed green colour more when compared to that of Cd group. The EA treated group [Fig. 17 (D)] shows excess number of green coloured sperms indicating the normal DNA. The lowest normality score was found in the Cd treated group when compared to all the other groups (Table 12).

Table 12: DNA damage score (%)

Control	Cd	Cd + EA	EA	$F_{3,19}$	p value
12.74 ± 1.56a	64.74 ± 0.14b	61.08 ± 0.05c	30.48 ± 0.19d	998.28	<0.0001

Values are expressed as mean ± standard error mean (n = 5/treatment). Cd- Cadmium and EA- Ellagic acid. One way ANOVA with Tukey post hoc multiple comparison test; Column with similar letters is not significant at $p > 0.05$ while different letters means statistical significant difference at $p < 0.05$.

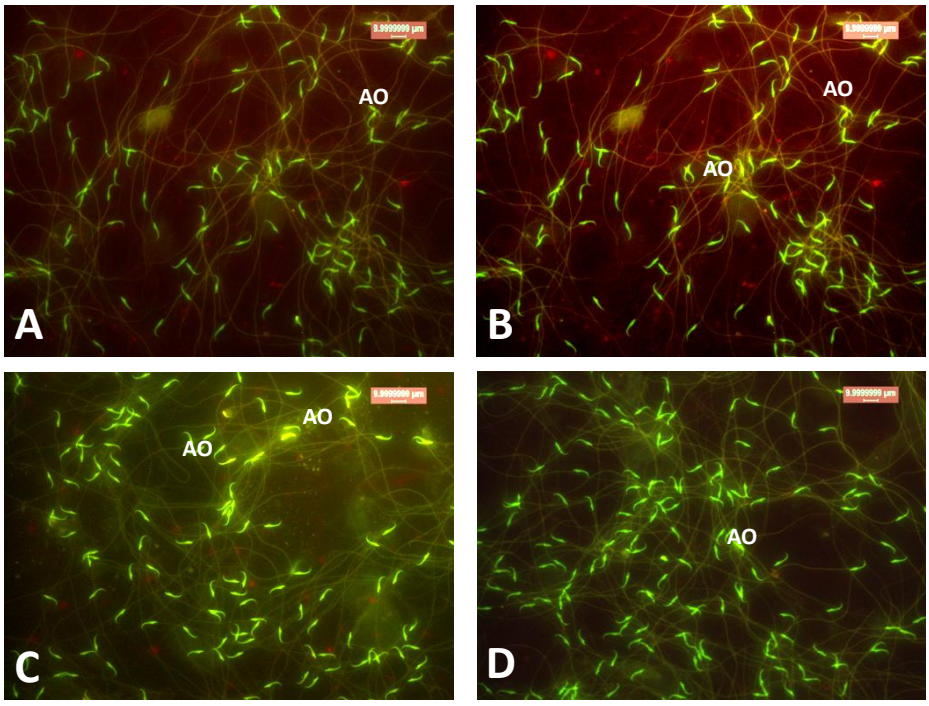


Fig. 17: Fluorescent photomicrographs of 60 days treated Acridine orange stained sperms (x40). (A) Control group, (B) $CdCl_2$ treated group, (C) Co-treated group and (D) EA group.

5.9. Expression of PCNA, Bcl-2 and Bax

5.9.1. PCNA

Immunohistochemical staining of sections from the Cd-treated rats [Fig. 18 (B)] revealed only PCNA positive spermatogonia. In rats treated with EA [Fig. 18 (D)], the PCNA staining pattern was identical to the control group [18 (A)].

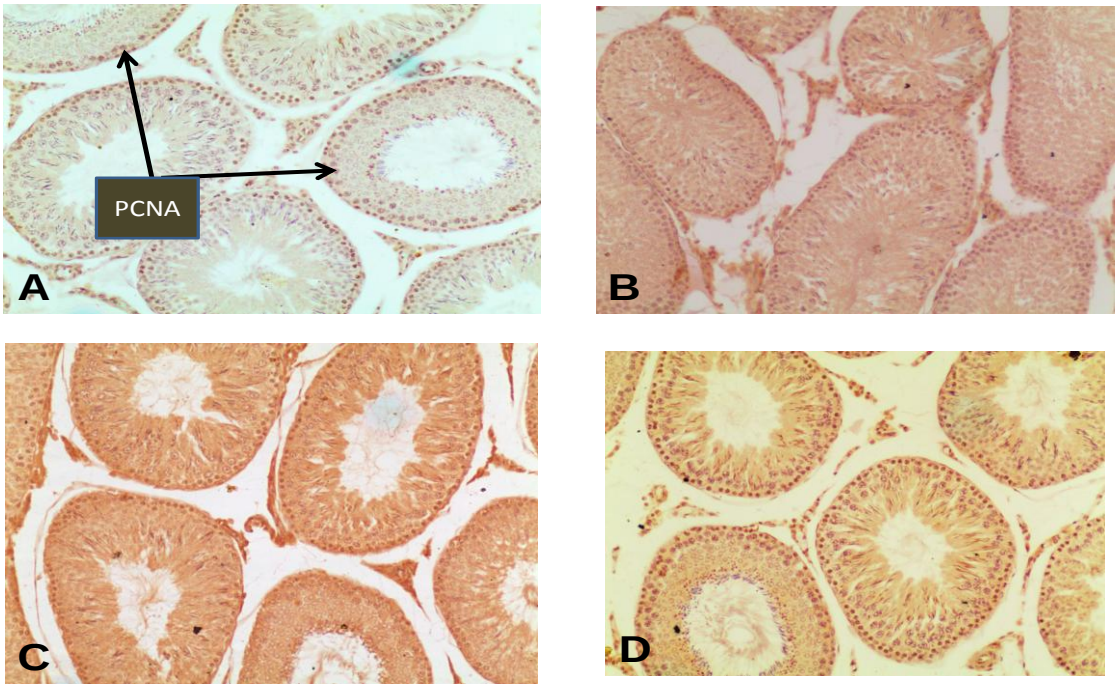


Fig.18: Testicular expression of PCNA was detected using immunohistochemical staining in (a) control, (b) $CdCl_2$, (c) EA+$CdCl_2$ and (d) EA treated rats. In the control and EA groups, apoptotic testicular cells, i.e., those immunostained with PCNA, were sparse and weakly stained. By contrast, where the rats were treated with $CdCl_2$, many testicular cells were apoptotic and the brown-stained positive cells were increased markedly. In the EA + $CdCl_2$ group, the number of apoptotic testicular cells was decreased markedly (400×).

5.9.2. Bcl-2

Testicular tissue obtained from Cd-treated [Fig. 19 (B)] rats showed weak expression of Bcl-2 in comparison with the control group [Fig. 19 (A)]. Treatment of animals with Cd+EA upregulated the expression of Bcl-2 [Fig. 19 (C)].

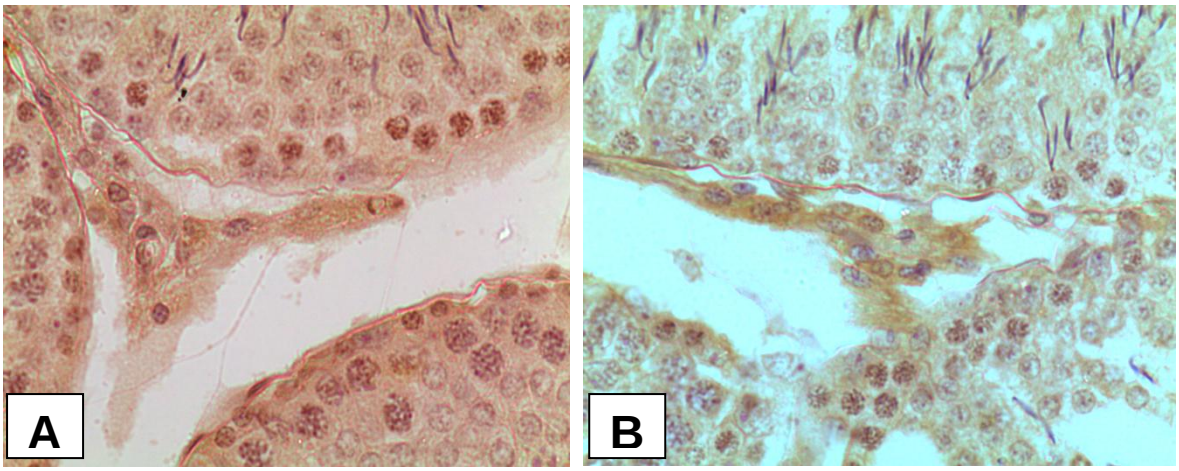


Fig.19: (A) Testicular section of Control rats showing positive staining of Bcl-2 and (B) Weak expression of Bcl-2 in germ cells of a rat treated with Cd (A and B, 40X).

