UNIVERSITI PUTRA MALAYSIA

EFFECT OF EDIBLE BIRD NEST ON TESTICULAR HISTOMORPHOLOGY, OXIDATIVE STRESS LEVEL AND APOPTOTIC BIOMARKER OF RATS EXPOSED TO CADMIUM TOXICITY

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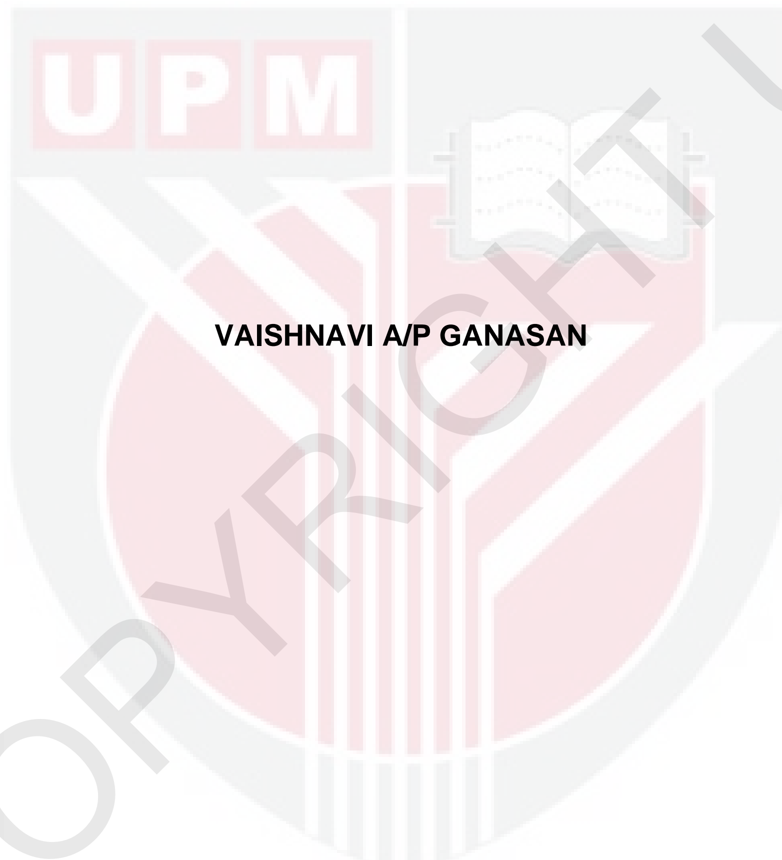

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A project paper submitted to the
Faculty of Veterinary Medicine, University Putra Malaysia
In partial fulfillment of the requirement for the
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CERTIFICATION

It is hereby certified that we have read this project paper entitled " Effect Of Edible Bird Nest On Testicular Histomorphology, Oxidative Stress Level And Apoptotic Biomarker Of Rats Exposed To Cadmium Toxicity", by Vaishnavi A/P Ganasan. In our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Final Year Project.

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LIST OF ABBREVIATIONS

Cd - Cadmium

EBN - Edible bird nest

SOD - Superoxide Dismutase

MDA - Malondialdehyde

mg - Miligram

µl - Microlitre

ml - Mililitre

g - Gram

kg - Kilogram

°C - Degree celsius

% - Percentage

hr – Hour

BW – Bodyweight

$CdCl_2$ - Cadmium chloride

PBS - Phosphate-buffered saline

DPX - Dibutylphthalate Polystyrene Xylene

ANOVA - Analysis of variance

DNA - Deoxyribonucleic acid

CAT - Catalase

TNFa - Tumour necrosis factor alpha

ELISA - Enzyme-linked immunosorbent assay

EAS - Endocrine active substances

EDC - Endocrine disrupting compounds

FSH – Follicle stimulating hormone

LH – Luteinizing hormone

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ROS – Reactive oxygen species

Zn - zinc

Hg – mercury

BTB – Blood-testes barrier

HRI - Heme-regulated inhibitor

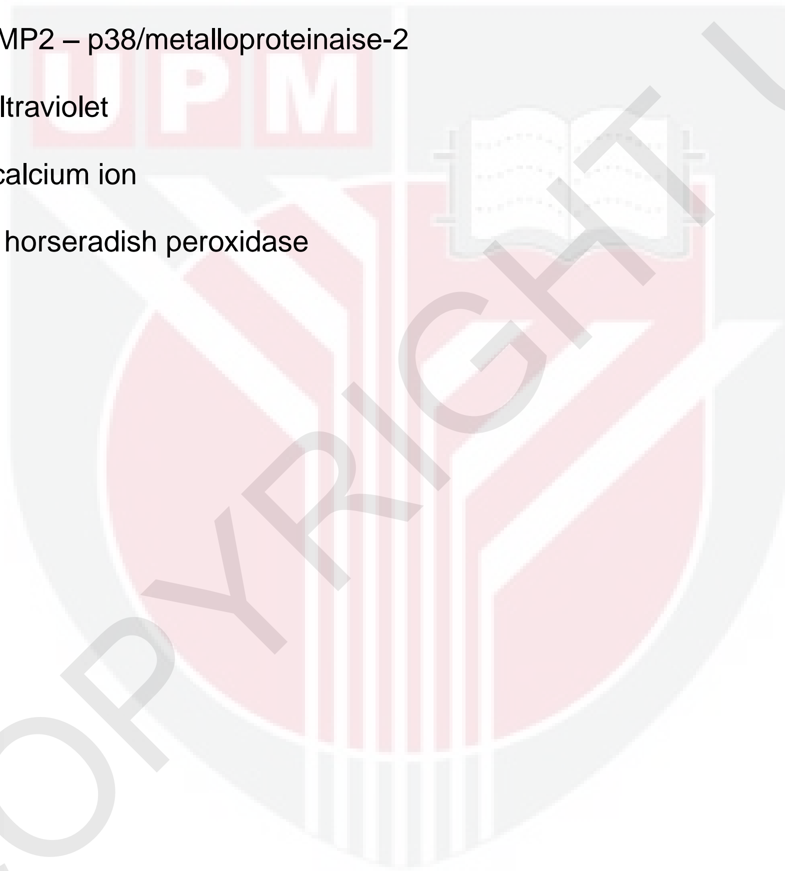
ISR – Integrated stress response

P38/MMP2 – p38/metalloproteinaise-2

UV – ultraviolet

Ca+ - calcium ion

HRP – horseradish peroxidase



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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Tahun Akhir.

Kesan Sarang Burung Boleh Dimakan Terhadap Histomorfologi Testis, Tahap Tekanan Oksidatif Dan Biomarker Apoptotik Tikus Yang Terdedah Kepada Ketoksikan Kadmium.

oleh

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2022

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Kadmium(Cd) ialah logam berat toksik yang terdapat di alam sekitar sebagai bahan pencemar yang berpotensi menyebabkan kerosakan pada organ pembiakan mamalia jantan. Sarang burung yang boleh dimakan (EBN) yang dihasilkan daripada air liur burung walit mempunyai ciri-ciri memperbaiki kesan toksik kadmium pada testis. Tiga puluh tikus Sprague Dawley berumur 10 minggu telah digunakan untuk mengkaji kesan perlindungan EBN terhadap ketoksikan testis yang disebabkan oleh kadmium. Lima tikus dipilih secara rawak dan dibahagi kepada 5: kumpulan kawalan (hanya Nacl 0.9%), empat kumpulan diberi 10mg/kg/hari CdCl2 secara oral dengan empat dos berbeza EBN 0, 60, 90 dan 120mg/kg/hari secara gavage oral. Selepas 21 hari rawatan, tikus dikorbankan dan testis dikumpulkan. Testis kiri digunakan untuk mengkaji perubahan histomorfologi dan homogenat testis kanan digunakan untuk menilai tekanan oksidatif (superoxidase dimutase, malondialdehyde) dan penanda bio apoptosis (caspase 3). Tahap superoksidase dimutase (SOD), malondialdehid (MDA), dan caspase 3 diukur menggunakan kit ujian. Kajian

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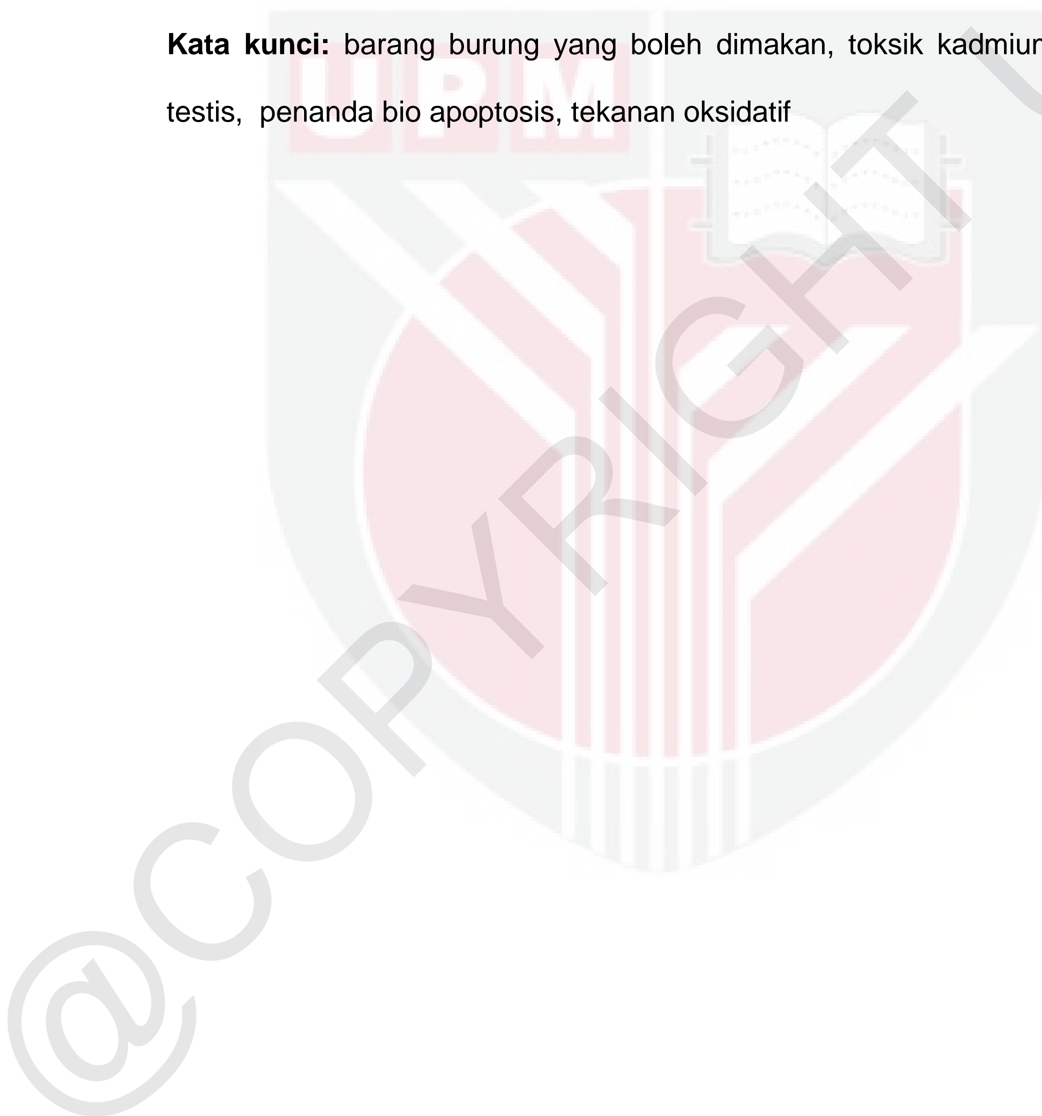
histomorfologi menunjukkan perbezaan yang ketara jika dibandingkan dengan kumpulan kawalan walaupun tahap SOD, MDA, dan caspase 3 tidak signifikan dalam eksperimen ini. Pemarkahan histopatologi untuk kumpulan yang dirawat dengan 0mg/kg EBN+10mg/kg $CdCl_2$ mendapat kedudukan paling rendah berbanding kumpulan rawatan 90mg/kg EBN.

