Age related changes in apoptosis of germ cells in normal and dibutyl phthalate-treated testes of Japanese quail (*Coturnix coturnix japonica*)

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Thesis submitted in fulfilment of the requirements for the degree

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Declaration

I, Musa Zakariah, hereby declare that this doctoral thesis titled: **Age-related changes in apoptosis of germ cells in normal and dibutyl phthalate-treated testes of Japanese quail (*Coturnix coturnix japonica*)** is my original work. The work has not been submitted to any higher institution of learning for award of degree elsewhere. University of Pretoria in consultation with the author reserves the right of permission for copying, duplication or printing of some parts or the whole thesis.

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Thesis summary

Age-related changes in apoptosis of germ cells in normal and dibutyl phthalate-treated testes of Japanese quail (*Coturnix coturnix japonica*)

Excess germ cells in normal and in pathological conditions are removed from testicular tissue by the mechanism of apoptosis. Studies on germ cell apoptosis in avian species are grossly lacking, and there are only a few reports of induced germ cell degenerations in the testicular tissue of birds. This study was designed to evaluate selected pro-apoptotic proteins, their mechanism of action and their involvement in germ cell apoptosis. Seminiferous tubule parameters such as seminiferous tubule diameter, seminiferous tubule epithelial height and seminiferous tubule lumen diameter were also investigated. In addition, the process of apoptosis of germ cells under normal conditions, and in pathological conditions induced by an endocrine disruptor, di-n-butyl phthalate (DBP) in the testes of Japanese quail (*Coturnix coturnix japonica*) was evaluated. Features of germ cell apoptosis were observed in birds of all age groups, namely pre-pubertal, pubertal, adult and aged under normal conditions. The morphological features of these apoptotic germ cells under normal conditions ranged from irregular nuclear membrane, ruptured nuclear membrane, condensed nuclear material and apoptotic bodies.

The treated birds were administered a daily dosage of 10, 50, 200 or 400 mg/kg/body weight of DBP (dissolved in corn oil) for 30 days intra-gastrically by lavage. Apoptosis of germ cells in the quail under normal conditions and those of the DBP treated groups was evaluated using various methods. These included haematoxylin and eosin (H&E)

staining, TdT dUTP Nick End Labeling (TUNEL) assay and electron microscopy, and results were similar to those observed in previous studies of germ cells and somatic cells of mammalian species. The observed morphological features of these apoptotic cells under pathological (DBP-treated testis) conditions were also similar to those under normal conditions, which ranged from irregular plasma and nuclear membranes in the early stage of apoptosis to rupture of the nuclear membrane, condensation of nuclear material, as well as fragments of apoptotic bodies, in later stages of apoptosis. These apoptotic germ cell features were similar to those observed in mammalian species, although the DBP doses were higher in reported data on mammalian species. Therefore, it is logical to propose that spermatogenic cells of Japanese quail seem to be more sensitive to DBP-induced germ cell degenerations compared to mammalian species. Therefore, more studies need to be conducted on the effects of DBP exposure on avian spermatogenesis.

CHAPTER ONE

General introduction

1.1 Overview on spermatogenesis in birds

Information on the biology of male reproductive systems is crucial to enhance the productivity of economically important animals, conservation, improving management practices, as well as refining artificial and natural breeding systems (Andrabi and Maxwell, 2007). The architecture and organization of the testis in all mammals are generally similar, but each species may show morpho-functional differences or characteristics related to reproductive behaviour and phylogenetic aspects (Kerr et al., 2006, Setchell and Breed, 2006; Costa et al., 2010).

Spermatogenesis is a complex process involving cell proliferation and differentiation from spermatogonia to mature spermatozoa (Aire, 2007). The process of spermatogenesis is similar in all mammals, as well as in all avian species studied to date. Nonetheless, few studies have investigated spermatogenesis in avian species compared to mammals. Spermatogenesis can be divided into the following three major stages: spermatocytogenesis, which consists of successive, rapid and incomplete cytoplasmic divisions of spermatogonia that result in a large population of germ cells (Lin and Jones, 1993). Secondly, spermatidogenesis is a decrease in the division of genetic material which includes both primary and secondary divisions of spermatocytes to form spermatids; and finally, spermiation (or spermiogenesis) occurs when undifferentiated haploid spermatids finally transform to mature spermatozoa (Bueno et al., 2014).

1.1.2 Spermatogonia

Spermatogonia lie on the basement membrane of seminiferous tubules but are not attached to it. Spermatogonia can be classified based on their activities into stem cell spermatogonia, proliferative spermatogonia and differentiating spermatogonia (Lin and Jones, 1993). The stem cell spermatogonia, as well as proliferative spermatogonia, are responsible for producing and renewing differentiating spermatogonia (Lin and Jones, 1993). Aire et al. (1980) identified two types of spermatogonia in guinea fowl (types A and B), while Lin and Jones (1992) identified four types of spermatogonia in Japanese quail (type A dark spermatogonia (Ad), type A pale spermatogonia (Ap1 and Ap2) and type B spermatogonia).

The early type A spermatogonia are identified by their pale-stained nuclei with clumps of chromatin granules spread across the nucleus. In Type B spermatogonia, dense chromatin is located on the nuclear membrane. This dense chromatin can be observed throughout the process of spermatogenesis (Bellve et al., 1977, Lin and Jones, 1992).

1.1.3 Spermatocytes

There are two types of spermatocytes, namely primary spermatocytes and secondary spermatocytes. The germ cells of primary spermatocytes are very large in size and they are present relatively longer than other germ cells during the process of spermatogenesis (Lin and Jones, 1993). Three types of primary spermatocytes were initially identified in Japanese quail and guinea fowls (Yamamoto et al., 1967, Aire et al., 1980). Subsequently, Lin and Jones (1992) reported eight different types of primary spermatocytes in Japanese quail. The different stages of secondary spermatocytes cannot be observed due to their very short life span compared to primary spermatocytes.

They are, however, relatively larger than spermatogonia in Japanese quail (Yamamoto et al., 1967).

Spermatocytes are produced from the last mitotic division of type B spermatogonia. The cells then undergo first meiotic division to form secondary spermatocytes. The second meiotic division follows immediately to form haploid spermatids (Bueno *et al*., 2014). When both the primary and secondary spermatocytes undergo meiotic divisions, they increase in size and change the appearance of chromatin nuclear material, which indicates consolidation of chromosomes as they get ready for meiotic division.

1.1.4 Spermatids

The haploid spermatids are formed from the second meiotic division of secondary spermatocytes. Spermatids are similar to secondary spermatocytes but with smaller nuclear diameter (Foster, 2015). In drakes there are four stages of spermatids, which include round nuclei spermatids, irregular nuclei spermatids, elongated nuclei with coarse chromatin granules spermatids, and condensed elongated nuclei spermatids (Martianov et al., 2005).

The evolution and formation of the acrosomic system and spermatids, especially in rats, takes place during four different phases of spermiogenesis (Abou-Haila and Tulsiani, 2000). Firstly, during the Golgi phase, several proacrosomal granules are formed from the Golgi apparatus and accumulate in the medullary region. These granules fuse to form a large single acrosomic granule. Secondly, the cap phase is formed when the spherical acrosomic granule is enlarged by adding glycoproteins synthesized from Golgi apparatus. Thirdly, during the acrosomal phase, the acrosomal granules attach themselves to the inner acrosomal membrane and change to hemispherical which remains distinct

throughout the maturation phase of spermiogenesis which represents the fourth phase and is the acrosome proper (Abou-Haila and Tulsiani, 2000). The acrosome shape is variable among species, ranging from sickle-shape in rodents to skullcap in humans (Clermont et al., 1993).

1.1.5 Spermatozoa

The round haploid spermatid is transformed through a complex process to an elongated spermatozoon during spermiogenesis, the last stage of spermatogenesis. In the process of maturation of spermatozoa, the cytoplasm and the nucleus go through a complex modelling and modification (Lin and Jones, 1993). In birds, sperm in the seminiferous tubules hang in seminiferous fluid and are transported to the cloaca through the excurrent ducts, comprising the rete testis (RT) which consists of intra and extra testicular regions, the connecting duct (CD), the efferent duct (ED), the epididymis and deferent duct (DD) (Froman and Kirby, 2005). Sperm is rapidly transported in the reproductive tract of Japanese quail (Clulow and Jones, 1982). During this time, sperm undergo maturation in the epididymis. In chickens, sperm in the seminiferous tubules, epididymis, and deferent duct lack motility (Ashizawa and Sano, 1990), but they acquire the potential for motility as they are transported in the excurrent ducts due to factors like glutamate secreted into fluids by the deferent duct (Froman and Kirby, 2005). The flagella movement in chicken sperm, and likely in other avian species, is temperature-dependent and sperm motility is inversely proportional to temperature at the time of ejaculation (Ashizawa and Sano, 1990). In addition, sperm motility is partly genetically dependent and influenced by mitochondrial function and morphology (Froman and Kirby, 2005).

1.2 General overview on phthalates

Phthalates are ubiquitous compounds that are used extensively in many products for daily use. Phthalates are not covalently bonded to polyvinyl chloride (PVC) and, as a result, they are released into the environment and accumulate in rivers, sewage sludge, air and soil (Zhao et al., 2015). Phthalates contaminate the environment, not only during production of plastics, but also through using products containing these compounds (Vikelsoe et al., 2002). The environment is contaminated through migration, oxidation, leaching, use and storage of products containing phthalates. Environmental contamination with phthalates has been reported in freshwater systems (Fatoki, 2010), effluent, treated water (Clara et al., 2010) and sewage sludge (Vikelsoe et al., 2002, Jonson et al., 2003). In addition, phthalate contamination has been reported in agricultural landfill leachate (Zhao et al., 2015), and water runoff from suburban roads and highways (Clara *et al*., 2010). Upon exposure to phthalates, the major part of phthalates are rapidly metabolized to their monoesters (Kalmykova et al., 2013, Zhao et al., 2015), while the remaining part is oxidized and released as free or conjugated products before excretion in urine (Calafat et al., 2006). Concentration of these compounds in biological specimens, such as urine, can be used as biomarkers for detecting levels of exposure (Control and Prevention, 2009). Analysis of phthalates and their metabolites can be done by gas chromatography and high-performance liquid chromatography (FDA, 2001). However, the most common method of analysing these compounds is by gas chromatography (Mount and Anderson-Carnahan, 1988).

1.2.1 Routes of exposure to phthalates

There are several routes through which individuals can be exposed to phthalates (Rudel et al., 2001). The most common route is through oral ingestion because of wrapping of food products in plastic materials containing phthalates (Aurela et al., 1999). Absorption through the skin is another route since they are lipophilic compounds. At elevated temperatures the phthalate compounds can be evaporated which makes inhalation possible, especially in the case of factory workers (FDA, 2001). In addition, polymer coated drugs (Chourasia and Jain, 2003), intravenous fluids, haemodialysis treatment, and medical devices formed of PVC linked to phthalates can serve as sources of exposure (FDA, 2001).

1.2.2 General effects of phthalates

Information on exposure to phthalates and its effects on humans is limited. Most of the available data compare the effect of phthalate exposure on laboratory animals in association with humans (Jones et al., 2009). General effects of phthalate exposure include irritation of the skin, throat and eyes (lacrimation), nausea, dizziness, weakness, spasms in legs and arms, as well as reproductive effects (FDA, 2001). In addition, the increasing rate of bronchial asthma is associated with the use of plastics containing phthalate compounds over the few years (Wang et al., 2015). In animal studies, different effects of phthalates have been reported, such as peroxisomal proliferation, liver injury, liver cancer, teratogenicity and anti-androgenic activity (FDA, 2001).

In vitro studies show that dibutyl phthalate (DBP) and butylbenzylphthalate (BBP) have anti-estrogenic effects on breast cell lines. In addition, high doses of these phthalate esters have also anti-androgenic effects in laboratory animals (FDA, 2001). The effects

of phthalates in humans and animals are species-specific, dose-related as well as route-specific. For example, the hepatic changes observed in laboratory animals following exposure to phthalates, including liver injury or cancer, are apparently not evident in humans (Wang et al., 2015).

Phthalate compounds and their metabolites have different molecular weights and as such their effects also tend to differ. However, despite these differences, they are all known to be moderately toxic to cells (Ventrice et al., 2013). Phthalates disrupt male fertility by decreasing sperm concentration and inducing abnormal semen morphology. Low concentrations of phthalates hamper the development of reproductive organs in animals through inducing lymphocyte infiltration, vacuolization of Sertoli cell cytoplasm, vas deferens underdevelopment and gonadal dysgenesis (Przybylińska and Wyszkowski, 2016). Phthalates with high molecular weight evaporate at elevated temperature and induce allergic reactions by increasing the presence of inflammatory cells and bronchial fluid (Ventrice et al., 2013).

1.2.3 Endocrine disrupting effects of phthalates

Phthalates are classified as endocrine disrupting chemicals (Biro et al., 2013). The chemical structures of phthalates are closely related to hormones and as such, they interfere with hormone receptors of the nuclear receptor family (Swedenborg et al., 2009). These compounds can bind directly either as agonists or antagonists to hormone receptors because of structural similarities, thereby disrupting or enhancing the effect of the hormones (Swedenborg et al., 2009). These chemicals may cross the placenta and affect offspring in mammals (Diamanti-Kandarakis et al., 2009). Phthalate activities against hormone receptors are determined by their carbon skeleton length. In males,

exposure to phthalates is associated with the increase of hormone-associated diseases, such as genital abnormality, abnormal sperm morphology and prostate cancer (Radke et al., 2018). In addition, phthalates and their metabolites can hamper motility, concentration, and morphology of sperm, as well as increasing the rate of DNA damage (Karačonji et al., 2017).

Furthermore, phthalates induce hypospadias, or abnormal urethral opening, in young males (Cai et al., 2015). In females, exposure to phthalates is associated with early puberty induced by estrogenic EDCs (Biro et al., 2013), breast cancer (Crain et al., 2008), ovarian dysfunction, fibroids and infertility (Jefferson et al., 2012). Phthalate compounds are also associated with increased numbers of egg abnormality in women (Souter et al., 2013) and animals (Uzumcu et al., 2012).

1.3 Choice of Japanese quail as a research model

All over the world, and for several years, Japanese quail have been used as a laboratory model. Padgett and Ivey first described the Japanese quail as a research model in 1959 in the United States. They also reported its practicality as a laboratory model for avian species developmental studies. As a research model, these birds have a small body size that favours ease of housing, reduced cost of feeding, and they also have a short life cycle. They adapt favourably to laboratory manipulations, and their physiological maturing and aging processes are fast due to their short life span. They are sensitive to environmental changes such as lighting and fluctuations in temperatures. Hence, this influenced the choice of Japanese quail as a research model for investigating apoptosis of germ cells in normal and in pathological conditions in this study.

1.4 Motivation and problem statement

Presently, little is known about the specific molecular targets of di n-butyl phthalate (DBP). In addition, the mechanism of actions of DBP on avian species have not been well characterized. Several studies have only focused on the deleterious effects of low, medium and high doses of DBP on adult and pre-pubertal laboratory animals such as rats (Gray and Gangolli, 1986, Dostal et al., 1988, Mylchreest et al., 2000). Also, little literature exists on the effects of environmentally relevant concentrations of DBP on wildlife. It is now generally accepted that many species of amphibians and wild birds that were exposed to very low doses of EDCs throughout life, via contaminated food and water, are on the decline, and some may face extinction (Houlahan et al., 2000, Stuart et al., 2004). Several mechanisms have been proposed to explain the induction of testicular atrophy by DBP, but the exact cellular targets of DBP and the molecular mechanisms of induced spermatogenic cell apoptosis in both humans and wildlife are still poorly understood (Alam et al., 2010).

1.5 Aim and objectives of the study

1.5.1 Aim of the study

To determine the effects of DBP on spermatogenic cells in male Japanese quail (*Coturnix coturnix japonica*).

1.5.2 Specific objectives

1. To provide a general overview on pro-apoptotic genes: mechanisms of action and involvement in germ cell apoptosis.

2. To determine the histomorphometry of seminiferous tubules in four reproductive phases of Japanese quail.
3. To determine the rates of apoptosis of germ cells in the normal testis of Japanese quail (*Coturnix coturnix japonica*) using light microscopy, transmission electron microscopy, and TdT dUTP Nick End Labeling (TUNEL) assay.
4. To determine the effects of DBP on the rates of apoptosis of spermatogenic cells in the testis of Japanese quail using light microscopy and transmission electron microscopy.

Hypothesis 1: There are effects of DBP on germ cells in the testis of Japanese quail.

Hypothesis 0: There no effects of DBP on germ cells in the testis of Japanese quail.

1.6 Benefits that will arise from the study

I. The study will provide information on pro-apoptotic proteins and their mechanisms of action.
II. The study will provide information on the rates of apoptosis of spermatogenic cells in the normal testis of the Japanese quail.
III. The study will provide information on the effects of exposure to different doses of DBP on the histological and ultrastructural appearance of spermatogenic cells in the male Japanese quail.

1.7 Study Design

An outline of the study is provided in Figure 1.1.

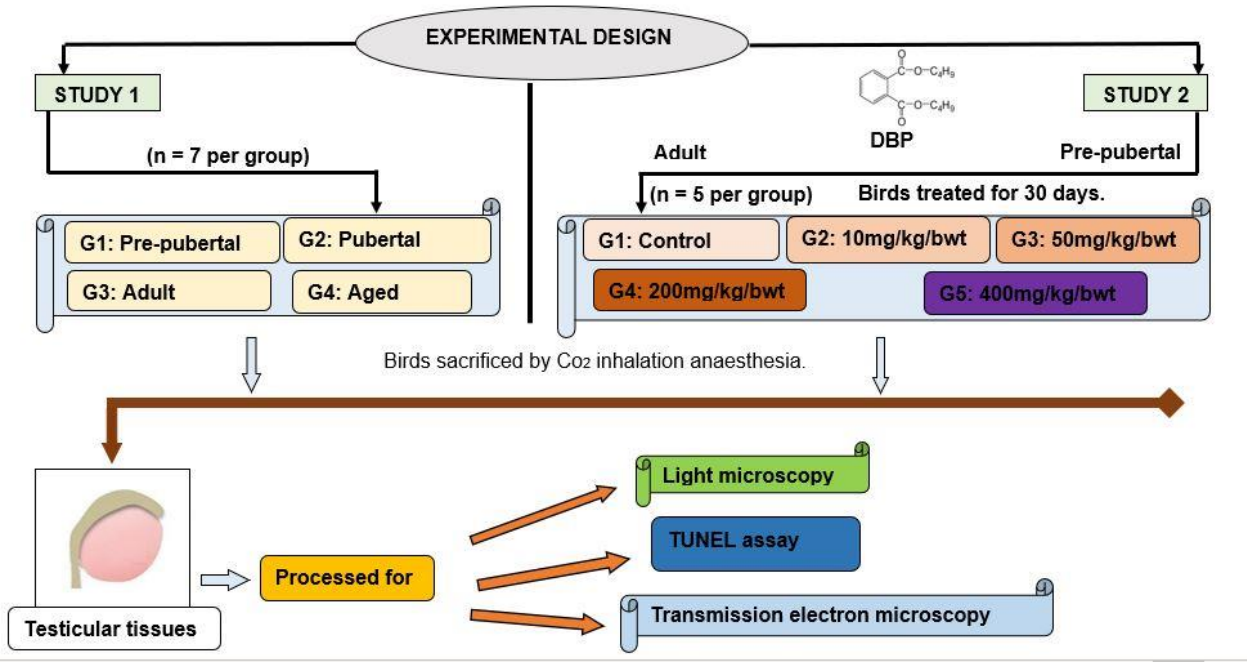

Figure 1.1. Study design for the age-related and DBP dose-related changes.

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CHAPTER TWO (REVIEW CHAPTER)

Pro-apoptotic proteins: mechanisms of action and involvement in apoptosis of spermatogenic cells

Abstract

Apoptosis of germ cells is an important feature of spermatogenesis, as this process allows the removal of excess germ cells from testicular tissue. This is crucial to control the number of germ cells that can be supported and nourished by the Sertoli cells. It has been established that up to 75% of germ cells are lost during the development of spermatogonia. Apoptosis also occurs during maturation divisions of spermatocytes and spermatids, but to a lesser extent. The present review focuses on pro-apoptotic proteins and their mechanisms of action, with special emphasis on their involvement in germ cell apoptosis. Many proteins have been associated with important roles in apoptosis. The most vital ones are caspase-3, B-cell lymphoma 2 (Bcl-2), truncated BH3 interacting death domain (tBID), tumour suppressor protein (p53), and Bcl-2-associated X protein (BAX). Execution of apoptosis may either be triggered by the intrinsic or the extrinsic pathway. The extrinsic pathway is initiated by death receptors and death ligands. Death receptors trigger pro-apoptotic proteins such as caspase-3 for the execution of apoptosis. The intrinsic pathway of apoptosis, conversely, is triggered by nutrient deprivation, stress or DNA damage, which in turn activates Bcl-2 families of pro-apoptotic proteins that foster apoptosis. This review aims to evaluate and summarize reported experimental data over the last few years on pro-apoptotic markers and their involvement in germ cell apoptosis.

