UNIVERSITI PUTRA MALAYSIA

EFFECTS OF EDIBLE BIRD'S NEST ON SPERM QUALITY AND TESTOSTERONE PRODUCTION IN SPRAGUE DAWLEY RATS EXPOSED TO CADMIUM TOXICITY

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It is hereby certified that we have read this project paper entitled "Effects of Edible Bird's Nest on Sperm Quality and Testosterone Production in Sprague Dawley Rats Exposed to Cadmium Toxicity", by Elisha Linus and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 - Final Year Project.

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DEDICATION

To Almighty God for the guidance, wisdom and strength throughout this journey. To my beloved parents for the continuous support, encouragement, inspiration and prays of day and night that have brought me this far as well as to my siblings and friends for the advice and words of encouragement.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Celsius
mg	Microgram
µL	Microlitre
ABTS	2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis Of Variance
AP-1	Activator protein-1
BTB	Blood testis barrier
BW	Body weight
Ca	Calcium
Cd	Cadmium
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
EBN	Edible Bird's Nest
ELISA	Enzyme-linked immunosorbent assay
FSH	Folicle stimulating hormone
GSH	Glutathione
g	Gram
H_2O_2	Hydrogen peroxide
HADSCs	Human Adipose Derived Stem Cells

$HEPG_2$	Hepatoblastoma cell line
HRP	Horseradish peroxidase
hCG	Human chorionic gonadotropin
INH-B	Inhibin B
IL-6	Interleukin-6
JAK/STAT	Janus kinase/Signal transducer and activator of transcription pathway
Kg	Kilogram
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinases
MSCs	Mesenchymal stem cells
mg	Milligram
ml	Milliliter
Na	Sodium
NF-κB	Nuclear factor kappa B
NOS	Nitrous oxide
ng	nanogram
nm	nanometer
ROS	Reactive Oxygen Species
RPM	Revolutions per minute

SD	Standard deviation
SEM	Standard error of the mean
T	Testosterone
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor

ABSTRAK

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

KESAN SARANG BURUNG WALIT PADA KUALITI SPERMA DAN TESTOSTERON DALAM TIKUS SPRAGUE DAWLEY YANG TERDEDAH KEPADA KERACUNAN KADMIUM

Oleh:

Elisha Linus

2022

Penyelia: Dr. Nurhusien Yimer Degu

Kadmium (Cd) ialah salah satu logam berat yang merupakan toksik kepada sistem reproduktif lelaki, melalui kesannya terhadap fungsi endokrin, spermatogenesis, kualiti sperma dan kesuburan. Sarang burung walit mampu mengurangkan kesan toksik plumbum asetate (LA) dalam rahim tikus disebabkan oleh sifatnya terhadap proliferasi sel dan sebagai antioksidan. Walaupun demikian, belum pernah ada kajian akan kesan rawatan sarang burung walit terhadap kesan toksik kadmium dalam tikus jantan. Kajian ini bertujuan untuk mengkaji kesan sarang burung walit pada kualiti sperma dan testosterone dalam tikus Sprague Dawley (SD) yang terdedah kepada keracunan kadmium. Sebanyak dua puluh lima tikus Sprague Dawley (SD) dibahagikan secara

rawak kepada lima kumpulan; Kumpulan 1 (normal saline), Kumpulan 2 (CdCl 10 mg/kg BW secara oral), manakala kumpulan 3, 4 dan 5 menerima rawatan $CdCl_2$ (10 mg/kg BW) dengan masing-masing dirawat dengan dos sarang burung yang berbeza iaitu 60, 90 dan 120 mg/kg BW selama 16 hari. Tikus itu kemudiannya disuntik mati, lalu testis diambil dan ditimbang untuk mengira organ koefisien relatif. *Cauda epididymis* diambil untuk analisis kualiti sperma (kuantiti sperma dan morfologi) dan sampel darah digunakan untuk analisis hormon testosteron menggunakan ELISA. Keputusan menunjukkan tidak ada perbezaan yang ketara dalam berat testikular relatif di antara kumpulan itu. Terdapat pengurangan signifikan kuantiti sperma dan penghasilan hormon testosteron, serta kenaikan yang ketara peratusan sperma abnormal dalam kumpulan yang dirawat dengan Cd berbanding dengan kumpulan kawalan dan kumpulan yang menerima EBN ($p < 0.05$). Berbanding dengan kumpulan yang dirawat dengan Cd dan EBN 60 mg/kg BW, EBN 120 mg/kg BW menunjukkan kuantiti sperma yang lebih tinggi dan peratusan sperma abnormal yang lebih rendah ($p < 0.05$). Kumpulan EBN 120 mg/kg BW juga menunjukkan kenaikan ketara dalam penghasilan hormon testosteron ($p < 0.01$) berbanding dengan kumpulan dirawat dengan Cd. Kesimpulannya, EBN mampu mengurangkan kesan Cd ke atas kualiti sperma dan penghasilan hormon testosteron pada dos 120 mg/kg BW.

Kata kunci: *keracunan kadmium; sarang burung walit; kualiti sperma; hormon testosterone; tikus*

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine, University Putra Malaysia in partial requirement for the course VPD 4999 - Final Year Project.

EFFECTS OF EDIBLE BIRD'S NEST ON SPERM QUALITY AND TESTOSTERONE PRODUCTION IN SPRAGUE DAWLEY RATS EXPOSED TO CADMIUM TOXICITY

By:

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2022

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Cadmium (Cd) is one of the major heavy metals exerting male reproductive toxicity, affecting endocrine function, spermatogenesis, semen quality and fertility. Edible Bird Nest (EBN) has proven to mitigate the toxic effects of Cd and lead acetate (LA) on the uterus of rats due to its positive proliferative and antioxidant properties. However, there is no study of EBN's role in heavy metal toxicity in male rats. This study aimed to evaluate the EBN's effect on sperm quality and testosterone production in rats. Twenty-five male Sprague-Dawley (SD) rats were randomly divided into five groups; Group 1 – control (NaCl 0.9%), Group 2 ($CdCl_2$ 10 mg/kg BW orally), while groups 3, 4, and 5 received CdCl2 (10 mg/kg BW) plus graded concentrations of 60, 90 and 120 mg/kg

BW of EBN, respectively for 16 days. Rats were then euthanized, and testicles were weighed for organ coefficients. Cauda epididymis were collected for sperm quality evaluation (sperm concentration and sperm morphology), and blood samples were used for testosterone analysis using ELISA. Results showed no significant differences in organ coefficient between the groups. There was a significant drop in sperm concentration and testosterone, plus a significant elevation in the abnormal sperm rate in the Cd-treated group compared to the control and EBN-treated groups ($p < 0.05$). Compared to the Cd-treated group and EBN 60 mg/kg group, EBN 120 mg/kg group demonstrated a significantly higher sperm concentration and a lower percentage of abnormal sperm ($p < 0.05$). Likewise, the EBN 120 mg/kg group showed a significant increase in testosterone levels ($p < 0.01$) compared to the Cd-treated group. Therefore, EBN can avert the effect of Cd on sperm parameters and testosterone production at a dosage of 120 mg/kg.

Keywords: *cadmium toxicity; edible bird's nest; sperm quality; testosterone; rats*

1.0 Introduction

Cadmium (Cd) is a heavy metal commonly found as an environmental contaminant. It is highly toxic and exposure to this metal exerts detrimental effects on multiple organs such as the lungs, pancreas, thyroid, spleen, liver, kidneys, and testes (Wang *et al*., 2021). Cd causes extensive damage to the male reproductive system, particularly the testes by impairing spermatogenesis, semen quality, and endocrine function, resulting in reduced male fertility (de Angelis *et al*., 2017). Other toxic effects of Cd include a reduction in sperm count, motility, and activity and a decrease in antioxidant activity (Jahan *et al*., 2019).

Edible Bird Nest (EBN) is known for its numerous health benefits and has been reported to have a protective role against heavy metal toxicity. Previous research has established that EBN could mitigate the toxic effects of lead acetate (LA) on the uterus of rats (Albishtue *et al*., 2019). In histological examination, LA-induced rats demonstrated an increased number of uterine cells and glands after being treated with EBN at an oral dosage between 60 to 120 mg/kg BW (Albishtue *et al*., 2019). In the most recent study on the protective effect of EBN on the histomorphology of rat's uterus against Cd toxicity, there was a significant reduction in Cd concentration within the uterine tissue of rats with EBN supplements, and the group treated with the highest dose of EBN (120 mg/kg BW) were reported to have the lowest Cd accumulation in uterine tissue (Quddus *et al.*,

2021). Hence, EBN has ameliorative effects against Cd and LA-induced reproductive disorders in female rats.

Currently, there is no detailed study on the protective role of EBN against heavy metal toxicity in male rats. Therefore, this study aims to evaluate the effect of EBN supplements on male reproductive parameters especially the sperm quality and testosterone production level in male rats with Cd toxicity. This will help build a better understanding of the potential treatment of Cd toxicity in male reproductive problems in animals and humans. It was hypothesized that there would be a decrease in sperm concentration, motility, and viability and an increase in sperm abnormalities percentage in Cd-induced groups. Serum testosterone level in Cd-induced groups is expected to reduce. EBN is able to protect the testes from the toxic effect of Cd, thus the sperm parameters of the group treated with a higher dosage of EBN will not be significantly affected.