Kata kunci: barang burung yang boleh dimakan, toksik kadmium, histomorfologi testis, penanda bio apoptosis, tekanan oksidatif



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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Final Year Project.

Effect Of Edible Bird's Nest On Testicular Histomorphology, Oxidative Stress Level And Apoptotic Biomarker Of Rats Exposed To Cadmium Toxicity.

by

Vaishnavi A/P Ganasan

2022

Supervisor: Assoc Prof Dr Nurhusien Yimer Degu

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Cadmium(Cd), a toxic heavy metal that is found in the environment as a pollutant, has the potential to cause damage to the male reproductive organs of mammals. Edible bird's nest (EBN) produced from the swiftlet's saliva has the properties of ameliorating the toxic effect of cadmium on testicles. Thirty Sprague Dawley rats aged 10 weeks old were used in the study to evaluate the protective effect of EBN on cadmium-induced testicular toxicity. Twenty-five rats randomly selected and grouped into 5: control group (given only Nacl 0.9%), and four other groups administered with 10mg/kg/day $CdCl_2$ and four graded doses of 0, 60, 90 and 120mg/kg/day of EBN by oral gavage. After 16 days of treatment, the rats were sacrificed and testicles were collected. The left testis was used to study histomorphological changes and the right testis tissue homogenates were used to evaluate oxidative stress (superoxidase dimutase, malondialdehyde) and apoptotic biomarkers (caspase 3). Levels of superoxidase dimutase (SOD), malondialdehyde (MDA), and caspase 3 were measured using ELISA. Histomorphological studies showed significant differences in pathological changes

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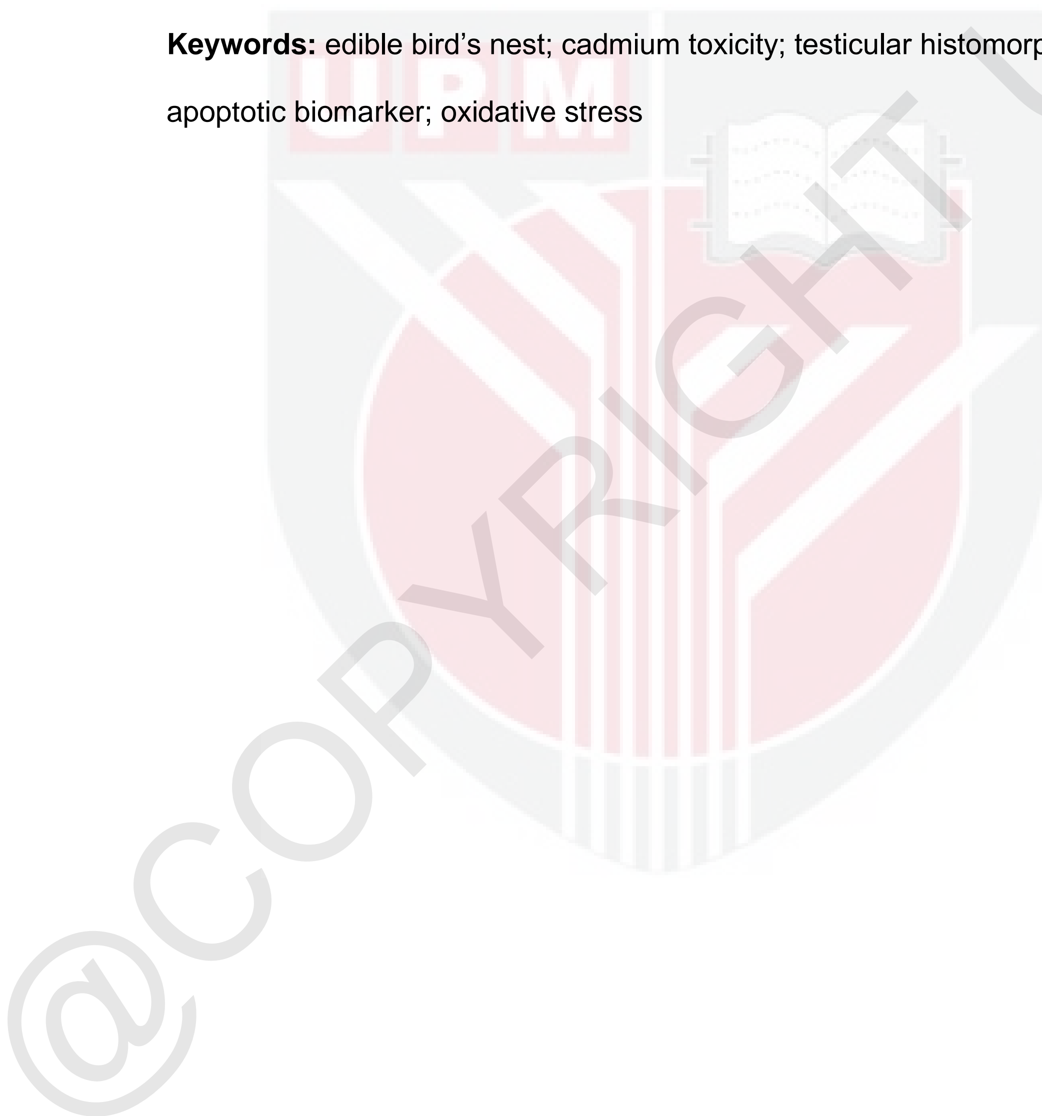
in treated groups when compared with the control group. However, differences in the SOD, MDA, and caspase 3 levels were not significant. Histopathological scoring for groups treated with 0mg/kg EBN+10mg/kg $CdCl_2$ ranked the least compared to 90mg/kg EBN treatment group suggesting a protective role of EBN against Cd toxicity effect on testicular tissues.

Keywords: edible bird's nest; cadmium toxicity; testicular histomorphology; apoptotic biomarker; oxidative stress



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1.0 INTRODUCTION

In industrialized countries, one of the most hazardous environmental and occupational pollutants is cadmium (Järup *et al.*, 2015; Lane *et al.*, 2015). Cadmium is known as toxic heavy metal that generally relates to diseases involving the reproductive system of mammals (Quddus *et al.*, 2021). Cadmium causes toxicity in multiple organs such as the liver, kidney, and several studies found that the testis is very sensitive to cadmium toxicity (Andjelkovic *et al.*, 2019; Waalkes, 2000; Wang *et al.*, 2017; Wang *et al.*, 2021, Babaknejad *et al.*, 2017). Studies conducted in vivo and in vitro have proven that cadmium leads to interruption of the blood-testis barrier, depletion of germ cells, edema of testicles, hemorrhage, necrosis, and deprivation of sperm cells, Leydig cells, and Sertoli cells in mammalian testis (Siu *et al.*, 2009).

Compounds derived from several plants and animals are described to exhibit a mechanism of averting heavy metal toxicity (Dailiah Roopha and Padmalatha, 2011). For decades, Edible Bird Nest (EBN) has been used as beneficial food and tonic which is made of salivary secretion of swiftlet birds. EBN provides a powerful medical effect in boosting the immune system, activating growth factors, and preventing viral infection. EBN function also correlates with cellular division, proliferation, and enrichment in new cell formation due to the presence of EGF as a component in EBN (Albishtue *et al.*, 2018). A recent study proved that cadmium toxicity in the reproductive system is alleviated by supplementation of EBN in female rats and a favorable success rate in reproductive efficacy.

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Edible bird nests expressed antioxidant and proliferating properties by raising the level of Superoxide Dismutase (SOD) activity and improved the VEGF expressions in ovarian tissues and developing structures of ovaries, separately (Quddus *et al.*, 2021). There is a lack of studies on the role of EBN in protecting the male reproductive system against exposure to cadmium toxicity. Therefore, in this study, the effectiveness of EBN supplement in alleviating the adverse effect of Cadmium toxicity on the male reproductive system was evaluated based on assessment of testicular histomorphology, oxidative stress (MDA, SOD level), and Apoptotic biomarker (Caspase 3) using rats as a model.

1.1 Objective

To determine the effect of EBN supplementation on testicular histomorphology, oxidative stress (MDA, SOD level), and apoptotic biomarker (caspase 3) of rats exposed to cadmium toxicity.

1.2 Hypothesis

H_O: Edible Bird Nest supplementation has no effect on the histomorphology, oxidative stress, and apoptosis of testis of rats exposed to cadmium toxicity.

H_A: Edible Bird Nest supplementation has effect on the histomorphology, oxidative stress, and apoptosis of testis of rats exposed to cadmium toxicity.

1.3 Justification

There are several studies showing that EBN ameliorates toxicity of heavy metal such as lead and cadmium in the reproductive system of female rats. However, there is no studies done to prove that EBN is a non-pathological and economical therapeutic approach to the Cadmium toxicity in the male rat reproductive system. Therefore, information in this

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study will enhance the understanding of the effectiveness of EBN supplements in reversing the adverse effect of Cadmium toxicity on male reproductive system which includes testicular histomorphology, oxidative stress (MDA, SOD level), and Apoptotic biomarker (Caspase 3). This study will be supportive evidence to evaluate the efficacy of EBN in treating reproductive disorders due to cadmium toxicity in animals as well as humans.

2.0 LITERATURE REVIEW

2.1 Rats

Rats are selected in several of the experimental studies as research models including research involving cardiovascular, toxicology, and behavioural studies (Jacob, 1999). The majority of in vivo investigations regarding endocrine active substances (EAS) and endocrine disrupting compounds (EDC) have been conducted on rats, which are remarkably similar to humans in terms of the physiological regulation of reproductive functions (Saghir *et al.*, 2012).

2.1.1 The reproductive anatomy and physiology of male rats

Chawla and Jena, (2021) stated that reproductive system of rat comprise of a pair of testes, extra-testicular ducts consisting of efferent ducts, epididymis, vas deferens, accessory sex glands such as prostate, seminal vesicles, ampullary and preputial glands along with urethra and penis (Fig.1). Male gametes which known as spermatozoa is produced from the testicles of the rat. The epididymis covers each testicle which comprises of the head of epididymis, followed by body of epididymis and tail of epididymis from which the ductus deferens emerges and passes through the inguinal canal to connect to the urethra. Inguinal canal of the rats are remain unclosed entire life

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of the rats. This provide a pathway for the testes to get through to the abdominal cavity. The rat testicles will descended into the two distinct scrotal sacs which made up of thin membrane by the age of rat between 7.5 weeks to 10 weeks old.