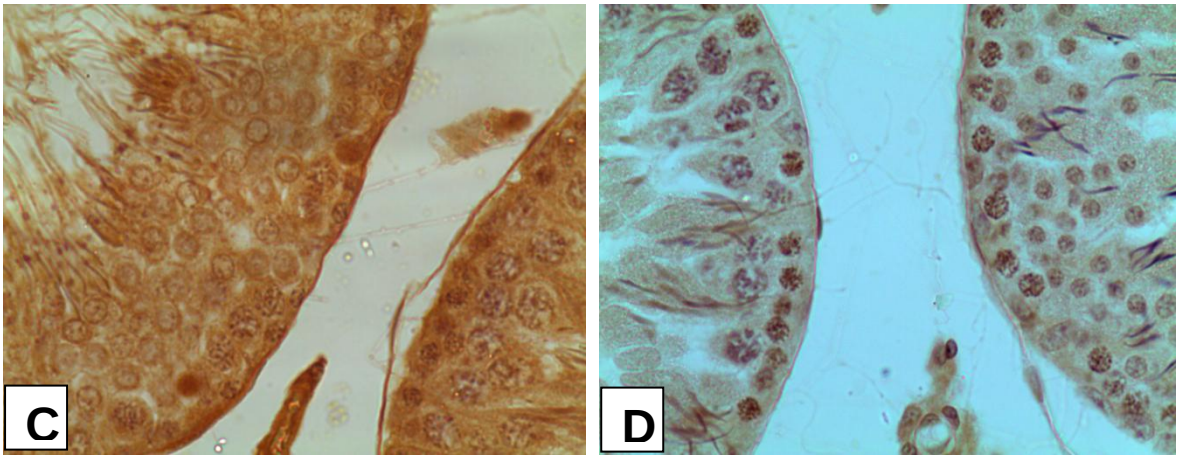


Fig.19: (C) An increased expression of Bcl-2 after treatment with Cd+*EA and (D) A regeneration of positive cells with EA treatment* (C and D, 40X).

5.9.3. Bax

Testicular expression in Cd [Fig. 20 (B)] treated rat showed marked increase in the Bax expression. Cd+EA showed weak expression [Fig. 20 (C)] when compared to that of the Cd-treated group [Fig. 20 (A)].

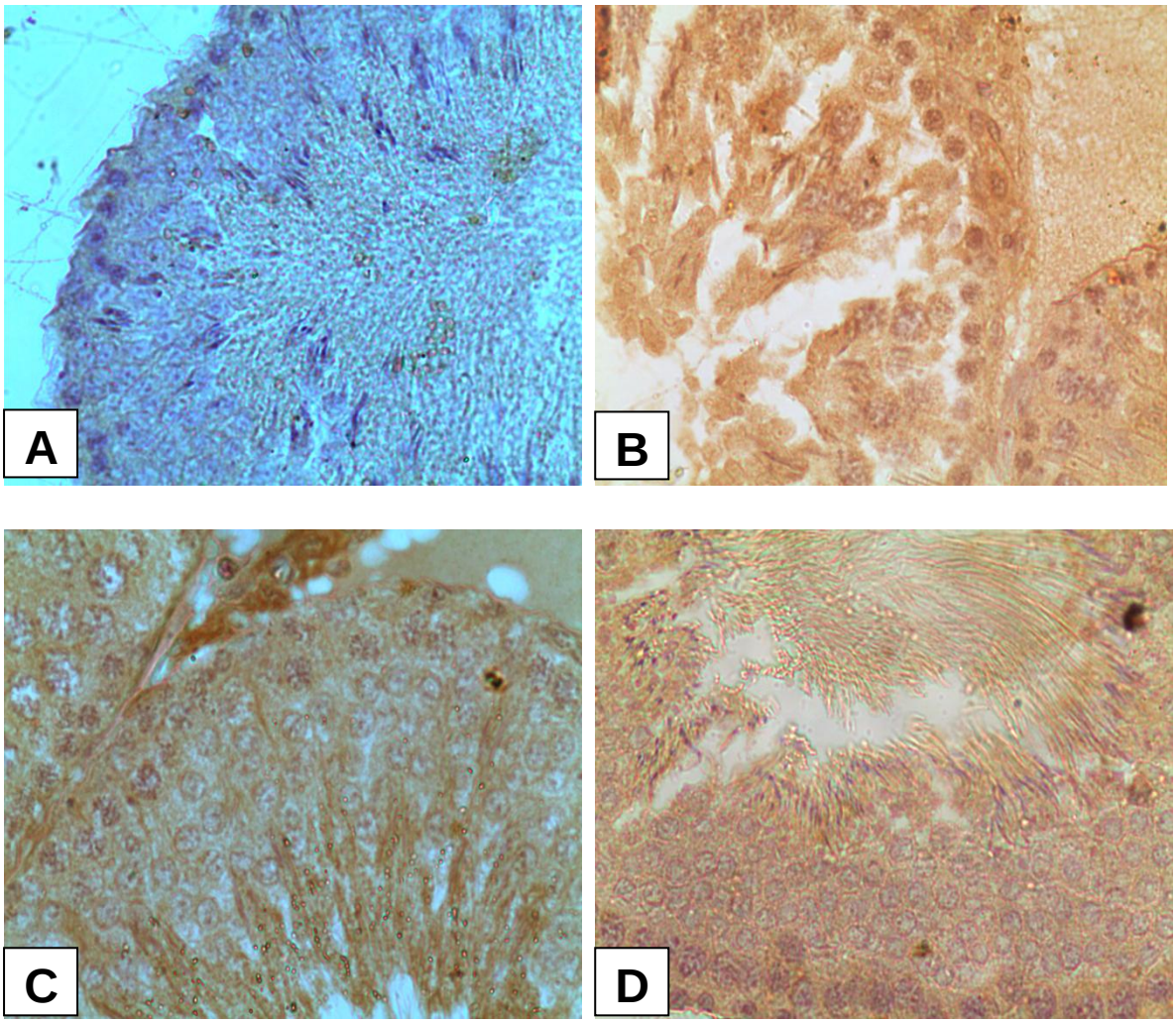


Fig.20: (A) Testicular section of a rat in control group showing very less bax expression (B) Section in testis of a rat treated with Cd showing increase of Bax expression in Leydig cells. (C) Section in testis of a rat treated with Cd+EA showing decrease of Bax expression (D) The expression in EA treated group showing an improved expression of Bax in the sertoli cells, spermatogonia and spermatocytes.

5.10. Western Blotting

The level of leydig and sertoli cell proteins [PCNA (Fig. 21) and Bcl-2 (22)] in the testis after the Cd exposure and EA administration. $CdCl_2$ (6.464 ± 0.03) significantly showed an increased expression when compared to control (0.558 ± 0.01), as seen in the figure 21 (B). The administration of EA (2.579 ± 0.02) significantly reduced the PCNA expression. Cd+EA (6.464 ± 0.04) treatment show no significant changes. As shown in Fig. 22, the quantification of Bcl-2 results showed that Cd dose-dependently (1.701 ± 0.13) lowered the levels of all these proteins when compared to those of the EA (18.795 ± 0.36) alone and control (18.771 ± 0.12) treatment which were in parallel with those of their respective mRNA levels. The co-

treated group (1.977 ± 0.42) showed a non-significant protein levels with Cd treated group. This result further confirmed that cadmium impaired the Leydig and sertoli cell functions in the testis.