2.0 Introduction

Apoptosis is a programmed cell death, with features such as membrane blebbing, chromatin condensation, and DNA laddering (Kerr et al., 1972). Several factors are known to play a critical role in the process of apoptosis. These factors are mostly pro-apoptotic proteins such as caspase-3, B-cell lymphoma 2 (Bcl-2), truncated BH3 interacting death domain (tBID), tumour suppressor protein (p53), and Bcl-2-associated X protein (BAX). The tissue in the vertebrate's body with a high incidence of apoptosis is the testis. This is where most of the germ cells produced are removed by the process of apoptosis (Shaha et al., 2010). Germ cell apoptosis is one of the most important features of spermatogenesis. It occurs mostly during spermatogonia proliferation, in which up to 75% of the tissue is lost, even though it can also occur during maturation divisions of spermatocytes and spermatids, albeit to a lesser extent (Hikim et al., 1995, Aitken et al., 2011).

Germ cell apoptosis plays a vital role in controlling sperm output and has been implicated in human male infertility (Weikert et al., 2004). The role of germ cell apoptosis is made evident when pro-apoptotic genes such as BAX and tBID are deleted. Such conditions are linked to infertility due to the stoppage of spermatogenesis just before the onset of meiosis (Yamamoto et al., 2001, Russell et al., 2002). Apoptosis is one of the most studied forms of cell death, but the molecular mechanisms and physiological significance are still largely unclear. It has been shown that pro-apoptotic proteins such as Bcl-2 can also act to prevent cell death in some cases. This suggests that there are multiple pathways of apoptosis that are regulated by the Bcl-2 gene (Furuchi et al., 1996). Even

though apoptosis of germ cells has been well documented in mammalian species using various animal research models, the exact mechanism of action is still not very clear.

2.1 Scope of the review

Spermatogenesis is a complex process involving germ cell proliferation and differentiation. During this process, errors are created that will require the induction of apoptosis to remove excess germ cells or germ cells with genetic defects. In this review, an overview is provided of the mechanism of action of some pro-apoptotic proteins, their involvement in germ cell apoptosis, as well as a brief overview of spermatogenesis. Unlike differentiation of somatic cells, germ cells are the only cells that undergo meiotic division, and an understanding of this process is crucial as it preserves and conveys parental genes to their offspring. Other information reviewed includes the general process of germ cell apoptosis and its two major pathways, namely extrinsic and intrinsic.

2.2 Pro-apoptotic protein pathways

2.2.1 Caspase-3

Caspase-3 is one of the members of the family of caspases (cysteine-aspartic-proteases), which are proteolytic enzymes known for their key roles in apoptosis and controlling inflammation (Shalini et al., 2015). Apoptosis is a major component of the biological process of an organism to destroy or remove unwanted cells during normal homeostasis, animal development, and in disease states (Jacobson et al., 1997). In all the enzymes or proteins that are known to be implicated in the execution and activation of apoptosis, caspase-3 appears to be crucial for this process (Thornberry and Lazebnik, 1998). Caspase-3 is a constantly activated death enzyme, even though the exact requirement of this protein in apoptosis is largely unknown (Porter and Jänicke, 1999). Some

experiments have shown that caspase-3 is crucial for the death of cells in an exceptional cell-tissue type or death stimulus-specific pattern. It is also important in certain biochemical changes associated with the initiation and completion of apoptosis (Porter and Jänicke, 1999). The mitochondrial disruption and the release of cytochrome enzyme are some of the important events associated with apoptotic cell deaths (Kluck et al., 1997). Caspase-3 is also essential for survival, as caspase-3-knockout animals die a few weeks after birth, aside from low birth frequency. These animals show some defects like masses of ectopic cells that suggest the failure of programmed cell death (Woo et al., 1998). Therefore, caspase-3 works in a selective tissue manner and contributes significantly to apoptosis.

There are extrinsic and intrinsic pathways of caspase activation in mammalian cells. Members of the family of Tumour Necrosis Factor (TNF) and apoptotic antigen (FAS) are the inducers of the extrinsic pathway. These proteins recruit other pro-apoptotic proteins to their cytosolic death domains (DDs) which bind to other pro-caspases such as pro-caspase 8. The intrinsic pathway can also be initiated by pore-forming pro-apoptotic protein members of the B-cell lymphoma 2 (Bcl-2) family. When cytochrome c is released, they bind and form complexes with Apoptotic Protease Activating Factor-1 (Apaf-1), which further activates caspase-9. Caspase-8 and caspase-9 are some of the initiators of apoptosis and they have shown to cleave directly to effector caspase-3 in the late stages of apoptosis (Reed, 2000).

2.2.2 B-cell lymphoma 2 (Bcl-2)

B-cell lymphoma 2 (Bcl-2) belongs to a family of proteins that are crucial in apoptosis regulation. This group of proteins is an integral part of core apoptotic machinery preserved in many species such as mammals and the free-living nematode *Caenorhabditis elegans* (Willis et al., 2003). The Bcl-2 related proteins functionally either promote or inhibit apoptosis, and interactions between either pro-apoptotic or anti-apoptotic members of other proteins determine whether a cell dies or lives (Willis et al., 2003). This has been studied in detail in the model organism *C. elegans,* results of which indicated that two Bcl-2 related proteins (pro-survival CED-9 and pro-apoptotic EGL-1) are vital in controlling programmed somatic cell deaths (Horvitz, 1994). The intrinsic pathway of apoptosis is controlled by the Bcl-2 protein family, which forms a tri-partied (three-point circuit) regulatory cascade that binds a distinctive component of DNA. The Bcl-2 homology (BH) domains are pro-apoptotic members of proteins activated by stress signals (Adams and Cory, 2007). There are two general classes of the Bcl-2 pro-apoptotic protein family: (a) those that share homology regions such as BH1, BH2 and BH3, called multidomain proteins; and (b) those that share the only sequence conserved for the BH3 domain, called 'BH3-only' proteins (Youle and Strasser, 2008).

The two pathways of coupling of apoptosis and autophagy are Bcl-2 homologs and Beclin (the protein responsible for autophagic cell death) in the nuclear envelope of the endoplasmic reticulum (Adams and Cory, 2007). In the presence of cytotoxic drugs and other chemicals, it induces autophagic cell death, whereas, under circumstances of nutrient limitation, it will prolong survival before eventual apoptosis. While on the outer

surface of mitochondria, Bcl-2 causes direct apoptosis when activated and binds to other apoptotic proteins (Adams and Cory, 2007).

2.2.3 tBID protein

Truncated BH3 interacting death domain (tBID) is a member of the BCL-2 family belonging to the BH3-domain-only subgroup. It is believed to connect survival and death signals to the intrinsic apoptotic pathway at the level that bears diverse BH domains (Adams and Cory, 2007). This was indicated in a model of tBID that moved from cytosol to the mitochondrial membrane to activate other apoptotic proteins that served as a death ligand (Wang et al., 1996). It has also been revealed that the cytosolic tBID can be activated by other caspases like caspase-8 after the engagement of apoptotic antigen receptors on the cells (Luo et al., 1998). Even though the exact mechanism by which cytochrome c is released from mitochondria is still not very clear, different observations have been noted with different death signals and cell types. Mitochondrial swelling has been observed following growth factor withdrawal with defective ADP exchange leading to hyperpolarization of an inner membrane, matrix volume increase and rupture of the outer membrane, causing the release of some proteins including cytochrome c (Vander Heiden et al., 1999). There are two major mechanisms by which tBID might cause the release of cytochrome c from mitochondria. Firstly, tBID might serve as a death signal to activate other protein receptors on the mitochondria to release cytochrome c, and secondly, tBID might function as an effector participating in pore formation in an intramembranous space of mitochondria causing the release of cytochrome c (Wei et al., 2000).

Also, two other types of models have been reported showing outer membrane permeability of mitochondria during apoptosis. In one model, mitochondria treated with tBID in buffer undergo efflux of endogenous cytochrome c. In the other model, mitochondrial exposure to cytosol induces high permeability, leading to exogenous cytochrome c due to permeability enhancement factor (PEF). The exchange of endogenous cytochrome c is lower than the rate of cytochrome c efflux following tBID treatment. PEF acts immediately following tBID-induced permeabilization but can be inactivated over an hour by other pro-apoptotic proteins such as caspases (Kluck et al., 1999).

2.2.4 Tumour suppressor protein (P53)

The p53 is a nuclear protein known to be implicated in apoptosis and is highly expressed in the testis (Almon et al., 1993). The presence of p53 and other proteins in primary spermatocytes indicates that p53 plays a vital role in the meiotic prophase of cell division. This suggests that p53 is key in apoptosis regulation in spermatocytes and spermatogonia and its deficiency might be associated with infertility (Ohta et al., 2003). The p53 protein is responsible for genome integrity (Lane, 1994). During stress conditions such as hypoxia or DNA damage, an increased level of p53 occurs, thereby leading to cell cycle arrest in dividing cells (Graeber et al., 1996). P53 transcriptional activity is elevated in proliferating testicular tissue and during organogenesis (Almon et al., 1993). High levels of p53 protein are found in testicular tissue, especially germ cells, and in particular primary spermatocytes (Ohta et al., 2003). During conditions of increased temperature, p53 is translocated from the nuclear envelope to the nucleus in association with apoptosis of testicular germ cells (Yin et al., 1997). Triggers for p53 dependent

apoptosis are growth factor deprivation, DNA damage, and cellular stress. The biochemical mechanism of p53 is not clearly understood, but the DNA break is believed to be the best stimulus of p53 dependent apoptosis (Bellamy, 1997). Analysis of cells and tissues of p53 knockout mice has shown that p53 is required for apoptosis in DNA damage-induced cells and tissues of cortical thymocytes, keratinocytes, cerebellar granule neurons and proliferating crypts of epithelial cells of small and large intestines. Under these conditions (DNA damage and cellular stress), p53 may be a DNA damage sensor (Bellamy, 1997).

2.2.5 Bax protein

Bcl-2-associated X protein (BAX) is a pro-apoptotic protein that is encoded by the BAX gene in humans. The pathway of intrinsic apoptosis is activated in response to different types of signals caused by stress or chemicals (Willis and Adams, 2005). It is the interplay of complex proteins of Bcl-2 family members that sends signals to the outer membrane of mitochondria to initiate activation of BAX protein. Once Bax protein is activated, it will cause the release of cytochrome C which in turn will activate other pro-apoptotic proteins to cause cell death (Youle and Strasser, 2008). The Bax pro-apoptotic protein has multiple domains for both pro-survival and the BH3-only groups. These domains induce mitochondrial outer membrane permeabilization (MOMP), commonly called a point of no return in apoptosis. A Bax protein is vital for the MOMP major checkpoint, as Bax gene knockout mice showed severe apoptosis impairment during development (Lindsten et al., 2000).

Upon receiving the signal of apoptosis by death receptors, the BH3 domain of Bax is exposed before binding to other pro-apoptotic molecules. This creates pores for the

release of cytochrome C from the outer surface of the mitochondria to form complexes with other pro-apoptotic proteins, known as apoptotic protease activating factor (Apaf1) to cause cell death (Dewson and Kluck, 2009).

2.3 Spermatogenesis in birds

Spermatogenesis in birds has been poorly studied, even though they constitute more than 50% of all vertebrates (Aire, 2007). It is a process in which stem spermatogonia undergo mitotic division to produce spermatocytes (primary and secondary spermatocytes), which in turn undergo meiosis to produce spermatids (round and elongating spermatids) which then further differentiate into spermatozoa (Lin and Jones, 1993). In birds, spermatozoa are produced in a large number over a short period. It is a continuous process that depends on the availability and renewal of spermatogonia which are the progenitor germ cells, from which other germ cell series originate (Aire, 2018). In general, as they transform, germ cells migrate from the basement membrane to the tubular lumen of seminiferous tubules. It is an organized, careful, unique and precise biological event that, even though similar in all vertebrates, has unique elements for each animal species (Aire, 2007).

2.4 Germ cell apoptosis

Apoptosis of germ cells is a regular occurrence during testicular development of male gonads, having a very important role in both foetal and adult testicular tissue. It is essential for normal spermatogenesis, and is believed to be responsible for cellular homeostasis, maintaining a balance between germ cells and Sertoli cells (Said et al., 2004). It also plays an important role in controlling the number of spermatogenic cells (Kerr et al., 1992, Hikim and Swerdloff, 1999) and preserving the genetic integrity of male

germ cells (Gartner et al., 2018). Also, apoptosis has a major role in semen quality and output (Schaller et al., 2008) as well as germ cell maturation (Brill et al., 1999). Among the germ cells that are more prone to undergo apoptosis are spermatogonia (Allan *et al*., 1992). Apoptosis has been considered as the key regulator of the development of germ cells by eliminating germ cells carrying DNA mutations or defective genes (Russell et al., 2002). In seasonal breeding vertebrates, apoptotic germ cells are high during the quiescent or non-breeding period (Lancaster et al., 2014) suggesting that apoptosis could be the mechanism responsible for testicular atrophy in those animals (Young and Nelson, 2001).

It has been reported that Sertoli cell numbers differ during pre-pubertal development to have an appropriate ratio with germ cells (Print and Loveland, 2000). These differences are a result of cell differentiation and death. To understand the spermatogenic cell apoptosis by Sertoli cells, *in vitro* phagocytosis is ideal where both Sertoli cells and spermatogenic cells can be individually isolated from cell cultures (Wang et al., 2006). In the seminiferous tubule, apoptosis has been reported to occur near the basement membrane, which shows that most of these cells are spermatogonia (specifically types A2, A3 and A4 spermatogonia) (Allan et al., 1992).

2.4.1 The extrinsic pathway of germ cell apoptosis

The extrinsic pathway of apoptosis initiation involves transmembrane receptor-mediated interactions. It involves death receptors that are family members of tumour necrosis factor (TNF) (Locksley et al., 2001). These receptors have cytoplasmic domains known as death domains (Ashkenazi and Dixit, 1998). The death domains play a very important role in conveying the death signal from the outer cell surface to intracellular space signalling

pathways. The extrinsic pathway also initiates apoptosis in response to external stimuli. These stimuli are binding ligands to a death receptor on the surface of the cell. There are many ligands and their corresponding death receptors that define the phase of extrinsic apoptosis such as Fas ligand/Fas receptor (FasL/FasR) and tumour necrosis family alpha/tumour necrosis factor receptor-1 (TNF-α/TNFRI) (Elmore, 2007). It has been reported that Fas receptor upregulation in the rat has been associated with apoptosis of spermatocytes during phase one of spermatogenesis (Lizama et al., 2007). Despite the evident requirement of Fas and Fas ligand for germ cell-induced apoptosis, mutant mice have been reported with normal spermatogenesis (Lee et al., 1997).

2.4.2 Intrinsic pathway

Unlike the extrinsic pathway of apoptosis, initiation of the intrinsic signalling pathway involves diverse ways of non-receptor mediated stimuli that lead to intercellular signals that act directly within the cell. These events are mitochondrial initiated, and the signal can either act positively or negatively (Shaha et al., 2010). Negative signalling can occur in the absence of hormones, certain growth factors and cytokines, which can lead to failure to suppress death ligands, thereby activating apoptosis. Stimuli such as hyperthermia, toxins, radiation, free radicals and viral infections can positively initiate apoptosis. Either positive or negative signalling may cause changes in the mitochondrial inner membrane, leading to the creation of mitochondrial permeability transition (MPT) pores (Saelens et al., 2004). The intrinsic pathway of apoptosis can also be activated by a variety of endogenous and exogenous stimuli such as ischemia, DNA damage and oxidative stress (Loreto et al., 2014). It is influenced by pro-apoptotic proteins that are bound to the membrane of mitochondria. The significance of pro-apoptotic signalling in

the intrinsic pathway is the perturbation of mitochondrial membrane to release cytochrome C, which triggers the formation of the apoptosome. This complex dissolves adenosine triphosphate to activate and cleave initiator caspase-9, and further activates and cleaves executioner caspase-3, leading to apoptosis (Rowinsky, 2005).

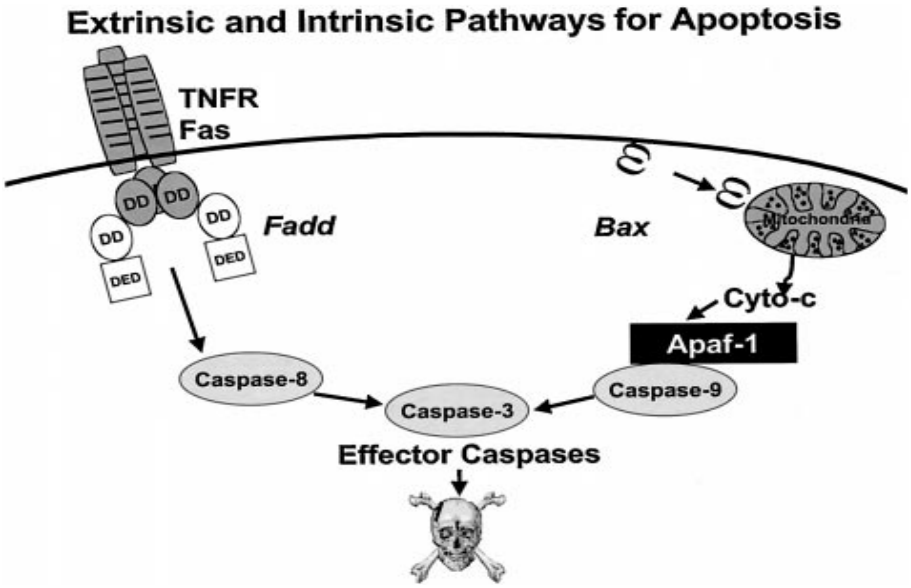

Figure 2.1. Schematic representation of apoptotic pathways (Reed John, 2000).

Members of the family of Tumor Necrosis Factor (TNF) like TNFR1 and apoptotic antigen (Fas) are the inducers of extrinsic pathway. These proteins recruit other pro-apoptotic proteins to their cytosolic (death domains) DDs which binds to pro-caspases especially pro-caspase-8. The intrinsic pathway can be initiated by pore-forming pro-apoptotic protein members of Bcl-2 family like Bax. When cytochrome c is released, they bind and form complexes with Apoptotic protease activating factor 1 (Apaf-1), which further activate caspase-9. Caspase-8 and caspase-9 which are initiators of apoptosis have shown to cleave directly to effector caspase-3 in the late stage of apoptosis.

2.5 Conclusions

The available information on pro-apoptotic protein mechanisms and their involvement in germ cell apoptosis have greatly improved the understanding of the mechanisms of germ cell apoptosis over the past few years. However, there are still unanswered questions on the mechanism of germ cell apoptosis. Although several pro-apoptotic proteins have been identified and studied, the molecular mechanisms of activation of these proteins are not fully understood. These should continue to be a focus of research.

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CHAPTER THREE

Histomorphometry of seminiferous tubules in four reproductive phases of Japanese quail (*Coturnix coturnix japonica*)

Abstract

Information on histomorphometry of seminiferous tubules of avian species is inadequate compared to data concerning mammalian species. The present study aimed to evaluate some parameters of seminiferous tubules of Japanese quail (*Coturnix coturnix japonica*) as a model avian species. A total of 28 birds were used for the study, comprising 7 birds per each of the 4 reproductive phases, namely pre-pubertal (4 weeks old), pubertal (6 weeks old), adult (12 weeks old) and aged (52 weeks old). Body and testicular weights varied ($P<0.001$) among the four age groups. For the seminiferous tubule diameter, the aged group had the highest mean diameter (305.974 ± 26.24) even though there was no significant difference between the age groups ($P>0.05$). However, a significant difference ($P<0.005$) for the aged groups was observed for both the seminiferous tubule lumen diameter (116,273 ± 14.47) and the mean epithelium height (121.017 ± 8.89) than the pre-pubertal, pubertal, and adult age groups. The cross-sections of the seminiferous tubules of the pre-pubertal, pubertal, adult and aged birds were similar in appearance. The shape and size of the seminiferous tubules appeared to be uniform. The smooth walls of the seminiferous tubules containing various types of germ cells from the basement membrane to the tubular lumen with clusters of Sertoli cells in between were observed. There were no obvious morphological differences observed between the age groups. The present study showed that spermatogenesis gradually declines in aged birds

by presenting wider seminiferous tubule lumens, which could be due to the high rate of germ cell apoptosis and decreased androgen concentrations, which are common in aged birds and mammals.