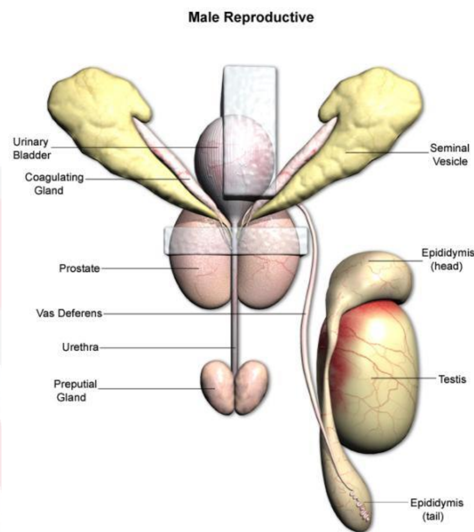

Figure 1: Male reproductive system of male rat (from Male Reproductive and Urinary System - Revised Guides for Organ Sampling and Trimming in Rats and Mice, 2001)

2.1.2 Histomorphology of rat's testis

According to Nahari and Eisa (2016), the histological transverse section of a normal rat testis consist of seminiferous tubules, spermatogonium, primary spermatocytes, secondary spermatocytes, spermatids, matured spermatozoa, Sertoli cells and Leydig cell (Fig.2). Connective tissues present in the interstitial space between the seminiferous tubules which arranged in consistent size and shape (Dollah *et al.*, 2015). Seminiferous tubules are distributed with various stages of cells such as spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa (Nahari and Eisa, 2016). Firman and Simmons (2009) stated that most muroid rats have an apical hook at the anterior part of the head of their sperm.

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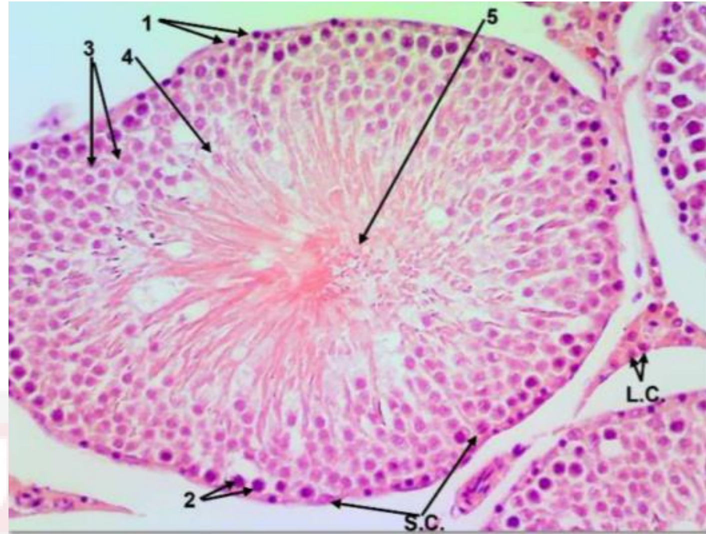

Figure 2: A transverse section in rat testis from the control group shows layers distribution of seminiferous epithelium cells in their different stages inside the seminiferous tubule: 1) Spermatogonia. 2) Primary spermatocytes. 3) Secondary spermatocytes. 4) Spermatids. 5) Mature Spermatozoa, Sertoli cells (S.C.) and Leydig cells (L.C.). X400, H&E (from Nahari and Eisa, 2016).

2.2 Edible Bird's Nest (EBN)

A glutinous salivary secretion from the sublingual glands of male swiftlet birds during the breeding season is known as Edible Bird's Nest (Guo *et al.*, 2006). According to Babji *et al*, (2011), the White nest swiftlet (Aerodramus fuciphagus) and the Black nest swiftlet (Aerodramus Maximus) are the two swiftlet species that are extensively and economically exploited species for the most of the global EBN trading. Since the 16th century, EBN has been traditionally consumed for health benefits in Chinese traditional medicine (Medway, 1969). Nutritional analysis of EBN from the Aerodramus genus nest indicates that the nest primarily composed of proteins (62.0–63.0%), carbs (25.6–27.26%), and fats, lipid (0.14–1.28%) and ash (2.1%) (Yu-Qin *et al.*, 2000). Sialic acid is the major carbohydrate found in EBN. Other important and fundamental components

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of EBN include the trace elements such as calcium, phosphorus, iron, salt, potassium, iodine, and essential amino acids. EBN contains 18 of the 20 different kinds of amino acids that are required by humans (Lee *et al.*, 2017). Moreover, proteases may hydrolyze the glycoproteins in the raw EBN into glycopeptides, resulting in an enzyme-mediated EBN hydrolysate with high bioactivities. This enzymatically hydrolysed EBN proven to provide high quality proteins compared to raw EBN (Ng *et al.*, 2020). EBN exists in two varieties: cave-EBN and house-EBN, which are both commercially available worldwide (Looi and Rahman, 2016).

EBN is scientifically accepted as it has some beneficial properties to treat osteoarthritis, and osteoporosis and also boost the immune system (Chua *et al.*, 2013; Matsukawa *et al.*, 2011; Zhao *et al.*, 2016). According to Roh *et al.*, (2012), stem cells derived from human adipose and fibroblasts showed some convincing proliferative effects of EBN. Therefore, these proliferative properties of EBN could have the potential to enhance the multiplication of spermatogonia and facilitate the spermatogenesis process in the testis (Quddus *et al.*, 2021). EBN also consists of male reproductive hormones such as testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) (Ma and Liu, 2012). Moreover, it was found that EBN administration in male rats which has been castrated had high testosterone and LH level in the serum (Ma *et al.*, 2012). Schulster et al., (2016) demonstrated that another predominant hormone involved in the female reproductive system, estradiol discovered in the extraction of EBN. Notably, estradiol also acts an important part in the male reproductive system where it facilitates spermatogenesis, and regulates libido and erectile function.

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2.3 Cadmium

In the Periodic Table of the elements, naturally produced cadmium (Cd) with chemical properties same as Zn placed in between zinc (Zn) and mercury (Hg). Cd mostly remains as a divalent cation by forming bonds with other elements. For example, it exists as $CdCl_2$. Cd formed naturally about 0.1 part per million of the earth's crust (Hans Wedepohl, 1995). Earlier in the 1960s, Cd has been used commercially in making television screens, lasers, batteries, and pigments of paint, cosmetic products, in a process of galvanizing steel, as a boundary in nuclear fission, and was used together with zinc in weld seals in pipes made of lead (Bernhoft, 2013).

Inhalation and ingestion are the main routes of exposure of humans to cadmium. The most powerful source of cadmium exposure in humans is cigarette smoke (Friberg, 1983). Exposure to cadmium through intake of food that is contaminated with cadmium such as crustaceans, organ meats, leafy vegetables, rice from some parts of Japan and China, or contaminated water due to either mixture of pollutants from industry or from the old Zn/Cd sealed pipes can affect the health for a long period of time (Abernethy *et al.*, 2010).

2.3.1 Cadmium toxicity

The fundamental metabolic characteristic of cadmium is the remarkably longer biological half-life which leads to a nearly irreversible build-up of metal in the body for the entire life. Cd remains in an organism for around 30 years due to its exceptionally long biological half-life, which emphasizes the necessity of treating its toxic effect on the organism (Brzóska *et al.*, 2016). It has been indicated that the mechanism of cadmium toxicity involves the generation of free radicals and reactive oxygen species (Manca *et*

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al., 1994; Stohs *et al*., 2001). Cadmium exposure via inhalation, ingestion, and injection to animals has been reported to activate peroxidation of lipid and oxidative damage within the lungs, liver, and kidneys (Bagchi *et al*., 1997; Manca *et al*., 1994; Stohs *et al*., 2001; Vincent *et al*., 1989). Even though no record shows the generation of free radicals by cadmium in vivo studies, the detailed studies conducted over the past two decades have shown that cadmium has the potential characteristics to stimulate the activation of different signaling pathways and generate free radicals which could impair the DNA and lead to the oxidation of lipid and protein (Liu *et al*., 2008).

Nasiadek (2011) stated that through a range of experimental investigations on female rats, it was discovered that with a relatively long half-life, cadmium accumulates in the reproductive system. Cadmium triggers oxidative stress, germ cell death, and cadmium-induced autophagy in as well as reducing testicular physiological function and spermatogenic activity in the testis (Fig 3). Besides, excess activation of autophagy occurs when the expression of protein receptors that involves autophagy increase due to exposure to cadmium (Ali *et al*., 2022).

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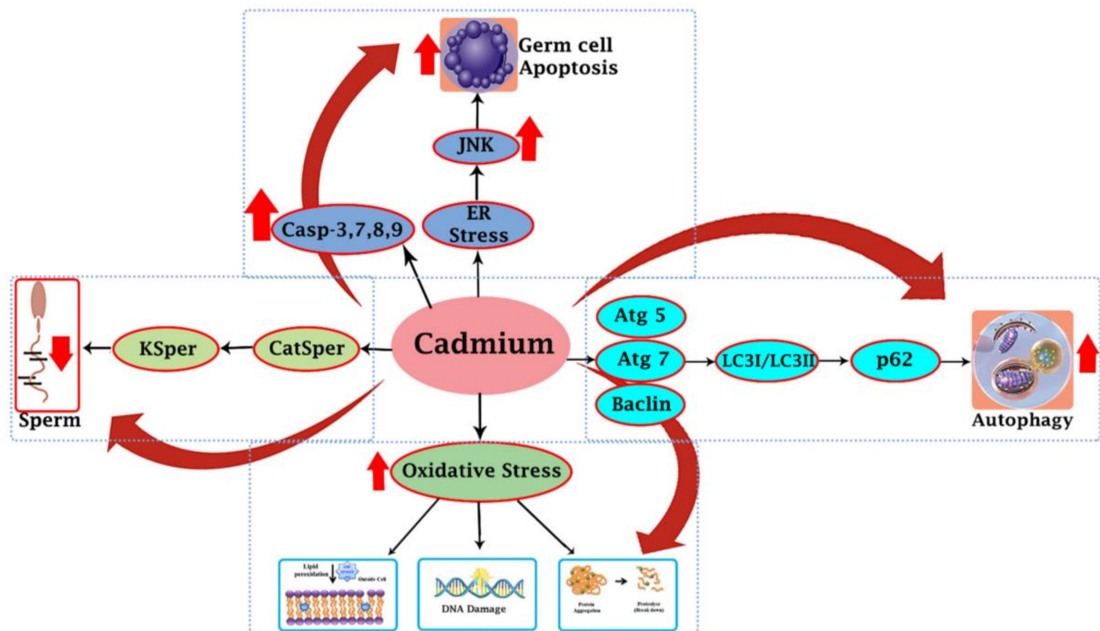

Figure 3 : Mechanism of toxicity of Cd on male reproductive system which involves the germ cell apoptosis, activation of autophagy, reduction in quality of semen (from Ali *et al.*, 2022).

Previous study demonstrates that blood-testis barrier (BTB) is disrupted upon exposure to cadmium (Fig.4). Heme-Regulated Inhibitor (HRI) responsive mitochondrial stress will be activated when the Sertoli cells exposed to cadmium. This stress response in mitochondria will stimulate the Integrated Stress Response (ISR) and p38/matrix metalloproteinase-2 (p38/MMP2) pathway which leads to decrease in tight junction protein known as occludin. Cd- induced reduction in occludin causes loss of function and structure of the BTB (Zhou *et al.*, 2022).