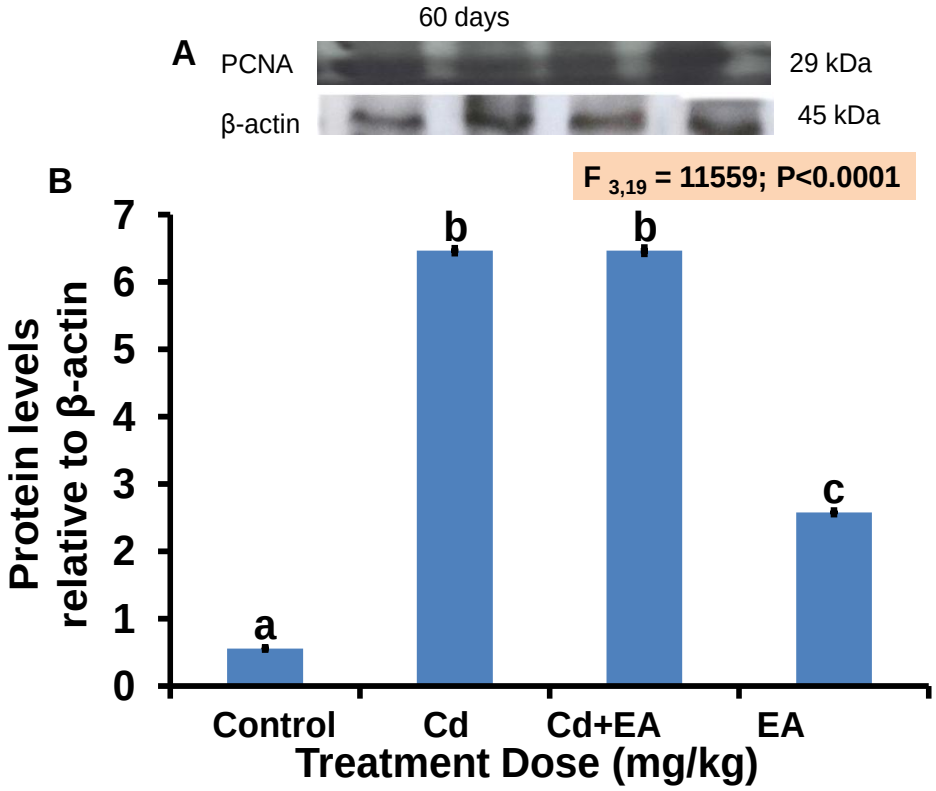


Fig.21: Western blot analysis in the rat testis showing transformation in the cell proliferation. (A) Western blot band. (B) Quantification of protein levels expressed as Mean ± SEM, n = 5. The different alphabetical letters indicate significant differences when compared to control at $p<0.0001$.

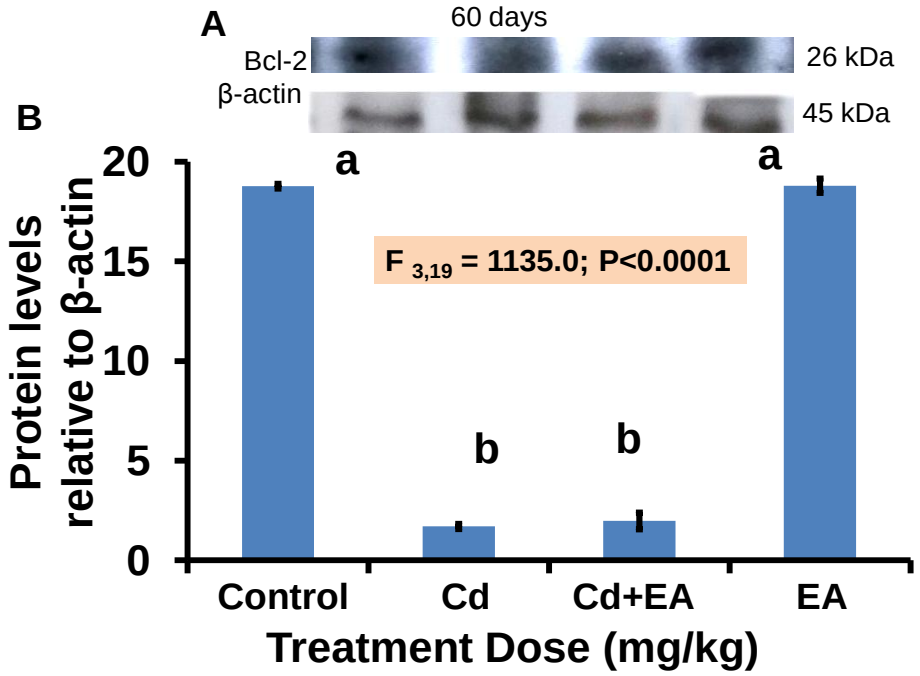


Fig.22: Protein levels of Leydig and Sertoli cells in rat testis. (A) Western blot band. (B) Quantification of protein levels expressed as Mean ± SEM, n = 5. The different alphabetical letters indicate significant differences when compared to control at p<0.0001.

VI. DISCUSSION

6.1. The Oxidative Stress And The Antioxidant level

Testicles are the potent target organs for oxidative stress due to their high content of polyunsaturated membrane lipids (Drasga et al., 1983) and a number of mechanisms of cadmium toxicity have been recommended with cell adhesion and signalling, oxidative stress, apoptosis, genotoxicity and cell cycle disturbance (Kawai et al., 2002; Amara et al., 2008). Recent evidences suggest that cadmium-induced testicular toxicity occurs as a result of oxidative stress and increased production of reactive oxygen species (Mohamed et al., 2014; Malik et al., 2017). The present work, in agreement with previous studies, confirmed that oxidative stress, increased lipid peroxidation and reduction of antioxidant defences in the pathogenesis of cadmium-induced testicular toxicity (Chandra et al., 2010; Farombi et al., 2012; Fouad et al., 2013). Also, the present study demonstrated that Cd treatment provided a significant protective effect against testicular injury caused by cadmium in rats as indicated by the improvement in the disturbed biochemical parameters and amelioration of testicular tissue damage observed by histopathological and immunohistochemical examinations.

The cadmium-induced decreases in serum testosterone and testicular reduced glutathione, superoxide dismutase and glutathione-S-transferase activity and significantly decreased the elevations of testicular malondialdehyde which is in agreement with previous studies (Fouad et al., 2013). Ellagic acid improved the body and testis weights of the animals thereby, enhancing sperm count which is one of the most sensitive tests for spermatogenesis and is highly correlated with fertility.

6.2. Enhancement Of Anti-Proliferative And Anti-Apoptotic By Ellagic Acid

$CdCl_2$ downregulated the anti-apoptotic gene Bcl-2 and upregulated the expression of pro-apoptotic genes Bax (Eleawa et al., 2014) and also showed PCNA status of spermatogonia. EA protected against and partially reversed $CdCl_2$ testicular toxicity via upregulation of Bcl-2 and downregulation of Bax gene expression. The result indicates the antioxidant activity of EA protects against $CdCl_2$ testicular toxicity and partially reverses its effect via upregulation of BCl2 and downregulation of Bax expression and the proliferative status of spermatogonia (germ cells).

The mechanism involved in the protective effect of ellagic acid against Cd-induced reproductive toxicity is unknown. Cd can disrupt the cellular mechanisms in different ways which may lead to toxicity inducing free radical formation and lipid peroxidation, which are

chemical mechanisms capable of disrupting the structure and function of testis. The antioxidant and free radical scavenging properties of ellagic acid may play an important role in preventing the toxic effects of drugs. $CdCl_2$ has been shown to have apoptosis-promoting effect on human and rat granulosa cells by increasing PCNA activity. The compounds that have anti-apoptotic properties like EA may be beneficial against testicular toxicity. Further, future studies are needed to investigate the exact protective mechanism of EA.

In conclusion, this study suggests that cadmium causes testicular toxicity, but the flavonoid compound ellagic acid is protective in terms of sperm count, motility and morphology. The histo-architecture of testis was also restored by ellagic acid confirming its protective effect. This action of ellagic acid may be closely related to its antioxidant and antiapoptotic property, which needs further research. Therefore, ellagic acid may be useful in epileptic patients on long-term cadmium treatment if proved effective in clinical trials.

VII. SUMMARY

- $CdCl_2$ administration significantly decreased sperm count, sperm motility and sperm morphology of rats. The changes observed in the above agree with the reports, which demonstrated that cadmium impairs testicular function.

- The significant reduction in sperm count, motility and morphology observed in this study following $CdCl_2$ administration may be associated to impairment of steroidogenesis consequent to reduced secretion of testosterone (from testis) caused by administration of $CdCl_2$.

- Regardless of the mechanism of induction of oxidative stress in cells by cadmium, increase in ROS occurs, which leads to the damage and changes in their structure and metabolism.

- Studies have shown that exposure to cadmium causes lipid peroxidation, which leads to oxidative stress. SOD is an enzymatic antioxidant that converts superoxide radicals into hydrogen peroxide and oxygen, which is further converted into water.