2.0 Literature Review

2.1 Laboratory Rats

Laboratory rats fall under the domesticated descendants of Rattus norvegicus. Individual stocks and strains have been used in biomedical research since the late 1800s and early 1900s. The two primary genetic groups of rats are inbred and outbred. When homozygosity is least important, Wistar and Sprague Dawley (SD) are outbred rats that are often used for general research. They are also known for their hybrid vigor and good reproductive performance (Sharp *et al*., 1998). Laboratory rats have been widely used in almost every aspect of biomedical and behavioral research for over the past 80 years. They are useful particularly in toxicology, teratology, experimental oncology, experimental gerontology, cardiovascular research, immunology, dental research, immunogenetics, and experimental parasitology (Sengupta, 2013). A comparative assessment of sexual maturation timing in male Wistar Han (WH) and SD rats had been conducted by Campion *et al.*, (2013) based on the increased number of testicular spermatids head counts as well as the sperm motility and morphology consistent with the qualitative sexual maturity. From this study, it was concluded that both WH and SD rats exhibited nearly identical trends of these endpoints and were proven to be sexually mature at an average PND of 70.

2.2 Anatomy of Male Rat Reproductive System

Generally, the male reproductive system in mammals consists of the internal structures: the testes, epididymis, vas deferens, prostate, and the external structures: the scrotum and penis (Gurung *et al*., 2022). Figure 1 illustrates the anatomy of the male reproductive organs. There are two functional units in the testes which are the tubules for sperm production and transportation to the excretory-ejaculatory ducts and interstitial or Leydig cells that are responsible for androgens production. Germ cells and Sertoli cells are located within the spermatogenic tubules.

The two functional compartments, basal and adluminal are divided by the tight junctions between the Sertoli cells, forming a diffusion barrier between the spermatogonia and primary spermatocyte (Griffin, 1993). This is also known as the blood-testis barrier (BTB) that limits the entry of macromolecules, analogous to the blood-brain barrier and other epithelial barriers (Neaves, 1977). The Leydig cells or interstitial cells are located in the basal compartment where spermatogonia originated. The adluminal compartment contains the primary spermatocyte and more advanced stages of spermatogenesis (Griffin, 1993). The seminiferous tubules are connected to a network of ducts known as the rete testes (Hudson *et al.*, 1985). The epididymis is a highly convoluted duct that receives spermatozoa from the ductules efferents and conducts them to the vas deferens. The three important

parts of the epididymis are the caput (head), corpus (body), and cauda (tail) (Cosentino & Cockett, 1985.) The vas deferens is a tubular structure that begins at the cauda epididymis and terminates at the ejaculatory ducts near the prostate (Griffin, 1993). The paired lobulated glands located symmetrically above the prostate and behind the bladder are known as seminal vesicles. It contains viscous proteinaceous fluid consisting of fructose and prostaglandins (Hudson *et al*., 1985)

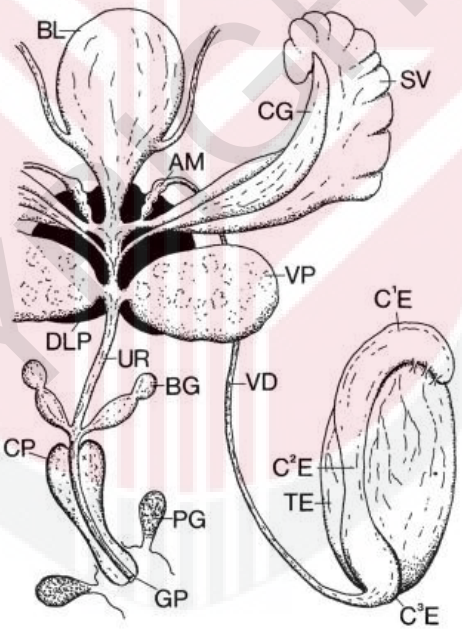

Figure 1: Illustration of male reproductive organs in rats. BL, bladder; AM, ampulla; CG coagulating gland; SV, seminal vesicles; VP, ventral prostate; DLP, dorsolateral prostate; UR, penile urethra; BG, bulbourethral glands; VD, vas deferens; CP, corpus penis; GP, glans penis; PG, preputial gland; TE, testis; C1/C2/C3E, caput/corpus/cauda epididymis (Haschek *et al*., 2010)

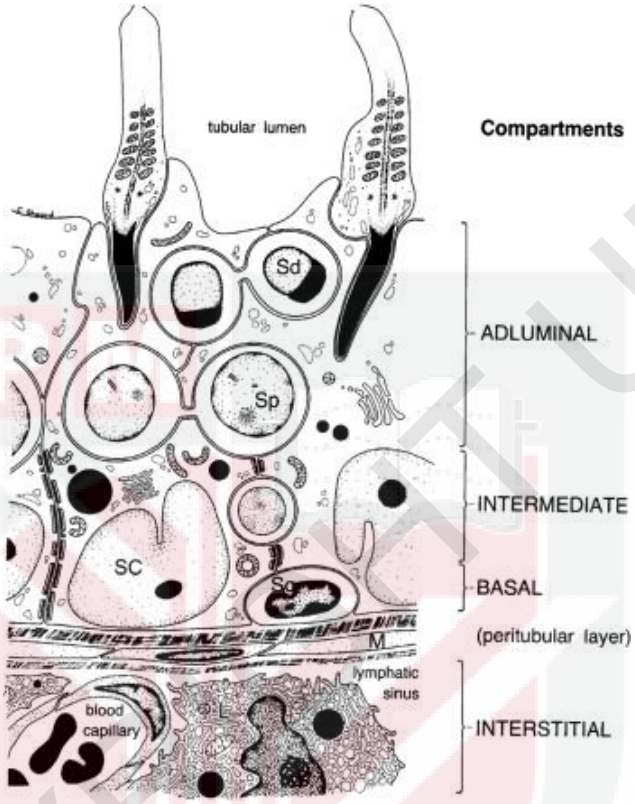

Figure 2: Diagrammatic representation of a portion of a seminiferous tubule. L, Leydig cell; SC, Sertoli cell; Sg, spermatogonium; Sp, spermatocyte; Sd, spermatid; M, myoepithelial cell (Haschek *et al.*, 2010).

2.3 Physiology of Male Rat Reproductive System

2.3.1 Spermatogenesis

Spermatogenesis is the production of sperm from primordial germ cells (Gilbert, 2000), in which spermatogonia, diploid stem cells at the base of seminiferous tubules undergo a series of mitotic and meiotic divisions followed by differentiation into haploid spermatids, resulting in the release of mature spermatozoa. The three major events involved are spermatocytogenesis, meiosis, and spermiogenesis (Cunningham & Klein, 2007). This process occurs in the tubules of Sertoli cells.

Spermatogenesis begins with the mitotic division of type A spermatogonia, a group of cells from self-regenerating spermatogonial stem cells (SCCs) to produce 16 cells. The transformed type A1 spermatogonia will further divide into types A and type B. In that type, A will be subdivided into types A1, A2, A3, and A4 while type B spermatogonia will divide to form resting primary spermatocytes (Johnson, 2013).

Each primary spermatocyte undergoes DNA replication and is then pushed into the adluminal intratubular compartment during prophase (Johnson, 2013). Meiotic division occurs as homologous chromosomes pair up, facilitating the genetic

material exchange. This process produces two haploid cells, which are also known as secondary spermatocytes with duplicated chromatids. The secondary spermatocytes will further divide during the second meiosis to form immature spermatozoa or spermatids (Cunningham & Klein, 2007).

Spermiogenesis is a process of differentiation of the spermatids into mature spermatozoa without dividing which occurs before spermiation. Acrosome formation from the Golgi apparatus, nucleus condensation and elongation, flagellum formation, and cytoplasmic shedding are the major features of the process (Cunningham & Klein, 2007). The sperm tail is formed initially to help it gain motility. Then, the middle piece, which contains mitochondria is developed to generate energy for the tail movement. After that, an acrosome is formed which contains hydrolytic enzymes that are required as an enzymatic knife to facilitate oocyte penetration The nucleus contains compact packaged haploid chromosomes that are necessary for fertilization. Spermiogenesis is completed with the formation of a spermatozoon that consists of a head, middle piece, and tail (Johnson, 2013).

2.3.2 Hormonal control of spermatogenesis

Two different cell types in the testes that produce hormones are the Sertoli cells and Leydig cells. Sertoli cells are located within the seminiferous tubules near the spermatogonia, while Leydig cells are found between the seminiferous tubules, called interstitial cells. Sertoli cells are stimulated by follicle-stimulating hormones (FSH) from the anterior pituitary and produce androgen-binding protein, inhibin, and estrogen. Testosterone is explicitly secreted from acetate and cholesterol in the smooth endoplasmic reticulum of the interstitial Leydig cells under stimulation of the luteinizing hormone (LH). It is secreted daily in sexually matured mammals and leaves the testis by three routes: blood, lymph, and exocrine. The lipid-soluble hormone passes into the tubule lumen and binds to the androgen-binding protein (ABP).