Keywords: Histomorphometry; Japanese quail; Reproductive phases; Seminiferous tubules

3.0 Introduction

Spermatogenesis in birds has been poorly evaluated, even though birds constitute more than 50% of all vertebrates (Aire, 2007). The efficiency of spermatogenesis can generally be evaluated by several quantitative parameters. The measurements of seminiferous epithelium height and the tubular diameter are among the most important parameters in estimating the efficiency of spermatogenesis (Tripathi et al., 2015, Kalwar et al., 2020). Even though avian gonadal morphometry has been well investigated, most emphasis has been placed on the endocrinological and physiological aspects of their reproductive biology. The few studies on the morphology of avian reproductive biology are mainly on the testicular structure, testicular weight and germ cell differentiation (Artoni et al., 1999, Deviche et al., 2011, Aire et al., 2019). The seminiferous tubules of avian species are similar to those of mammals. Histologically, the tubule is lined with stratified epithelium comprising various types of germ cells that are nourished and supported by Sertoli cells (Deviche et al., 2011). The advent of stereological study methods provides quantitative information that is crucial in establishing associations between biochemical and physiological data (Bordbar et al., 2013). The most commonly used parameter in evaluating seminiferous tubules is the mean tubular diameter. This is imperative because

the seminiferous tubule diameter changes in testicular disorders (Wit et al., 2007). The study of testicular morphometry in animals allows the establishment of behavioural and physiological patterns that are crucial in understanding aspects of reproductive biology of different species, aiding development of protocols for assisted reproduction (Caldeira et al., 2010). In relation to the features of male reproductive biology, a strong correlation between sperm production and testicular weight has been reported (Kenagy and Trombulak, 1986). Also, the relative size of the testis can guide the biology of reproduction and the system of mating. Therefore, the size of the testis is directly proportional to reproductive behaviour, since monogamous animals have a lower gonadosomatic index compared to species with polyandrous mating (Kenagy and Trombulak, 1986, Caldeira et al., 2010). The most copious portion of the testicular parenchyma is the seminiferous tubules. All quantitative parameters associated with seminiferous tubule evaluation, such as seminiferous epithelium thickness and tubular diameter have a strong correlation with spermatogenic activity (Franca and Russell, 1998). Measurement of seminiferous tubules is therefore the most reliable traditional approach employed as an indicator of spermatogenic activity in testicular function investigations (Mascarenhas, 2006). Morphometric studies on seminiferous tubules of mammalian species are well documented (Neves et al., 2002, França and Godinho, 2003, Caldeira et al., 2010, Kalwar et al., 2020). However, not much has been done on the seminiferous tubules of avian species compared to mammals. The present study aimed to evaluate the seminiferous tubule diameter, seminiferous epithelium height and seminiferous tubule lumen diameter in four stages of the reproductive cycle of male Japanese quail. As a research model, these birds have a small body size that favours ease of housing and reduces the cost of

feeding, and they also have a short life cycle. They adapt favourably to laboratory manipulations, and their physiological maturing and aging processes are fast due to their short life span.

3.1 Materials and methods

3.1.1 Animals used for the study

A total of 28 birds were used for the study, comprising 7 birds for each of the 4 reproductive phases, namely pre-pubertal (4 weeks old), pubertal (6 weeks old), adult (12 weeks old) and aged (52 weeks old) according to previously classified reproductive phases (Zakariah et al., 2020). The birds were purchased from the Agricultural Research Council (ARC), Irene, Pretoria, South Africa. The protocol for euthanasia, using carbon dioxide ($C0_2$) inhalation anaesthesia, was approved by the Institutional Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, University of Pretoria, South Africa (issued vide No. AEC/A065-12).

3.1.2 Tissue samples for light microscopy

Tissue samples from both testes of each bird were collected following the opening of the thoracoabdominal cavity. The tissue samples were fixed for 5 days in 10% buffered formaldehyde and were processed conventionally for paraffin wax embedment, sectioned at 5 µm thick, and were stained with haematoxylin and eosin (H&E) for light microscopy.

3.1.3 Morphometrical analysis of body weight, testis and seminiferous tubules

The body and testicular weights of each bird in all the age groups were measured using a digital precision balance (MII-300 digital precision weighing balance, Algen Scale Corporation^R Bohemia, NY). The seminiferous tubule diameter, seminiferous epithelium

height and the tubular lumen diameter were evaluated were as described (Valença et al., 2013, Chiarini-Garcia et al., 2017). Seminiferous tubule and the tubular lumen diameters were measured across major and minor axes, and the average recorded. Fifteen cross-sections of seminiferous tubules that were round or nearly round were randomly selected per bird and measured using a linear graticule micrometer, with an image analyzer system (CellSens dimension software) tethered to an Olympus BX-63 microscope.

3.2 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare, among age groups, morphometric parameters for bodies, testes and seminiferous tubules.

Age groups were used as independent variables. The body and testicular weights, the tubular diameter, seminiferous epithelium height and the seminiferous tubule lumen diameter were used as the dependent variables. All analyses were performed using IBM SPSS version 26 software. A Tukey's Honest Significant Difference (HSD) post hoc test was used to measure the extent of difference between groups at a 5% significant level ($p>0.05$). Data were presented as mean ± standard error of the mean (SEM) and bar plots were generated using SPSS version 26.

3.3 Results

3.3.1 Body and testicular weights

Body and testicular weights differed among ages, but there was no significant difference between the mean of the right and left testes (Table 3.1 and Figures 3.1-3.3).

Table 3.1. Mean body weight, right and left testes, as well as paired testicular weight.

Age group	Mean body weight (g) ±SE	weight (g) of left testes ±SE	weight (g) of right testes ±SE	Overall testicular weight (g) ±SE	P-value
Pre-pubertal	81.060 ± 8.900	0.182 ± 0.740	0.097 ± 0.025	0.280 ± 0.817	
Pubertal	131.900 ± 4.100	1.507 ± 0.160	1.731 ± 0.193	3.230 ± 0.308	
Adult	160.940 ± 7.000	2.995 ± 0.440	2.626 ± 0.193	5.621 ± 0.601	
Aged	167.031 ± 7.700	3.075 ± 0.450	2.813 ± 0.231	5.889 ± 0.626	0.0001

Significant differences (P< 0.001) between the age groups were sequentially observed (from the pre-pubertal to the aged birds) for the body and testicular weights.

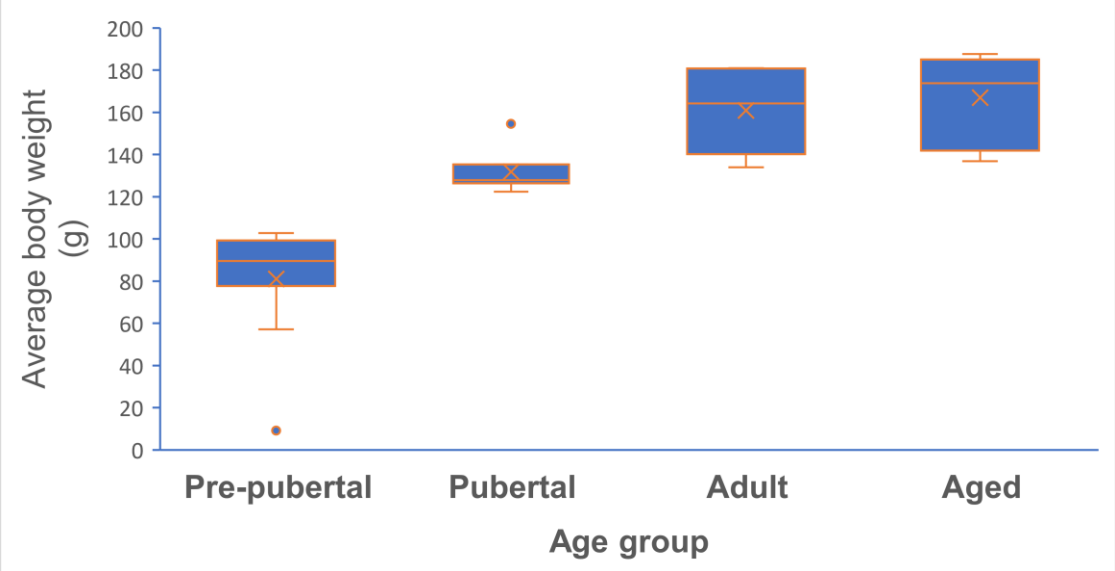

Figure 3.1. Mean body weight of all the age groups with an overall significant difference between the age groups (P<0.01).

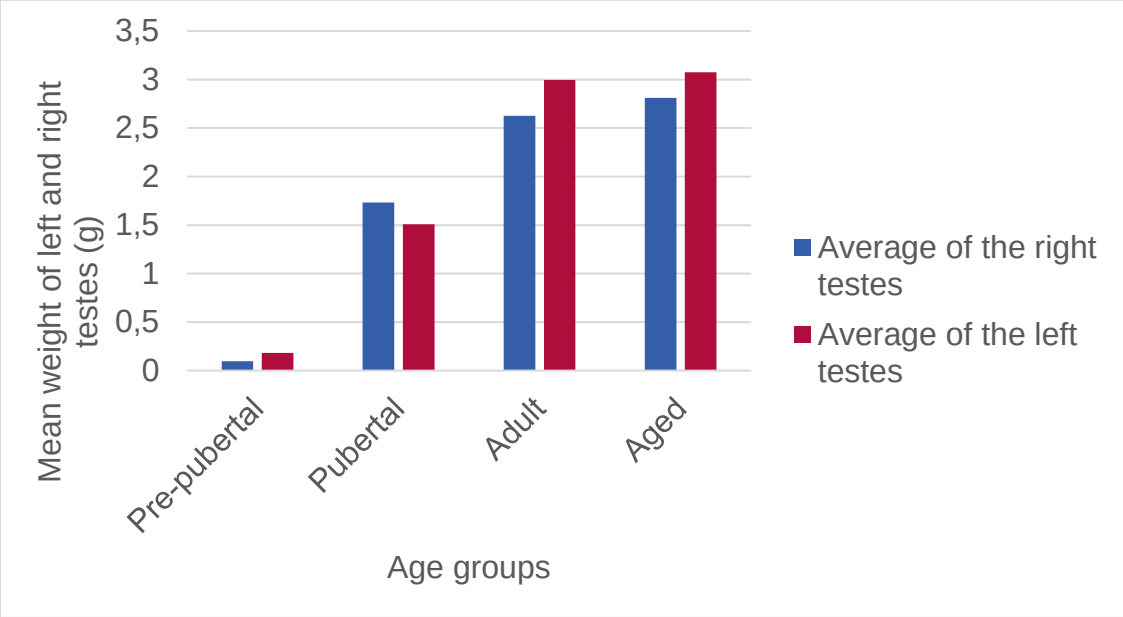

Figure 3.2. Mean weight of the right and the left testes of all the age groups. There was a significant difference of mean testicular weight between the age groups ($p<0.05$), but no significant difference ($p>0.05$) between right and left testes.

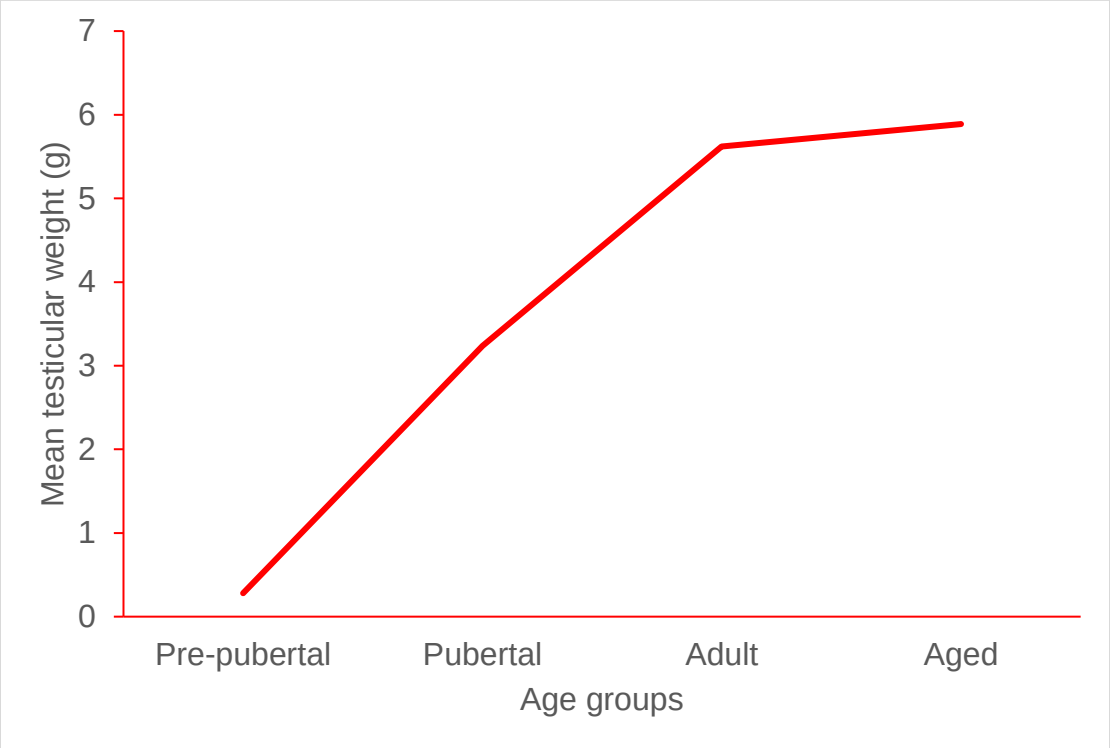

Figure 3.3. Graph showing the mean testicular weight of both the right and left testes of all the age groups. There was a significant difference of mean testicular weight between the age groups ($P<0.05$), sequentially from pre-pubertal to aged birds.

3.3.2 Seminiferous tubules parameters

Morphometric evaluation of the seminiferous tubules was performed to determine the mean difference between the age groups for the seminiferous tubule diameter, seminiferous tubule lumen diameter, and seminiferous tubule epithelium height (Table 3.2 and Figure 3.4). For the seminiferous tubule diameter, the aged group had the highest mean diameter (305.974 ± 26.24) even though there was no significant difference between the groups ($P>0.05$) (Table 3.2). However, there was a significant difference between the groups for both the seminiferous tubule lumen diameter (116.273 ± 14.47)

and the mean epithelium height (121.017 ± 8.89) with the aged group being significantly higher ($P<0.005$) than pre-pubertal, pubertal, and adult age groups (Table 3.2). Following the post hoc test for multiple comparisons, there was no significant difference between all the groups compared in terms of the seminiferous tubule diameter.

Table 3.2. Mean seminiferous tubule diameter, seminiferous tubule lumen diameter and seminiferous epithelium height.

Age groups	**Seminiferous tubule diameter (µm) ±SE**	**P-value**
Pre-pubertal	244.030 ± 28.92	
Pubertal	259.3514 ± 22.81	
Adult	210.352 ± 25.08	
Aged	305.974 ± 26.24	*0.131*
	Seminiferous tubule lumen diameter (µm) ±SE	
Pre-pubertal	64.675 ± 5.60	
Pubertal	92.444 ± 12.78	
Adult	70.468 ± 9.32	
Aged	116.273 ± 14.97	*0.006**
	Seminiferous tubule epithelium height (µm) ±SE	
Pre-pubertal	91.457 ± 6.01	
Pubertal	80.237 ± 7.65	
Adult	116.744 ± 13.39	
Aged	121.017 ± 8.89	*0.014**

Significant difference between the age groups for both the seminiferous tubule lumen diameter and the mean epithelium height, with the aged group being significantly higher ($P<0.005$) than pre-pubertal, pubertal and adult age groups.

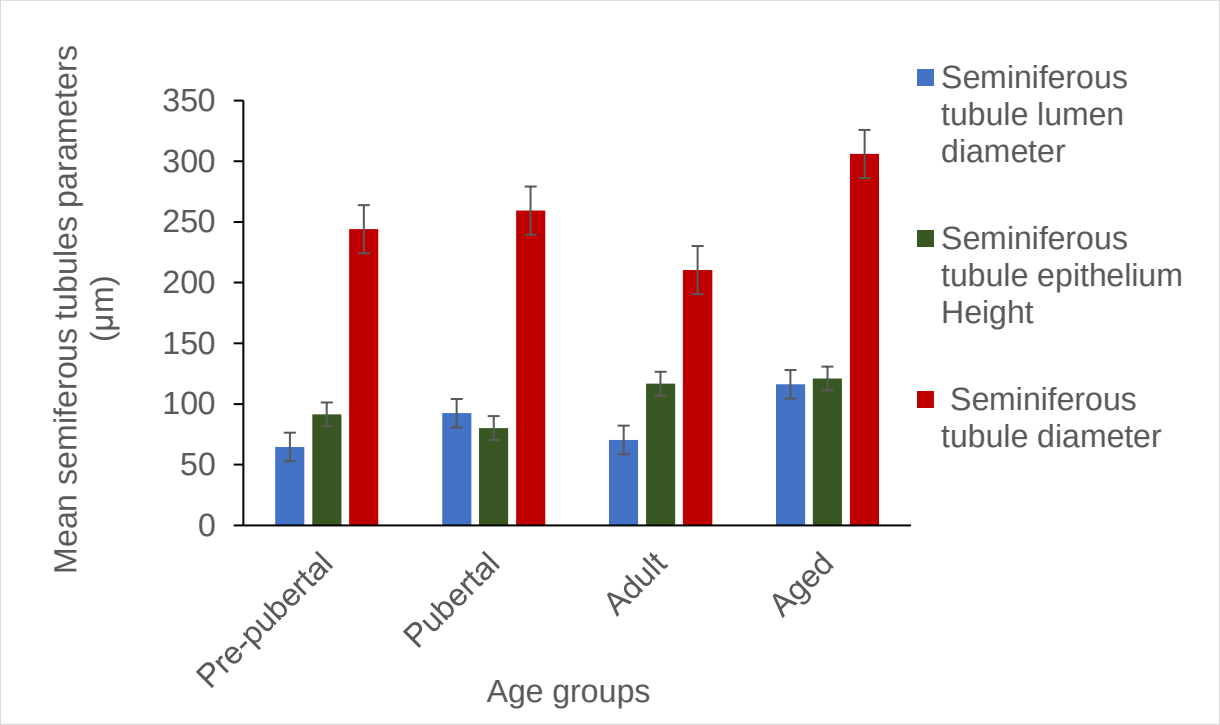

Figure 3.4. Mean seminiferous tubules parameters of all the age groups. There was a significant difference ($P<0.05$) between the groups for both the seminiferous tubule lumen diameter and epithelium height but no significant difference ($P>0.05$) for seminiferous tubule diameter among the age groups.

3.3.3 Seminiferous tubules morphology

The cross sections of the seminiferous tubules of the pre-pubertal, pubertal, adult and aged birds were similar in appearance (Figures 3.5 and 3.6). The smooth walls of the seminiferous tubules contained various types of germ cells from the basement membrane to the tubular lumen with clusters of Sertoli cells in between. There were no obvious morphological differences between the age groups. The seminiferous tubule diameters of all the age groups were not significantly different from each other. However, the seminiferous epithelium height and the seminiferous tubule lumen diameter were significantly different among the age groups, sequentially from pre-pubertal to aged birds (Table 3.2). The seminiferous tubule parameters were measured and demonstrated (Figure 3.7).