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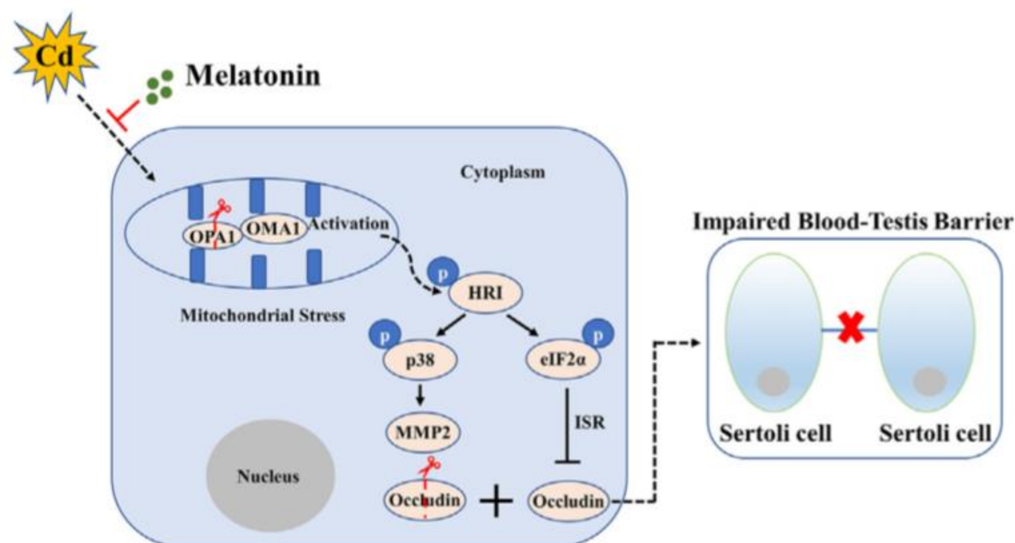

Figure 4: Mechanism of blood-testes barrier impairment due to cadmium toxicity (from (Zhou *et al*., 2022)

2.4 Oxidative stress

Generally known as Reactive Oxygen Species (ROS), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and superoxide radicals (O_2•) are formed as the by-product of the metabolic process by biological systems (Sato *et al*., 2013; Navarro-Yepes et al., 2014). Harm caused by the ROS to the cells is prevented by the antioxidant defence system of the cell that is mostly dependent on enzyme components, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Deponte, 2013). Although ROS are normally formed as by-products of oxygen metabolism and can play a wide range of physiological roles, including cell signaling, environmental stressors like UV, ionising radiation, pollutants, and heavy metals, as well as xenobiotics such as anticlastic drugs play a significantly role in increasing ROS production, which creates the imbalance that results in cellular damage. A disequilibrium between the generation and build-up of ROS in tissue and cells along with the inability

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of a biological system to eliminate the excess reactive species is a condition known as oxidative stress. For example, an over production of hydroxyl radical and peroxynitrite leads to peroxidation of lipid to the degree it damages the cell membrane and lipoproteins. Malondialdehyde (MDA) and conjugated diene compounds, which are known to be cytotoxic and mutagenic, will then emerge as a result of this process (Pizzino *et al.*, 2017). Wang *et al.* (2019) stated that oxidative stress can be identified using the biologically derived indicator, MDA.

2.5 Apoptosis

Programmable cell death is known as apoptosis, which encompasses a variety of cytoarchitecture and biochemical changes, such as loss of cell volume, damage of DNA material, blabbing of membrane, decondenstation of chromatin and the generation of cell fragments (Fadeel and Orrenius, 2005; Taylor *et al.*, 2008). Cadmium leads to apoptosis through numerous signalling pathways, including the nuclear factor-Kb system, the Ca2+ pathway, the mitogen-activated protein kinase pathway, and the phosphatidylinositol-3-kinase pathway (Yu *et al.*, 2022; Zou *et al.*, 2015).

2.5.1 Apoptotic biomarker (Caspase 3)

Caspase is cysteine aspartic proteases that promote apoptotic cell death and engaged in immunity. In cells, caspases are existing as inactive forms that need to be activated in order to break particular target substrates, like cytokines that promote inflammation in the process of innate immune reactions. A greater proportion of cellular proteins are cleaved by pro-apoptotic caspases, which then activate apoptosis (Teng and Marie Hardwick, 2015). When the cell stimulated by death signal, the initiator caspases (caspase-8, -9 and -10) are activated. Activated initiator caspase will cleave and trigger

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the effector caspases (caspase-3 and -7) which lead to cleavage of cellular substrates during the apoptosis (Walsh *et al.*, 2008). Research has revealed that caspase-3 is vital for cell death in a tissue-, cell type-, or death stimulus-specific manner, and is considered necessary for some of the aspect changes in cell structure and certain biochemical processes related to the initiation and completion of apoptosis (Porter and Jänicke, 1999). Abu-Qare and Abou-Donia (2001) reported that apoptotic biomarkers have been studied through the discharge of cytochrome C by mitochondria into the cytoplasm and the trigger of caspase 3 in the cells encountering programmed cell death.

3.0 MATERIAL AND METHODS

All the methods and procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) with reference no.: UPM/IACUC/AUP-U033/2022

3.1 Laboratory Animals and experimental design

Thirty Sprague Dawley rats (10 weeks old) were purchased from Salim Reptile Centre. Animals were acclimatized for one week. A commercialized rodent diet (Gold Coin Brand Animal Feed) and filtered water were given ad libitum throughout the experiment. All the animals were kept in a controlled conditions with standard humidity at 25 ± 1 °C and a 12/12hr light/dark cycle. As shown in figure 5, rats were housed in a transparent glass cage with metal lids (four per cage). Rats' physical conditions were examined and monitored daily. The weights of the rats were measured twice a week using an electronic balance.

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Figure 5: 10 weeks old male Sprague Dawley rats in transparent glass cage with free excess of feed and filtered water.

3.2 EBN and Cadmium preparation

Edible Bird Nest was purchased as raw powdered EBN from Nest Excel Resources Sdn Bhd. It was stored at room temperature in an air-tight plastic container. EBN solution was prepared by dissolving the EBN raw powder in distilled water with a ratio of 1: 100. 100ml of distilled water was used to dissolve 1g of raw powdered EBN and heated up at a temperature of 60° C in the water bath for 45 minutes for a complete dissolution of the solid particles of EBN. It was cooled down to room temperature before administering to rats according to the treatment group dosage and body weight of the rat. Cadmium chloride was prepared by adding 0.5g of cadmium powder into 250ml of distilled water. Cadmium chloride, $CdCl_2$ solution was stored at room temperature.

3.3 EBN and Cadmium supplementation

Twenty-five rats were randomly divided into 5 experimental groups as follows: group 1 - negative control (NC) which received only 1.5ml of normal saline; group 2- positive

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control (PC) 10mg/kg BW of $CdCl_2$ only was given orally; Group 3, Group 4, Group 5 were administered with 10mg/kg $CdCl_2$ and different doses of EBN (60mg/kg, 90mg/kg, 120mg/kg) respectively. The volume of normal saline, $CdCl_2$ dosages, and the routes of administration was in accordance to Iqbal *et al.*, (2021). While the EBN dosages and route of administration were in accordance with the previous study by Quddus *et al.*, (2021). Before administration of the treatments, body weight of each rats was measured twice a week for dose calculation for EBN and CdCl2.

Table 1: Animal grouping and dose of cadmium chloride and Edible bird nest administered via oral gavage.

GROUP	GROUP ASSIGNED	NUMBER OF RATS	TYPE OF FEED AND DOSE
Control	G1 (NC)	5	Normal diet, ND + 15ml normal saline PO
Treated	G2 (Cd) (PC)	5	ND + $CdCl_2$ (10mg/kg) PO
	G3 (Cd +EBN 60)	5	ND + EBN (60 mg/kg BW) PO + $CdCl_2$ (10 mg/kg BW) PO
	G4 (Cd + EBN 90)	5	ND + EBN (90 mg/kg BW) PO + $CdCl_2$ (10mg/kg BW) PO
	G5 (Cd + EBN 120)	5	ND + EBN (120 mg/kg BW) PO + $CdCl_2$ (10mg/kg BW) PO

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3.4 Sample collection

After 16 days of the treatment period, the rats were euthanized by exsanguination under general anesthesia, which includes ketamine 30mg/kg and xylazine 10mg/kg body weight rats according to Albishtue *et al*., (2018). Rat's testes were removed and weighed. The left testicle was sectioned longitudinally and kept in 10% buffered formaldehyde solution for histopathological studies. The right testicle was rinsed with ice-cold PBS (pH7.4) to remove the excess blood. PBS was added to the tissue based on the weight of the right testicle at a ratio of 9:1 = PBS: weight of testicle and kept stored at -80 °C.

3.5 Histology slide preparation

3.5.1 Specimen fixation

The left testicle was placed in a 10% buffered formaldehyde solution for 24 hours.

3.5.2 Specimen sectioning and dehydration

The left testicles were sectioned longitudinally and placed in cassettes. The sectioned testicles were processed for dehydration in ethanol and clearing overnight using an automated tissue processor.

3.5.3 Embedding

Wax was added to the testis at 60 °C and cooled immediately at -20 °C for 30 to 40 minutes to embed the tissues in paraffin blocks.

3.5.4 Sectioning using a microtome

The formed block was clamped to a microtome for sectioning. The testis infiltrated

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with wax and was sectioned to the thickness of 5µm to form the ribbon. Then the ribbon was placed on a water bath to stretch out. The tissue was collected using the glass slide. The glass slide was then labeled and allowed to dry.

3.5.5 Staining with H&E stain

Staining of the slide was done based on Harris' hematoxylin and eosins staining method. The slides were submerged in xylene (hydrocarbon solvent) for 5 minutes to remove the wax. After de-waxing, the slides were submerged in alcohol 100% for 5 mins followed by 70% alcohol for another 5 minutes to remove the xylene so that other aqueous reagents could penetrate the testicular tissue. After the slides were rinsed in water, slides were submerged in Haematoxylin to stain the nucleus for 5 minutes and rinsed 3 to 5 times. The slides were dipped in 1% acid alcohol for 3 seconds and rinsed in running tap water for 5 minutes to remove excess background stain. Slides were submerged in Eosin stain for 1 minute to stain non-nuclear elements in the cells. Then the slides were sprayed with 95% of alcohol, cleaned, and let the slides dry for a few minutes. Lastly, mount with DPX and coverslip.

3.6 Histomorphological examination of of testis

All the slides were observed under a light microscope at x20 magnification and scored the histomorphological changes of the testis.

3.6.1 Scoring of seminiferous tubules lesion

Seminiferous tubule scored based on modified Johnson testicular biopsy score (Johnson, 1970).