- A significant reduction in the activities of both SOD and GST, but significant rise in the MDA levels in testicular tissues of rats administered with $CdCl_2$. The derangement in the lipid peroxidation and anti-oxidant status of testicular tissues might be a contributing factor in the reduction of testosterone secretion with resultant poor sperm quality.

- EA significantly increased SOD and GST activities, thus enhancing the anti-oxidant system in the testicular tissues. This is in agreement with the previous studies.

- EA improved sperm quality in rats. Pre-treated with EA also prevented $CdCl_2$ – induced poor sperm quality by inhibiting lipid-peroxidation.

- EA enhances testicular oxidative status. The study also shows that EA pre-treatment prevents cadmium chloride – induced testicular toxicity by increasing the tissue enzymatic antioxidant activities (SOD and GST) and reducing lipid peroxidation (MDA).

- Immunohistochemical staining of sections from the treated rats revealed only PCNA-positive spermatogonia. Bax expression was also assessed in sertoli cells, spermatogonia and spermatocytes where deficiency of Bax results in increased apoptosis and testicular atropy.

- Bcl-2 an antiapoptotic gene was significantly downregulated, confirming the apoptotic effect due to cadmium exposure.

- Varied effects of Cadmium under different conditions in different cell lines have rendered the possibility of a specific mechanism evasive. Such being the case, it is necessary to profile the effects comprehensively and identify the common themes in cadmium toxicity for further progress to be made in this regard.

- The findings in the study showed that pre-treatment of rat model with ellagic acid prevented $CdCl_2$ – induced reproductive toxicity by improving sperm quality and enhancing testicular lipid peroxidation status.

- To date information of the pharmacological properties of EA in the context of hepatoprotection is very limited; therefore it is important to extend the knowledge about the action mechanisms of this flavonoid and to perform additional preclinical studies in vitro and in vivo models.

- Also, it is worth mentioning that the effects of EA and pomegranate juice (containing EA) are being evaluated in phase I, II and III clinical trials (ClinicalTrials.gov Identifier: NCT00455416; NCT02263378; NCT01916239), though these studies are focused mainly on its anticarcinogenic activity and in hyper-pigmentation disorders treatment.

- These pioneer studies might be a breakthrough in the search for natural compounds with beneficial activities to humans and serve as a basis in the near future to begin tests seeking to prevent, reduce or eliminate testicular damage caused by the exposure to xenobiotics or disease.

- By better defining the problems, learning about the mechanisms responsible for adverse effects, and developing panels of relevant biomarkers, we will make progress towards preventing future adverse effects on male reproductive health.

VIII. REFERENCES

Adaramoye OA, Akanni OO (2016) Protective effects of *Artocarpus altilis* (Moraceae) on cadmium-induced changes in sperm characteristics and testicular oxidative damage in rats. *Andrologia* 48: 152-163.

Agarwal A, Said TM (2003) Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod* 9:331–345.

Aitken RJ, Curry BJ (2011) Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal* 14:367-381.

Alkhedaide A, Alshehri ZS, Sabry A, Abdel-Ghaffar T, Soliman MM, Attia H (2016) Protective effect of grape seed extract against cadmium-induced testicular dysfunction. *Mol Med Rep* 13: 3101-3109.

Amara S, Abdelmelek H, Garrel C, Guiraud P, Douk T, Ravanat JL, Favier A, Sakly M, Rhouma KB (2008) Preventive effect of zinc against cadmium-induced oxidative stress in the rat testis. *J Reprod Dev* 54:129–134.

Angenard G, Muczynski V, Coffigny H, Pairault C, Duquenne C, Frydman R, Habert R, Rouiller-Fabre V, Livera G (2010) Cadmium increases human fetal germ cell apoptosis. *Environ Health Perspect* 118:331–337.

Asada K, Takahashi M, Nagate M (1974) Assay and inhibitors of spinach superoxide dismutase. *Agric Biol Chem* 38: 471–474.

Atessahin A, Turk G, Yilmaz S, Sonmez M, Sakin F, Ceribasi AO (2010) Modulatory effects of lycopene and ellagic acid on reproductive dysfunction induced by polychlorinated biphenyl (Aroclor 1254) in male rats. *Basic Clin Pharmacol Toxicol* 106: 479–489.

ATSDR. Agency for Toxic Substances and Disease Registry. U.S. Department of Health and Human Services; Atlanta, GA (2007) The 2007 CERCLA priority list of hazardous substances.

Bairy L, Paul V, Rao Y (2010) Reproductive toxicity of sodium valproate in male rats. *Indian J Pharmacol* 42: 90–94.

Bancroft JD and Gamble A (2002) General stains. In: Theory and Practice of Histological Techniques. 5th edition. Churchill Livingstone, Edinburgh, pp 116-117.

Bu T, Mi Y, Zeng W, Zhang C (2011) Protective effect of quercetin on cadmium-induced oxidative toxicity on germ cells in male mice. *Anat Rec* 294:520–526.

Ceribasi AO, Turk G, Sonmez M, Sakin F, Atessahin A (2010) Toxic effect of cyclophosphamide on sperm morphology, testicular histology and blood oxidant–antioxidant balance, and protective roles of lycopene and ellagic acid. *Basic Clin Pharmacol Toxicol* 107:730–6.

Chandra AK, Chatterjee A, Ghosh R, Sarkar M (2010) Vitamin E supplementation protect chromium (VI)-induced spermatogenic and steroidogenic disorders in testicular tissues of rats. *Food Chem Toxicol* 48:972-979. DOI: 10.1016/j.fct.2010.01.008

Cheng H, Lu C, Tang R, Pan Y, Bao S (2017) Ellagic acid inhibits the proliferation of human pancreatic carcinoma PANC-1 cells in vitro and in vivo. *Oncotarget* 8:12301-12310. DOI:1081632/oncotarget.14811

Cifuentes M, Albala C, Rojas C (2005) Cadmium sensing receptor expression in human adipocytes. *Endocrin* 146:2176-2179.

Clark BJ, Wells J, King SR, Stocco DM (1994) The purification, cloning and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells— characterization of the steroidogenic acute regulatory protein (StAR). *J Biol Chem* 269:28314 –28322.

Correia S, Vaz CV, Silva AMS, Cavaco JE, Socorro S (2017) Regucalcin counteracts tert-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis. *J Appl Toxicol* 37: 159–166.

Deng X, Xia Y, Hu W, Zhang H, Shen Z (2010) Cadmium induced oxidative damage and protective effects of N-acetyl- L-cysteine against cadmium toxicity in Solanum nigrum L. *J Hazard Mater* 180:722–729.

Drasga RE, Einhorn LH, Williams SD, Patel DN, Stevens EE: Fertility after chemotherapy for testicular cancer. *J Clin Oncol,* 1, 179-183, 1983. DOI: 10.1200/JCO.1983.1.3.179

Eleawa SM, Alkhateeb MA, Alhashem FH, Bin-Jaliah I, Sakr HF, Elrefaey HM, Elkarib AO, Alessa RM, Haidara MA, Shatoor AS, Khalil MA (2014) Resveratrol reverses cadmium chloride-induced testicular damage and subfertility by down regulating p53 and Bax and up regulating gonadotropins and Bcl-2 gene expression. *J Reprod Dev* 60: 115–127.

Farbood Y, Sarkaki A, Dianat Mahin, Khodadadi A, Haddad MK, Mashhadizadeh S (2015) Ellagic acid prevents cognitive and hippocampal long-term potentiation deficits and brain inflammation in rat with traumatic brain injury. Life Sci 124:120–127.

Farombi EO, Adedara IA, Akinrinde SA, Ojo OO, Eboh AS (2012) Protective effects of kolaviron and quercetin on cadmium-induced testicular damage and endocrine pathology in rats. *Andrologia* xx:1–12.

Fouad A, Jresat I (2015) Thymoquinone therapy abrogates toxic effect of cadmium on rat testes. *Andrologia* 47:417–426.

Fouad AA, Jresat I (2014) Thymoquinone therapy abrogates toxic effect of cadmium on rat testes. *Andrologia* 11:1–10.

Goyer RA, Clarkson TW (2001) Toxic effects of metals. In: Klaassen CD, editor. Casarett and Doull's Toxicology: *The Basic Sci Poi* 6:811-837.

Goyer RA, Liu J, Waalkes MP (2004) Cadmium and cancer of prostate and testis. *Biometals* 17:555-558.

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 249:7130–7139.

IARC (1993) Cadmium and cadmium compounds. *IARC Monogr Eval Carcinog Risks Hum* 58:119–237.