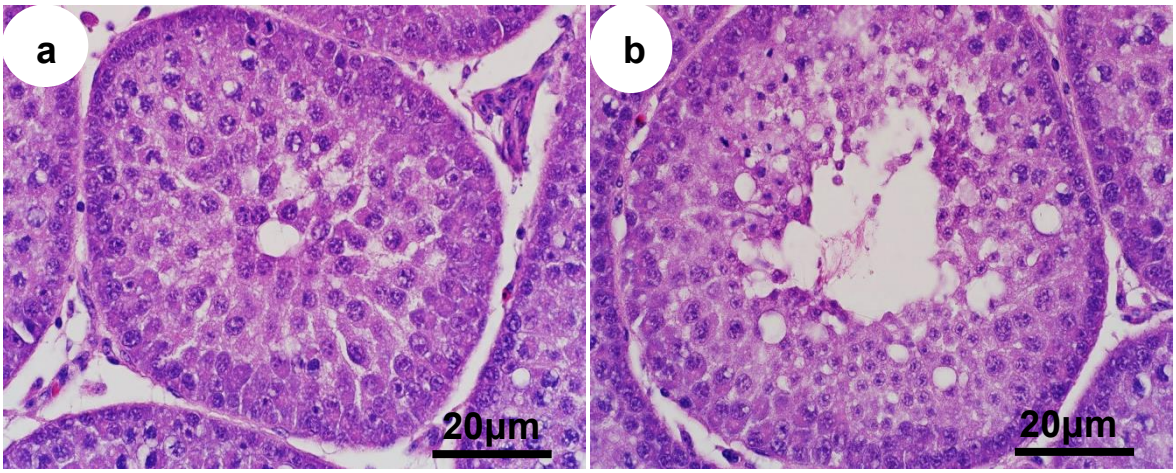

Figure 3.5. Transverse sections of seminiferous tubules of pre-pubertal (**a**) and pubertal (**b**) Japanese quail.

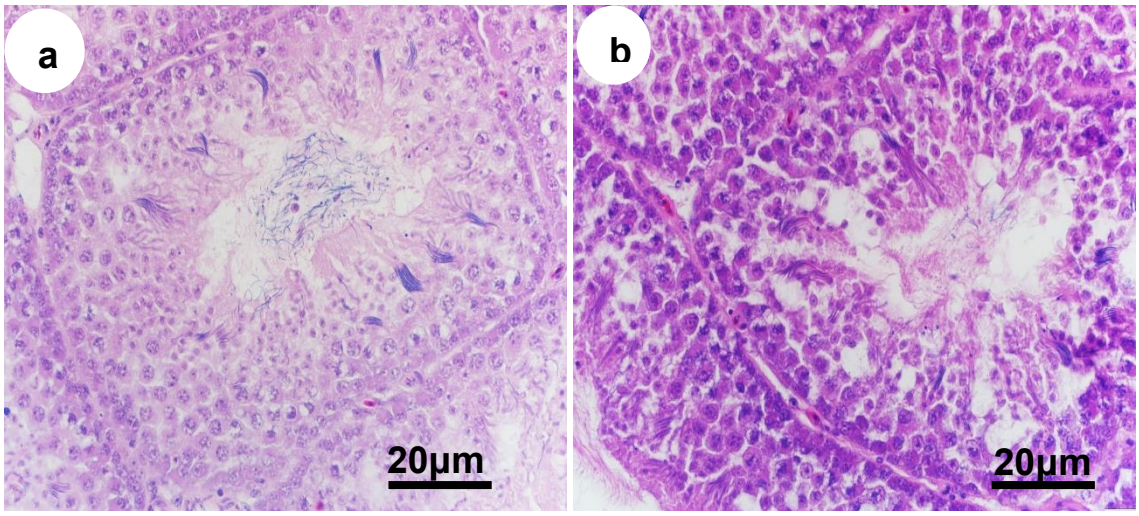

Figure 3.6. Transverse sections of seminiferous tubules of adult (**a**) and aged (**b**) Japanese quail.

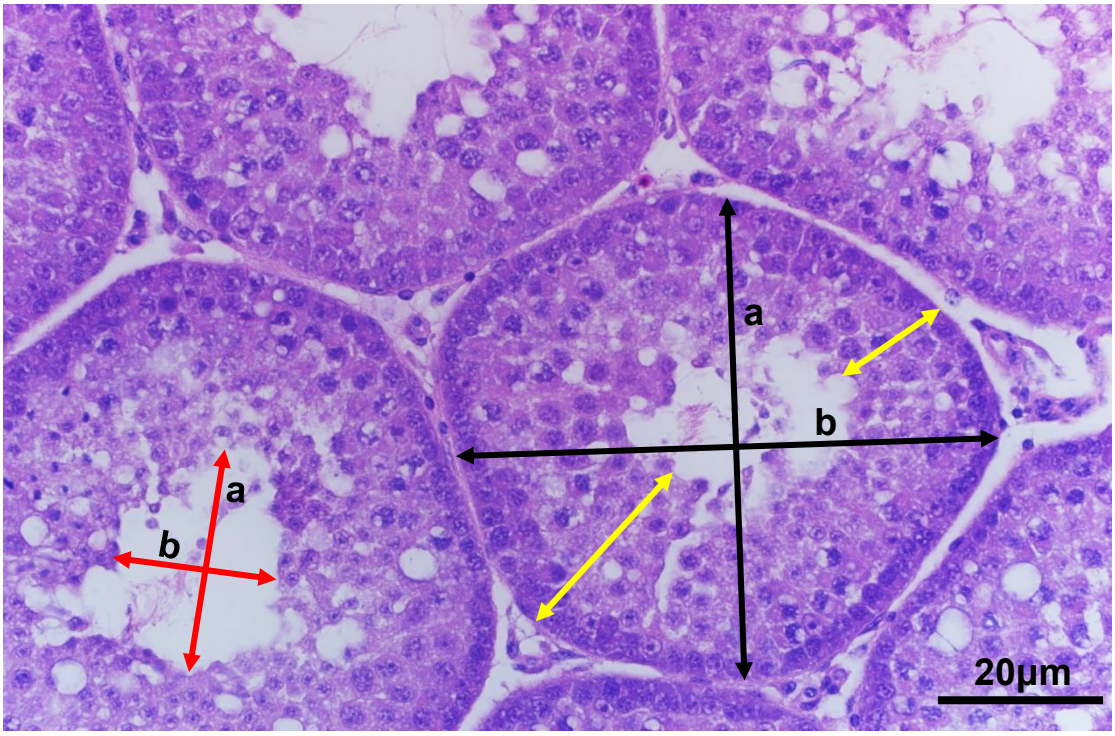

Figure 3.7. Transverse sections of seminiferous tubules showing how the components of the tubules were measured. Black double-head arrows: seminiferous tubule diameter,

Red double-headed arrows: seminiferous tubule lumen diameter, Yellow double-headed arrows: seminiferous tubule epithelial height, **a**: major axis**, b**: minor axis.

3.4 Discussion

The present study showed a significant change in the body weight, testicular weight, seminiferous tubule epithelium height and seminiferous tubule lumen diameter between the age groups. However, seminiferous tubule diameter was not significantly different among the age groups. This is in accordance with reports of previous studies in boys (Müller and Skakkebæk, 1983), rats (Gaytan et al., 1986) and Japanese quail (Artoni et al., 1999). Muller and Skakkebaek, (1983) observed that testicular weight increases with age which was highly influenced by the size of the germ cell proliferation. They also found no significant difference in the mean tubular diameter during the 0 – 10 year period of pre-pubertal boys. Most of the morphometric studies on mammalian and avian testes have measured testicular parameters under experimental conditions. To our knowledge, this is the first study that reports morphometric changes in body weight, testicular weight, seminiferous tubule diameter, seminiferous epithelium height and tubular diameter in four reproductive phases of Japanese quail. Shil et al. (2015) evaluated the morphometry of testes of one reproductive cycle of Japanese quail and reported positive correlations between mean body and testicular weights, which is similar to the observations in one of the reproductive phases (adult age group) of the present study.

In birds, testes are located deep in the abdominal cavity, and are only visible after removal of other organs such as the intestine. They are surrounded by a fibrous tissue capsule comprising contractile fibres and connective tissue (Aire and Ozegbe, 2007). The main

component of the testes is seminiferous tubules, where spermatogenesis takes place. The left and the right testes in the present study were symmetrical, as no significant differences were observed. This is in agreement with the reports of Kempenaers et al. (2002) in the tree swallow (*Tachycineta bicolar*). The cross-sections of the seminiferous tubules observed in the present study are similar to the previously described testicular structure of curve-billed thrashers (*Toxostoma curvirostre*) (Deviche et al., 2011). They reported that seminiferous tubules are the major component of testicular tissue and these are surrounded and anastomosed by a basal lamina consisting of myoepithelial cells, fibroblasts and connective tissue. They also described the epithelium where germ cells proliferate and differentiate as they move from the basement membrane to the tubular lumen. The changes in shapes and sizes of the seminiferous tubules in avian species as observed in the present study were originally attributed to changes in androgen levels. Older animals secrete more androgen than younger ones, which stimulates testicular activity and maintenance of the seminiferous tubule integrity (Thurston and Korn, 2000). The significant increase in the seminiferous tubules lumen diameter observed in the aged birds in the present study could be due to high rates of germ cell apoptosis (Zakariah et al., 2020) and a fall in androgen levels (Zirkin and Chen, 2000). Germ cell deaths create intervening spaces in the seminiferous tubules, thereby leading to the larger tubular lumen.

Even though evaluation of spermatogenesis can be highly inconsistent (Briskie and Montgomerie, 2007), testicular size is often used to measure sperm production, as greater components of testicular tissue are dedicated to spermatogenesis. Generally, sperm production is proportional to testicular size as demonstrated in the zebra finch

(Birkhead et al., 1993) and the house sparrow (Birkhead et al., 1994). However, not much information is available concerning sperm production in all avian species, especially wild birds. Therefore, the correlation between testicular size and sperm production cannot be generalized since several factors can alter the process of spermatogenesis (Briskie and Montgomerie, 2007). Age-related studies in relation to testicular size reporting larger testes in older animals than younger ones, as observed in Japanese quail in the present study, have also been documented in other avian species (Deviche et al., 2000, Graves, 2004, Laskemoen et al., 2008). The differences in testicular size in relation to age may be due to younger birds secreting less gonadotropic hormones (GTHs) than older birds (Silverin et al., 1997). Also, the differences in testicular size in relation to age could be due to a lower level of testosterone in younger adults compared to older ones (Deviche and Sharp, 2001). Inter- and intraspecies comparisons indicate an allometric relationship between testis and body size, but the strength of this relationship also differs (Pitcher et al., 2005, Gunn et al., 2008). It is common knowledge that climate change can affect factors that are responsible for the reproductive development of birds (Deviche et al., 2011). This is evident as the breeding season of some avian species has been shifted from the historic breeding season as observed through a long-term study (Pearce-Higgins et al., 2005, Visser et al., 2006). However, in Japanese quail, seasonal variations do not have significant effects on live body weight (Shil et al., 2015). As previously observed, the mean diameter of the seminiferous tubule and the seminiferous tubule epithelial height in mammals is directly proportional to the spermatogenic activity (Franca and Russell, 1998, Valença et al., 2013). This is because the seminiferous tubule lumen diameter and seminiferous epithelium height are determined by the number of germ cells present in the

seminiferous tubules. Even though these values can vary, as germ cell apoptosis is not sequential, it can be affected by several factors because of its complexity (Billig et al., 1995). In conclusion, the degree of variation in the morphometric data of the present study is similar to mammalian species studied. However, it is not sufficient to draw an inference for the reproductive phases of Japanese quail, as more studies are still needed to validate the findings of the present study. These results can be used as baseline data for future studies.

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CHAPTER FOUR

Apoptosis of germ cells in the normal testis of the Japanese quail (*Coturnix coturnix japonica*)

Preface

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Abstract

It has been established that excess germ cells in normal and in pathological conditions are removed from testicular tissue by the mechanism of apoptosis. Studies on germ cell apoptosis in avian species are grossly lacking, and there are only a few reports on induced germ cell degenerations in the testis tissue of birds. This study was designed to investigate the process of apoptosis of germ cells in the Japanese quail (*Coturnix coturnix japonica*). Germ cell degenerations were investigated in birds of all age groups, namely pre-pubertal, pubertal, adult and aged. Apoptosis of germ cells in the quail, as shown by haematoxylin and eosin (H&E), TdT dUTP Nick End Labeling (TUNEL) assay and electron microscopy, was similar to that observed in previous studies of germ cells and somatic cells of mammalian species. The observed morphological features of these apoptotic cells ranged from irregular plasma and nuclear membranes in the early stage of apoptosis to rupture of the nuclear membrane, condensation of nuclear material, as

well as fragments of apoptotic bodies, in later stages of apoptosis. In the TUNEL-positive cell counts, there was a significant difference between the mean cell counts for the four age groups ($P<0.05$). Post hoc analysis revealed a highly significant difference in the aged group relative to the pubertal and adult age groups, while the cell counts of the pre-pubertal group were significantly higher than those of the pubertal group. However, there was no significant difference between cell counts of the pre-pubertal and the adult, and between the pre-pubertal and the aged groups.

Keywords: Apoptosis; Germ cells; Japanese quail; TUNEL

Abbreviations: TUNEL, TdT dUTP Nick End Labeling, BM, basement membrane, N, nucleus, M, mitochondria, LD, lipid droplets, sgn, spermatogonia, SPC, spermatocyte, spd, round spermatid, H&E, haematoxylin, and eosin, TEM, transmission electron microscope.

4.0 Introduction

There are few studies on spermatogenesis in avian species, compared to mammals (Aire et al., 2019). Spermatogenesis is a complex process involving the transformation of spermatogonia to mature spermatozoa. This process is similar in all vertebrates. The process can be divided into three progressive stages: a mitotic division of spermatogonia, meiotic division of spermatocytes, and spermiogenesis (Aire, 2007). Degeneration of germ cells occurs mainly during mitosis and meiosis (Nihi et al., 2017). It is thought to be a mechanism aimed at removing or eliminating chromosomes with abnormalities (Ehmcke and Schlatt, 2006), and reducing the number of germ cells that can be supported and nourished by Sertoli cells (Oatley and Brinster, 2008).

Apoptosis is a programmed and genetically controlled process that causes selective cell death (Allan et al., 1992). It is a normal physiological process that occurs during spermatogenesis, embryogenesis, immune system regulation, and maintenance of tissue homeostasis (Saraste and Pulkki, 2000). Apoptosis plays an important role in controlling the number of spermatogenic cells that can be supported by Sertoli cells (Kerr et al., 1992, Hikim and Swerdloff, 1999) and preserving the genetic integrity of male germ cells through the removal of germ cells with defective genes (Bailly and Gartner, 2013). The most common germ cells that are easily removed by programmed cell death are spermatogonia (Allan et al., 1992).

Apoptosis is also known to occur mainly near the basement membrane of seminiferous tubules, which indicates that these cells are predominantly spermatogonia, specifically types A2, A3, and A4 spermatogonia (Allan et al., 1992). Apoptotic germ cells can be detected by the terminal deoxynucleotidyl transferase TdT dUTP Nick End Labeling (TUNEL) assay, a technique widely used to detect cells undergoing stages of apoptosis in which there is extensive DNA fragmentation (Soldani and Scovassi, 2002, Gavrieli et al., 1992). Biochemical and morphological features demonstrated in the stallion testis have confirmed that the TUNEL assay is an acceptable technique for identification and estimation of germ cell apoptosis in histological tissue sections (Heninger et al., 2004).

Although the TUNEL staining technique is very sensitive and has been accepted universally as a method of choice for detecting apoptosis *in situ,* it is always more accurate to use another independent method such as transmission electron microscopy (TEM) for further confirmation. This is imperative because DNA fragmentations are not

unique features of apoptosis alone, as the TUNEL assay has been reported to detect non-apoptotic DNA damage in necrotic cell death (Ansari et al., 1993, Loo, 2011).

Germ cell apoptosis in mammalian species has been well documented (Heninger et al., 2004, Shaha, 2008, Tapia and Pena, 2009, Dadhich et al., 2010, Sharma et al., 2014, Jiménez et al., 2015, Massoud et al., 2018), but not much is known about the rates of apoptosis during spermatogenesis in avian species. The aim of this study was to document rates of germ cell apoptosis in the male Japanese quail, as a model avian species. As a research model, they have a small body size that favours ease of housing and reduces the cost of feeding, and they also have a short life cycle. They adapt favourably to laboratory manipulations, and their physiological maturing and aging processes are fast due to their short life span.

4.1 Materials and methods

4.1.1 Animals used for the study

A total of 28 birds were used for the study, comprising 7 birds in each of the 4 reproductive phases, namely pre-pubertal (4 weeks old), pubertal (6 weeks old), adult (12 weeks old) and aged (52 weeks old) according to previously classified reproductive phases (Ottinger et al., 2004, Huss et al., 2008). The birds were purchased from the Agricultural Research Council (ARC), Irene, Pretoria, South Africa. The protocol for euthanasia, using carbon dioxide ($C0_2$) inhalation anaesthesia, was approved by the Institutional Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, University of Pretoria, South Africa (issued vide No. AEC/A065-12).

4.1.2 Tissue samples for light microscopy

Tissue samples from both testes of each bird were collected following the opening of the thoracoabdominal cavity. The left testes of each bird were used for light microscopy and the TUNEL assay, and the right testes were used for electron microscopy. The tissue samples were fixed for 5 days in 10% buffered formaldehyde and were processed conventionally for paraffin wax embedment, sectioned at 5 um thick, and were stained with haematoxylin and eosin (H&E) for light microscopy.

4.1.3 TUNEL labeling technique

Detection of degenerated germ cells was done by TUNEL labelling. The assay was carried out on 5 µm-thick sections, using an apoptosis detection kit (ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit #S7101 Millipore, USA), following the manufacturer's instructions.

4.1.4 TUNEL-positive cell counting

The TUNEL-positive cell counting was performed according to previously described methods (Dutta et al. 2012, Madekurozwa and Mpango, 2020). An image analyzer system (CellSens dimension software) tethered to an Olympus BX-63 microscope was used to count the TUNEL-positive cells*.* The number of TUNEL-positive cells in 100 µm lengths of seminiferous tubules from 10 randomly selected microscopic fields of each testicular cross-section per bird was counted. One TUNEL-positive cell was selected from each microscopic field to identify the positive cell's intensity to be counted using the software tool. All positive cells were counted and grouped according to their reproductive phases (pre-pubertal, pubertal, adult, and aged birds) without separating them according to cell

types. The data generated were presented as mean ± standard error of mean (SEM) (n=7).

4.2 Statistical Analysis

All data generated were analyzed using IBM SPSS version 26 software. The number of TUNEL-positive cells was analyzed using one-way analysis of variance (ANOVA). Age groups were used as the independent variables while the frequency of apoptotic cells was the dependent variable. A Tukey's Honest Significant Difference (HSD) post hoc test was performed on the results between the age groups. The differences were considered as significant at $p<0.05$. Data were presented as mean ± standard error of mean (SEM).

4.3 Transmission Electron Microscopy

The right testicular tissue samples for electron microscopy were immediately fixed following collection in 3% glutaraldehyde in Millonig's phosphate buffer, at pH 7.4. The tissue samples were later post-fixed for 2 h in 1% osmium tetroxide, rinsed in 0.1M Millonig's buffer, dehydrated in graded concentrations of alcohol, and embedded in epoxy resin overnight. Semi-thin sections of 1µm thickness were cut and stained with toluidine blue. Ultra-thin (50-90nm thick) sections of selected areas were conventionally cut and stained with Reynold's lead citrate on copper grids. The sections were then counterstained with uranyl acetate and examined in a Phillips CM10 transmission electron microscope (GmbH, Munster, Germany), at 80kV.

4.4 Results

4.4.1 Haematoxylin & Eosin sections

Apoptosis of germ cells was detected in all the age groups, and these cells were irregular in shape, and contained eosinophilic cytoplasm with condensed nuclei (Figure 4.1). There were no other visible alterations in the seminiferous tubules in any of the age groups. However, all the types of germ cells, ranging from spermatogonia (dark, pale, and type B spermatogonia), spermatocytes, round and elongating spermatids were also seen in the adult bird, aside from the apoptotic germ cells (Figure 4.2).