Testosterone has critical functions in the Sertoli cells. This includes the maintenance of blood-testis barrier integrity between the basal and adluminal compartments, involved in Sertoli–spermatid adhesion and essential to release mature sperm at spermiation. The testosterone secretion also acts as negative feedback, which inhibits LH and FSH secretion (Johnson, 2013). Apart from that, it also stimulates the development of male

reproductive organs (including accessory sex glands and scrotum) and also stimulates male behavior. The release of LH is stimulated by gonadotropin-releasing hormone (GnRH), which acts upon the pituitary gland (Long, 2006.) It is produced in the hypothalamus which lies below the thalamus at the base of the brain.

2.4 Epididymal sperm collection

In the cauda epididymis, mature spermatozoa are stored until ejaculation (Jones, 2004). Several methods have been used to recover epididymal spermatozoa, including cutting, floating, mincing, and flushing (Fickel *et al*., 2007). In the cutting method, used in bulls (Martins *et al*., 2007) and goats (Jindal, 1984), epididymal cells were separated from the testis, and spermatozoa were recovered. The floating method was applied to bulls (Turri *et a*l., 2012), canines (Yu and Leibo, 2002), felines (Thuwanut *et al*., 2010; Filliers *et al*., 2008), rams (Lone et al., 2011), and stallions (Cary et al., 2004). For the mincing method in bull (Kang *et al*., 2016), feline (Vernocchi *et a*l., 2014), and stallion (Neuhauser *et al*., 2013), spermatozoa-containing fluid was immediately extracted after finely chopping the cauda epididymis. The cutting method was described by Kempinas & Lamano-Carvalho (1988) to evaluate sperm concentration in Wistar rats. The cauda epididymis was

cut using a pair of fine-pointed scissors and compressed with forceps to release the spermatozoa into the medium. The spermatozoa will swim to the fluid within the cauda epididymis tubules, where they will be recovered. Sperm analysis in rats focuses primarily on male reproductive assessment. The methods used to recover epididymal spermatozoa may affect sperm motility, sperm concentration, and blood contamination (Kang *et al*., 2018)

2.5 Sperm Quality Evaluation

2.5.1 Sperm concentration

The concentration of spermatozoa is measured using a hemocytometer (Youngquist, 1997). The sperm concentration is expressed as the number of sperm per ml and the number of sperm per ejaculate. Several reports on the number of spermatozoa ejaculated by male albino rats have been published: 83 x 106 (Farris, 1946) or 58 x 106 (Blandau & Odor, 1949) upon normal copulation; 63 x 106 (Scott & Dziuk, 1959) or 55 - 60 x 106 (Mauss et al., 1970) upon electroejaculation; and 2 - 6 x 106 (Agmo, 1976) upon spontaneous ejaculation. The concentration of spermatozoa in the epididymis was determined in the Sprague-Dawley albino rat via micropuncture technique (Turner, Hartmann & Howards, 1977). They detected 184 (±

0.10 SEM) x 106 spermatozoa/ml in the cauda epididymis of adult rats.

2.5.2 Sperm Morphology

The abnormalities in sperm morphology as well as the differentiation of live sperm from dead sperm can be observed and counted by examining the stained semen smears. The commonly used stain for this procedure is eosin-nigrosin and Wright's stain. Dry preparations can be made in which an aliquot of the sperm suspension is placed on a slide and air-dried. Usually, the slide is observed using eosin-nigrosin staining under a light microscope. The assessment of rat sperm morphology is rather subjective and there is no generally accepted classification scheme.

An individual sperm can be classified into more than one category (Seed *et. al*., 1996). Overall, the abnormality morphology classification is based on the three important anatomical locations of the sperm which are the head, mid-piece, and tail. For instance, some studies classified sperm abnormalities as double head, flattened head (reduced hook or banana head), bent neck, bent tail, and multiple abnormalities (Figure 3). In another study by Wendmu *et al*., 2020 using

Giemsa staining, additional abnormal morphology was included such as curved midpiece and coiled tail, hook-less head, decapitated head, and headless sperm (Figure 4).

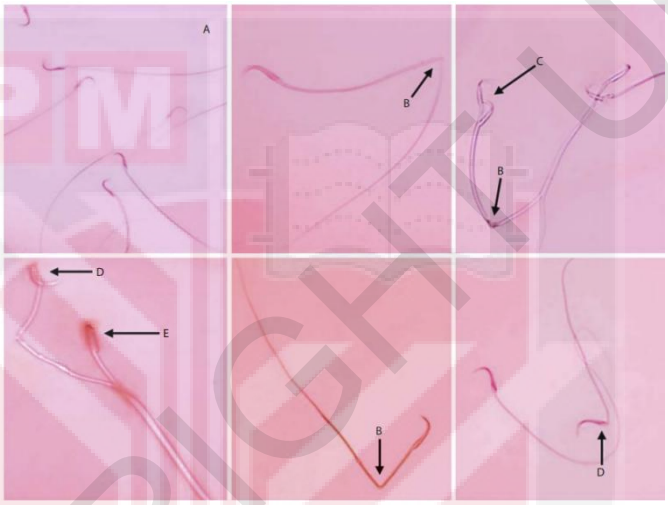

Figure 3: Photomicrographs of Wistar rats' semen stained with Eosin-nigrosin at 40x magnification A: Normal morphology; B: bent tail; C: double head; D: bent neck; E: flattened head (Abbasi *et al.,* 2009)

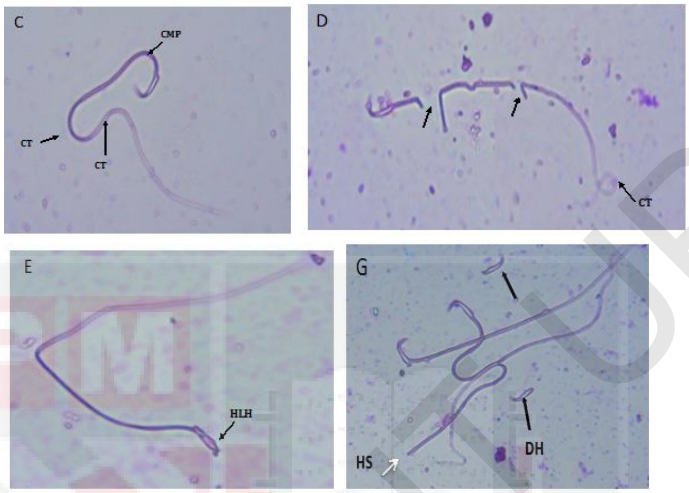

Figure 4: Photomicrographs of SD rats' semen stained with Giemsa at 40x magnification. C: Curved mid-piece (CMP) and curved tail (CT), D: Broken sperm (arrowed) with coiled tail (CT), E: Hookless head (HLH), G: Decapitated head (DH) and headless sperm (HS) (Wendmu *et al.*, 2020)

2.5.3 Sperm Motility

Various laboratories collect samples for sperm motility analysis from the vas deferens, the cauda epididymidis, and specific regions of the vas deferens or cauda epididymis of the rats. There was no difference between the percentage of sperm motility collected from the cauda epididymis and vas deferens reported. Slott et al. (1991) found that sperm obtained from proximal cauda epididymidis had significantly greater motility, progressive motility, progressive velocity, and path velocity than sperm from distal cauda epididymidis, with a magnitude of about 15% higher. Recovery and motility of the sperms collected from four segments of vas deferens were 95 ± 2, 94 ± 3, 92 ± 3, and 72 ± 9% respectively in which the highest value was the distal portion closest to the urethra and the lowest value was the proximal portion closest to the epididymis. Motility was also greatest for samples obtained from the four segments of the vas deferens distal portion with values of 8.5 ± 2, 81 ± 2, 74 ± 1, and 72 ± 4% respectively.

The diffusion method is often used in sperm extraction from the cauda epididymis. The cauda epididymis was placed in 2 ml buffer in a petri dish on a slide warmer. The tubules were pierced using a scalpel blade and tissue was removed. The sperms were allowed to swim into the medium for about 5 minutes and the

medium was withdrawn for analysis under a light microscope. Temperature control is crucial as cooling slows sperm motility. Several buffers that can be used are M199, a modified Hanks' Balanced Salt Solution, Dulbecco's PBS, and Dulbecco's PBS plus glucose 1 mg/ml (Chapin *et al*., 1992)

2.4 Measuring plasma testosterone using ELISA

The measurement of testosterone production is necessary for the evaluation of endocrine disorders in Cd-induced testicular toxicity. Referring to a study by Elder and Lewis (1985), assay procedures for all steroid assays, including testosterone, have improved over the past two decades, with several different techniques being explored, including gas-liquid chromatography (GLC), high-performance liquid chromatography (HPLC), and [3+] radioimmunoassays (RIA) as well as enzyme immunoassays (EIA). In a study by Wilkinson et al., (2000) using RIA to measure whole blood testosterone concentration, there was no significant difference in testosterone production between the three rat strains-Wistar, Sprague-Dawley (outbred strains), and Dark Agouti, DA (an inbred strain). They reported that the testosterone concentration of DA, SD, and Wistar rats are 3.84 ± (0.19 SE) ng/ml, 4.14 (± 0.22 SE) ng/ml, and 3.89 (± 0.22 SE) ng/ml respectively.