Jahan S, Khan M, Ahmed S, Ullah H (2014) Comparative analysis of antioxidants against cadmium induced reproductive toxicity in adult male rats. *Syst Biol Reprod Med* 60:28–34.

Jamsai D, O'Bryan MK (2011) Mouse models inmale fertility research. Asian J Androl 1:139-151.

Janero (1990) Malondialdehyede and thiobarbituric cid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic Biol Med* 9:515-540.

Ji YL, Wang H, Liu P, Wang Q, Zhao XF, Meng XH, Yu T, Zhang H, Zhang C, Zhang Y, Xu DX (2010) Pubertal cadmium exposure impairs testicular development and spermatogenesis via disrupting testicular testosterone synthesis in adult mice. *Reprod Toxicol* 29:176-183.

Johnsen KP, Hawkins JL (1970) Perception of behaviuoral conformity, imputation of census and martial satisfaction. *J Marriage Fam* 31:507-511.

Kawai M, Swan KF, Green AE, Edwards DE, Anderson MB, Henson MC (2002) Placental endocrine disruption induced by cadmium: effects on P450 cholesterol sidechain cleavage and 3b-hydroxysteroid dehydrogenase enzymes in cultured human trophoblasts. Biol Reprod 67:178–183.

Khojastehfar A, Aghaei M, Gharagozloo M, Panjehpour M (2014) Cadmium induces reactive oxygen species-dependent apoptosis in Mcf-7 human breast cancer cell line. *Toxicol Mech Methods* 18:1–18.

Malik A, Muhammad I, Ramli R, Muhammad L Md Isa, Nur Aizura MA, Ainin Azwani AR, Mohd Nur NA (2017) Male Infertility: The Effect of Natural Antioxidants and Phytocompounds on Seminal Oxidative Stress. Diseases 5: 9. doi: 10.3390/diseases5010009.

Mallya R, Chatterjee PK, Vinodini NA, Chatterjee P, Mithra P (2017) Moringa oleifera Leaf Extract: Beneficial Effects on Cadmium Induced Toxicities - A Review. *J Clin Diag Res* 11:CE01-CE04.

Mohamed SO, Ahmed N, Hassan SZ, Ahmed E, Abdel M (2014) Effect of Physalis peruviana L. on Cadmium-Induced Testicular Toxicity in Rats. *Biol Trace Elem* Res 159:278–287. doi 10.1007/s12011-014-9955-1.

Mohammed IYE, Manal FE, Ebtesam MAO, Ahmed EAM (2017) Protective Effect of Fragaria ananassa Crude Extract on Cadmium-Induced Lipid Peroxidation, Antioxidant Enzymes Suppression, and Apoptosis in Rat Testes. *Int J Mol Sci* 18:E857. doi:10.3390/ijms18050957.

Moron MA, Depierre JW, Mannervick B (1979) Levels of glutathione, glutathione reductase and glutathione-s transferase activities in rat lung and liver. *Biochem Biophys Acta* 582:67-78.

Narayana K (2008) An aminoglycoside antibiotic gentamycin induces oxidative stress, reduces antioxidant reserve and impairs spermatogenesis in rats. *J Toxicol Sci* 33: 85–96.

Ogawa Y, Itoh M, Hirai S, Suna S, Naito M, Qu N, Terayama H, Ikeda A, Miyaso H, Matsuno Y, Komiyama M, Mori C (2013) Cadmium exposure increases susceptibility to testicular autoimmunity in mice. *J Appl Toxicol* 33:652–660.

Oguzturk H, Ciftci O, Aydin M, Timurkaan N, Beytur A, Yilmaz F (2012) Ameliorative effects of curcumin against acute cadmium toxicity on male reproductive system in rats. *Andrologia* 44:1-7.

Ponnusamy, Pari M, Pari L (2011) Protective role of diallyl tetrasulfide on cadmium-induced testicular damage in adult rats: A biochemical and histological study. *Toxicol Indus Health* 5:407-416.

Predes Fde S, Diamante MAS, Foglio MA, Dolder H (2016) Effects of *Arctium lappa* on cadmium-induced damage to the testis and epididymis of adult wistar rats. *Biol Trace Elem Res* 173:362–371.

Robb GW, Amann PR, Killian GJ (1978) Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *J Reprod Fert* 54: 103-107.

Roy VK, Krishna A (2011) Regulation of leptin synthesis during adipogenesis in males of males of a vespertilionid bat, Scotophilus heathi. *J Exp Biol* 214:1599-1606.

Santos FW, Oro T, Zeni G, Rocha JBT, doNascimento PC, Nogueira CW (2004) Cadmium induced testicular damagr and its response to administration of succimer and diphenyl diselenide in mice. *Toxicol Lett* 152:255-63.

Satoh K (1978) Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clin Chim Acta J* 90:37–44.

Shivji KK, Kenny MK, Wood RD (1992) Proliferating cell nuclear antigen is required for DNA excision repair. Cell 69:367–374.

Shukla R, Kumar M (2009) Role of Panax ginseng as an antioxidant after cadmium-induced hepatic injuries. *Food Chem Toxicol* 47:769-773.

Srivastava PM, Calofiore P, Macisaac RJ, Patel SK, Thomas MC, Jerum G, Burrel LM (2008) Prevelance and predictors of cardiac hypertrophy and dysfunction in parients with type 2 diabetes. *Clin Sci (Lond)* 114:313-320.

Syed U, Ganapasam S (2017) Beneficial influencial effects of ellagic acid in biochemical indexes associated with experimentally induced colon carcinogenesis. *J Can Res Ther* 13:62-68.

Syed U, Ponnuraj N, Ganapasam S (2014) Antiproliferative and apoptotic-inducing potential of ellagic acid against 1,2-dimethyl hydrazine-induced colon tumorigenesis in Wistar rats. *Mol Cell Biochem* 388:157–172. DOI 10.1007/s11010-013-1907-0

Syed U, Ponnuraj N, Ganapasam S (2015) Ellagic acid inhibits proliferation and induced apoptosis via the Akt signaling pathway in HCT-15 colon adenocarcinoma cells. *Mol Cell Biochem* 399:303–313. DOI 10.1007/s11010-014-2257-2

Tasaki M, Umemura T, Maeda M, Ishii Y, Okamura T, Kuroiwa TIY, Hirose M, Nishikawa A (2008) Safety assessment of ellagic acid, a food additive, in a sub-chronic toxicity study using F344 rats. *Food Chem Toxicol* 46:1119–1124.

Tejeda RI, Mitchell JC, Norman A, Marik JJ, Friedman S (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil and Steril* 42:87-91.

Tremellen K (2008) Oxidative stress and male infertility-a clinical perspective. *Hum Reprod* 14:243-258.

Turk G, Ceribasi, AO, Sakin F, Sonmez M, Atessahin A (2010) Antiperoxidative and antiapoptotic effects of lycopene and ellagic acid on cyclophosphamide-induced testicular lipid peroxidation and apoptosis. *Reprod Fertil Dev* 22:587–596.

Waalkes MP, Rehm S, Devor DE (1997) The effects of continuous testosterone exposure on spontaneous and cadmium-induced tumors in the male Fischer (F344/NCr) rat: loss of testicular response. *Toxicol Appl Pharmacol* 142:40-46.

Wang D, Chen Q, Liu B, Li Y, Tan Y, Yang B (2016) Ellagic acid inhibits proliferation and induces apoptosis in human glioblastoma cells. *Acta Cir Bras* 31:143-149. DOI: 10.1590/s0102-865020160020000010

Wang D, Chen Q, Tan Y, Liu B, Liu C (2017) Ellagic acid inhibits glioblastoma growth in vitro and in vivo. *Oncol Rep* 37:1084-1092.

Wang YJ, Yan J, Yin F, Li L, Qin YG, Meng YC, Lu RF, Guo L (2016) Role of autophagy in cadmium-induced testicular injury. *Hum Exp Toxicol* 21:1–10.

Wyrobek AJ, Watchmaker G, Gordon L (1984) Sperm morphology testing in mice. In: Hand-book of mutagenecity test procedures. *Amsterdam: Elsevier Science* 733–735.

Xu LC, Sun H, Wang S, Song L, Chang HC, Wang XR (2005) The roles of metallomethionein on cadmium-induced testes damages in Sprague-Dawley rats. *Environ Toxicol Pharmacol* 20:83-87.

Yari A, Asadi MH, Bahadoran H, Dashtnavard H, Imani H, Naghii MR (2010) Cadmium toxicity in spermatogenesis and protective effects of L-carnitine in adult male rats. *Biol Trace Elem* Res 137:216-225.