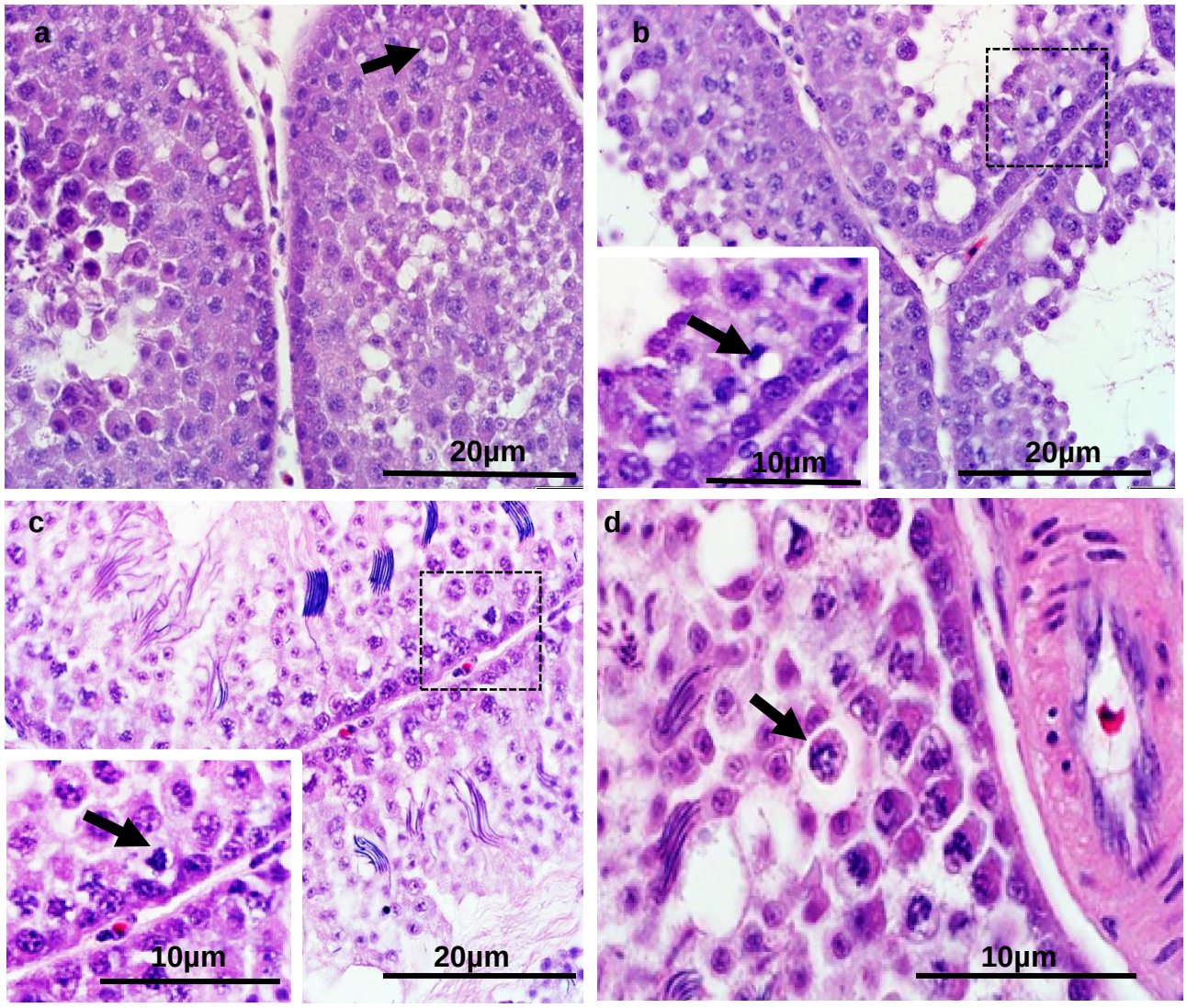

Figure 4.1 Transverse sections of seminiferous tubules of the Japanese quail (a) pre-pubertal, (b) pubertal, (c) adult, and (d) aged. Arrows showing apoptotic germ cells.

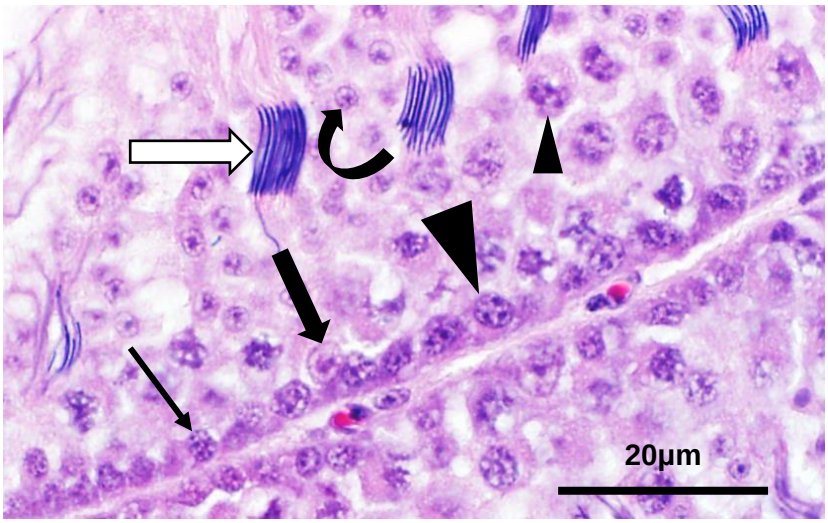

Figure 4.2. Transverse sections of seminiferous tubules of an adult Japanese quail. Thin black arrow = dark spermatogonia, thick black arrow = pale spermatogonia, thick arrowhead = type B spermatogonia, thin arrowhead = spermatocyte, curve arrow = round spermatid, white arrow = elongating spermatids.

4.4.2 TUNEL-positive sections

TUNEL staining of germ cells in the cross-sections of seminiferous tubules in all the age groups and the positive control from mammary gland tissue were immuno-positive (Figure 4.3). The mean and standard deviation of TUNEL-positive cell counts were as follows: Pre-pubertal, 23.67± 0.67 (N = 7); Pubertal, 8.00 ±0.70 (N = 7); Adult, 15.30 ± 0.33 (N = 7); and Aged, 25.0± 4.00 (N = 7). In the TUNEL-positive cell counts, there was a significant difference between the mean cell counts for the four age groups ($P<0.05$). The post hoc test revealed a highly significant difference in the cell counts of the aged group relative to those of the pubertal and adult age groups, while cell counts for the pre-pubertal

group were significantly higher than those of the pubertal group. However, there was no significant difference between the pre-pubertal and the adult, and between the pre-pubertal and the aged group cell counts (Figure 4.8).

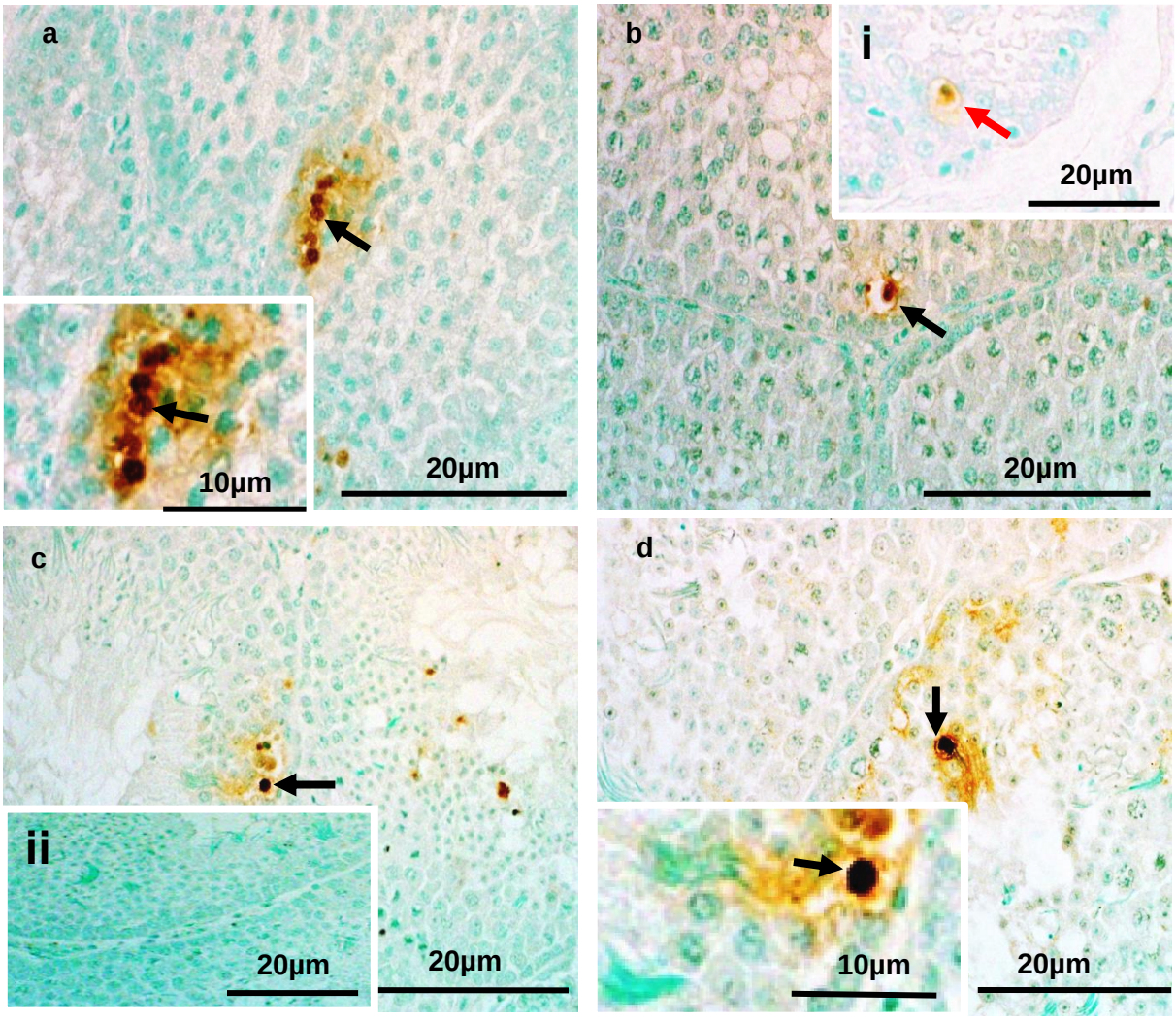

Figure 4.3 TUNEL labelling of germ cells of Japanese quail of varying ages; **a** = pre-pubertal, **b** = pubertal, **c** = adult, **d** = aged, **i** = positive control from the tissue of mammary gland, and **ii** = negative control. Black arrows = TUNEL-positive cells from all the age

groups; red arrow = TUNEL-positive cell from the positive control (mammary gland tissue).

4.4.3 Transmission electron microscopy

4.4.3.1 Pre-pubertal birds

In the pre-pubertal birds, germ cell degenerations were observed. They were seen close to the basement membrane, and they were most likely to be apoptotic spermatogonia or spermatocytes, as other germ cells such as spermatids are yet to develop at this stage (Figure 4.4).

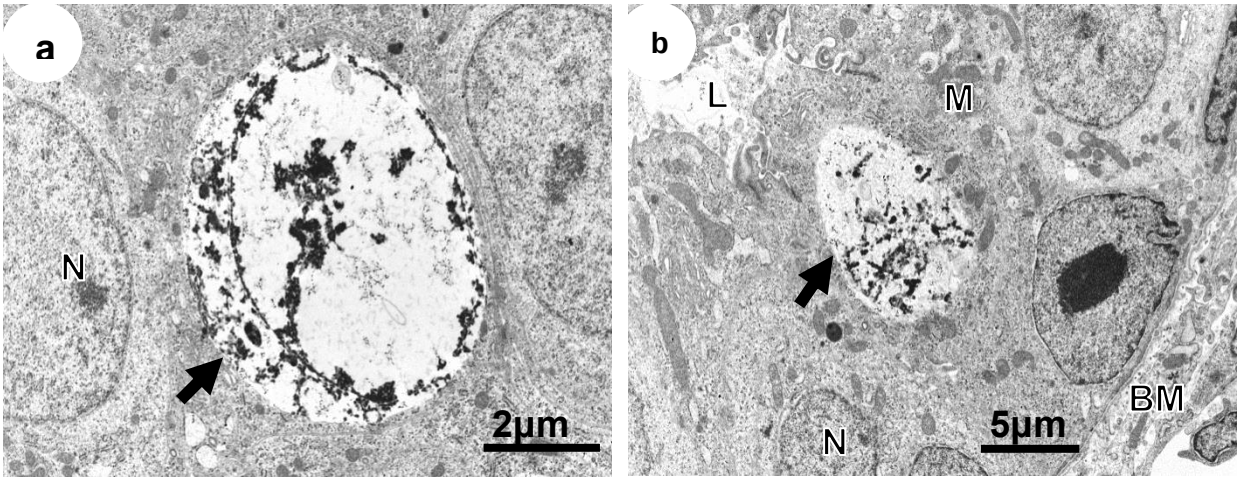

Figure 4.4. Electron micrographs of seminiferous tubules of pre-pubertal birds. **(a, b)**, arrows = apoptotic bodies. BM = basement membrane; M = mitochondria; N = nucleus; L = lumen.

4.4.3.2 Pubertal birds

In the pubertal birds, the main finding was the rupture of nuclear membrane of the germ cells at varying degrees. In addition, clusters of round mitochondria were observed in the cytoplasm of the apoptotic germ cells (Figure 4.5).

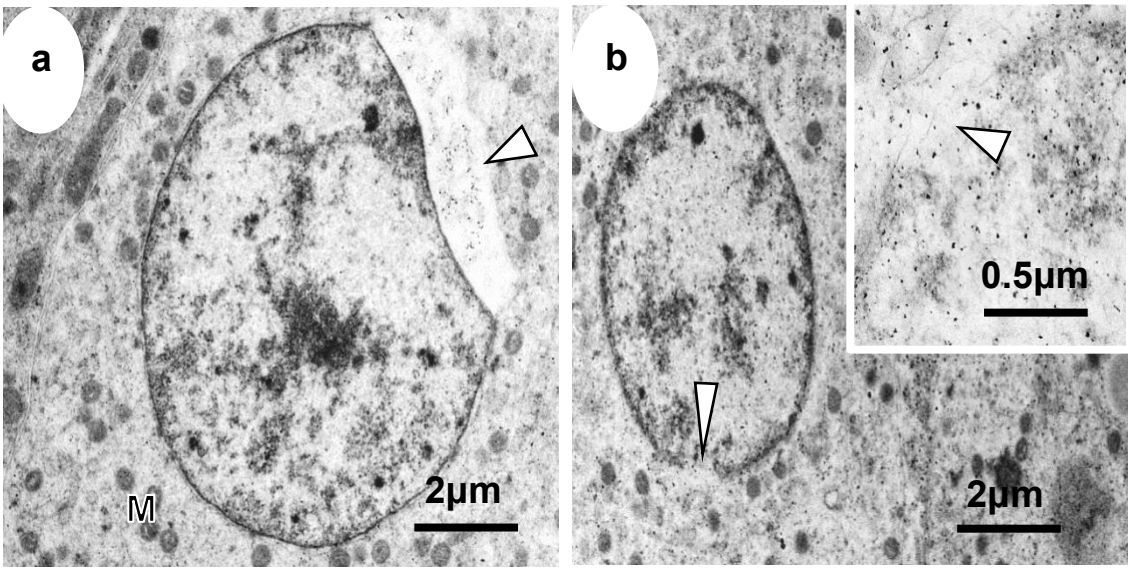

Figure 4.5. Electron micrographs of seminiferous tubules of pubertal birds. **(a, b)**, white arrowheads = disruption of nuclear membrane (also in inset). M = mitochondria.

4.4.3.3 Adult birds

Apoptotic germ cells in the adult birds showed the same appearance as in the pubertal age group. However, no organelle was seen, and only lipid droplets in the cytoplasm of the apoptotic germ cells were observed (Figure 4.6).

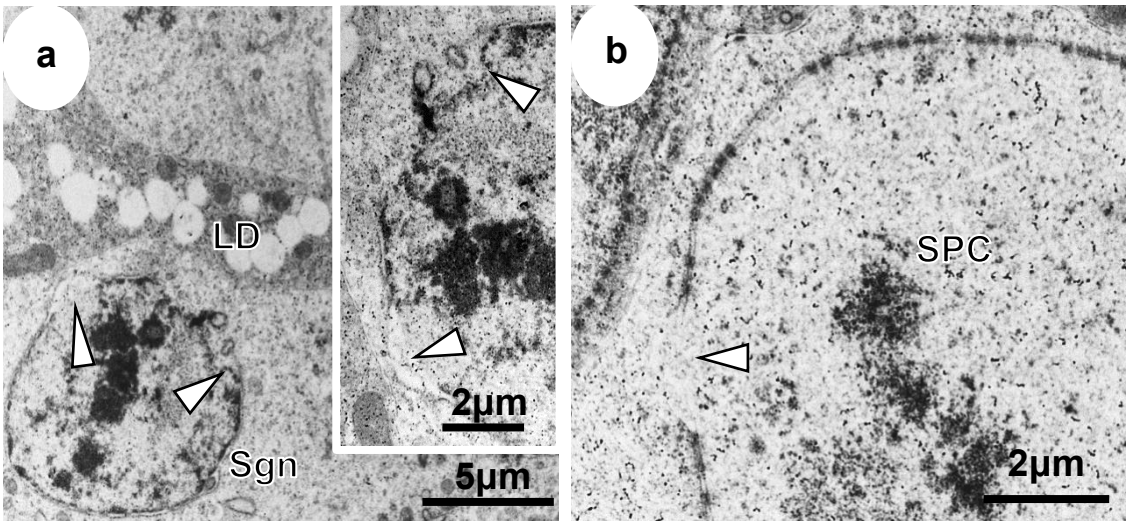

Figure 4.6. Electron micrographs of seminiferous tubules of adult birds. **(a, b),** white arrowheads = rupture of nuclear membranes (also in inset). Sgn = spermatogonia; SPC = spermatocyte; LD = lipid droplets.

4.4.3.4 Aged birds

In the aged birds, germ cells that were normally located near the basement membrane degenerated, leaving numerous lipid droplets in the cytoplasm of the degenerated germ cells (Figure 4.7a). Some germ cells with intact nuclear membranes, however, displayed dilated intercellular spaces (Figure 4.7b). Rupture of the nuclear membrane was also observed, as in the other age groups (Figure 4.7c, d).

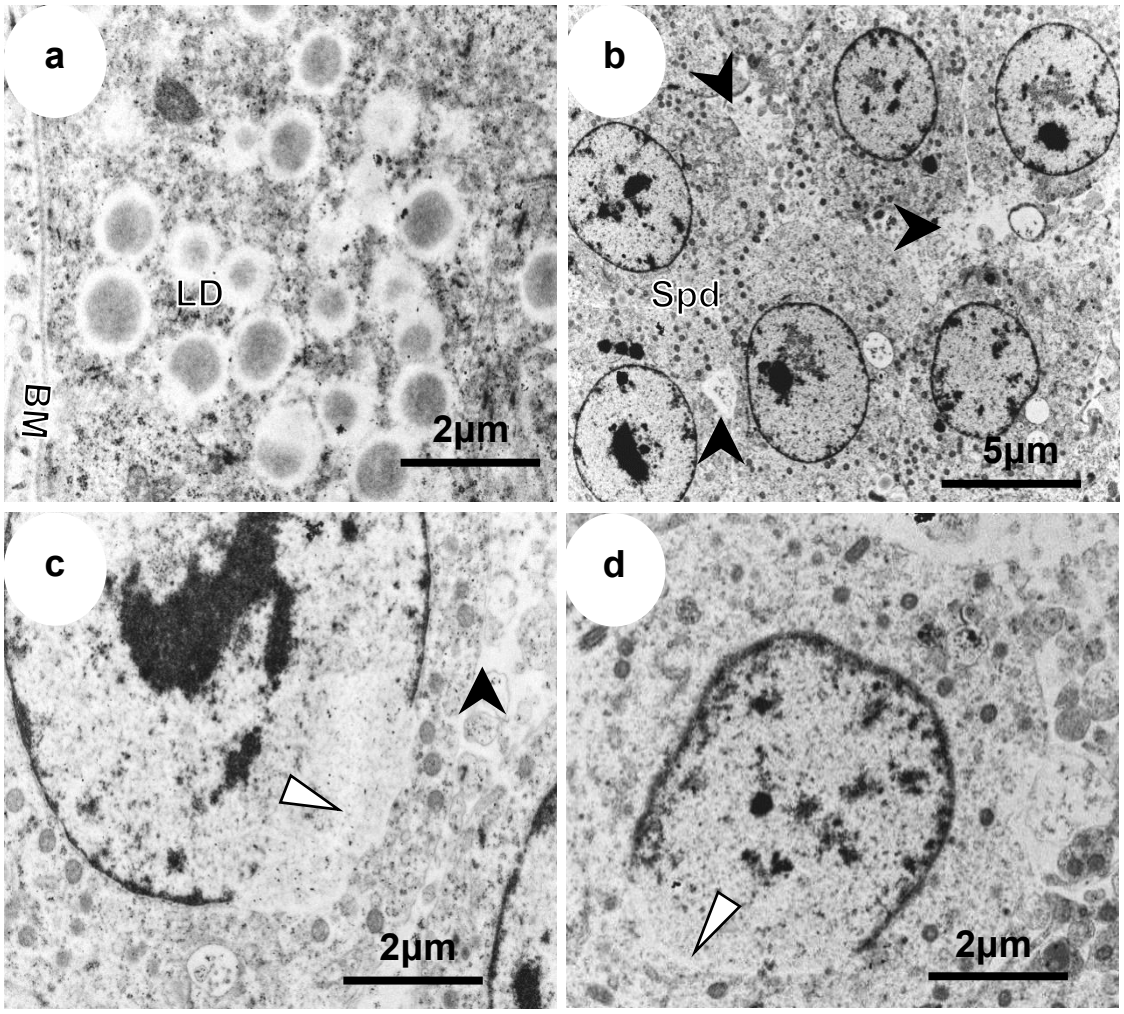

Figure 4.7. Electron micrographs of seminiferous tubules of aged birds. **(a),** BM = basement membrane; LD = lipid droplets. **(b),** Spd = round spermatids, black arrowheads = dilation of intercellular spaces. **(c and d),** white arrowheads = rupture of nuclear membrane, black arrowhead = dilation of intercellular space.