Table 2 Modified Johnsen testicular biopsy score (Johnsen, 1970)

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Score	Histological Symptoms
10	Tubules with no cells and calcification
9	Tubules containing Sertoli cell but no germinal cell
8	Tubules containing only spermatogonium / primary and secondary spermatocytes degenerated
7	Tubules without spermatozoa and spermatid, and containing focal sloughing of spermatogonia
6	Tubules containing spermatocytes but no spermatozoa and spermatid
5	Tubules without spermatozoa, and containing less than 10 spermatids
4	Tubules with a large number of spermatids, but containing no spermatozoa
3	Tubules containing single-layered germinal epithelium but containing less than 10 spermatozoa in their lumens
2	Tubules containing spermatozoa, but the order of the spermatogonia is impaired and packed towards the lumen
1	Tubules with single-layered and smooth germinal epithelium and containing numerous spermatozoa

3.6.2 Scoring of interstitial space lesion

The parameters for edema, number of Leydig cells and infiltration of inflammatory cells in the interstitium assessed based on the intensity and criteria given in the table below according to modified interstitial scoring method report by Bozkurt (2018).

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Table 3 Modified interstitial lesion scoring (Bozkurt, 2018)

Score	Histological symptoms
1	0% - no lesion
2	< 25% lesion
3	25-75% lesion
4	> 75% lesion

3.7 Analyses of superoxide dismutase, malondialdehyde and caspase 3

Enzyme-linked immunosorbent assay (ELISA) test was used to evaluate the level of superoxide dismutase, malondialdehyde and caspase 3 in the testicular tissues.

3.7.1 Sample preparation

Left testicles were kept in PBS of the ratio of 9:1; PBS: testicular weight. Then the testicles were homogenized using a hand homogenizer by maintaining the temperature at 4°C using an ice bath. The homogenates were collected in a 1.5 ml centrifuge tube and centrifuged for 20 minutes at speed of 2000-3000 rpm while maintaining the temperature at 4-6 °C. The supernatant was collected using micropipette and kept at -20°C. The samples were thawed completely before using it for assay.

3.7.2 Preparation of diluted washing buffer

Diluted washing buffer was prepared by adding 20ml of washing buffer into 380ml of distilled water.

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3.7.3 Standard preparation for MDA, SOD, and Caspase 3

The standard was diluted with standard diluents in the method of multiple proportion dilution. The concentrations of standard MDA were as follows: 2000, 1000, 500, 250, 125, 0 pmol/ml. The standard concentration for SOD were 400, 200, 100, 50, 25, 0 ng/ml. The standard concentration for Caspase 3 were 50, 20, 12.5, 6.25, 3.12, 0 ng/ml.

3.7.4 ELISA test for SOD, MDA and Caspase 3

Firstly, blank wells, standard wells and sample wells were determined and set up. Blank wells were left empty. Then 50µl of standards were added to the standard wells. 40µl of special diluent added to the sample wells followed by 10µl of sample. Next, 50µl of horseradish peroxidase (HRP) was added into each well, except the blank well. The plate gently shook after being sealed. The plate was incubated at 37 ℃ for 60 minutes.

After discarding the excess liquid, the wells filled with washing buffer and soaked for 30 seconds. The washing liquid discarded and tapped on absorbent paper to remove the excess liquid. Then the plate washing method is repeated 4 more times. Next, 50µl of chromogen solution A added to each well followed by 50µl chromogen solution B in a dark room away from light. The plate gently shook after being sealed. The plate was incubated at 37 ℃ for 10 minutes. Stop solution was added into each well and within 15 minutes the optical density (OD) was measured at 450nm wavelength using microplate reader and Tecan's Magellan Microplate Reader Software. Using the website Arigobio.com the 5 points standard curve plotted according to a 4-parametric logistic (4 PL) to obtain the regression equation. The concentration of MDA was calculated by applying the OD values of the sample to the regression equation. All the ELISA operation steps were repeated for evaluation of SOD and caspase 3 concentration in testicles with their respective standard concentration and ELISA kit.

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3.8 Statistical analysis

IBM SPSS 28.0 for Windows was used to conduct the data analysis. Statistical comparisons were made using one-way analysis of variance (ANOVA) between the experimental groups for the evaluation of body weight gain, relative testicular weight, level of caspase 3 in testis, and scoring of lesion of seminiferous tubules. Kruskal-Wallis test was used for statistical comparisons between the experimental groups for the evaluation of level of MDA and SOD in testis and scoring of lesions of interstitial space of testis. Data prersented as mean ± standard error. Differences were accepted as statistically significant at $P < 0.05$.

4.0 RESULTS

4.1 Effect of Cd and EBN on body weight gain by the rats

No significant difference in body weight gain between the experiment groups and control group was identified, $P > 0.05$. Magnitude wise the mean body weight gain notably reduced in the groups that were exposed to Cd in comparison with control group (Fig.6). However, the group co-administered with 60, 90 and 120 mg/kg BW of EBN showed slightly higher mean body weight gain than Cd only treated group.

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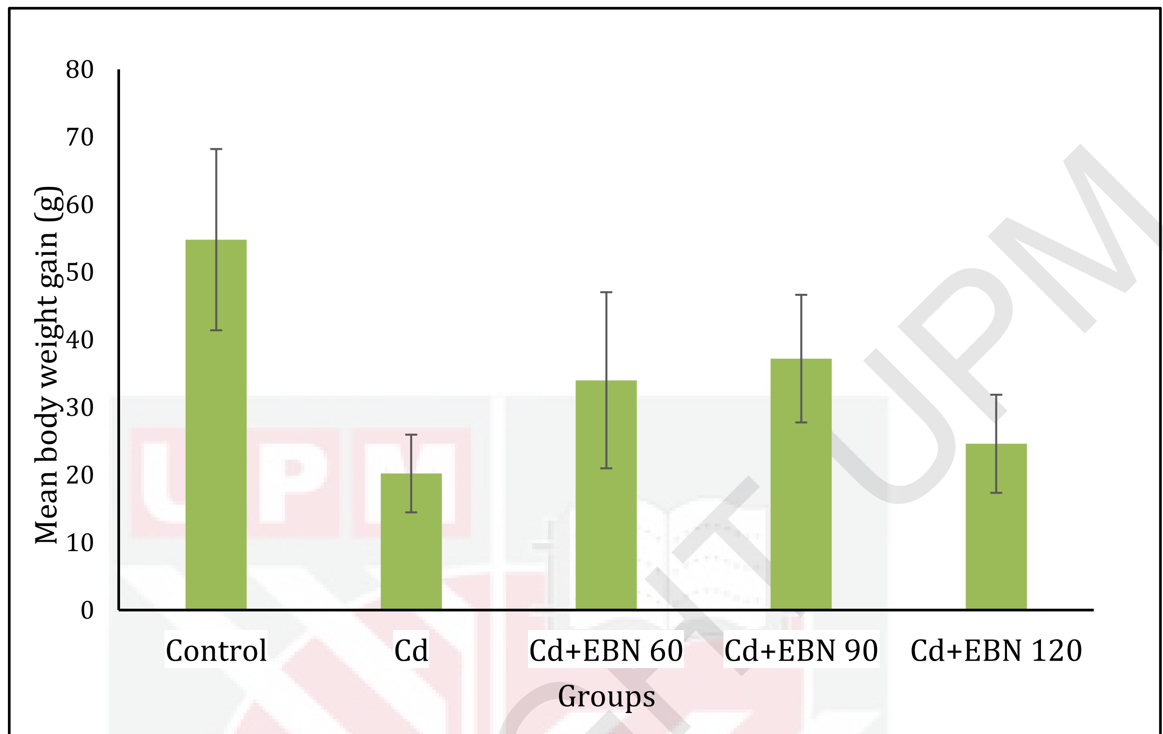

Figure 6: Differences in mean body weight gain (g) among experimental groups of rats

4.2 Effect of Cd and EBN on the relative testicular weight of the rats

Statistically, the difference in mean relative testicular weight between the control and experimental group is not significant. The mean relative testicular weight of the cadmium treated group is the lowest compared to control and Cd + (60,90,120)mg/kg BW EBN treated groups (Fig.7).

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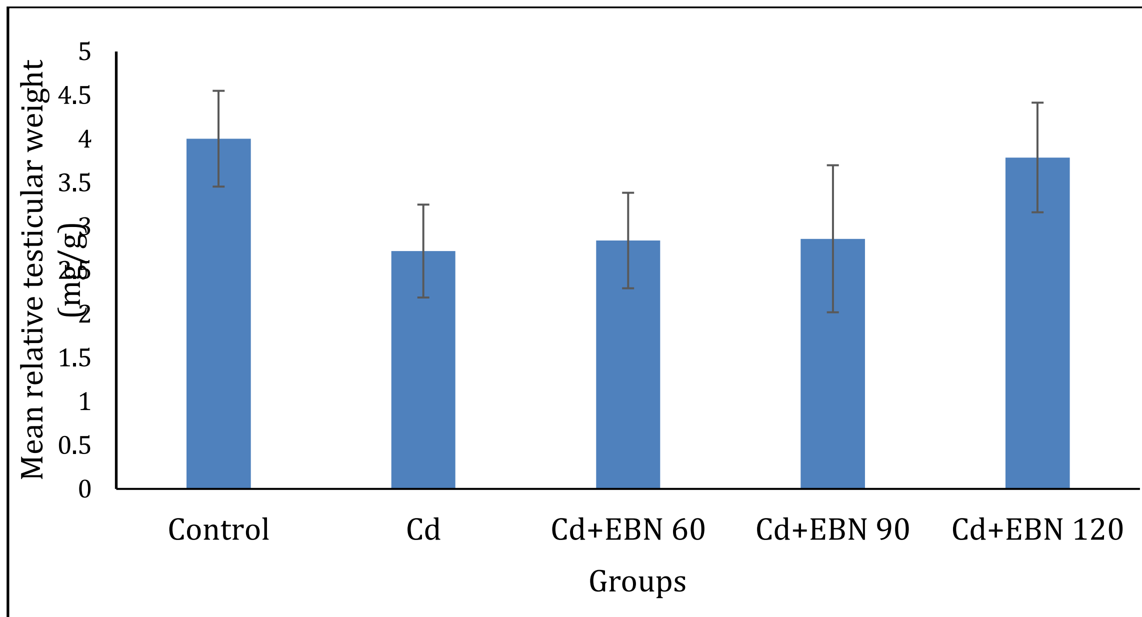

Figure 7: Differences in mean relative testicular weight (mg/g) among the experimental groups of rats

4.3 Gross examination of the testicles

The testes of rats that were exposed to cadmium only were smaller than the control group. Testes of Cd only and Cd + EBN 60 treated group appeared congested along with caseous material seen grossly (Fig. 8A). Moreover, the consistency of the testicles of the rats that were exposed to cadmium only (Fig. 8A & 8B) were hard and firm while the testicles of the other experimental groups supplemented with EBN were soft same as the control group (Fig. 8C).

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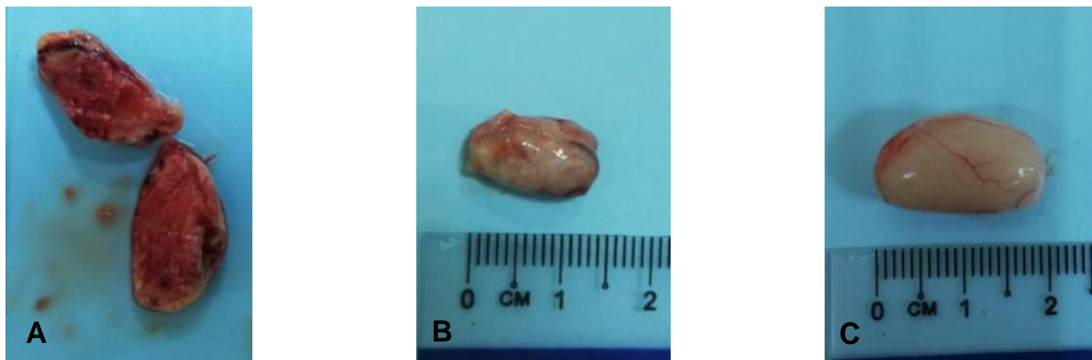

Figure 8: Gross morphological appearance of testes of experimental rats. A-Testis of rats that treated with Cd only with congestion and caseous material, B- Atrophied testis of rat that treated with Cd only, C- Normal gross testis of rat from the control group.