Yari A, Sarveazad A, Asadi E, Raouf Sarshoor J, Babahajian A, Amini N, Amidi F, Bahadoran H, Joghataei MT, Asadi MH, Shams A (2016) Efficacy of *Crocus*

sativus L. on reduction of cadmium-induced toxicity on spermatogenesis in adult rats. *Andrologia* 48:1244-1252.

Yousef AI, El-Masry OS, Yassin EH (2016) The anti-oncogenic influence of ellagic acid on colon cancer cells in leptin-enriched microenvironment. *Tumor Biol* 37:13345-13353. DOI 10.1007/s13277-016-5284-7

Zhao M, Tang SN, Marsh JL, Shankar S, Srivastava RK (2013) Ellagic acid inhibits human pancreatic cancer growth in Balb c nude Mice. *Cancer Lett* 337:210–217.

VIII. APPENDIXES

1. List of Acronyms

%	Percentage
°C	Degree celsius
°F	Degree fahrenheit
µg	microgram
µl	microlitre
µm	micrometer
ABC	Avidin-biotinylated peroxidase complex
AO	Acridine Orange
ATSDR	The Agency for Toxic Substances and Disease Registry
Bax	Apoptotic regulator
Bcl-2	B-cell lymphoma
CAT	Catalase
Cd	Cadmium
$CdCl_2$	Cadmium Chloride
CDNB	1-Chloro-2,4-dinitrobenzene
DAB	3,3′-diaminobenzidine tetrachloride
DNA	Dioyribonucleic acid
DTNB	5,5’-dithiobis-(2-nitrobenzoic acid)
EA	Ellagic acid
EDTA	Ethylene diamine tetraacetic acid
ECL	Enhanced chemiluminescence
FSH	Follicular Stimulating Hormone
G2	Second Growth Phase
GLUT4	Glucose transporter type 4
GLUT8	Glucose transporter type 8
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
GS-TNB	Glutathione adduct of GSH
H_2O_2	Hydrogen Peroxide
IARC	The International Agency For Research on Cancer

IU/L	International unit per litre
K_2HPO_4	Dipotassium phosphate
Kg	Kilogram
KH_2PO_4	Monopotassium Phosphate
Ki	Potassium iodide
LH	Leutinising Hormone
LOO	Lipid peroxidation
M	Molar concentration
M	Motile
MAPK	Mitogen-activated protein kinase
MDA	3,4-Methylenedioxyamphetamine
Mg	milligram
Min	minute
Ml	millilitre
mM	millimolar
MW	Molecular weight
Na_2HPO_4	Disodium phosphate
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
Nm	Nanomole
NM	Non Motile
NOAEL	No-observed adverse effect level
NOEL	No-observed effect level
p53	Tumor protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
pH	Power of Hydrogen
PVDF	Polyvinylidene fluoride
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
RT-PCR	Reverse Transcriptase Polymerase Chan Reaction
SDS	Sodium dodecyl sulphate
Sec	Second
SOD	Superoxide dismutase

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Tetracyclic acid
Tris-HCl	Tris hydrochloride
UV-VIS	Ultraviolet visible
Wt	Weight

2. List of Seminar/Workshop/Program attended and participation

Sl. No.	Seminar/Workshop/Program attended and participation	Date
1	National Seminar on Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India (BCUNRNEI) organized by Department of Botany, Mizoram University, Aizawl. Participated in oral presentation on "Protective effect of Ellagic acid against Cadmium Chloride-induced testicular toxicity in rats: A behavioural, biochemical and molecular approach".	30th-31st March, 2017
2	The Workshop on Mechanisms of adaptation in the Temporal Environment organized at Department of Zoology, Mizoram University, Aizawl, Mizoram was attended.	23rd May, 2017
3	Participation in the Science Communication Workshop (SciComm 101) organized by Department of Biotechnology, Mizoram University Sponsored by The Wellcome Trust/DBT India Alliance.	6th June, 2017
4	Participation in the National Level Workshop on Biostatistics and Bioinformatics organized by Department of Biotechnology, Mizoram University sponsored by Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi.	01-07th Sept., 2016
5	Participation at technical Session/Exhibition in Mizoram Science Congress held at Mizoram University.	14th Oct., 2016
6	The Outreach Program on Human Health and Biological Timing organized at Department of Zoology, Mizoram University, Aizawl, Mizoram was attended.	22nd May, 2017

National Seminar
on

Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India (BCUNRNEI)

30th - 31st March 2017

Certificate

This is to certify that Prof./Dr./Mr./Mrs./Ms. Bidanchi R. Momin of Dept. of Zoology, MZU has participated in the National Seminar on Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India and chaired/delivered an oral presentation/invited lecture on Protective effect of Ellagic acid against cadmium chloride-induced testicular toxicity in rats: A behavioural, biochemical and molecular approach.
organized by Department of Botany, Mizoram University, Aizawl.

Vice Chancellor
Mizoram University

Convenor
BCUNRNEI, Mizoram University

CERTIFICATE OF ATTENDANCE

This is to certify that

Bidanchi R. Momin

has attended
The Workshop on
Mechanisms of adaptation in the Temporal Environment
May 23, 2017

Organized at: Department of Zoology, Mizoram University, Aizawl, Mizoram

Prof. Vinod Kumar	***Prof. G Gurusubramanian***	***Prof. N. Senthil Kumar***	***Dr. Amit Kumar Trivedi***
President, InSC	***Chairman***	***Coordinator, State Biotech Hub***	***Convener***

The Wellcome Trust/DBT India Alliance

presents

Certificate of Participation

to

Bidanchi R Momin

in recognition of participation in the ***Science Communication Workshop (SciComm 101)***

held on 6 June, 2017 at Mizoram University, Aizawl

Dr. Shahid Jameel
Chief Executive Officer

BIOINFORMATICS INFRASTRUCTURE FACILITY (BIF)
DEPARTMENT OF BIOTECHNOLOGY
Mizoram University
(Accredited with 'A' Grade by NAAC)
Aizawl – 796004

Certificate

Certified that Bidanchi R. Momin *Participated / acted as a Resource person in the Workshop/ Training on* National Level Workshop on Biostatistics and Bioinformatics *held during* 01-07 Sept, 2016 *organized by Department of Biotechnology, Mizoram University sponsored by Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi.*

(Prof. R. Lalthantluanga)
Vice-Chancellor
Mizoram University

Professor
जीवविज्ञानविभाग
Department of Zoology
मिजोरम विश्वविद्यालय
Mizoram University
आइजल-796 004
Aizawl-796 004

(Prof. N. Senthil Kumar)
Coordinator, DBT-BIF Centre
Mizoram University

CERTIFICATE

THIS IS TO CERTIFY THAT

Bidanchi R. Momin

has participated at Technical Session/ Exhibition in Mizoram Science Congress held at Mizoram University during 13th - 14th October, 2016

(Dr. R. LALFAKZUALA)	(Dr. LALDINPUIA)	(Dr. R.K. LALLIANTHANGA)
Secretary	Jt. Secretary	Chairman

MIZORAM SCIENCE CONGRESS 2016 ORGANISING COMMITTEE

Organized by: MISTIC, MSS, MAS, STAM, MMS, GSM & BIOCONE
Sponsored by: NEC, DST (SERB) & MISTIC

CERTIFICATE OF ATTENDANCE

This is to certify that

Bidanchi R. Momin

has attended
The Outreach Program on
Human Health and Biological Timing
May 22, 2017

Organized at: Department of Zoology, Mizoram University, Aizawl, Mizoram

Prof. Vinod Kumar	*Prof. G Gurusubramanian*	*Prof. N. Senthil Kumar*	*Dr. Amit Kumar Trivedi*
President, InSC	*Chairman*	*Coordinator, State Biotech Hub*	*Convener*

GONADOPROTECTIVE EFFECT OF ELLAGIC ACID IN CADMIUM-INDUCED TESTICULAR TOXICITY IN WISTAR ALBINO RATS

Abstract submitted in partial fulfilment of the requirements for the degree of Master of Philosophy in Zoology

By

Bidanchi R. Momin

Registration No: MZU/M.Phil/394 of 26.05.2017

Under the Supervision of

Prof. G. Gurusubramanian

Department of Zoology

Department of Zoology
School of Life Sciences
Mizoram University
Aizawl-796004, Mizoram

2017

INTRODUCTION

Cadmium is a toxic heavy metal element, which probably cause infertility by impairment in spermatogenesis (Yari et al., 2016). With high levels of exposure to toxic chemicals including heavy metals, released into the environment and introduced in the food chain, the health of living organisms is affected, sometimes with fatal consequences (Predes et al., 2016). In fact, the International Agency for Research on Cancer classified Cd as a known human carcinogen in 1993 (IARC, 1993) and Cd is ranked the 7^{th} toxicant in the Priority List of Hazardous Substances of the Agency for Toxic Substances and Disease Registry (ATSDR, 2007). Recent studies have demonstrated that the testis is sensitive to cadmium, but studies investigating cadmium-induced testicular injury have not yet clearly revealed the underlying mechanisms (Wang et al., 2016). Also, it has been shown that successful spermatogenesis and the achievement of optimal sperm function requires protection from increased levels of oxidative stress (Correia et al., 2016).