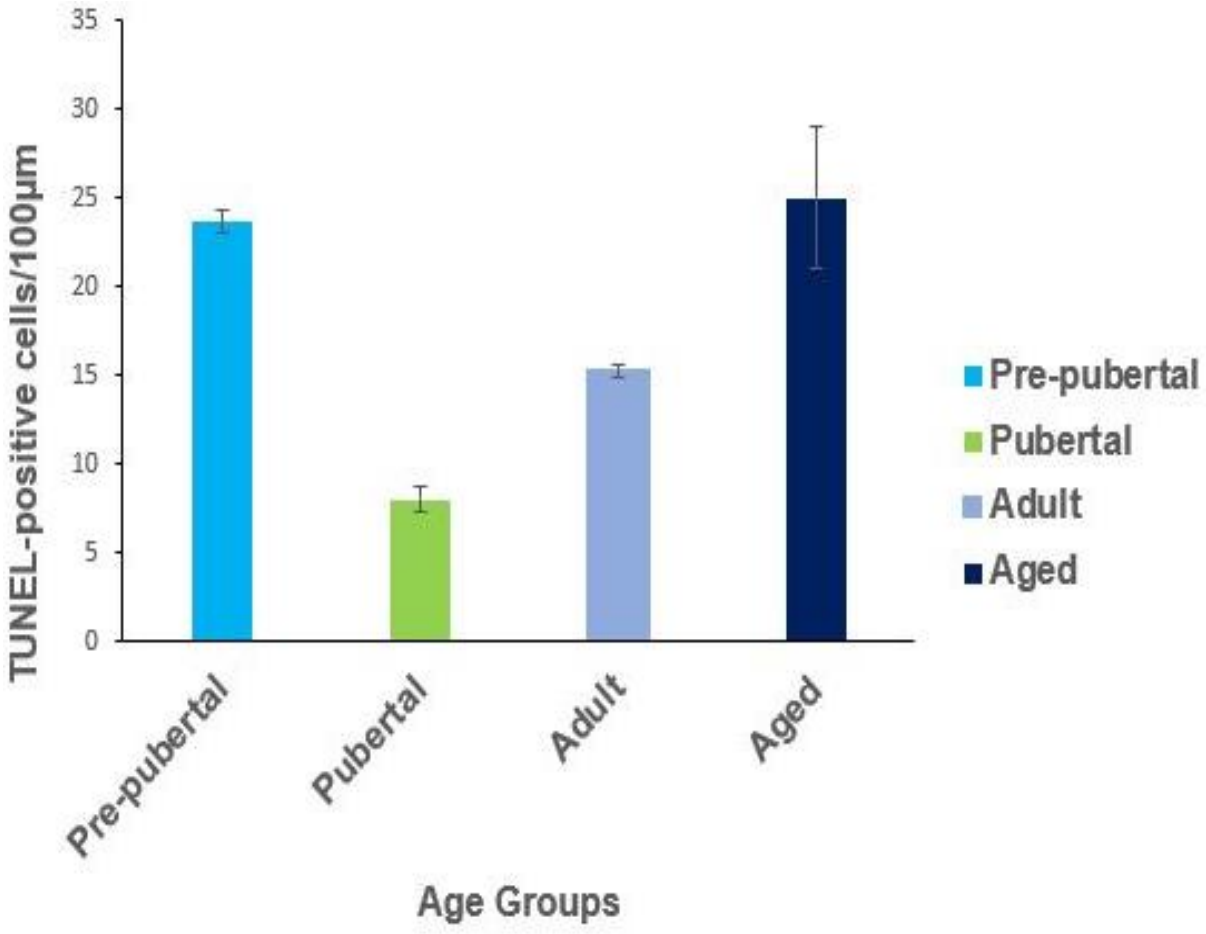

Figure 4.8. Bar chart showing the frequency of TUNEL-positive cells between the different age groups. TUNEL-positive cell counts were significantly higher in pre-pubertal and aged than other age groups ($P<0.05$).

4.5 Discussion

This appears to be the first study on apoptosis of germ cells in the normal testis of Japanese quail of different age groups. The irregular cell shapes and eosinophilic cytoplasm along with condensed or fragmented nuclei are in accord with reports by Cai et al. (2000) in rats and Liu et al. (2006) in mice. TdT dUTP Nick End Labeling of TUNEL-

positive cell counts were detected in all age groups but with higher frequencies in pre-pubertal and aged birds. Even though the pattern of germ cell apoptosis is not sequential as observed in rats (Billig et al., 1995), the higher frequencies of TUNEL-positive cell counts in pre-pubertal and aged birds in the present study agrees with findings in pre-pubertal boys (Berensztein et al., 2002), pre-pubertal rats (Palacios et al., 2016) and in aged mice (Jara et al., 2004).

During pre-pubertal life in mammalian species, up to 75% of germ cells produced are lost by the mechanism of apoptosis (Aitken et al., 2011). This is crucial to reduce the number of germ cells that can be supported and nourished by Sertoli cells (Oatley and Brinster, 2008). Also, a high frequency of germ cell apoptosis associated with aging was reported by Levy and Robaire (1999) in rats and Pentikäinen et al. (1999) in humans. They observed that both steroidogenesis and spermatogenesis decreased with age, apparently due to increased rates of apoptosis. These changes could be due to testicular tissue oxidative stress and a fall in androgen levels as observed in Brown Norway rats (Zirkin and Chen, 2000). Even though germ cell apoptosis in mammalian species has been well studied, the exact mechanism responsible for programmed germ cell death is still not very clear. However, based on DNA fragmentation and estimation of TUNEL-positive nuclear counts, it has been noted in rats that there is a steady increase of germ cell apoptosis in pre-pubertal and pubertal animals, and an abrupt decrease in adults (Billig et al., 1995).

In normal spermatogenesis, the underlying mechanism of programmed germ cell death is apoptosis, as has been observed by Sinha Hikim et al. (1997) in rats, Lue et al. (1997) in hamsters and Shanna et al. (2011) in humans. This was also evident in the present study. Several reports have shown that apoptosis plays a crucial role in the normal and

pathological coordination of male germ cells and ejaculated spermatozoa (Tapia and Pena, 2009). This is necessary because apoptosis is required to achieve an equilibrium between germ cells and somatic cells (Print and Loveland, 2000).

Downregulation of germ cell density in testicular tissue by the mechanism of apoptosis guarantees germ cell homeostasis, as each Sertoli cell can support and nourish only a certain number of germ cells (Griswold, 1998). Even though the importance of apoptosis is well established, there are some conflicting reports about apoptosis markers in mature spermatozoa. It is poorly understood whether pro-apoptotic proteins detected in matured spermatozoa resulted from a failed process of apoptosis or whether the process started later, before spermatozoa maturation (Sakkas et al., 2004, Grunewald et al., 2005). Germ cell degeneration as revealed by electron microscopy in the present study exhibited the characteristic features of apoptosis, such as margination of nuclear chromatin, irregular or ruptured nuclear membranes, as well as phagocytosis of apoptotic bodies. These features are similar to spermatogonial degeneration in rats (Allan 1992).

A common techniques that has been universally accepted in detecting DNA fragmentation is TUNEL labeling, although there are conflicting reports among authors on apoptotic changes based on DNA fragmentation (Tornusciolo et al., 1995, Orita, 1999). It has been reported that, unless DNA damage is severe, it will not be detected by TUNEL methods (Brum et al., 2008). Anzar et al. (2002) demonstrated that apoptotic germ cell numbers could be underestimated, especially in the early stage of apoptosis (Aoki et al., 2020). This is possible because DNA damage usually occurs during the late stage of apoptosis (Borges et al., 2008).

Normal seminiferous tubule architecture maintenance is essential for spermatogenic efficiency. The maintenance of spermatogenesis is achieved by a balance between the degeneration and regeneration of germ cells. This equilibrium is regulated by Sertoli cells, each of which can nurture a certain number of germ cells (Johnson et al., 2008). It has been established in mammals that loss of germ cells occurs normally and necessarily during spermatogenesis (Richburg, 2000, Royere et al., 2004). The various features of avian germ cell apoptosis as shown in this study correlate with those reported in mammalian species studied.

4.6 Conclusion

The current study demonstrated some degenerative changes of germ cells in the normal testis of birds in all age groups (pre-pubertal, pubertal, adult and aged) for the first time. The apoptosis of germ cells in avian species seemed to correspond to that of mammalian species that have been widely studied. The germ cell degenerations revealed by haematoxylin and eosin staining, the TUNEL assay and electron microscopy in the present study reflected the characteristic features of apoptosis similar to those previously reported in mammalian species. These apoptotic features ranged from the condensation of nuclear chromatin, irregular shape or rupture of the nuclear membrane as well as phagocytosis of apoptotic bodies. Also, numerous lipid droplets were found in the cytoplasm of degenerated germ cells, and dilation of intercellular spaces was apparent, especially in the aged birds. This study therefore contributes to understanding of apoptosis in normal avian germ cells using Japanese quail as a model.

4.6 Ethics approval

This study was approved by the Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, the University of Pretoria, South Africa (issued vide No. AEC/A065-12).

4.7 Competing interests

The authors declare that there are no competing interests.

4.8 Acknowledgment

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CHAPTER FIVE

Alterations of spermatogenic cells in the testis of Japanese quail (*Coturnix coturnix japonica*) exposed to dibutyl phthalate (DBP).

Abstract

Studies on the effects of dibutyl phthalate (DBP) on spermatogenic cells in birds are grossly lacking. This study was designed to determine the effects of DBP on spermatogenic cells in the testis of Japanese quail (*Coturnix coturnix japonica*). The birds were randomly divided into five dosage groups. The control group was administered a corn oil vehicle only (a dose of 1 ml/kg body weight), while the other four experimental groups were administered intra-gastrically, a daily dosage of 10, 50, 200, 400 mg/kg/body weight of DBP (dissolved in corn oil), respectively with the aid of gastric lavage, for a period of 30 days. Histopathological evaluation revealed vacuole formation, germ cell degenerations, necrosis, and absence of spermatogenic cell series. These features have not been reported in mammalian species at the same dosage level. In the transmission electron microscopy evaluation, germ cells with chromatin clumps, ruptured nuclear membranes, degenerated germ cells resting on distorted basement membranes, intracytoplasmic vacuoles, and dense apoptotic bodies were observed. These features have also not been detected in mammalian species at the same dose. The findings of the present study reveal that spermatogenic cells of Japanese quail seem to be more sensitive to DBP-induced degeneration compared to mammalian species studied.

Keywords: Dibutyl phthalate; Degeneration; Japanese quail; Spermatogenic cells

5.0 Introduction

Several studies have shown that there is a decline in male reproductive health due to the deleterious effects of environmental chemicals, especially endocrine-disrupting chemicals such as phthalates, on the testis and its excurrent ducts (Wong and Cheng, 2011). Dibutyl phthalate (DBP), is one of the phthalates that have attracted serious concerns because millions of tons of this chemical are produced annually and used for the manufacturing of plastics (Guerra et al., 2010).

Phthalates are classified as endocrine-disrupting chemicals (EDCs) (Biro et al., 2013). The chemical structures of phthalates are closely related to those of hormones, and that is why they are capable of interfering with hormone receptors (Swedenborg et al., 2009). These compounds can bind directly either as agonists or antagonists to hormone receptors because of structural similarities, thereby disrupting or enhancing the effect of the hormones (Swedenborg et al., 2009). These chemicals may cross the placenta and affect the offspring (Diamanti-Kandarakis et al., 2009).

Phthalate activities against hormone receptors are determined by their carbon skeleton length. In males, exposure to phthalates is associated with the increase of hormone-associated diseases, such as genital abnormality, abnormal sperm morphology, and prostate cancer (Radke et al., 2018). In addition, phthalates and their metabolites can hamper motility, concentration, and morphology of sperm, as well as increasing the rate of DNA damage (Karačonji et al., 2017). Furthermore, phthalates induce hypospadias - abnormal urethral openings - in young males (Cai et al., 2015).

Phthalates such as DBP have been used as plasticizers since 1921 and are added to polyvinyl chloride (PVC) to increase its strength, flexibility, and durability (FDA, 2001).

DBP is usually added in the manufacturing of several products, such as children's toys, cosmetics, car interiors, food packaging materials, inks, adhesives and paints (Przybylińska and Wyszkowski, 2016).

Interestingly, phthalates used in PVC are not covalently bonded and, as a result, they leach easily into the environment (FDA, 2001). They are soluble in fat and organic solvents such as chloroform and hexane, but they are hardly soluble in water (Becker et al., 2004). Therefore, exposure to DBP occurs through oral ingestion, the dermal route, or inhalation. Metabolites of phthalate have been found in urine, seminal fluid, saliva and breast milk (Swan, 2008, Wittassek et al., 2011). Specifically, spermatogenesis disruption by phthalates in mammals has been linked to disturbance of oxidative balance, which has been suggested to occur via peroxisome proliferator-activated receptor (PPAR) activation (Onorato et al., 2008).

Spermatogenesis in avian species is similar to mammalian species; in the sexually active and mature testis, it is made up of germ cells in varying degrees of development (Aire, 2007). The germ cells usually proliferate and migrate from the basement membrane towards the lumen of seminiferous tubules. In the process, they grow older until they mature into spermatozoa. Germ cells develop in close association with one another by maintaining close linkages through incomplete cytoplasmic division development (Aire, 2007). This close association can render them vulnerable to degeneration by endocrine-disrupting chemicals such as phthalates.

It has also been shown that Sertoli cells, germ cells and interstitial Leydig cells are the targets of phthalates (Ward et al., 1998). The modes of action of phthalates are not clearly understood, but their effects might occur via multiple pathways. Dibutyl phthalate reduces

the production of testosterone and insulin-like factor 3 (insl3) mRNA in fetal rat testes, thereby disrupting the spermatogenic process (Wilson et al., 2004). Even though the exact mechanism of action of DBP is still unclear, the literature on the effects of DBP in the male reproductive system of mammalian species is well documented (Park et al., 2002, Spade et al., 2015, Aly et al., 2016, Nelli and Pamanji, 2017).

Spermatogenic cells are similar in all vertebrates, but there are noteworthy variations, especially in birds (Aire et al., 2019). As a result, there are insufficient data on the effects of DBP on the biology of spermatogenic cells in avian species. Therefore, the present study was focused on the effects of DBP on developing germ cells in the testis of Japanese quail.

5.1 Study Methodology

5.1.1 Chemical used for the study

The test compound, Di (n-butyl) phthalate DBP (CAS Number 84-74-2 technical grade, purity>99.8%, PN61840625001730 was purchased from Sigma-Aldrich (Pty) Ltd (Johannesburg, South Africa).

5.1.2 Experimental animals and management

A total of twenty-five newly hatched, pre-sexed male Japanese quail (*Coturnix coturnix japonica*) procured from the Aviary Unit, Irene Animal Improvement Research Station, Pretoria were used for the study. Prior to the experiment, the quail were acclimatized at the poultry facility (brooder cages) located in the Poultry Research Unit of the Department of Production Animal Studies (University of Pretoria) for 2 weeks, before transfer into battery cages (49 x 95 x 51 cm). At hatch, the temperatures were first maintained at 35-

37^0C and then slowly decreased by 0.5^0C/day until a temperature of 16^oC to 23^0C was reached at 4 weeks of age. Thereafter, the birds were maintained under a controlled photoperiod (16L: 8D, schedule of light-dark cycle), at 25^0C ± 2^0C with a relative humidity of 50% ± 5%, until the age of 10 weeks (the age at the onset of the experiment).

The birds were individually identified by means of wing-tags and fed on a standard commercial high protein diet (Obaro Feeds, Pretoria, South Africa). Tap water was provided *ad libitum*. All procedures were carried out in accordance with the guidelines for the care and use of laboratory and research animals (SANS Guidelines, 2008) and the experiment was approved by the institutional Animal Ethics Committee (AEC) of the University of Pretoria (issued vide No. AEC/A065-12).

5.1.3 Animal exposure and dosing regimen

The experiment was conducted in accordance with the guidelines for avian toxicity testing studies as stipulated by the Organization for Economic Co-operation and Development (OECD guideline, 2010). DBP (the test compound) was dissolved in a vehicle corn oil base and used for the treatment groups. The birds were randomly divided into five dosage groups. The control group was administered a corn oil vehicle only (at a dose of 1 ml/kg body weight), while the other four experimental groups were administered intra-gastrically, with a daily dosage of 10, 50, 200, 400 mg/kg body weight of DBP (dissolved in corn oil), respectively, for a period of 30 days. Throughout the dosing period, food intake, body weight and clinical signs or any signs of abnormal behaviour were monitored daily.

5.1.4 Necropsy and organ harvesting

After the 30 day experimental period, the quail (n=5) each from the control and experimental groups were weighed, using a digital precision balance UWE Digital precision weighing balance (Algen Scale Corporation® Bohemia,NY), and immediately euthanized, using carbon-dioxide ($C0_2$) inhalation anaesthesia. At necropsy, the location of the testes were noted and the detunicated (isolated) testes (left and right) from both control and DBP-exposed groups, were removed from the remainder of the adherent tissues and visually inspected for evidence of gross morphology and symmetry, and then weighed.

5.1.5 Tissue processing for light microscopy

Testicular tissue samples were processed for histology using an automated tissue processor (Shandon Excelsior Thermo Scientific, city, Germany). Tissue processing included dehydration in ascending series of alcohol concentrations (50, 60, 80, 95 and 100%), clearance in xylene (two changes), infiltration, as well as embedding using molten paraffin wax.

5.1.6 Transmission electron microscopy

Tissue samples were placed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.4) for 24 hours. Thereafter, the tissue samples were post-fixed in 0.5% osmium tetroxide for 2 hours. The tissue samples were then rinsed in phosphate buffer (pH 7.4), dehydrated in a series of ethanol concentrations and embedded in epoxy:resin at a ratio of 1:2 for 1 hour, 1:1 for 2 hours and 100% resin overnight. Semi-thin sections were cut using a diamond knife and stained with toluidine blue and analyzed using light microscopy. Ultra-

thin sections were cut using a diamond knife, stained with lead acetate and counter-stained with uranyl citrate. The samples were viewed with a Philips CM10 transmission electron microscope (FEI, The Netherlands), fitted with an Olympus Mega View III imaging system.

5.2 Results

5.2.1 Light microscopy

5.2.1.1 The DBP control group

In the control birds, the spermatogenic cells in the seminiferous tubules (ST) were morphologically normal. The shape and size of the seminiferous tubules appeared to be uniform. The spermatogonia, spermatocytes, round and elongated spermatids all appeared to be normal and were arranged in an orderly fashion along the smooth wall of seminiferous tubules (Figure 5.1).