4.4 Effect of Cd and EBN on testicular histomorphology

In the control group, the seminiferous tubules were found arranged in an orderly manner and close together. Germinal epithelium, spermatogonium, primary spermatocytes, secondary spermatocytes, numerous spermatids and matured spermatozoa, and sertoli cells were found in the tubules of seminiferous. Interstitial space between the seminiferous tubules was normal and numerous leydig cells found in the interstitial space of control group (Fig 9A).

However, testicular histomorphology of cadmium only treated group showed infiltration of inflammatory cells in the interstitium (Fig 9B). The interstitial space between the tubules wider compared to control group. The structure of the seminiferous tubules were severely distorted and presence of calcification are noteworthy (Fig 9C). Testicular cells were absent in cadmium only treated group.

All the groups that co-administered with EBN were structurally with a higher integrity than cadmium only treated group. The Cd + EBN 60 group presented with edema, reduced

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Leydig cells and wider interstitial space compared to control group. The seminiferous tubules were disordered and shrunken. The layers of cells in the tubules including spermatids, spermatozoa were reduced. Some of the tubules were presented with no germinal cells, spermatogonium and cells sloughing off at the lumen of the seminiferous tubules (Fig 9D).

The Cd + EBN 90 group preserved the normal size, shape, and the arrangement of the seminiferous tubules (Fig 9E). The interstitial space in Cd + EBN90 group was narrower which is almost similar to control group even though it presented with some degree of lesion. Layers of cells which includes germinal epithelium, spermatogonium, primary spermatocyte, secondary spermatocyte, spermatids and matured spermatozoa were found in the tubules.

Although Cd + EBN 120 group have administered with the highest dose of EBN, the histomorphology of Cd + EBN 90 is still more consistent with the control in comparison with Cd + EBN 120. In the Cd + EBN 120 group, the interstitial space is larger and less number of Leydig cells present. Cell layers were disrupted in some of the seminiferous tubules even though the size, shape, and order of tubules remain normal (Fig 9F). Spermatids and spermatozoa still present in tubules of Cd + EBN 120 group.

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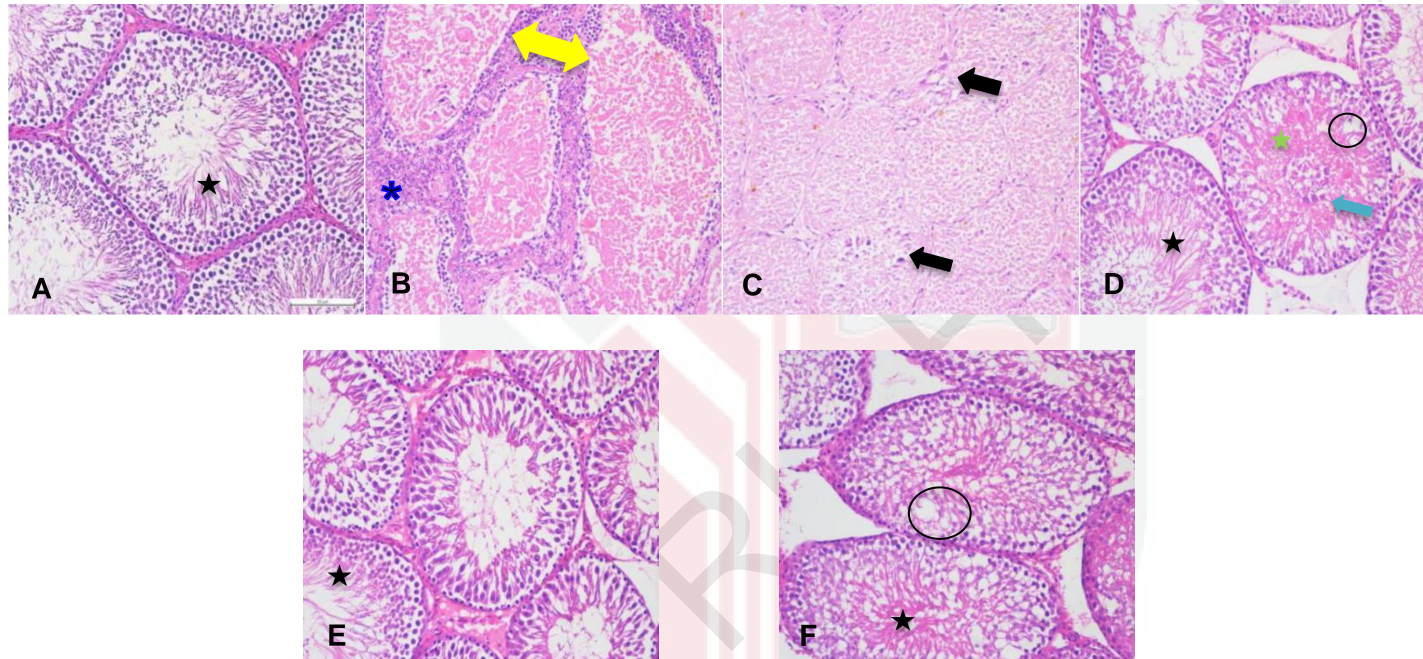

Figure 9: Photomicrographs of the testicular sections of rats under x20 magnification after staining with H&E stain. A: control; B & C: Cd group; D: Cd+EBN60 group; E: Cd+EBN90 group and F: Cd+EBN120 group. Black star illustrates spermatozoa, black arrow shows calcification, yellow arrow demonstrates increased interstitial space, blue asterisk infiltration of inflammatory cells, blue arrow shows sloughing off cells in the lumen, green star illustrates edema, black arrow illustrates cell necrosis.

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Statistically the mean histopathological scoring of seminiferous tubule and interstitial space is significant (P<0.05). The mean histopathological scoring of seminiferous tubule and interstitial space for Cd group and Cd + EBN 60 is statistically higher in comparison with control group. Cd group scored the highest for the mean histopathological scoring of seminiferous tubule compared to other groups. Co-administration of EBN showed lower mean histopathology scoring than Cd only treated group (fig.10) but the mean histopathological scoring for seminiferous tubule still relatively higher than control group.

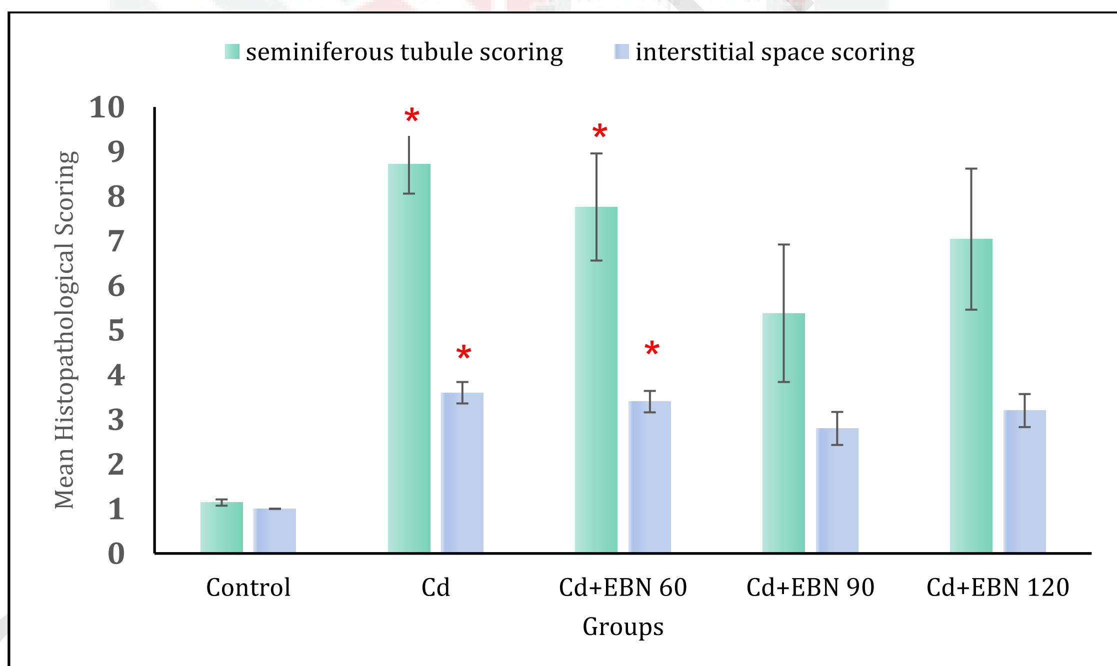

Figure 10: Differences in mean histopathology scoring among the experimental groups of rats

4.5 Analysis of concentration of caspase 3 in testes

In comparison with the control, the caspase 3 level in the testes of all the treatment groups does not significantly differ (P > 0.05). Based on the mean value of caspase 3 concentration as shown in the figure 11, Cd administrated group has a higher concentration of caspase 3 compared to the control group. All the Cd groups that co-administered with EBN have slightly lower concentration of caspase 3 in comparison with Cd only treated group. The mean concentration of caspase 3 of Cd + EBN 90 and Cd + EBN 120 has only slight difference compared to control group.

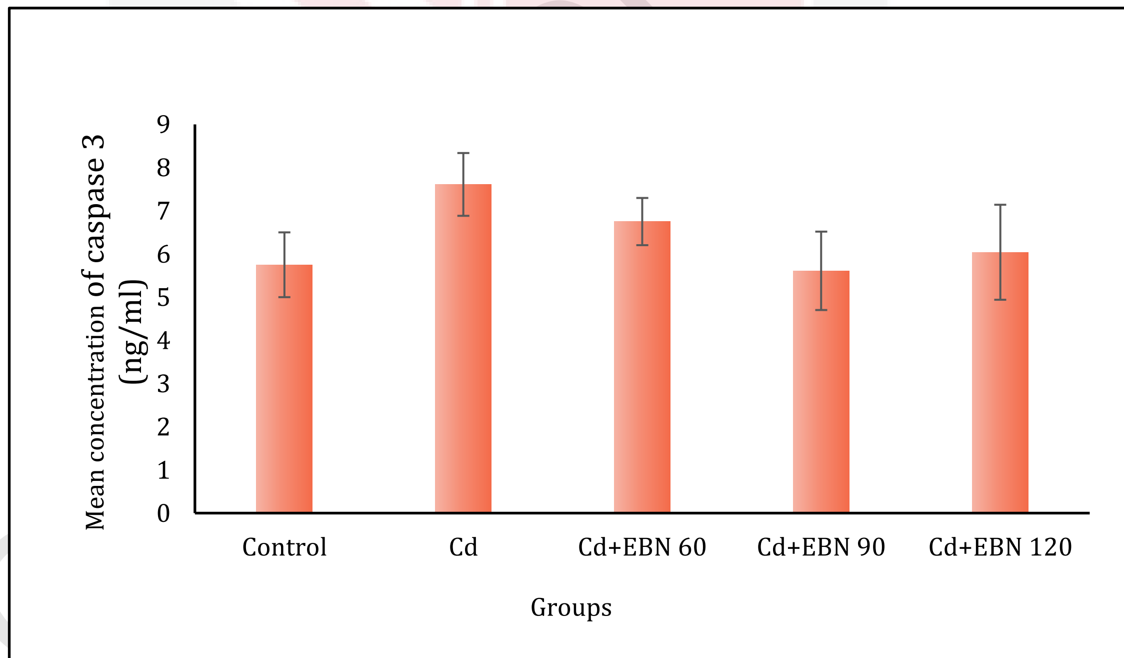

Figure 11: Differences in mean concentration of caspase 3 (ng/ml) in testis among the experimental groups of rats.