Enzyme-linked immunosorbent assay (ELISA) is a labeled immunoassay that is considered the gold standard of immunoassays. It is a very sensitive immunological test that detects and quantifies antibodies, antigens, proteins, glycoproteins, and hormones. These products are detected by complexing antibodies and antigens to produce measurable results. An ELISA is typically performed on polystyrene plates, 96 wells, coated with a very strong binding agent for protein. Different types of ELISA tests require a primary and/or secondary detection antibody, analyte/antigen, coating antibody/antigen, buffer, wash, and substrate/chromogen. Primary detection antibodies bind exclusively to the protein of interest, while secondary detection antibodies react with primary antibodies that are not enzyme-conjugated. In the ELISA protocol, usually, a serial dilution of concentrations is placed in the wells of the plate. Detection is carried out by adding a color-generating substrate (chromogens). The most commonly used are horseradish peroxidase (HRP) and alkaline phosphatase (ALP). The substrate for HRP is hydrogen peroxide which results in a blue color change. After the results are measured, a standard curve from the serial dilutions data is plotted with a concentration on the x-axis using a log scale and absorbance on the y-axis using a linear scale. The four major types of ELISA are direct, indirect, sandwich, and competitive ELISA (Alhajj & Farhana, 2022).

2.5 Edible Bird's Nest

Edible Bird's Nest is the nest of the swiftlets, small insectivorous birds generally found throughout Southeast Asia and the South Pacific. The nest is built from their starch-like saliva secretion that hardens on exposure to air. Most of the bird's nests were built by male swiftlets (Marcone, 2005). However, a report published by Lim et al. (2002) found that both males and females participate in nest building. The edible bird's nest is produced by several different swiftlet species in the genus Aerodramus and Collocalia (Ma & Liu, 2012). The white-nest swiftlet (Aerodramus fuciphagus) and the black-nest swiftlet (Aerodramus maximus) are among the species in Malaysia that produce the most traded edible bird's nest worldwide compared to other species, which are Hydrochus gigas, Collocalia esculent (White Belly Swifts) and Cypsiurus balasiensis (Asian Palm Swifts) (Ibrahim *et al*., 2009). Malaysia is situated in the center of the swiftlet's habitat and is one of the leading producers in the EBN industry (Daud *et al*., 2019). The swiftlets build the nest in about 35 days (Marcone, 2005). The white nests are mostly produced in the bird premises and only a little amount is found in the caves, whereas the black nests are only harvested in caves. The white nest swiftlet's EBN has greater economic value considering the fact that the white nests are almost entirely made from saliva (Sims, 2008) with only a small amount of impurities. In contrast,

black nest swiftlets contain more impurities, 45–55% feathers, and small dried leaves (Zulkifli *et al*., 2019). Thus, the tedious cleaning process makes it extremely expensive compared to white nest EBN and it is still heavily harvested. A recent report by Manan and Othman (2012) revealed that raw pre-processed EBN was sold at RM 3000/kg to RM4500/kg in the market from 2010 to 2011. The market process, however, is always doubled after the laborious and time-consuming cleaning process (Lim, 2006). EBN harvested from house farms is much lower priced in the market than those harvested from caves.

Figure 5: An overview of white (A), black (B) and red (C) EBNs (Lee *et al.,* 2021)

2.5.1 Chemical compositions of edible bird's nest and its benefits

Glycoproteins are the main components in EBN followed by proteins and carbohydrates, covering 60% and 30% of total mass respectively (Ma & Liu, 2012a; Marcone, 2005). Protein is known for its positive effect on cells and tissue growth as well as other metabolic functions. Based on previous studies, the average

protein content in EBN ranges from 50 to 55% of the dried weight (Wong *et al*., 2018c). It has been reported that EBN contains a higher percentage of humin nitrogen and cysteine nitrogen compared to pure protein due to the presence of some carbohydrate radicals and fine feathers. Carbohydrates EBN of 9% sialic acid contains N-acetyl-4-O-acetylneuraminic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fucose (Kathan & Weeks, 1969). Interestingly, ingestion of it can enhance and improve the neurological and intellectual ability of infants. According to Wang and Brand-Miller (2003), sialic acid facilitates the development of ganglioside structures in the brain. In addition, a myriad of glutinous glycoproteins, such as chondroitin glycosaminoglycan (GAGs) are found abundant in EBN and are crucial in bone and dermal development. This is supported by a study by Matsukawa *et al*. (2011), in which EBN can resist osteoporosis and increase dermal thickness in rats.

Some trace of micronutrients can be found in EBN, including fat (< 2%) and minerals such as sodium, calcium, and magnesium (Norhayati, Azman & Wan Nazaimoon, 2010; Quek, Chin, Yusof, Law & Tan, 2018), phosphorus, iron, potassium, iodine, and essential amino acids (Hun *et al*., 2015). The humidity condition in the caves activates the hydrolytic cleavage of

triacylglycerols, causing the EBN to have a high amount of mono- and diglycerides. Another possible factor is the action of enzymes in EBN (Marcone, 2005). The amino acids detected in EBN include nine essential amino acids (phenylalanine, valine, threonine, histidine, tryptophan, isoleucine, methionine, lysine, and leucine) which are required by the human body for tissue growth and reparation (Azmi *et a*l., 2021). Phenylalanine and tyrosine are the most common aromatic amino acids found in the white nest (Chua, Chan, Bloodworth, Li, & Leong, 2015; Marcone, 2005). The EBN was proven scientifically to possess high medicinal benefits in enhancing complexion, strengthening the immune system, stimulating epidermal growth, depressing the production of TNF-α, inhibiting viral infection, and improving respiratory and digestive problems.

The different environments in the caves and house farms may have a significant impact on the mineral profile of EBN (Seow *et al*., 2016). Cave EBN has higher Ca content as compared to house EBN. This is because cave EBN is normally found as a self-supporting nest that is attached to the vertical or concave surface of the calcium carbonate-rich cave wall. According to Northup and Lavoie (2010), mineral dissolution and precipitation processes in caves are microbially mediated reactions involving the iron-,

sulfur- and manganese-oxidizing bacteria which lead to pH reduction.

Apart from that, Na content in house EBN was also found higher than in cave EBN samples. Referring to a report by Norhayati *et al*. (2013), this could have originated from the marine aerosols (sea sprays) through atmospheric deposition into EBN. The high sea salt concentration in the air as a result of persistent on-shore winds at these locations may contribute to the accumulation of seawater droplets and marine aerosols (sea sprays). This hypothesis is made based on the swiftlet's unique ability to capture airborne water droplets. Thus, Na content in marine aerosol (swiftlet's saliva) is assumed to contribute to nest Na content. Differences in the elemental profile of both types of EBN could also be partly affected by the swiftlet diets. According to Lourie and Tompkins (2000), their diets vary and depend on their foraging regions and food availability. White nest swiftlet's diet was predicted to survive and adapt well in urban areas (shop lot house farms) which lead to the different minerals composition patterns in EBN harvested from different origins.

2.5.2 Effect on compositions of edible bird's nest on the reproductive system and fertility

EBN is recently found to enhance the DNA synthesis in 3T3 fibroblasts and mitogenic responses in human peripheral blood monocytes. Cellular communication relies on growth factors such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and cytokines such as interleukin-6 (IL-6). These molecules act as intracellular mediators to regulate their survival, growth, differentiation, and effects. The IL-6 family of cytokines has been implicated in maintaining both embryonic and adult stem cells as well as increased placenta-derived multipotent mesenchymal stem cell (MSCs) proliferation. MSCs are adult progenitor cells derived from bone marrow or fat tissue and able to differentiate into several cell lineages, including osteoblasts, chondrocytes, adipocytes, and myoblasts which are involved in the repair of injured tissue through the secretion of cytokines, chemokines, and growth factors. Angiogenic and antiapoptotic growth factors, such as VEGF and hepatocyte growth factor (HGF) are produced by human adipose stromal cells in response to hypoxia. The conditioned media containing these growth hormones show positive cell growth and suppress cell apoptosis. Other than that,

EBN promotes the proliferation of human adipose stromal cells (hADSCs) by upregulating the expression of IL-6 and VEGF by activating NF-κB and AP-1 (p44/42 MAPK and p38 MAPK). IL-6 has been shown to activate the JAK/STAT, MAPK, and AKT pathways. It has been implicated as a potent mediator of numerous critical biological processes, including differentiation, apoptosis, and proliferation whereas, VEGF stimulates cell proliferation in human adipose stromal cells' response to hypoxia. The unique nature of stem cells lies in their ability to proliferate and specialize, as well as their ability to self-renew. EBN enhances stem cell potency by increasing the proliferative capacity of hADSCs (Roh et al., 2012). Hence, this effect may also promote the proliferation of spermatogonia during spermatogenesis in the testis (Farah *et. al*., 2020).