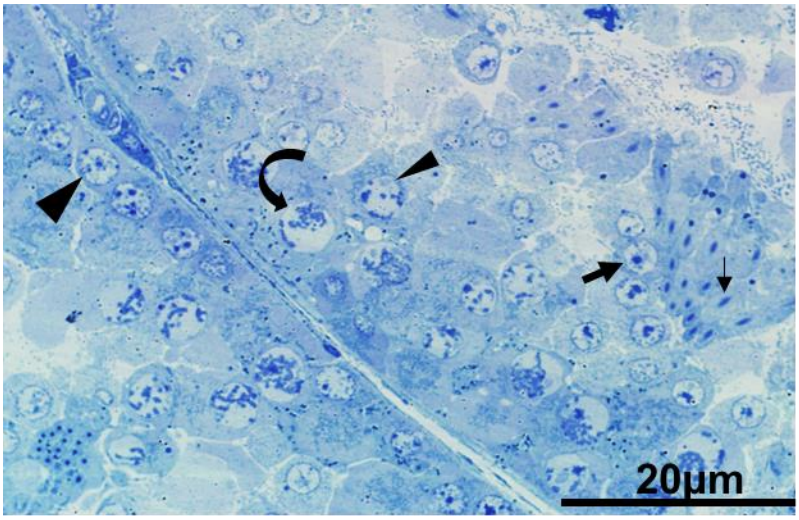

Figure 5.1. Light photograph of seminiferous tubules of male Japanese quail from the control group showing normal germ cells. Thick arrowhead = spermatogonia, curved arrow = primary spermatocyte, thin arrowhead = secondary spermatocyte, thick arrow = round spermatid, thin arrow = elongated spermatid.

5.2.1.2 Low- and medium-dose DBP treatments(10 and 50 mg/kg body weight, respectively).

There were no obvious morphological differences between the low and medium dose exposed groups (10 mg/kg and 50 mg/kg body weight of DBP) respectively. Evidence of spermatogenic cell series was observed in the smooth wall of the seminiferous tubule with less frequent vacuolation and intercellular dilation (Figure 5.2).

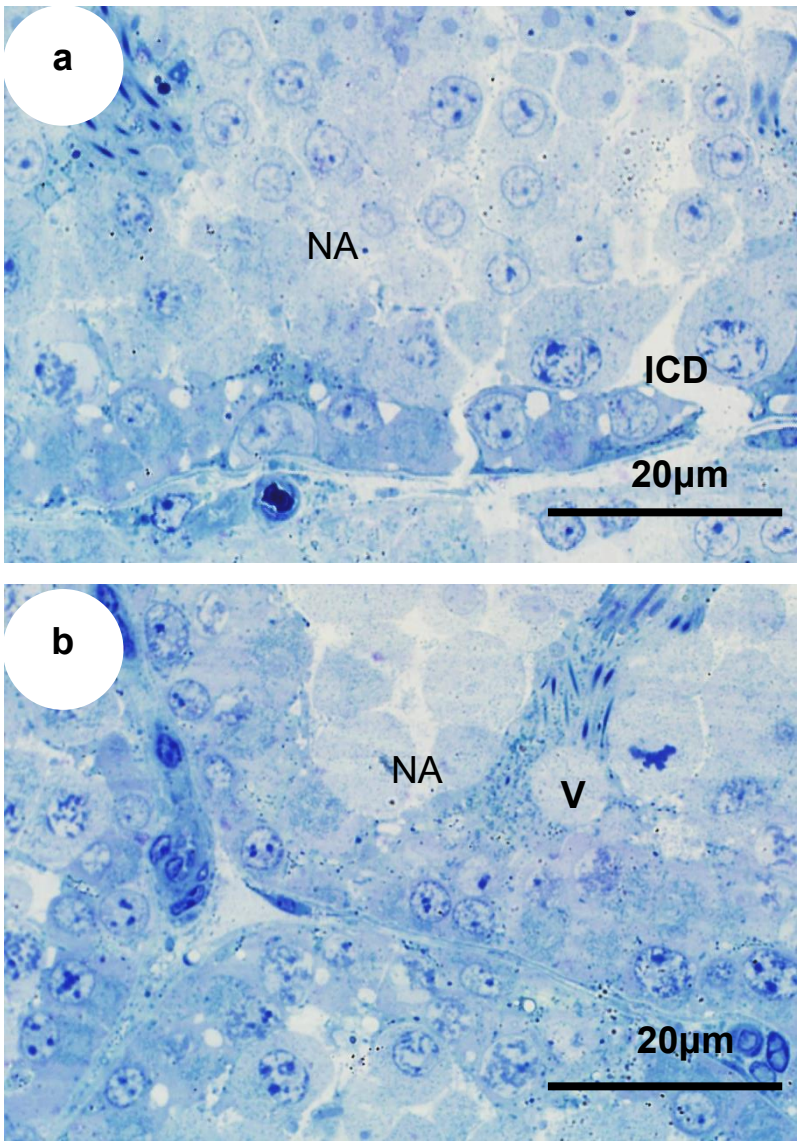

Figure 5.2. Light photographs of seminiferous tubules of male Japanese quail from the DBP 10 mg/kg body weight treatment group (**a**), and from the DBP 50 mg/kg body weight treatment group (**b**). NA = necrotic area, ICD = intercellular dilation, V = vacuole. H&E X Bar = 20µm.

5.2.1.3 High dose of DBP treatment groups (200 mg/kg body weight and 400 mg/kg body weight).

In birds given 200 mg/kg body weight, there was an absence of spermatogenic cell series due to germ cell degeneration (Figure 5.3a). In the group given 400 mg/kg body weight, there were fewer germ cells, but similar degenerative changes and other features as observed in the 200 mg/kg group (Figure5.3b).

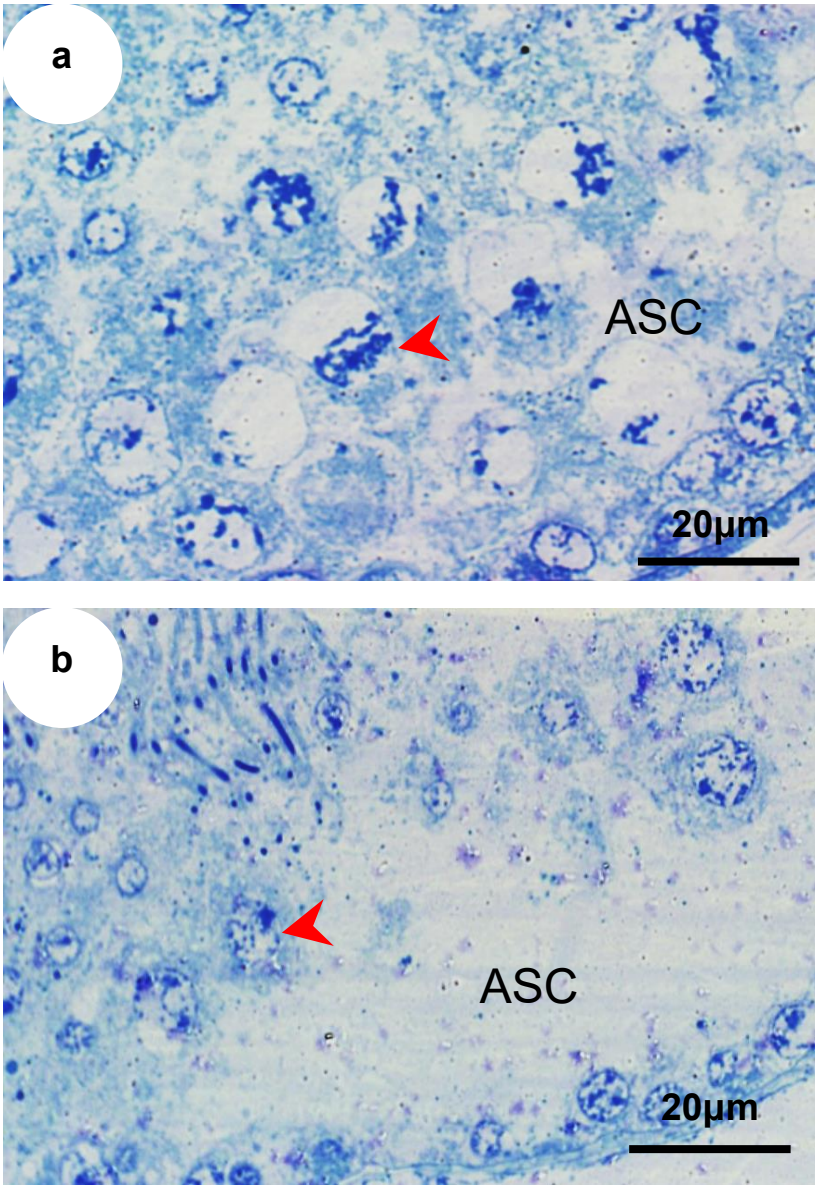

Figure 5.3. Light photographs of seminiferous tubules of male Japanese quail (**a**), from the DBP 200 mg/kg body weight treatment group and (**b**), from the DBP 400 mg/kg body weight treatment group. Arrow heads = apoptotic germ cells, ASC = absence of spermatogenic cells.

5.2.2 Transmission electron microscopy

5.2.2.1 The DBP control group

In the control birds, the architecture of seminiferous tubules was normal with spermatogonia resting on the basement membrane, other germ cells such as spermatocyte, round and elongated spermatids all appear to be normal (Figure 5.4).

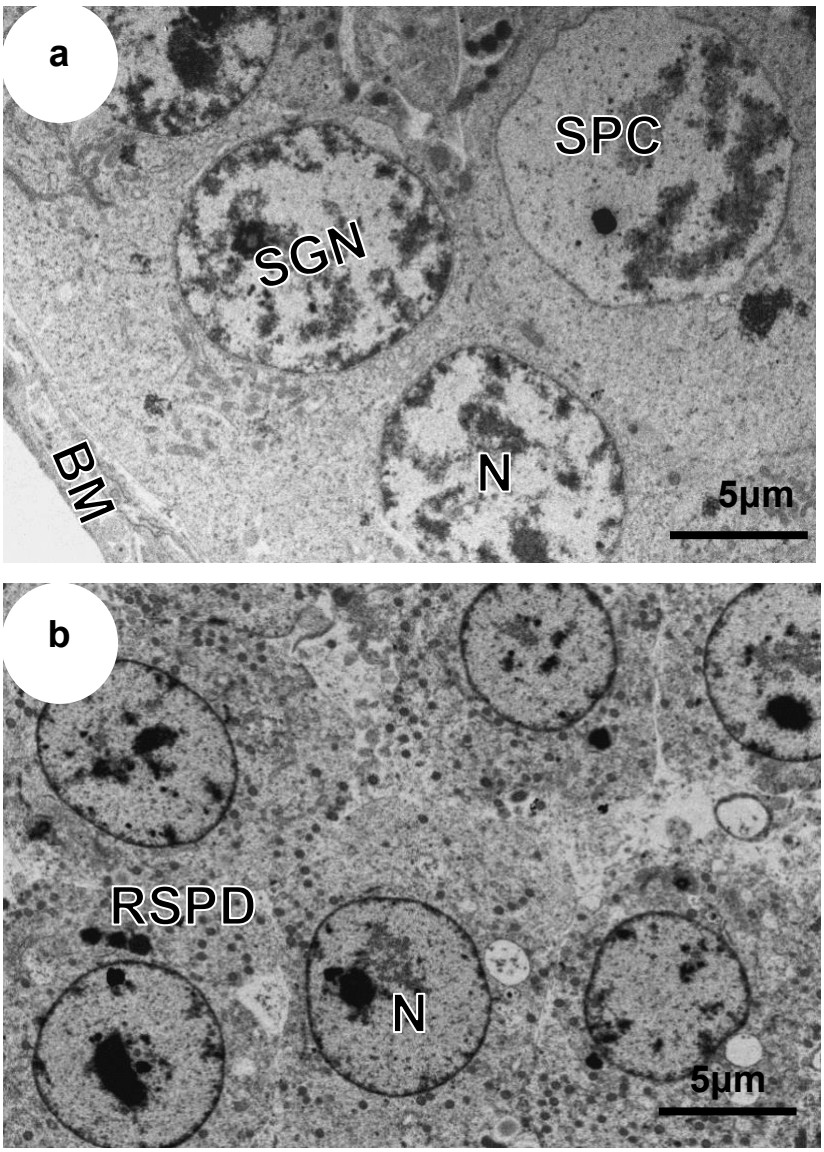

Figure 5.4. Electron micrographs of seminiferous tubules of control birds (**a, b**). SGN = spermatogonia, SPC = spermatocyte, RSPD = round spermatid, spermatid, N = nucleus, BM = basement membrane.

5.2.2.2 Low and medium dose of DBP treatment groups (10 mg/kg body weight and 50 mg/kg body weight).

The low- and medium-dose treatment groups showed some normal germ cells resting on an irregular basement membrane. Degenerative features such as irregular nuclear membranes of spermatocytes, ruptured nuclear membranes of spermatogonia, and intercellular dilations were observed (Figure 5.5).

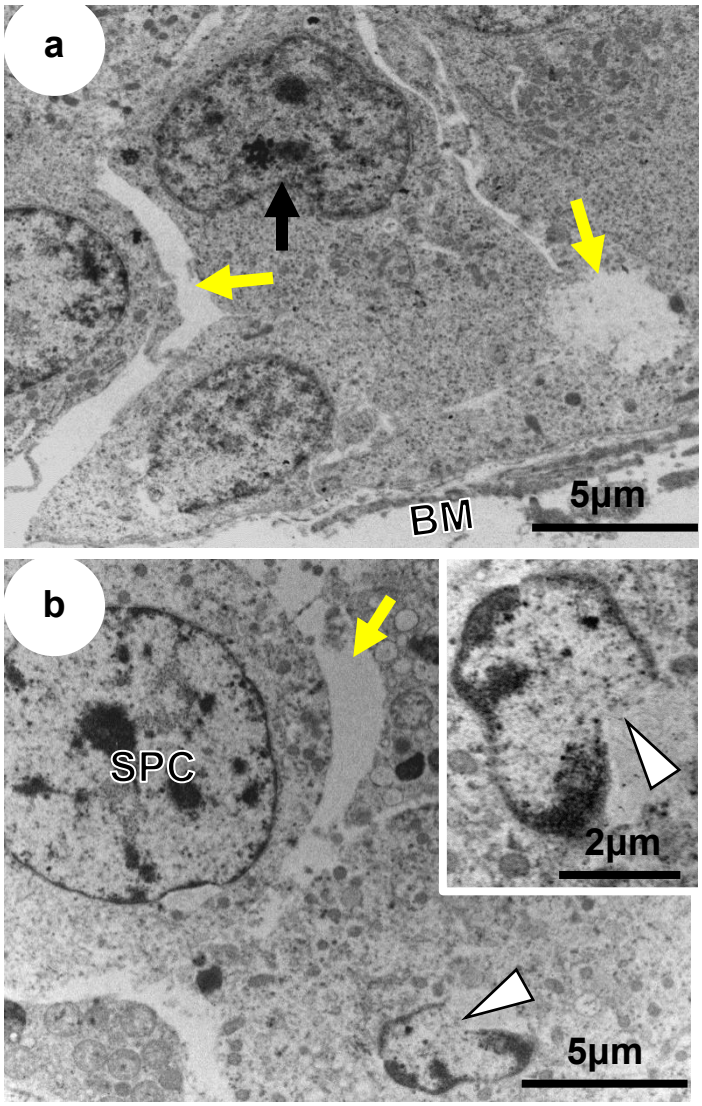

Figure 5.5. Electron micrographs of seminiferous tubules of birds exposed to DBP (**a),** 10 mg/kg body weight treated group and (**b),** 50 mg/kg body weight treated group. Black arrow = irregular nuclear membrane, yellow arrows = intercellular dilations, white arrow heads = rupture of nuclear membrane, SPC = spermatocyte, N = nucleus, BM = basement membrane.

5.2.2.3 High dose of DBP treatment groups (200 mg/kg body weight and 400 mg/kg body weight).

In the 200 mg/kg body weight group, obvious degenerative changes were observed, such as degenerated spermatogonia resting on irregular basement membranes, and chromatin clumps in spermatocytes (Figure 5.6). In the 400 mg/kg body weight of DBP, cellular degenerations were more severe, as dense apoptotic bodies and intracytoplasmic vacuoles were observed (Figure 5.7).

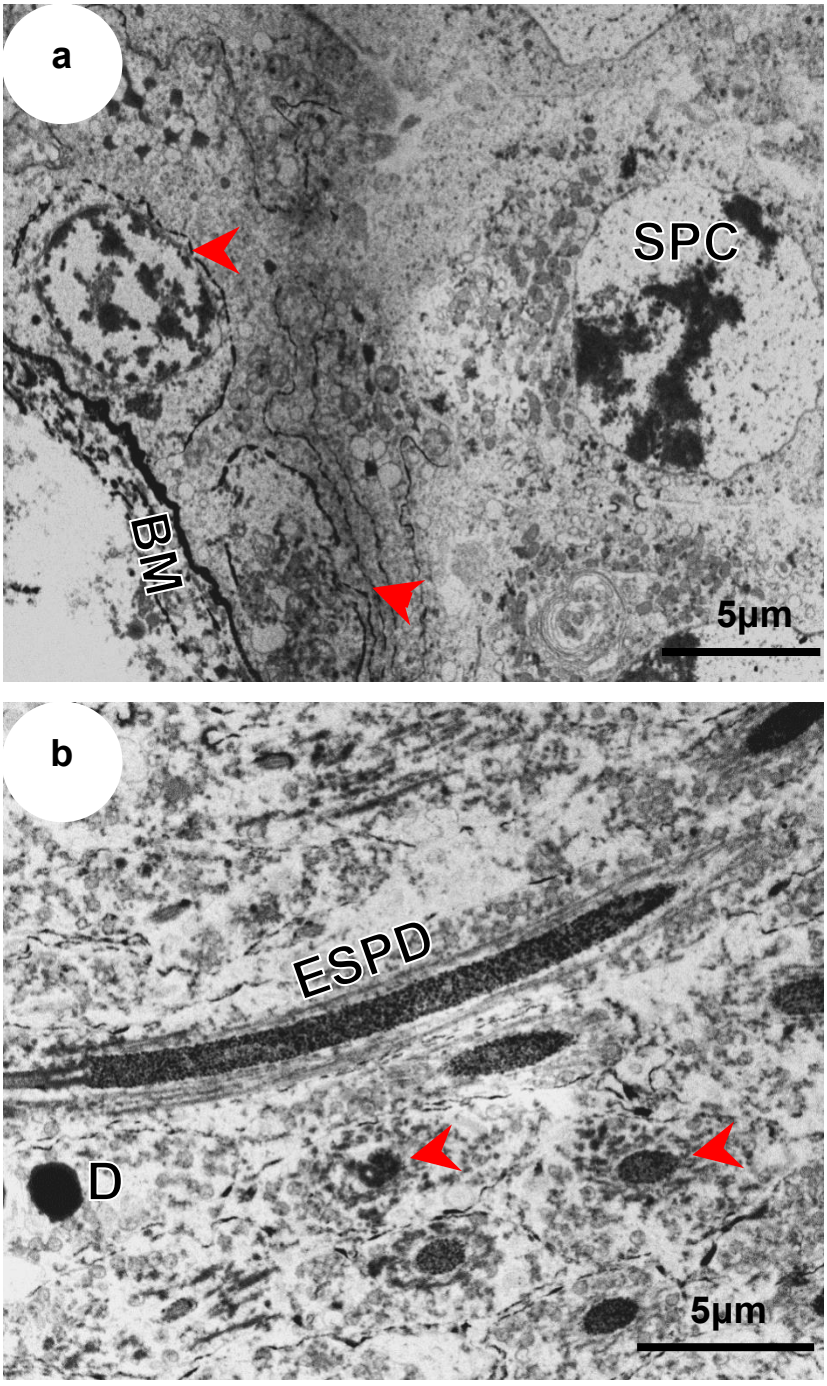

Figure 5.6. Electron micrographs of seminiferous tubules of birds exposed to DBP at 200 mg/kg body weight treated group (**a**, **b**). SPC = spermatocyte, double arrows = chromatin

clumps, ESPD = elongating spermatid, BM = basement membrane, red arrowheads = degenerated spermatogonia, D = dense body.