4.6 Analysis of Malondialdehyde (MDA) and Superoxidase dismutase (SOD) concentration in testes

Differences in both the levels of MDA and SOD of the testes are statistically not significant ($P > 0.05$). As shown in the figure 12, the mean MDA level is lowest in the Cd group compared to other groups. All the EBN-treated groups resulted in slightly lower mean MDA concentration than the control group. The mean concentration of SOD is highest in testis of Cd treated group while the control group has the lowest concentration of SOD. Groups that treated with cadmium and different doses of EBN have slightly higher mean concentration of SOD in comparison with the control group (Fig. 13).

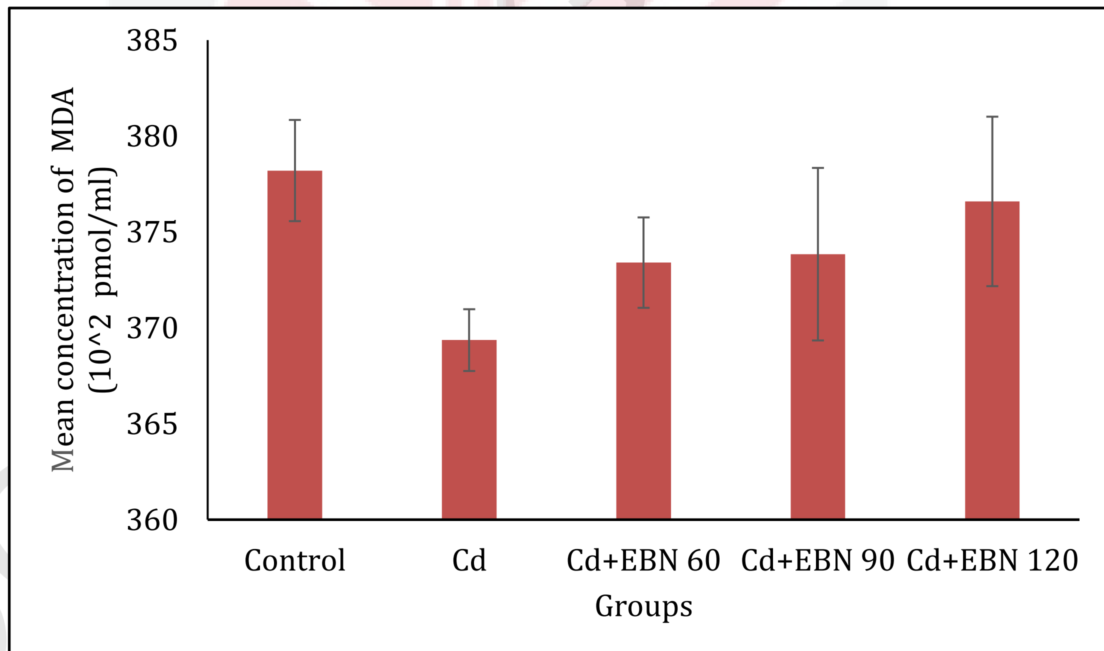

Figure 12: Differences in mean concentration of MDA ($x10^2$ pmol/ml) in testis among the experimental groups of rats

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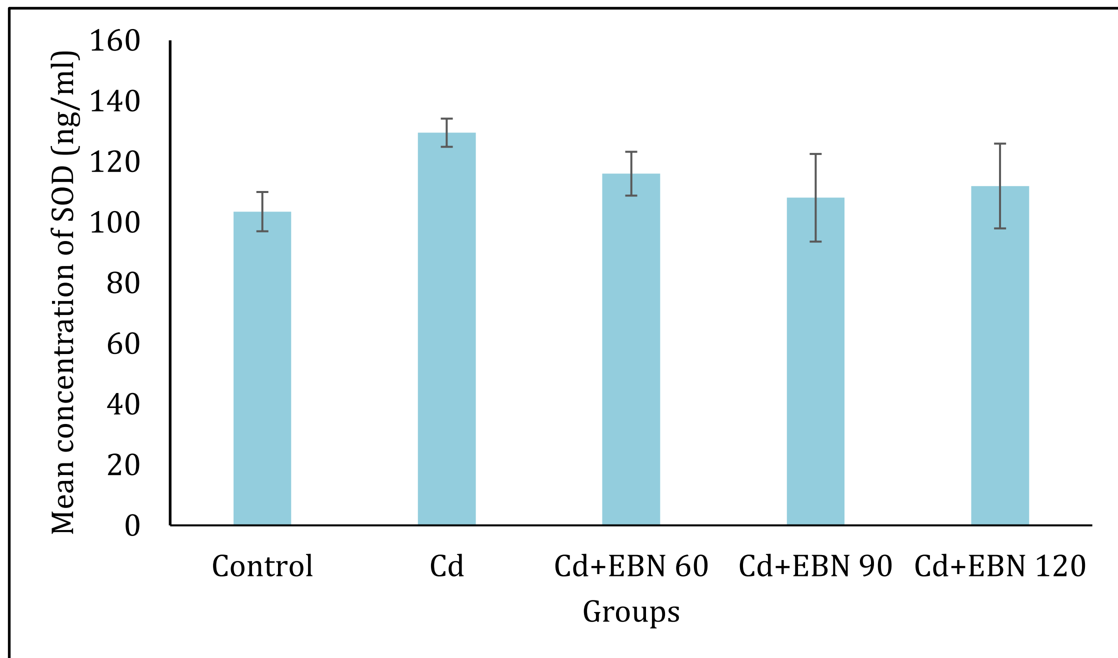

Figure 13: Differences in mean concentration of SOD (ng/ml) in testis among the experimental groups of rats

5.0 DISCUSSION

Based on the experiment conducted, the mean weight gain and mean relative testicular weight of the rats between the groups showed no statistical difference however, a slight difference was noticed in magnitude between the control and the treatment group. Cd only treated group showed the lowest mean body weight gain and mean relative testicular weight among the other groups. This is consistent with the previous study that reported cadmium has some negative effect on weight gain and testicular weight of rats that exposed to cadmium for 4 weeks. Cadmium can suppress the activity of digestive enzymes which will reduce feed efficiency in rats that treat with cadmium. Cadmium inhibits growth of testicular cells by reducing the absorption of nutrients from the feed (Wang *et al.*, 2021). Therefore, in this experiment, rat testis that exposed to cadmium toxicity has exert negative outcome in mean weight gain and mean relative testicular weight.

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Moreover, gross examination of testicles revealed that testicles that exposed to cadmium only smaller in size than control group which could also be due to Cd-induced inhibition of testicular growth and cell atrophy. This gross examination results supports the outcome of the lowest mean relative testicular weight and histopathology findings of Cd group compared to control group. Even though gross lesions of testicles are not reported in previous studies, comparatively testicles of Cd group had severe gross lesions than other experimental groups which clearly indicates that Cd has negative effect on the testicles and EBN have some positive effect on reducing the testicular cadmium toxicity.

Testicular histomorphology results proved that cadmium can cause severe damage to the testes. The results of histomorphology obtained in this experiment are similar to a previous study by Babaknejad *et al.* (2017), which mentioned that calcification, prominent degenerative seminiferous tubules, and necrosis were observed in rats administered with cadmium 1mg/kg for 3 weeks. The absence of spermatogenesis and infiltration of inflammatory cells can be related to oxidative damage due to cadmium toxicity. Excessive peroxidation of lipids can disrupt the structure and the cell loses its function (Agarwal and Saleh, 2002). Cadmium influences the series of inflammatory reactions in the cells with greater amount of pro-inflammatory cytokines especially TNFa which generate ROS in the tissue. This process leads to damage to testicular tissue due to lipid peroxidation (Al-Azemi *et al.*, 2010). Cd and Cd+EBN 60 group presented with wider interstitial space and a reduced number of Leydig cells. This result suggested that the Cd toxicity effect is exceeding the antioxidant defense properties which lead to impairing the integrity of the cells. In an earlier experiment, lower Leydig cell number and larger interstitial space were observed in the Cd-treated group (Nna *et al.*, 2017; Yi *et al.*, 2022).

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The integrity of seminiferous tubules was preserved and spermatogenesis was evident in the testicular histomorphology of rats co-administered with 90 and 120 mg/kg BW EBN. EBN increased the level of antioxidants that could reduce the imbalance between the radicals production and the antioxidant defense system. According to Albishtue *et al.* (2019), EBN prevented the cellular redox balance from being changed by lead acetate by raising antioxidant potential and boosting one of the antioxidant defense enzymes, SOD, which plays the role of free radical blockers. The introduction of enzymatic antioxidants may lead to greater membrane integrity, thereby enhancing the rigidity of the membrane to toxic metals. Numerous Chinese medicinal herbal substances, including EBN, have indeed been discovered to improve the proliferation of adult stem cells during the regeneration of tissue (Wong *et al.*, 2012).

Statistical scoring of histomorphology of Cd + EBN 90 and Cd + EBN 120 did not significantly differ from the control group suggesting that EBN provided some degree of protection to the cells from the toxic effect of cadmium which is consistent with previous studies. Quddus *et al.* (2021) stated that EBN 90mg/kg and 120mg/kg doses resulted in significantly higher ($p<0.05$) number of corpus luteum and surviving follicles in female rats that were exposed to cadmium toxicity.

However, administration of 90mg/kg BW dose EBN still provided more protection against cadmium-induced damage than 120mg/kg BW dose EBN. This could be because increased doses can be less beneficial and the toxicity differs from organ to organ. This can be related to the study mentioned that EBN 250 mg/kg dose showed slow proliferation of cells compared to EBN 60 and 120mg/kg doses that exhibited a protective role on hepatocytes that were exposed to acetaminophen toxicity (Muhammad-Azam *et al.*, 2022).

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EBN administration reduces the caspase 3 activity in testis cells. Therefore, the apoptotic biomarker, caspase 3 in EBN treated group is lower than the cadmium only treated group. This indicates apoptotic cells due to oxidative stress has been overcome by the antioxidant properties of EBN which supports the studies conducted previously. Vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) present in the EBN are known to protect the embryonic neurons from programmed cell death and suppress the apoptotic cells by interrupting with stimulation of caspase 3 (Roh *et al.*, 2012).

In comparison with the control group, the content of SOD and MDA levels in the testis of the other group does not significantly differ ($P>0.05$). The antioxidant enzyme (SOD) level was lowest in the control group and highest in the cadmium group. The result of MDA indicates that the control group has higher lipid peroxidation compared to all the treatment group while the Cd group have the lowest lipid peroxidation. In this study, the results contradict the previous studies.