Ma and Liu (2012) discovered the presence of six kinds of hormones as bioactive in EBN which are testosterone (T), estradiol (E2), progesterone (P), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (PRL). Another study done by Ma *et al*. (2012) also demonstrated that EBN supplementation in castrated male rats had a significant increase in serum testosterone and LH. This finding suggested that EBN can become a potential erectile dysfunction treatment. The bioactive

component for enhancing sexual function is testosterone (T). In animal studies, T plays critical physiological (activity of NOS and phosphodiesterases), biochemical (endothelial-independent pathway and androgenic tonicity), and structural (change of fibro-elasticity and hollow cell accumulation) roles in erectile function (Hwang and Lin, 2008). In the experiment, it was suggested that T increased after administration of EBN at 9 mg/kg/day which contributed to the increase in penis and prostate and seminal vesicle indexes and the protein expression of eNOS in male castrated rats.

The presence of antioxidants has been reported in EBN for a long time (Ghassem *et al*., 2017). However, the effect of its antioxidants after oral administration is not fully known. An important component of EBN's antioxidative properties is the pool of bioactive compounds such as amino acids, sialic acid, triacylglycerol, vitamins, lactoferrin, fatty acids, minerals, and glucosamine (Liu *et al*., 2012; Zainab *et al.*, 2013; Lee *et al*., 2020). Moreover, ovotransferrin and lactoferrin also contribute to the anti-oxidative effect of EBN (Hou *et al*., 2015). The authors reported their protective effects against H2O2-induced toxicity on the human neuroblastoma cell line, SH-SY5Y. Oxygen Radical Absorbance Capacity (ORAC) and 2,2-azinobis-(3-

ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays were used in a study by Yida *et al.* (2014) to evaluate the in vitro bioaccessibility and antioxidant properties of water extracts of EBN. It was observed that there was low antioxidant activity (about 1% at 1,000 μg/ml) on the undigested EBN water extract for both ABTS and ORAC assays. On the contrary, the digested EBN samples using pepsin, pancreatin, and bile extract at similar concentrations showed improved antioxidant activities at around 38 and 50% for ABTS and ORAC assays respectively. Besides, the EBN extracts showed non-toxicity toward human hepatocellular carcinoma (HEPG2) cells and protected HEPG2 cells from H2O2-induced toxicity. In short, the enhancement of antioxidant activities of EBN after digestion highlighted some of its functional effects after consumption.

2.6 Cadmium Toxicity

2.6.1 Effects of cadmium chloride on the male reproductive system

Cadmium (Cd) is a heavy metal commonly found as an environmental contaminant. Cd and Cd compounds are by-products of human activity, including Cd-chloride (CdCl2), Cd-acetate, and Cd-carbonate. These compounds are found in batteries, pigments, plastic stabilizers, pesticides, fertilizers, and photovoltaics, along with rubber processing, galvanizing, burning fossil fuels, and incinerating waste. The primary targets of Cd toxicity are the liver and kidneys. Cd intoxication manifests itself earliest in these organs following ingestion. Additionally, Cd accumulates in the ovaries and placenta of women, as well as in the testis and epididymis of men. Approximately half of the total human Cd intakes are through cigarette smoking in non-Cd-polluted areas, with 0.2 - 1.0 mg per cigarette assimilated. There is a relatively high bioavailability of inhaled Cd oxide, with 10% depositing in lung tissues and another 30 - 40% absorbed into the systemic circulation of smokers (de Angelis *et al*., 2017). In a study by Lim *et. al*., (2022), the current prevalence of smoking at baseline in Malaysia was 21.1% and it was significantly higher among males compared to females (39.1% vs 2.1%). Cadmium has been identified as a human carcinogen by the International Agency for Research on Cancer and the

National Toxicology Program. However, the detailed mechanism of carcinogenesis caused by Cd remains unknown.

Cadmium's harmful effects on reproduction can be irreversible and long-lasting when administered as a single high dose, or chronic low doses. When animals are treated during fetal development, in early life, or before pubertal years, severe damage may occur to proliferating and differentiating Sertoli cells, which play a crucial role in the formation of functional testis and spermatogenesis. Cd causes functional impairment of Sertoli and Leydig cells, as well as oxidative stress in somatic and germ cells in the testes. Additionally, Cd severely affects testis structure by damaging vascular endothelium and blood-testis barrier (BTB) integrity and inducing inflammation and apoptosis within the testis. In germ cells, these effects are primarily mediated by mimicry and interference with essential ions. Lastly, disturbance of the hypothalamus-pituitary-gonadal axis is also reported after Cd exposure (de Angelis *et al*., 2017).

Oxidative stress

Oxidative stress is the main mechanism in reproductive toxicity that specifically target the Leydig cells, germ cells, and spermatozoa. Cd increases the reactive oxygen species (ROS) and induces oxidative stress via two indirect mechanisms: the first

involves Cd binding to the sulfhydryl groups of ROS scavengers, resulting in a shift in their regulatory activity. Secondly, it interferes with the important ions needed for ROS scavengers to work, leading to the depletion of GSH. This is most likely due to the ROS production at a rate that exceeds the capacity to regenerate reduced GSH. Both processes produce ROS, including superoxide ions, hydrogen peroxide, and hydroxyl radicals. Indirect Cd-induced elevation of ROS can cause excess protein oxidation, lipid peroxidation, DNA damage, and, ultimately, cell death. The consequences of oxidative stress are particularly dramatic in oxidative damage-sensitive cells, such as spermatozoa, mainly when lipid peroxidation within the plasma membrane occurs, by strongly contributing to the reduction of sperm motility and fusogenic potential with the oocyte. A well-known cause of male infertility is oxidative stress-induced DNA damage in spermatozoa as it causes the transfer of abnormal genomic information to the embryo, which increases the likelihood of miscarriage and offspring problems (de Angelis *et al.,* 2017).

Blood-testis barrier disruption

The BTB is made up of the tight junction of Sertoli cells, which bisect the seminiferous epithelium into the basal and the apical compartments. This unique structure prevents not only the passage of cytotoxic agents from the blood into the seminiferous tubules but also the passage of antigenic products of germ cell maturation into the circulation, which might generate autoimmunity against germ cells. Cd triggers the down-regulation of the integral membrane protein of the tight junction, namely occludin. Besides preventing cytotoxic agents from passing into the seminiferous tubules, this structure also prevents antigenic products from maturing germ cells from moving into the circulation, which could lead to autoimmunity (de Angelis *et al*., 2017).

Inflammation within the testis

In testis homogenates from Cd-induced rats, multiple inflammation markers were significantly increased, including inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-, nuclear factor-kB, and heme oxygenase-1. Seminiferous epithelium cells became necrotic and vacuolized due to Cd accumulation, resulting in interstitial edema and hemorrhage and

consequently, the impairment of spermatogenesis (de Angelis *et al.*, 2017).

Apoptosis within the testis

It has been shown that treatment with Cd strongly induces germ cell apoptosis, mainly by downregulating B-cell lymphoma extra-large (Bcl-XL) and boosting Bax and caspase-3 (de Angelis *et al.,* 2017*)*.

Direct cytotoxicity on testis cells

In addition to showing significant structural and functional changes in response to Cd exposure, Sertoli cells seemed to be more sensitive to Cd toxicity. Exposure to CdCl2 causes reductions in INH-B and anti-Mullerian hormone (AMH) secretion and disruption of FSH receptor responsiveness, assessed by E2 production, along with apoptosis in Sertoli cells. Cd compromises Leydig cell function by reducing its responsiveness to hCG stimulation as demonstrated by the reduction of T production. The cytotoxicity of Leydig cells may act as a mediator for the toxic effect of Cd on testis endocrine function (de Angelis *et al.,* 2017*)*.

Disruption of the hypothalamus-pituitary-gonadal axis

Cd significantly affects the endocrine system and causes hormonal imbalance via alteration of the concentrations of gonadotropin, T, and INH-B, in experimental models. Cd affects the expression of steroidogenesis enzymes, such as StAR, cholesterol C20-22 desmolase, 17a-hydroxylase, 17 - hydroxysteroid dehydrogenase, and suppresses the expression of LH receptors in the testis of mice and rats (Gunnarsson et. al., 2007). In male rats exposed to Cd, the circadian release of noradrenaline is compromised, which greatly influences gonadotropin-releasing hormone production from the hypothalamus, LH, and prolactin secretion from the pituitary, and T circulating concentrations. All of these impacts on the endocrine system are facilitated by direct effects on target organs and cell addition to this (de Angelis *et. al.*, 2017).

The testosterone concentration can be affected by Cd without the involvement of Leydig cell necrosis. A study by Laskey and Phelps (1991) found changes in Leydig cell viability were not observed during 3 hours of incubation with Cd2+ and other metal cations (Co2+, Cu2+, Hg2+, Ni2+, and Zn2+) in vitro treatment. There was dose-response depletion in both hCG- and db-cAMP-stimulated testosterone production in conjunction with

the cation treatment. Surprisingly, the same cation treatment caused significant increases in HCHOL- and PREG-stimulated testosterone production over untreated and similarly stimulated cultures. This implies that these cations may exert their effects across different sites of Leydig cell membranes. Compared to progesterone and 17b-estradiol, testosterone was more sensitive to Cd exposure, indicating that this metal has multiple sites of action in steroidogenesis. Reduced activity of essential enzymes that are involved in the biosynthesis of testosterone may occur due to impaired testosterone synthesis (Knazicka *et. al*., 2014).