Experimentally, it is known that cadmium is toxic to both the reproductive and immune systems, in addition to its nephrotoxicity and hepatotoxicity (Ogawa et al., 2013). It will be determined that acute $CdCl_2$ exposure caused a significant reproductive damage via increased oxidative stress (increased TBARS levels and decreased SOD, CAT, GPx and GSH levels), histological alterations (necrosis, oedema etc.) and spermatological damage (decreased sperm motility and sperm concentration and increased abnormal sperm rate) in male rats (Oguzturk et al., 2012) . Also, exposure to low dose of $CdCl_2$ induces no significant disturbance in spermatogenesis, but, it does change the immunological microcircumstances in the testis, resulting in increased susceptibility to testicular autoimmunity (Ogawa et al., 2013). So, in order to neutralize the damaging ROS, aerobic cells are provided with extensive antioxidant defence mechanisms consisting mainly of antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidase etc.) and small molecule antioxidant (e.g., glutathione, N-acetyl-cysteine, vitamin E and vitamin C).

Androgenesis in the testes involves multi-critical steps that start with the synthesis of cholesterol, followed by its transport within the steroidogenic testicular tissues and its metabolism to form steroid biosynthesis. StAR (steroidogenic acute regulatory protein) protein an essential and limiting factor in testicular Testosterone synthesis, responsible for the transport of cholesterol into mitochondria. Testosterone synthetic enzymes in Leydig cells

include cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme, cytochrome P450 17-hydroxysteroid dehydrogenase ($P450_{17\alpha}$) and 17-αhydroxysteroid dehydrogenase (17-βHSD). P450scc converted cholesterol to pregnenolone that is catalyzed by $P450_{17\alpha}$ to produce 17-hydroxyprogesterone and androstenedione, with the latter then being converted to Testosterone by 17-βHSD). The 3β-HSD complex is responsible for the conversion of Pregnenolone to progesterone, 17α-Hydroxypregnenolone to 17α-hydroxyprogesterone, DHEA to androstenedione, Androstenediol to testosterone and Androstadienol to androstadienone (Ji et al., 2010; Alkhedaide et al., 2016).

The Bcl-2 family is the best characterized protein family involved in the regulation of apoptotic cell death, consisting of anti-apoptotic and pro-apoptotic members. The anti-apoptotic members of this family, such as Bcl-2 prevent apoptosis either by sequestering proforms of death-driving cysteine proteases called caspases (a complex called the apoptosome) or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and AIF (apoptosis-inducing factor) into the cytoplasm. In contrast, Bax is a proapoptotic marker, which is increased in the early stages of intoxication and disease. Bax also trigger the release of caspases from death antagonists via heterodimerization and also by inducing the release of mitochondrial apoptogenic factors into the cytoplasm via acting on mitochondrial permeability transition pore, thereby leading to caspase activation. Thus, the Bcl-2 family of proteins acts as a critical life-death decision point within the common pathway of apoptosis (Eleawa et al., 2014; Alkhedaide et al., 2016). The increased production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) allows the synthesis of cholesterol by activating the enzymes of cholesterol biosynthesis, including cholesterol ester hydrolase. Furthermore, LH and FSH production regulates the uptake of cholesterol esters. $CdCl_2$ administration down regulates the expression of 3β-HSD and 17β-HSD enzymes, and serves a critical role in steroidogenesis (Alkhedaide et al., 2016).

Ellagic acid, a member of flavanoids (EA; $C_{14}H_6O_8$; MW: 302.202; 3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1] benzopyran-5,10-dione) has been receiving the most attention because it has potent antioxidant activity, radical scavenging capacity, chemopreventive and antiapoptotic (Turk et al., 2010; Ceribasi, et al., 2010) properties. It is believed that EA functions either by countering the negative effects of oxidative stress by directly acting as an antioxidant or by activating / inducing cellular antioxidant enzyme systems (Atessahin et al., 2010). The no-observed-effect level (NOEL) will be estimated to

be 5% (3011 mg/kg b.w./day) for males and the no-observed-adverse-effect level (NOAEL) and NOEL in females will be estimated to be 5% (3254 mg/kg b.w./day) and <1.25% (778 mg/kg b.w./day), respectively (Tasaki et al., 2008). On the other hand, some studies have reported the failure of antioxidant therapy in limiting cadmium induced testicular damage (Alkhedaide et al., 2016). Hence, the present study will be designed to investigate the protective role of EA in cadmium induced testicular toxicity. However, to date, there is no available information on the impact of EA on testicular toxicity after Cd exposure in rats. Therefore, the aim of the present study will be to investigate whether ellagic acid (EA) has protective effect on cadmium chloride ($CdCl_2$)-induced testicular and spermatozoal toxicity associated with the oxidative stress.

AIMS AND OBJECTIVES

To evaluate the protective effect of ellagic acid against cadmium -induced testicular toxicity in rats with respect to:-

- Oxidative stress (MDA) and antioxidant enzymes (SOD, GST, GSH) concentrations.
- Sperm quality and serum hormone (LH, FSH, estradiol and testosterone) analyses.
- Histopathological analysis and DNA damage detection.
- Assessment of modulation of steroidogenic, apoptosis, cell proliferation and cytoprotection activities by immunohistochemical, Western blotting and RT-PCR.

REVIEW OF LITERATURE

Cadmium is a commonly used chemical in agricultural and industrial operations and is released into the environment causing atmosphere, soil, and water pollution. Cadmium can accumulate in the body for up to 40 years, so it can harm human health over an extended period of time (Wang et al., 2016). There has been a surge in Cd pollution of the environment partly due to urbanization resulting in increase in Cd exposure in human beings which is subsequently leading to higher incidence of Cd-related diseases (Adaramoye et al., 2016). Phenolic phytochemicals such as ellagic acid (EA) are important components of fruits and vegetables and are partly responsible for their beneficial health effects against oxidation-linked chronic diseases such as cancer and cardiovascular diseases (Atessahin et al., 2010). Ellagic acid (EA) has received particular attention because of its wide array of biological properties (Tasaki et al., 2008). Previous studies observed that EA significantly improved the

damages in sperm parameters, oxidant/antioxidant balance and testicular apoptosis induced by chemotherapeutics such as cisplatin (Turk et al., 2008) and cyclophosphamide (Turk et al., 2010a). Experimental studies have shown that Cd can induce oxidative stress and apoptosis of germ cells (Khojastehfar et al., 2014). Several agents have been reported to mitigate Cd-induced toxicity in testis (Jahan et al., 2014). The formation of multinucleated giant cells in Cd-intoxicated rats suggests continuous degeneration of spermatogenic epithelium and appears to represent a non-specific reaction to injury (Ponnusamy and Pari, 2011). In view of the above-mentioned, the expedition for safer and active agents that can alleviate cadmium-induced pathologies is required.

Bax is a proapoptotic marker, which is increased in the early stages of intoxication and disease. Conversely, Ki-67 expression is a marker for cell proliferation. High expression of Ki-67 has been reported in cells during G2 and early M stages of cell growth. The results of the present study confirmed that Bax markers will be increased in the spermatogenic cells of the $CdCl_2$-treated group, while Ki67 will be decreased. It has been reported that a decrease in StAR activity is associated with a decrease in steroidogenesis (Alkhedaide et al., 2016). Previous studies have shown that, $CdCl_2$ intoxication increases the expression of pro-apoptotic proteins p53 and Bax, while reducing the expression of Bcl-2, an anti-apoptotic protein. CdCl2 induced concomitant increases in expression of P53 and Bax but led to decreased expression of Bcl-2, suggesting their important roles in testis damage, apoptotic cell death and decreased semen parameters elicited by $CdCl_2$ (Eleawa et al., 2014).