Quddus *et al.* (2021) stated that SOD activity increased significantly in the EBN-treated group when compared to the groups that were administrated with cadmium only. Excess Cd inhibits antioxidant activity as well as raises the levels of ROS and MDA in the testes (Nna *et al.*, 2017). This could be due to technical errors that occurred when conducting the ELISA test to measure the level of SOD and MDA level in testis.

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6.0 CONCLUSION

Based on histomorphological studies, oral administration of EBN (90 and 120 mg/kg BW) ameliorated but not completely protect the Cd toxicity effects like necrosis, shrinkage of the seminiferous tubule, and inflammation on rat's testis. Even though the results of caspase 3, weight gain, and relative testicular weight of rat testis statistically not significant, it still showed some degree of therapeutic effect of EBN. Therefore, it can be concluded that EBN has protective effect on histomorphology, oxidative stress, and apoptosis of the testis of rats exposed to cadmium toxicity.

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RECOMMENDATION

It is recommended to repeat the test with a longer period of exposure to cadmium and EBN with a few more other additional parameters such as sperm count, motility, and viability. Several techniques and samples can be used for the detection of oxidative and apoptotic biomarkers like immunohistochemical expression of caspase 3 and use serum samples for SOD and MDA ELISA test. Moreover, further studies on effectiveness of different type of EBN and their prophylactic and therapeutic effect is recommended for better understanding on EBN nature. In addition, studies on EBN can be done especially to evaluate the toxic dose and effect of EBN on different type of tissue that exposed to various metal toxicity.

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APPENDICES

APPENDIX A: ANIMAL MONITORING SHEET

PROJECT TITLE:

Effect of edible bird nest (EBN) supplementation on testicular histomorphology, oxidative stress level, apoptotic biomarker, semen quality and testosterone production in Sprague Dawley rats exposed to cadmium toxicity.

PI : DR. NURHUSIEN YIMER DEGU

RESEACHER/PERSON IN CHARGE:

DR. NURHUSIEN YIMER DEGU

APPROVAL AUP NO:

PHONE NUMBER:+60397693983

DATE OF EXPERIMENTATION :

PROCEDURE:

SPECIES/STRAIN/SEX/AGE: Sprague-Dawley Rats

CAGE/ANIMAL ID:

OBSERVATION	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
DATE							
ACTIVITY Normal =0 Isolated =1 Inactive= 2 Moribund = 3							
MOVEMENT Normal = 0 Slight incoordination = 1 Tiptoewalking/reluctanttomove=2 Staggering/limbdragging/ paralysis=3							
COAT CONDITION Normal/groomed=0 Rough=1 Ruffled/unkempt=2 Bleeding or infected wounds or self mutilation=3							
EATING/DRINKING Normal=0 Decrease intake during the 1st 24 hrs =1 Decreased intake more than1day=2 Decreased intake over 48hrs=3							
BREATHING Normal=0 Rapid, shallow=1 Rapid, abdominal breathing=2 Laboured,irregular,skinblue=3							
OTHER COMMENTS							
MONITORED BY/INITIAL							

APPENDIX B: ANOVA FOR BODY WEIGHT GAIN

Tests of Normality

	group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
wt_gain	0	.196	5	.200*	.930	5	.595
	1	.186	5	.200*	.942	5	.680
	2	.208	5	.200*	.926	5	.569
	3	.233	5	.200*	.883	5	.325
	4	.213	5	.200*	.919	5	.523

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
wt_gain	Based on Mean	1.254	4	20	.321
	Based on Median	.678	4	20	.615
	Based on Median and with adjusted df	.678	4	13.828	.618
	Based on trimmed mean	1.305	4	20	.302

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wt_gain

	group	N	Subset for alpha = 0.05 1	2
Tukey HSD[a]	1	5	20.2000	
	4	5	24.6000	
	2	5	34.0000	
	3	5	37.2000	
	0	5	54.8000	
	Sig.		.159	
Duncan[a]	1	5	20.2000	
	4	5	24.6000	24.6000
	2	5	34.0000	34.0000
	3	5	37.2000	37.2000
	0	5		54.8000
	Sig.		.295	.069

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

APPENDIX C: ANOVA FOR RELATIVE TESTICULAR WEIGHT

Tests of Normality

	group	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
relative_wt	0	.266	5	.200*	.864	5	.244
	1	.213	5	.200*	.925	5	.560
	2	.187	5	.200*	.942	5	.681
	3	.342	5	.056	.797	5	.077
	4	.248	5	.200*	.874	5	.283

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
relative_wt	Based on Mean	1.345	4	20	.288
	Based on Median	.212	4	20	.929
	Based on Median and with adjusted df	.212	4	11.667	.927
	Based on trimmed mean	1.236	4	20	.328

ANOVA

relative_wt

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.302	4	1.825	.922	.470
Within Groups	39.577	20	1.979		
Total	46.879	24			

APPENDIX D: ANOVA FOR CASPASE 3

Descriptives

caspase3

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
.00	5	5.7500	1.67791	.75038	3.6666	7.8334	4.24	7.95
1.00	5	7.6100	1.62520	.72681	5.5920	9.6280	4.86	8.99
2.00	5	6.7520	1.22226	.54661	5.2344	8.2696	5.46	8.27
3.00	5	5.6130	2.02953	.90763	3.0930	8.1330	3.08	7.72
4.00	5	6.0460	2.45936	1.09986	2.9923	9.0997	3.04	8.44
Total	25	6.3542	1.85063	.37013	5.5903	7.1181	3.04	8.99

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	group	Statistic	df	Sig.	Statistic	df	Sig.
caspase3	0	.329	5	.081	.832	5	.144
	1	.285	5	.200*	.839	5	.163
	2	.236	5	.200*	.905	5	.440
	3	.302	5	.153	.871	5	.269
	4	.217	5	.200*	.883	5	.324

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
caspase3	Based on Mean	1.603	4	20	.212
	Based on Median	.472	4	20	.756
	Based on Median and with adjusted df	.472	4	16.946	.756
	Based on trimmed mean	1.529	4	20	.232

Robust Tests of Equality of Means

caspase3

	Statistic[a]	df1	df2	Sig.
Welch	.970	4	9.861	.466
Brown-Forsythe	1.002	4	16.582	.434

a. Asymptotically F distributed.

ANOVA

caspase3

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.724	4	3.431	1.002	.430
Within Groups	68.472	20	3.424		
Total	82.196	24			

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caspase3

	group	N	Subset for alpha = 0.05
			1
Tukey B[a]	3.00	5	5.6130
	.00	5	5.7500
	4.00	5	6.0460
	2.00	5	6.7520
	1.00	5	7.6100
Duncan[a]	3.00	5	5.6130
	.00	5	5.7500
	4.00	5	6.0460
	2.00	5	6.7520
	1.00	5	7.6100
	Sig.		.140

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

APPENDIX E: KRUSKAL - WALLIS TEST FOR SOD AND MDA

Tests of Normality

	group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
SOD	0	.199	5	.200*	.954	5	.765
	1	.309	5	.133	.824	5	.125
	2	.267	5	.200*	.942	5	.677
	3	.208	5	.200*	.923	5	.551
	4	.286	5	.200*	.837	5	.157
mda	0	.276	5	.200*	.890	5	.356
	1	.172	5	.200*	.933	5	.617
	2	.244	5	.200*	.938	5	.649
	3	.363	5	.030	.721	5	.016
	4	.256	5	.200*	.868	5	.257

Ranks

	group	N	Mean Rank
mda	0	5	17.00
	1	5	8.40
	2	5	13.00
	3	5	11.80
	4	5	14.80
	Total	25	
SOD	0	5	8.70
	1	5	17.90
	2	5	13.50
	3	5	12.00
	4	5	12.90
	Total	25	

Test Statistics[a,b]

	mda	SOD
Kruskal-Wallis H	3.862	4.042
df	4	4
Asymp. Sig.	.425	.400

a. Kruskal Wallis Test

b. Grouping Variable: group

APPENDIX F: KRUSKAL WALLIS TEST FOR INTERSTITIAL SCORING

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
interstial_score	25	2.8000	1.11803	1.00	4.00
group	25	2.00	1.443	0	4

Test Statistics[a,b]

	interstial_score
Kruskal-Wallis H	14.459
df	4
Asymp. Sig.	.006

a. Kruskal Wallis Test

b. Grouping Variable: group

Ranks

	group	N	Mean Rank
interstial_score	0	5	3.00
	1	5	18.10
	2	5	16.40
	3	5	12.30
	4	5	15.20
	Total	25	

APPENDIX G: ANOVA FOR SEMINIFEROUS TUBULE SCORING

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
S.TUBULE_SCORE	.00	5	1.1400	.15166	.06782	.9517	1.3283	1.00	1.30
	1.00	5	8.7200	1.47377	.65909	6.8901	10.5499	6.40	10.00
	2.00	5	7.7600	2.68198	1.19942	4.4299	11.0901	3.90	10.00
	3.00	5	5.3800	3.44703	1.54156	1.0999	9.6601	1.80	9.00
	4.00	5	7.0400	3.52817	1.57785	2.6592	11.4208	2.80	10.00
	Total	25	6.0080	3.61005	.72201	4.5178	7.4982	1.00	10.00

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
S.TUBULE_SCORE	Based on Mean	11.130	4	20	<.001
	Based on Median	1.556	4	20	.225
	Based on Median and with adjusted df	1.556	4	11.502	.251
	Based on trimmed mean	10.293	4	20	<.001

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
S.TUBULE_SCORE	Between Groups	177.906	4	44.477	6.595	.001
	Within Groups	134.872	20	6.744		
	Total	312.778	24			

Multiple Comparisons

Dependent Variable		(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
	Games-Howell	.00	1.00	-7.58000*	.66257	.001	-10.4940	-4.6660
			2.00	-6.62000*	1.20133	.024	-11.9430	-1.2970
			3.00	-4.24000	1.54305	.202	-11.0860	2.6060
			4.00	-5.90000	1.57930	.086	-12.9075	1.1075
		1.00	.00	7.58000*	.66257	.001	4.6660	10.4940
			2.00	.96000	1.36858	.949	-4.1153	6.0353
			3.00	3.34000	1.67654	.376	-3.1801	9.8601
			4.00	1.68000	1.70997	.854	-4.9981	8.3581
		2.00	.00	6.62000*	1.20133	.024	1.2970	11.9430
			1.00	-.96000	1.36858	.949	-6.0353	4.1153
			3.00	2.38000	1.95320	.743	-4.4684	9.2284
			4.00	.72000	1.98197	.995	-6.2483	7.6883
		3.00	.00	4.24000	1.54305	.202	-2.6060	11.0860
			1.00	-3.34000	1.67654	.376	-9.8601	3.1801
			2.00	-2.38000	1.95320	.743	-9.2284	4.4684
			4.00	-1.66000	2.20590	.937	-9.2818	5.9618
		4.00	.00	5.90000	1.57930	.086	-1.1075	12.9075
			1.00	-1.68000	1.70997	.854	-8.3581	4.9981
			2.00	-.72000	1.98197	.995	-7.6883	6.2483
			3.00	1.66000	2.20590	.937	-5.9618	9.2818