Other than Cd-induced decrease in total testosterone hydroxylase activity (Clark *et al*., 1994), decreased plasma levels of follicle-stimulating hormone and luteinizing hormone were an indication of low testosterone hormone (Davidson *et al*., 1993; Lafuente *et al*., 1999), which causes a dramatic decrease in testosterone hormone. In turn, this decline may be responsible for a significant decrease in the hCG-stimulated serum testosterone levels, (Laskey *et al*., 1984) interference of Cd with cAMP in testis, and depression of protein kinase (Singhal *et al*., 1976).

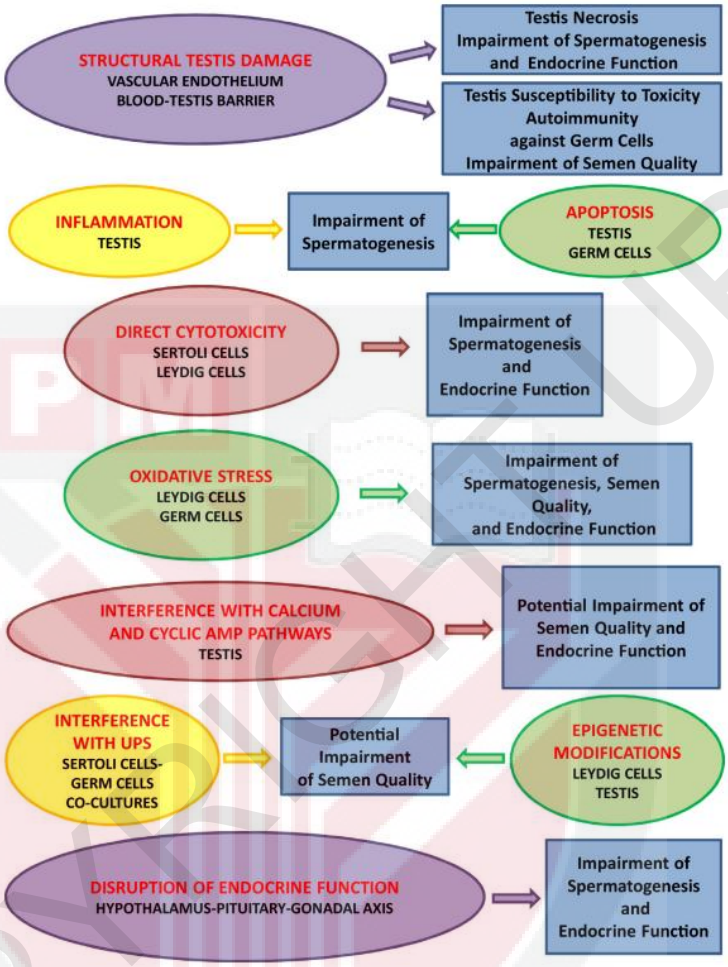

Figure 6: Overview of the Proposed Pathogenesis and Mechanisms of Cadmium Reproduction toxicity (de Angela *et al.,* 2017)

3.0 Materials & Methods

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

3.1 EBN preparation

Raw EBN powder was obtained from Universiti Kebangsaan Malaysia (UKM) and a solution was prepared by dissolving 1g of EBN powder in 100 ml of distilled water, followed by heating in a water bath at 60°C for 45 min. The EBN solution was cooled down to room temperature and administered to the rats at appropriate doses according to individual rat's bodyweights.

3.2 Cd solution preparation

A 0.5g of Cd powder was dissolved in 250 ml of distilled water and individual rats of treated groups were given orally at a dose of 10 mg/kg BW using a gavage tube.

Figure 7: Cadmium Chloride (CdCl2) used for experiment

3.3 Animals and experimental design

Twenty-five Sprague Dawley rats at the age of 10 weeks were used in this study. The rats were acclimatized in an animal house under standard laboratory conditions for 7 days with free access to water and standard diet (Gold Coin Brand Animal Feed).

Figure 8: 10 weeks old of Sprague Dawley rats used for the experiment

After 7 days of acclimatization, rats were randomly divided into five groups; Group 1 (Normal saline), Group 2 (CdCl2 10 mg/kg BW orally), while groups 3, 4, and 5 received CdCl2 (10 mg/kg BW) plus graded concentrations of 60, 90 and 120 mg/kg BW of EBN, respectively for 16 days. The choice of EBN doses was according to a previous report by Albishtue *et. al.* (2019). During the treatment period, the body weight in each group was measured and recorded twice a week. Rats were then euthanized under a general anaesthesia procedure, which included an injection of 30 mg ketamine/kg BW and 10 mg xylazine/kg BW, following intracardiac puncture for blood collection.

Table 1: Animal grouping and treatment regime of Cadmium and EBN via oral gavage

Group	Group Assigned	Number of rats	Type of Treatments (Dose)
Control	NC	5	Normal diet (ND) + Normal saline PO
Treated groups	T0	5	ND + $CdCl_2$ (10 mg/kg BW) PO
	T1	5	ND + EBN (60 mg/kg BW) PO + $CdCl_2$ (10 mg/kg BW) PO
	T2	5	ND + EBN (90 mg/kg BW) PO + $CdCl_2$ (10 mg/kg BW) PO
	T3	5	ND + EBN (120 mg/kg BW) PO + $CdCl_2$ (10 mg/kg BW) PO

3.4 Evaluation of body weight and relative weights of organs

The testes were collected and weighed at the end of the treatment. The relative weight of organs was calculated based on the previous study by Wang *et. al.* (2021), which is the ratio of the absolute weight of organs to the final body weight and expressed as mg/100g body weight.

3.5 Sperm evaluation

3.5.1 Evaluation of epididymal sperm count

The epididymis was cut using a pair pf pointed-end scissors in preheated normal saline (37 ◦C) and the resulting sperm suspension was left for 5 minutes. The suspension was diluted with formal saline at 1: 100 ratio and about 10µl of suspension was transferred into the hemocytometer chamber. The sperm number was counted under a light microscope (40x). Each square contains 16 small squares. The number of sperm was calculated from the top to the left of each small square in all five large squares using the following formula below as described previously by Rathje *et al.* (1995) with modification.:

$$\boldsymbol{Y} = \frac{(\boldsymbol{Y1} + \boldsymbol{Y2})}{\boldsymbol{2}}$$

Y_1 = Number of sperm in chamber A

Y_2 = Number of sperm in chamber B

Y = Total sperm

Then, the sperm concentration was calculated using the formula below:

Concentration = 50Y x 10^6 sperm/ml

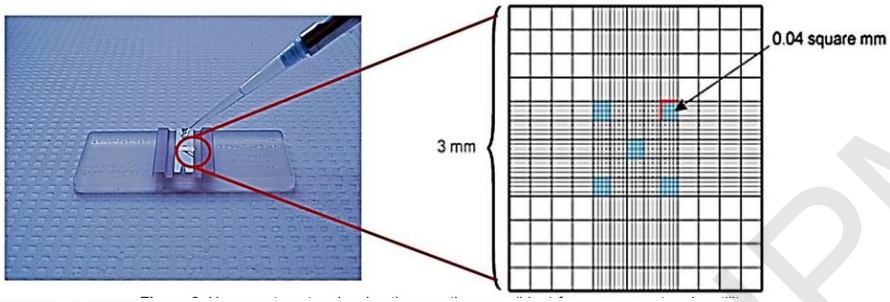

Figure 9: Haemocytometer showing the counting area (blue) for sperm count and motility (Parhizkar *et al*., 2013)

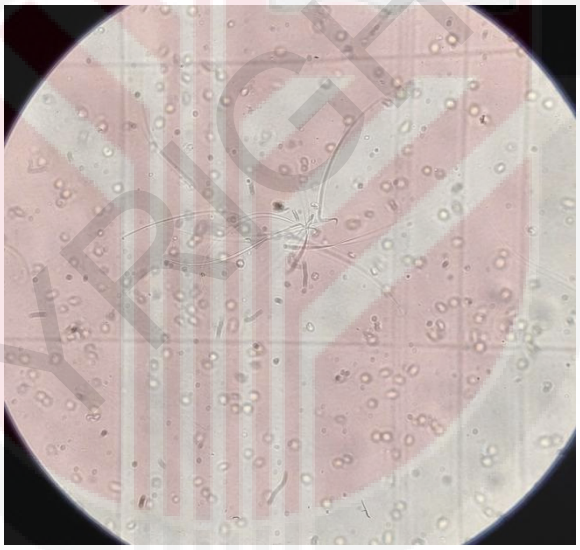

Figure 10: Counting sperms using haemocytometer under light microscope at magnification 40x

3.5.2 Evaluation of Sperm Morphological Abnormalities

Three to four drops of Eosin-nigrosin stain were placed on a clean glass slide using a dropper. Then, a drop of sperm suspension was added to the stain and mixed well. After 3 minutes, a thin smear was done and air-dried. The slide was then observed under a light microscope (40x). Spermatozoa were classified into normal and abnormal types as described previously (Narayana *et al.*, 2002) and the percentages of abnormal morphology were further classified into head, midpiece and tail abnormalities.