Hence, in the light of prospective antioxidant activity of flavonoids, we hypothesized that prophylactic treatment of EA may have protective effects against $CdCl_2$ induced on testicular toxicity by interfering with pathogenetic pathway and oxidative processes. Therefore the present study will be undertaken to investigate the protective role of EA against $CdCl_2$ induced deteriorated epididymal sperm characteristics, damaged oxidant/antioxidant balance and testicular apoptosis in rats.

MATERIALS AND METHODS

Animals and experimental design

The animal care and handling was done according to the guidelines approved by the Animal Ethics Committee of Mizoram University, Aizawl, India (MZUIAEC 17-18/1). Usually, three

months old male Wistar rats (150 - 180 g) was procured and maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and 12 h of light and dark cycle, respectively. The animals had free access to the standard food and water. The animals were divided into four groups where six animals each were housed in a polypropylene cage containing wood powder (procured locally) as bedding throughout the experiment:

Group I: Control where the animals were given any treatment.

Group II: Cadmium Chloride alone (5 mg/kg b.wt of cadmium chloride in distilled water for 30 and 60 consecutive days) (Alkhedaide et al., 2016).

Group III: Ellagic acid alone (2 mg/kg b.wt. of ellagic acid in alkaline solution) (Atessahin et al., 2010).

Group IV: $CdCl_2$+Ellagic acid (5 mg/kg b.wt. of cadmium first and then 2 mg/kg b.wt. of ellagic acid administration).

The animals from all groups were sacrificed after 60 days $CdCl_2$ and ellagic acid administration.

Sperm analysis

The technique of Wyrobek et al. (1984) was adopted for sperm abnormality. To evaluate the sperm abnormalities, the sperm suspension will be stained with eosin, dropped on slides to determine the motility, abnormality (using Olympus microscope by objective (40×). Abnormality was classified in head and tail. Abnormal sperm cells will be counted and the percentage calculated. To assess motility, the sperms was classified as motile sperm (M) and non-motile (NM). To evaluate the daily sperm production in testes, spermatozoa was counted as per the standard protocol of Robb et al. (1978). Motile sperm cells was counted and the percentage calculated.

DNA Damage – Acridine orange staining and TUNEL assay

Sperms with normal DNA fluorescence inhibit green colour and those with abnormal DNA fluorescence red or yellow. It is important to score DNA normality immediately after staining (Tejeda et al., 1984).

Determination of oxidative stress (lipid peroxidation) and Antioxidant enzymes

Lipid peroxidation (MDA) (Satoh et al., 1978), superoxide dismutase (SOD) (Asada et al., 1974), reduced glutathione (GSH) (Moron et al., 1979) and glutathione S-transferase (GST) (Habig et al., 1974) was estimated by following standard protocols.

Measurement of reproductive hormones in the serum

Concentration of different hormones (testosterone, leuteinizing hormone and follicle stimulating hormone) was analyzed using ELISA reader in the serum by following standard protocols as per the manufactures instructions.

Histopathological and immunohistochemical study of the testes

Sections (5 μM) were cut using a microtome from the left and right testes of the rats and will be processed for general histological staining using hematoxylin and eosin stain (Sigma-Aldrich), based on previously stated protocols (Bancroft and Gamble, 2002). The sections were deparaffinized in xylene and dehydrated in various ascending concentrations of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. Blocking of non-specific binding was conducted using normal horse serum (Sigma-Aldrich) for 20 min at room temperature. Subsequently, the testicular sections were incubated with the following primary antibodies for 30 min: 1) Anti-B-cell lymphoma 2-associated X protein (**Bax**), 2) Anti-**Bcl-2** (B-cell lymphoma 2), 3) anti-**PCNA**, 4) anti-**HSP 70**, and 5) anti-**3β-HSD** at dilutions of 1:1,000 for each antibody. Subsequently, the immunoperoxidase technique [avidin-biotin complex (ABC) kit; Lab Vision Corporation, Fremont, CA, USA) was used to stain the testicular sections and the binding sites will be detected with ABC chromogen. For rinsing between each step, phosphate-buffered saline was used. Finally, all sections were counterstained with hematoxylin and eosin stain and images were viewed microscopically and captured using a digital camera.

Western blotting method to detect Bcl_2 expression

Western blot analysis for Bcl_2 was carried out as previously described by Clark et al. (1994).

Statistical analysis

All values were presented as mean ± S.E.M. Differences were considered to be significant at $p < 0.05$. One-way ANOVA and post hoc Tukey-high significant differrence

(HSD) test was used to determine differences between groups. The SPSS / PC Program was used for the statistical analysis.

SUMMARY OF RESULT

- $CdCl_2$ administration significantly decreased sperm count, sperm motility and sperm morphology of rats. The changes observed in the above agree with the reports, which demonstrated that cadmium impairs testicular function.

- The significant reduction in sperm count, motility and morphology observed in this study following $CdCl_2$ administration may be associated to impairment of steroidogenesis consequent to reduced secretion of testosterone (from testis) caused by administration of $CdCl_2$.

- Regardless of the mechanism of induction of oxidative stress in cells by cadmium, increase in ROS occurs, which leads to the damage and changes in their structure and metabolism.

- Studies have shown that exposure to cadmium causes lipid peroxidation, which leads to oxidative stress. SOD is an enzymatic antioxidant that converts superoxide radicals into hydrogen peroxide and oxygen, which is further converted into water.

- A significant reduction in the activities of both SOD and GST, but significant rise in the MDA levels in testicular tissues of rats administered with $CdCl_2$. The derangement in the lipid peroxidation and anti-oxidant status of testicular tissues might be a contributing factor in the reduction of testosterone secretion with resultant poor sperm quality.

- EA significantly increased SOD and GST activities, thus enhancing the anti-oxidant system in the testicular tissues. This is in agreement with the previous studies.

- EA improved sperm quality in rats. Pre-treated with EA also prevented $CdCl_2$ – induced poor sperm quality by inhibiting lipid-peroxidation.

- EA enhances testicular oxidative status. The study also shows that EA pre-treatment prevents cadmium chloride – induced testicular toxicity by increasing the tissue

enzymatic antioxidant activities (SOD and GST) and reducing lipid peroxidation (MDA).

- Immunohistochemical staining of sections from the treated rats revealed only PCNA-positive spermatogonia. Bax expression was also assessed in sertoli cells, spermatogonia and spermatocytes where deficiency of Bax results in increased apoptosis and testicular atropy.

- Bcl-2 an antiapoptotic gene was significantly downregulated, confirming the apoptotic effect due to cadmium exposure.

- Varied effects of Cadmium under different conditions in different cell lines have rendered the possibility of a specific mechanism evasive. Such being the case, it is necessary to profile the effects comprehensively and identify the common themes in cadmium toxicity for further progress to be made in this regard.

- The findings in the study showed that pre-treatment of rat model with ellagic acid prevented $CdCl_2$ – induced reproductive toxicity by improving sperm quality and enhancing testicular lipid peroxidation status.

- To date information of the pharmacological properties of EA in the context of hepatoprotection is very limited; therefore it is important to extend the knowledge about the action mechanisms of this flavonoid and to perform additional preclinical studies in vitro and in vivo models.

- Also, it is worth mentioning that the effects of EA and pomegranate juice (containing EA) are being evaluated in phase I, II and III clinical trials (ClinicalTrials.gov Identifier: NCT00455416; NCT02263378; NCT01916239), though these studies are focused mainly on its anticarcinogenic activity and in hyper-pigmentation disorders treatment.

- These pioneer studies might be a breakthrough in the search for natural compounds with beneficial activities to humans and serve as a basis in the near future to begin tests seeking to prevent, reduce or eliminate testicular damage caused by the exposure to xenobiotics or disease.

- By better defining the problems, learning about the mechanisms responsible for adverse effects, and developing panels of relevant biomarkers, we will make progress towards preventing future adverse effects on male reproductive health.

- In conclusion, this study suggests that cadmium causes testicular toxicity, but the flavonoid compound ellagic acid is protective in terms of sperm count, motility and morphology. The histo-architecture of testis was also restored by ellagic acid confirming its protective effect.
- This action of ellagic acid may be closely related to its antioxidant and antiapoptotic property, which needs further research. Therefore, ellagic acid may be useful in epileptic patients on long-term cadmium treatment if proved effective in clinical trials.

Bidanchi R. Momin

20th Nov., 2017