3.6 Determination of Testosterone Production

Blood collected was transferred into an EDTA tube and centrifuged for 15 minutes, 3000 rpm. Plasma was collected and transferred into a 1.5 ml microcentrifuge tube. Plasma testosterone was measured by sandwich enzyme-linked immunosorbent assay (ELISA, Qayee-Bio, Shanghai, China). All the assay procedures were done according to the kit instruction.

Briefly, 50 µl standard, followed by 40µl of special diluent and 10µl of plasma were pipetted to a 96-well ELISA plate in duplicate. Then, 50 µl of horseradish peroxidase (HRP) was added into each well, except the blank well, sealed, gently shaken and incubated for 60

minutes at 37 ℃. All the solution was removed from the well before being washed five times. Colour development was done by adding 50 µl Chromogen A followed by Chromogen B, covered from light and further incubated for another 10 minutes at 37 ℃. The reaction was stopped by adding 50 µL Stop solution and the absorbance was measured at 450 nm. Testosterone levels were interpolated from 5 points standard curve plotted according to a 4-parameter logistic (4 PL) by using Arigobio.com.

3.7 Statistical Analysis

Statistical analysis was performed using SPSS version 26.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Games Howel and Tukey's posthoc analysis was conducted to compare the sperm concentration and morphology as well as testosterone production owing to the normal distribution and variance homogeneity of the data. A p-value < 0.05 was considered statistically significant.

4.0 Results

4.1 Effect of Cd on relative testicular weight

As shown in Figure 11, the relative weight of the testis in the Cd group was lower than in the control group. There was an incremental increase in the relative testicular weight with the increase in EBN dosage. The relative testicular weight in the control group remained the highest as compared to the other group. However, there was no statistically significant difference in testicular relative weight between all the groups.

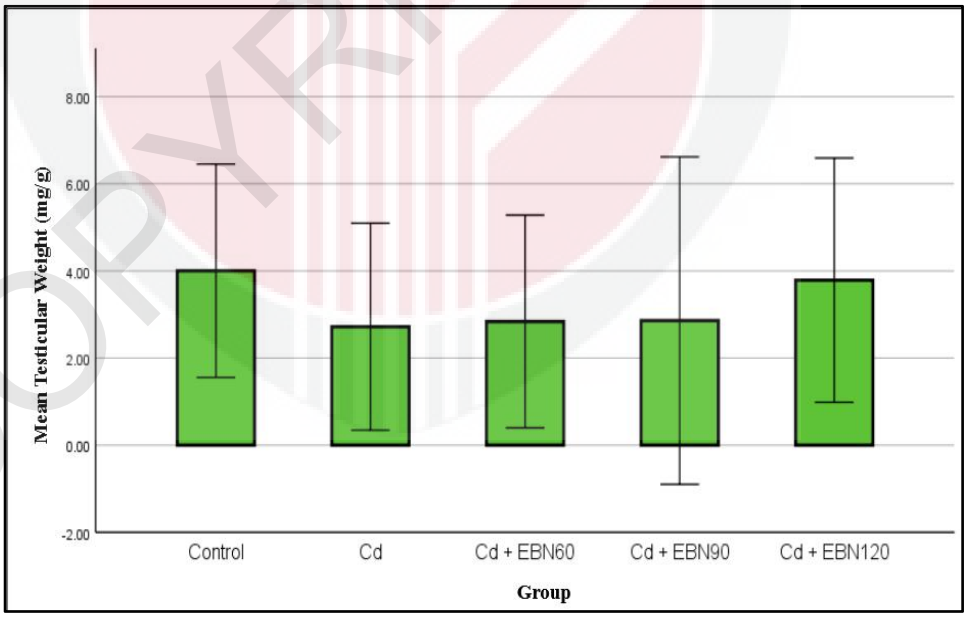

Figure 11: Relative mean testicular weight in each group. No statistically significant difference in mean testicular relative weight between the groups.

4.2 Effect on sperm concentration and morphology

Compared with the control group, sperm count in the Cd-treated group significantly decreased ($P < 0.05$) (Table 2). There was no significant difference in sperm concentration between the Control, 60 mg/kg BW/d EBN, and 90 mg/kg BW EBN. On the other hand, the sperm concentration in the 120 mg/kg BW of EBN treated group showed a significant increase compared to the Cd-treated and 60 mg/kg BW of EBN treated group.

For the percentage of sperm abnormal morphology, there was also no significant difference in sperm abnormal morphology between the Control, 60 mg/kg BW/d EBN, and 90 mg/kg BW EBN (Table 2). However, the sperm abnormal morphology in the 120 mg/kg BW/d EBN showed a significant drop compared to the Cd-treated ($P < 0.05$) and there was a significant increase in the Cd-treated group compared to the control group and 120 mg/kg BW/d EBN. Interestingly, the sperms retrieved from the Cd-treated group demonstrate higher percentages of head abnormalities compared to tail abnormalities (Figure 12). In contrast, the group treated with 120 mg/kg BW/d showed lower percentages of head abnormalities.

Table 2: Effect of CdCl2 on sperm concentration and sperm morphology. Different superscripts across each column represent significant differences ($p < 0.05$).

Group	Control	Cd	Cd + EBN60	Cd + EBN90	Cd + EBN 120
Sperm Concentration (x 10^6/ml) ± SD	121 ± 0.11[a]	91 ± 0.20[b]	103 ± 0.59[b]	100 ± 0.49[b]	118 ± 0.22[a,c]
Abnormal sperms (%) ± SD	6.0 ± 1.10[a]	15.6 ± 2.41[b]	15.4 ± 1.82[b]	12.8 ± 2.05[b,c]	10.4 ± 1.14

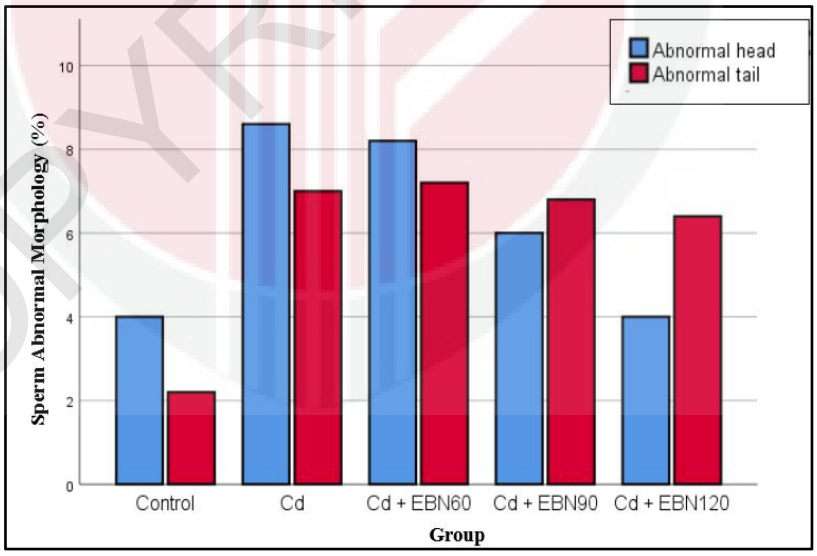

Figure 12: Percentages of sperms with abnormal heads and tails in different treatment groups

4.3 Testosterone hormone analysis

The testosterone in the plasma of Sprague Dawley rats showed a trend of gradual increase with increasing EBN dose (Figure 13). However, only the control, Cd-treated group and 120 mg/kg BW/d showed a significant result in which the Cd-treated group was significantly lower than the control group and 120 mg/kg BW/d EBN ($P < 0.01$), whereas the 120 mg/kg BW/d was significantly higher than the Cd-treated group ($P < 0.01$).

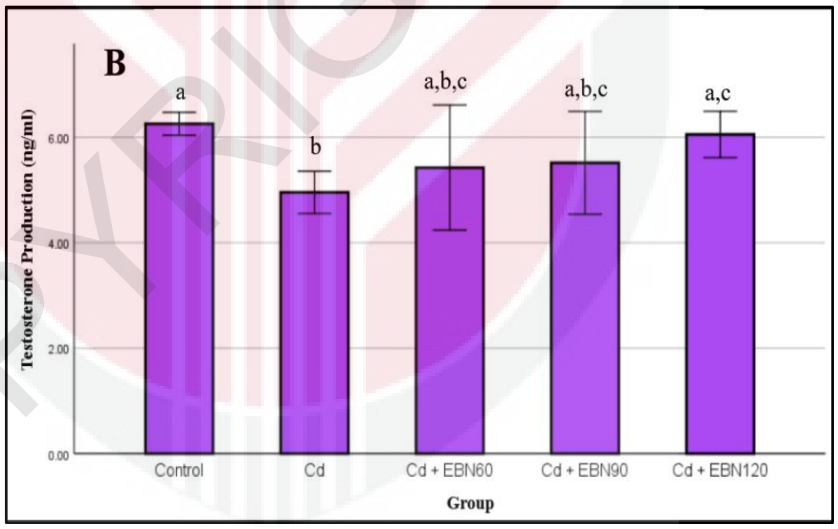

Figure 13: Comparison of testosterone production in rats supplemented with different doses of EBN. Bars with different letters indicate statistical significance ($p < 0.05$).

5.0 Discussion

Testicular toxicity is characterized by the reduction in the weight of the organs (Fallahzadeh *et al*., 2017; Nowrouzi *et al*., 2019). The Cd-treated group showed the lowest relative testicular weight. However, the difference among the groups was not significant. In a previous study, four weeks of oral exposure to Cd significantly decreased the relative testis weight in SD rats (Wang *et al.*, 2020). This study, however, was conducted for 16 days only which explains the less significant differences in organ coefficient between each group. From the results, Cd significantly reduced sperm count and markedly increased abnormal sperm morphology in Cd-treated rats. These findings may be related to the oxidative damage caused by Cd. Sperm is vulnerable to reactive oxygen species (ROS) as its cell membrane is rich in polyunsaturated fatty acids. Sperm morphology will be impaired if exposed to oxidative damage from heavy metals (Agarwal *et. al*., 2002). Both head and tail abnormalities were found in the treatment groups, however, the highest percentages of sperm head abnormalities were observed in the Cd-treated group. Previous research has shown a positive correlation between abnormal sperm heads and DNA damage which explains that sperm head abnormalities may be an indirect indication of mutagenic effects on sperm DNA. Hence, it is crucial to study sperm DNA structural integrity to evaluate sperm function and structural changes (Baysal *et al.,* 2017).

Improvements in sperm quality after EBN co-treatment are attributed mostly to the inhibition of free radicals over the production and/or enhancement of the ability of the testis antioxidant defense system to effectively restrain the oxidative impairment in testes induced by Cd. In addition, the increment in sperm concentration may also be related to the positive proliferative effect of EBN (Aswir and Wan Nazaimoon, 2011; Roh *et al*., 2012) which is highly associated with the presence of sialic acid (Aswir and Wan Nazaimoon, 2011). Since sialic acid is the most abundant glycoprotein in EBN, it may act synergistically with another factor present in EBN to promote spermatogonia proliferation during spermatogenesis. Similarly, a study by Albishtue *et al.* (2018) also reported that EBN has a protective effect on the rats' uterus against the effect of lead acetate (LA) toxicity. The treatment group of EBN at a dosage of 120 mg/kg BW showed normal uterine structures and the uterine morphology parameters were higher than LA-treated and control group. This is supported by earlier comparative studies which revealed that EBN had increased proliferation rate of human colonic adenocarcinoma cell line (Caco-2 cells) 135% - 215% compared to control and reduced percentage of tumor necrosis factor-alpha in vitro (Aswir and Wan Nazaimoon, 2011; Vimala *et al*., 2012)

Additionally, EBN supplementation promotes sperm maturation by enhancing sperm viability and motility. It was reported that there was a significant increase in motile and viable sperm percentage in the 250 mg/kg

BW EBN group compared to the 10 mg/kg BW/d EBN group (Farah *et al*. 2021). Sperm motility and viability were not evaluated in this study due to the unavailability of special media. Apart from collection methods, temperature and different regions of the epididymis, the type of sperm dilution medium may also affect sperm motility. Although rat sperm in the cauda epididymidis are considered motile, the high viscosity of the epididymal fluid makes them immobile. This ability to move does not manifest until the sperm are diluted in a special medium. Dulbecco's or plain phosphate-buffered saline (PBS) with or without Ca ++ and Mg ++, BSA, or glucose, Medium 199 with added BSA and modified HBSS are some of the different media used in motility analysis of rat sperm. Sperm diluted in PBS generally exhibited the highest motility and velocity over the brief period of the analysis, according to data presented at a prior workshop (Chapin *et al*., 1992). However, the pH of the medium may also influence sperm motility (Slott *et al*., 1991). Sperm viability assessment is used to determine whether non-motile sperm are alive or dead and is only implied when sperm motility is less than 5%–10% (Vasan, 2011), which in this case was not evaluated as well.

Sialic acid is also known as an antioxidative molecule that contributes to the antioxidative properties of EBN (Yadav *et al*., 2020). The EBN used in a previous study by Quddus *et al.* (2021) contains 10.7% sialic acid. In this study, there was a remarkable reduction in Cd accumulation in the uterine tissue of SD rats treated with the EBN, and the lowest Cd concentration was

found in rats treated with 120 mg/kg BW. Studies have shown that sialic acid has a high affinity to toxic heavy metals, especially Cd ions and that its deficiency is often associated with oxidative stress. Thus, high sialic content in EBN is proven to avert the oxidative stress caused by Cd toxicity (Saldini *et al*. 2002). A key mechanism by which EBN prevented the alteration of cellular redox states caused by LA was by increasing antioxidant capacity. SOD is one of the enzymes that contribute to antioxidant defense. These antioxidant enzymes can improve membrane integrity, blocking free radicals, thus making it more resistant to metal exposure. EBN supplementation at a dose as low as 30 mg/kg BW is sufficient to enhance the enzymatic antioxidant defense against LA toxicity and prevent disturbance of the redox system (Albishtue *et al*., 2018).

Hormones play an important role in the production and maintenance of androgens in male animals. The hypothalamus secretes GnRH, which can provoke the secretion of FSH and LH in the anterior pituitary gland and thus, has considerable activity potential. FSH plays an important role in nourishing the Sertoli cells of the testes; it plays a key role in spermatogenesis. In male animals, LH acts on the Leydig cells of the testes to promote testosterone production. Due to the effect of testosterone, sperm formation is completed during the development of secondary sexual characteristics. Low LH is a manifestation of testicular dysfunction (Wang *et al*., 2021). In the present study, the concentration of testosterone in Cd-treated rats was significantly

reduced. The concentration of other hormones was not measured. However, testicular Leydig cells secrete testosterone and the number of Leydig cells is usually positively correlated with the concentration of testosterone. The hypothalamus and pituitary hormone secretion is likely to be affected by Cd-induced oxidative stress since both are crucial for gonadal function. Considering EBN's antioxidative properties, the increase in testosterone concentration in the EBN-supplemented group may be a result of its protective effect on Leydig cells. EBN consumption is traditionally believed to increase libido. A variety of factors influence libido, but testosterone is closely related to it (Allan *et al*., 2008; Corona *et al*., 2014; Rizk *et al*., 2017). In addition, several male reproductive hormones such as FSH, LH, and testosterone were also found in EBN according to Ma and Liu (2012) and this may serve as a potential alternative treatment for erectile dysfunction (Ma *et al*., 2012). Therefore, the presence of hormones in the EBN itself contributes to the gradual increase in testosterone concentration in the EBN-treated group. This is consistent with a study by Farah *et al.* (2021) where testosterone serum of SD rats demonstrated a similar trend of gradual increase with increasing EBN dose.

6.0 Conclusion

Cd-exposed rats without EBN supplement demonstrated significantly low sperm concentration with a high percentage of abnormal sperm morphology and low testosterone production. There was an incremental increase in sperm parameters and testosterone production as the dose increased in the EBN-treated group. A simultaneous supplement of EBN 120 mg/kg with Cd exposure revealed significant differences in sperm quality and testosterone production. Thus, an oral supplement of EBN at 120 mg/kg BW is effective to mitigate the Cd toxicity-induced reproductive dysfunction in male SD rats. This might provide scientific evidence to traditional beliefs on the role of EBN in reproduction. The overall findings in this study also coincide with the traditional belief among consumers on the role of EBN consumption to enhance reproduction, fertility and ameliorating effect against the toxicity of Cd.

7.0 Recommendations

Long-term Cd exposure experiment is required to study whether the ameliorating effect of EBN supplement against Cd toxicity on the male reproductive system of rats persists. Larger sample sizes are required for precise means and less margin of error. In this study, only 25 male rats were used with 5 rats in each group. A maximum sample size of 30 to 40 rats would be sufficient for more accurate results. Studies on other male reproductive parameters should be conducted. In the present study, sperm motility, viability and DNA integrity were not evaluated. Other important hormones such as FSH and LH should be considered in the study to further investigate the direct hormonal-promoting effect of EBN. Further studies on EBN's effectiveness against various environmental toxicants is needed to elucidate the action of EBN on different toxicants that induce reproductive toxicity in animals. This study was conducted using only raw EBN. A different study should be conducted using hydrolyzed EBN to compare its effect to the raw EBN.

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9.0 Appendices

9.1 Animal Monitoring Form

With any abnormal findings – please give specifics.

PROJECT TITLE:

PI :

APPROVAL AUP NO:

RESEACHER/PERSON IN CHARGE:

PHONE NUMBER/EMAIL:

DATE OF EXPERIMENTATION :

SPECIES/STRAIN/SEX/AGE:

PROCEDURE:

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 Tiptoe walking/reluctant to move = 2 Staggering/limb dragging/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 than 1 day=2 Decreased intake over 48hrs=3							
BREATHING Normal=0 Rapid, shallow=1 Rapid, abdominal breathing=2 Laboured, irregular, skin blue=3							
OTHER COMMENTS							
MONITORED BY/INITIAL							