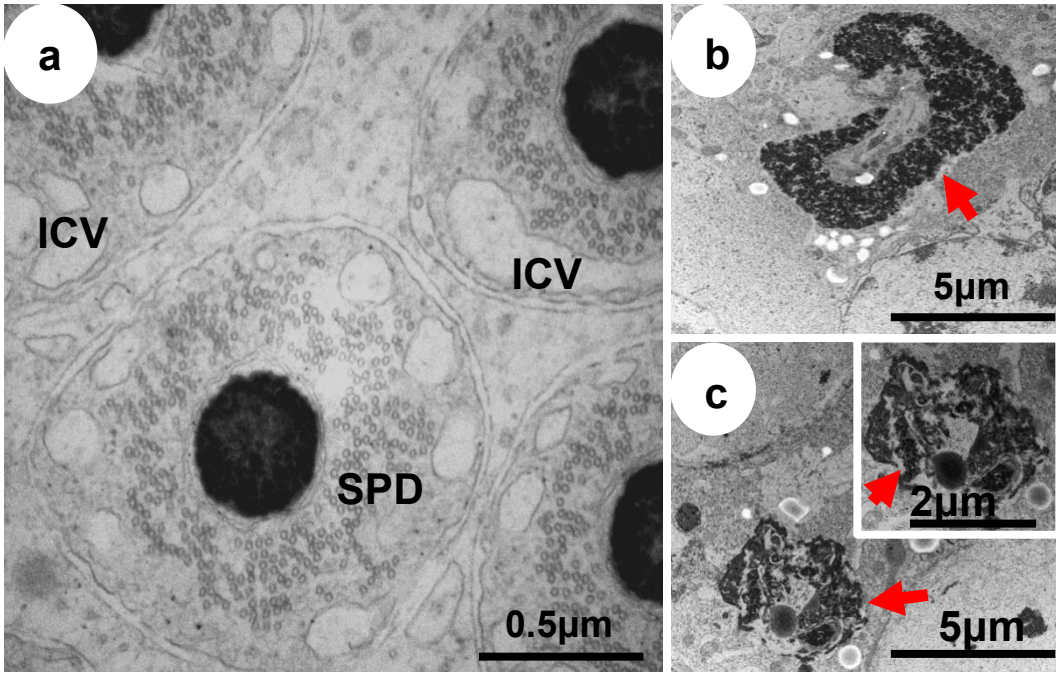

Figure 5.7. Electron micrographs of seminiferous tubules of birds exposed to DBP 400 at mg/kg body weight treated group (**a**, **b**, **c**). Red arrows = apoptotic bodies (also in inset), ICV = intra cytoplasmic vacuoles, SPD = spermatid.

5.3. Discussion

In this study, germ cells of male Japanese quails were highly susceptible to degenerative changes induced by DBP. These changes were dose-dependent and more pronounced than those reported for mammalian species (Park et al., 2002, Aly et al., 2016). There is considerable evidence that phthalates induce various reproductive effects in male

mammals, including disruption of reproductive development and spermatogenesis, and alteration of steroid hormone balance, all of which can lead to infertility (Howdeshell et al., 2008). Environmental toxicants such as DBP have been shown to cause testicular tissue injury and cell death (Arif and Khan, 2010).

In the present study, the dose-dependent histopathological changes in the seminiferous tubules of Japanese quail induced by DBP were associated with vacuole formations in the seminiferous epithelium, spermatogonia and spermatocyte degenerations, and loss of spermatogenic cells. This is in accord with the reports of Higuchi et al. (2003) in rabbits, Lee and Veeramachaneni (2005) in frogs, Bello et al. (2014) in Japanese quail and Elharoun and Bashandy (2014) in rats.

Unlike the reports of Bello et al. (2014) that focused on the effects of DBP on key enzymes that are involved in the pathways of testicular steroidogenesis, the present study focused on the effects of DBP on developing germ cells. The earlier findings of Yin et al. (2016) that DBP esters cause a reduction in germ cell numbers, which consequently creates large intervening spaces among spermatocytes in the seminiferous epithelium in rats, was also observed in the present study.

The birds exposed to low and medium doses of 10 and 50 mg/kg body weight of DBP respectively, displayed minor histopathological changes in the seminiferous tubules. Similar findings were reported by Bao et al. (2011) and Nelli and Pamanji (2017) in rats. They reported that male rats exposed to a low dose of DBP did not show obvious histopathological changes. However, in the birds on high dosage levels (200 and 400 mg/kg) in the present study, loss of germ cells, degenerative changes of spermatogonia

and spermatocytes were observed. Rats appear to be less sensitive to DBP because those administered with 250 mg/kg body weight of DBP, displayed no obvious histopathological changes (Zhou et al., 2010). However, severe alterations in the seminiferous tubules of adult rats treated with a higher dose of 500 mg/kg body weight of DBP were observed. The main histopathological changes noted were atrophy of seminiferous tubules, the disintegration of seminiferous tubule epithelial cells, and a marked decrease in spermatogenic cells. These changes are similar to those observed in the quail administered 400 mg/kg body weight of DBP in the present study.

The disparity in histopathological changes reported in the seminiferous tubule, due to similar dosage levels, in the mouse (Moody et al., 2013), rat (Zhou et al. 2010), and in the present study on the quail, could be due to species differences in DBP metabolism. Aly et al. (2016) reported atrophy of seminiferous tubules and, thus, the absence of germ cells in the testis of rats at a dose of 600 mg/kg body weight of DBP, which is similar to the observations in the present study. It is, therefore, logical to propose that the seminiferous tubule of the Japanese quail is more sensitive than the mammalian tubule to DBP administration.

Ultrastructurally, alterations in germ cells observed in the present study were also dose-dependent. In the low and medium (10 and 50 mg/kg) dose levels of DBP-treated groups, abnormalities such as condensed chromatin material, irregular nuclear membrane, rupture of the nuclear membrane, and intercellular dilations were observed. These are reminiscent of earlier findings by Yin et al. (2016) in rats, although the rats were administered a higher dose of 500 mg/kg body weight of DBP.

In the 200 mg DBP-treated group, degenerated spermatogonia resting on irregular basement membranes were observed. Similar findings have been reported by Alam et al. (2010), Aly et al. (2016), and Yin et al. (2016) in rats, albeit at a higher dose. However, a very distinct feature observed in the 400 mg/kg dosed birds was the severe structural derangement of all germ cells except the round spermatids which displayed intra-cytoplasmic vacuoles and debris of dense apoptotic bodies. This level of degeneration has not been described in mammals administered with a similar dose level of DBP. It is not known why round spermatids are relatively stable and unaffected by DBP. However, the chances of round spermatids not to be disintegrated like other germ cells at the concentration of 400 mg/kg body weight of DBP could be due to the compact nature of their nuclei – the nuclei are more compact than in higher, elongating and elongated spermatids. Aire (2018) reported that, during normal spermiogenesis in birds, round spermatids drastically reduce in size and lose 96% of their nuclear volume. This could possibly render them less susceptible to injury than other germ cells that are relatively larger in size.

The present observations indicate that low DBP dosages caused deleterious effects in the seminiferous epithelium of the quail but not in mammals. Therefore, the Japanese quail appears to be a good research model to study the effects of low doses of DBP on the seminiferous epithelium.

5.4 Ethics approval

This study was approved by the Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, the University of Pretoria, South Africa (issued vide No. AEC/A065-12).

5.5 Competing interests

The authors declare that there are no competing interests.

5.6 Acknowledgment

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CHAPTER SIX

6.0 General discussion, conclusion, and recommendations

6.1 Pro-apoptotic proteins: mechanisms of action and involvement in spermatogenic cell apoptosis.

The first objective of this study was to review some pro-apoptotic proteins and their involvement in germ cell apoptosis. Several factors have been implicated to play a very important role in the process of apoptosis. These factors are mostly pro-apoptotic proteins such as caspase-3, B-cell lymphoma 2 (Bcl-2), truncated BH3 interacting death domain (tBID), tumour suppressor protein (p53), and Bcl-2-associated X protein (BAX). The tissue in the vertebrate's body with a high incidence of apoptosis is the testis. This is where most of the germ cells produced are removed by the process of apoptosis (Shaha et al., 2010). Germ cell apoptosis is one of the most important features of spermatogenesis. It occurs mostly during spermatogonia proliferation in which up to 75% of tissue is lost, even though it can also occur during maturation divisions of spermatocytes and spermatids, but to a lesser extent (Hikim et al., 1995; Aitken et al., 2011). The role of germ cell apoptosis is made evident when pro-apoptotic genes such as BAX and tBID are deleted. Such conditions are linked to infertility due to the stoppage of spermatogenesis just before the onset of meiosis (Yamamoto et al., 2001; Russell et al., 2002). Apoptosis is one of the most studied forms of cell death, but the molecular mechanisms and physiological significance are still largely unclear.

Germ cell apoptosis is the main event in vertebrate's gonads during development. It plays a very important role in the molecular and physiological processes of both fetal and adult

testicular tissues. It is critical for normal spermatogenesis, cellular homeostasis and to maintain a balance between Sertoli cells and germ cells (Said et al., 2004). The factors responsible for the regulation of apoptosis in testicular cells are not fully understood, but it is evident that this change takes place before testosterone peak in gonadal tissue. It has also been reported that speedy apoptosis of spermatocytes might be responsible for germ cell loss in aging men (Berensztein et al., 2002; Kimura et al., 2003). Degeneration of germ cells occurs mainly during mitosis, meiosis and spermiogenesis (Nihi et al., 2017). It could be a mechanism to remove or eliminate chromosomes with abnormalities (Clermont, 1962), and reduce the number of germ cells that can be supported and nourished by Sertoli cells (Huckins, 1978). Moreover, greater degeneration of type B spermatogonia has been reported in the horse during the breeding season, after the number of type A spermatogonia is doubled (Johnson and Nguyen, 1986). Degeneration of germ cells during spermiogenesis is relatively lower than in mitosis of spermatogonia, as this was noted in horses (Johnson, 1986) and bulls (Amann, 1962).

6.2 Histomorphometry of seminiferous tubules in four reproductive phases of Japanese quail

The second objective was to evaluate seminiferous tubule parameters in four reproductive phases of Japanese quail. The significant increase in the seminiferous tubule lumen diameter observed in the aged birds in the present study could be due to high rates of germ cell apoptosis (Zakariah et al., 2020) and a fall in androgen levels (Zirkin and Chen, 2000). Germ cell deaths create intervening spaces in the seminiferous tubules, thereby leading to the larger tubular lumen.

Even though evaluation of spermatogenesis can be highly inconsistent (Briskie and Montgomerie, 2007), testicular size is often used to measure sperm production, as greater components of testicular tissue are dedicated to spermatogenesis. Generally, sperm production is proportional to testicular size as demonstrated in the zebra finch (Birkhead et al., 1993) and the house sparrow (Birkhead et al., 1994).

6.3 Germ cell apoptosis in the normal testis of Japanese quail

The third objective was to determine the rates of germ cell apoptosis in the normal testis of Japanese quail. Japanese quail have been described as a research model of avian species over the past 60 years. However, this is the first study on apoptosis of germ cells in the normal testis of this important species. It is important to generate baseline data that can be used as a comparison in further research. The study showed apoptosis of germ cells that were frequently observed in spermatogonia and in spermatocytes but to a lesser extent. Similar observations were previously reported (Allan et al., 1992, Rodriguez et al., 1997, Print and Loveland, 2000). It has been observed that excess germ cell removal in normal spermatogenesis is caused by apoptosis, as has been observed by Hikim et al. (1997) in rats, Lue et al. (1997) in hamsters, Shanna et al. (2011) in humans, and birds as shown in the present study.

Germ cell degeneration has been shown to occur in spermatogonia and spermatocytes. Hoewever, degeneration of spermatogonia seems to take the ideal form of apoptosis, such as margination of nuclear chromatin, irregular nuclear membranes, rupture of nuclear membranes, as well as phagocytosis of apoptotic bodies as observed in the present study and also reported by Allan (1992). The ultrastructural observation in the

present study revealed that spermatogonia and spermatocytes undergo degeneration more frequently and profoundly than other germ cells. The round and elongated spermatids were rarely observed undergoing degeneration in the present study. This is possibly due to the compact nature of their nucleus as also observed previously (Hasegawa et al., 1997, ITO et al., 1997, Henriksen and Parvinen, 1998).

TUNEL labeling, as carried out in the present study, is a sensitive technique that has been accepted globally for detection of apoptosis based on DNA fragmentation. All the age groups showed TUNEL-positive cell counts at various frequencies. However, unless DNA fragmentation is extensive, it will not be detected by TUNEL methods (Brum et al., 2008). Anzar et al. (2002) have shown that apoptotic germ cell numbers could be underestimated, especially in the early stage of apoptosis. In order to avoid underestimation of apoptotic germ cells, electron microscopy evaluation was employed in addition to light microscopy and TUNEL labelling. The irregular cell shapes and eosinophilic cytoplasm noted, along with condensed or fragmented nuclei were in accord with reports by Cai et al. (2000) in rats and Liu et al. (2006) in mice. In the TUNEL labelling experiments, the frequency of apoptosis of germ cells was high in aged birds. This is in agreement with the reports by Levy and Robaire (1999) in rats and Pentikäinen et al. (1999) in humans. They observed that both steroidogenesis and spermatogenesis decreased with age, apparently due to increased rates of apoptosis. These changes could be due to testicular tissue oxidative stress and a fall in androgen levels.

6.4 Alterations of spermatogenic cells in DBP-treated testis of Japanese quail.

The fourth objective was focused on the effects of DBP on the biology of developing germ cells in the testis of Japanese quail. Histopathological evaluation in the present study was observed to be associated with vacuole formations of different sizes, degeneration of germ cell apoptosis (mostly spermatocytes) and a decrease in germ cell number in the low and medium dose treated groups. These features have not been reported in mammalian species studied at the same dosage levels. In groups treated with doses of 200 and 400 mg/kg body weight, necrosis and absence of spermatogenic cell series were frequently observed.

This is in agreement with the reports of Higuchi et al. (2003) in rabbits, Lee and Veeramachaneni (2005) in frogs, Bello et al. (2014) in Japanese quail, and Elharoun and Bashandy (2014) in rats. Unlike the report of Bello et al. (2014) that focused on the effects of DBP on key enzymes that are involved in the pathways of testicular steroidogenesis, the present study targeted the effects of DBP on developing germ cells. Germ cell loss was observed in some treated groups with minor evidence of spermatogenesis. These observed features in the present study are good indicators of infertility as reported by Park et al. (2002) in Sprague–Dawley rats. The presence of vacuoles was noted in all the DBP treated groups at different frequencies. This supports the earlier findings of Yin et al. (2016) that DBP esters have been known to cause a reduction in spermatocytes, which consequently creates large intervening spaces among spermatocytes, leading to the creation of large vacuoles and lumens in the seminiferous tubules of rats.

The disparity in the doses of DBP inducing similar histopathological changes observed in the previous study by Zhou et al. (2010) in rats, Moody et al. (2013) in mice and birds in

the present study, are most likely due to species differences in DBP metabolism. Aly et al. (2016) reported necrosis of seminiferous tubules and absence of spermatogenic series in the testis of rats at the dose of 600 mg/kg body weight of DBP, which is similar to the observations in the present study but at the dose of 400 mg/kg body weight of DBP. Therefore, it is logical to propose that DBP induces more testicular lesions in the seminiferous tubules of birds than in mammalian species.

In the electron microscopy evaluation, alterations of germ cells observed in the present study were also dose dependent. In the low and medium (10 and 50) mg/kg body weight of DBP treated groups respectively, abnormalities such as dense apoptotic bodies, swollen mitochondria, rupture of the nuclear membrane, and spermatocytes with a karyopyknotic nucleus and chromatin clumps were observed. This is in agreement with earlier findings by Yin et al. (2016) in rats but at a much higher dose of 500 mg/kg body weight of DBP.

Distorted basement membranes and germ cells with chromatin clumps were also observed in the medium dose (50 mg/kg body weight) treated group. Similar observations in the components of basal lamina have been reported by De Kretser et al. (1975) in men with untreated hypothyroidism and Veeramachaneni et al. (1987) in bulls with hypoplastic testes. These alterations may well have been caused by DBP in the seminiferous tubules of Japanese quail in the present study, as they were absent in the control group. Unlike in the present study, the toxic effects of DBP at low doses were unable to cause significant germ cell alterations in mammalian species. Therefore, Japanese quail might be a good research model to study the low dose effects of DBP on spermatogenic cell alterations.

In the 200 mg/kg body weight of DBP treated group, degenerated spermatogonia resting on a degenerated basement membrane, spermatocytes with chromatin clumps, as well as degenerated round and elongating spermatids were observed. Similar findings were previously reported by Richburg and Boekelheide (1996), Aly et al. (2016) and Yin et al. (2016) in rats but also at higher doses of DBP. However, very distinct features were observed in the present study at the dose of 400 mg/kg body weight of DBP, as all germ cells were degenerated beyond recognizable features, except round spermatids at various degrees of degeneration. This could be due to the compact nature of their nuclei. Aire (2018) reported that during normal spermiogenesis in birds, round spermatids drastically reduce in size and lose 96% of their nuclear volume. This could render them less susceptible to injury than other germ cells that are relatively larger.

In some seminiferous tubules, the degenerative changes were more severe, as there was extensive desquamation of germ cells leading to the creation of empty spaces. Gray and Beamand (1984) reported similar observations in Sertoli-germ cell co-cultures treated with phthalic acid esters. They found out that persistent vacuolations might have resulted from denudation and death of differentiating germ cells. They further revealed by electron microscopic evaluations, just as in the present study, that apoptotic changes, disruption, necrosis, and eventual desquamation of germ cells were caused by phthalate esters.

It has been known for decades that DBP is a ubiquitous pollutant in the environment, although its exact mechanism of action is still poorly understood. The data in the present study show that a low dose of DBP can disrupt the proliferation of spermatogenic cells in Japanese quail. This suggests that avian species are highly susceptible and sensitive to

DBP compared to mammalian species studied. The dose-dependent effects observed from 10 to 400 mg/kg body weight of DBP, are lower than for previously reported data in other species. Therefore, Japanese quail should be considered as an ideal research model for testing low dose effects of endocrine-disrupting chemicals such as DBP.

Limitations of the study

Even though the effects of DBP (graded concentrations) on germ cells were observed, we were not able to carry out the expression of pro-apoptotic proteins as illustrated in Chapter 2 (the review chapter). This is largely due to the COVID-19 pandemic which led to the closure of most companies supplying these pro-apoptotic proteins.

6.5 Conclusions

The current study provides an overview of some pro-apoptotic proteins and their involvement in germ cell apoptosis. Evaluation of seminiferous tubule parameters in four reproductive phases of Japanese quail was also carried out, where tubular lumen diameter increased with age. In the normal testis, apoptosis of germ cells was observed in all age groups (pre-pubertal, pubertal, adult and aged) of Japanese quail for the first time. The histopathological evaluation in DBP treated birds showed abnormalities associated with vacuole formation, germ cell apoptosis and decrease in germ cell number in the 10 and 50 mg/kg body weight treated birds. These features have not been reported in mammalian species at the same dosage level. In the transmission electron microscopy evaluation, spermatocytes with a karyopyknotic nucleus, ruptured nuclear membrane and chromatin clumps were observed in the low and medium dose DBP treated groups. These features have not been detected in mammalian species at the same dose exposure.

However, very distinct features were observed in the present study at a dose of 400 mg/kg body weight of DBP. The spermatogenic cells were degenerated beyond recognizable features, aside from round spermatids undergoing various degrees of degeneration with inter and intra-cellular vacuoles of various sizes. The findings of the present study revealed that spermatogenic cells of Japanese quail seem to be more sensitive to DBP-induced degenerations compared to mammalian species studied to date.

6.7 Recommendations for further research

- Techniques for detecting apoptotic round and elongating spermatids should be explored and developed as these are difficult to identify due to the compact nature of their nuclei.
- There is a need for further study on the effects of endocrine-disrupting phthalates such as DBP on avian spermatogenesis, as birds may be more susceptible than mammalian species.
- Pro-apoptotic protein expression should be studied in both normal and induced germ cell apoptosis in the testis of birds.
- DBP low dose exposure research in avian species is grossly lacking, therefore there is a need for further investigation in this area.
- More approaches of determining long term *in ovo* exposure to EDCs in avian species need to be evaluated.
- Other techniques of screening EDCs from biological tissue such as high-performance liquid chromatography (HPLC) should be explored in different avian species.

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APPENDIX 1. Ethical approval certificates

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
ANIMAL USE AND CARE COMMITTEE
Private Bag X04
0110 Onderstepoort

Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aucc@up.ac.za

Ref: V058-12 (With Amendment 1)

25 September 2012

Prof MC Madekurozwa
Department of Anatomy and Physiology
Faculty of Veterinary Science
(mary.madekurozwa@up.ac.za)

Dear Prof Madekurozwa

V058-12 : The effects of di(n-butyl) phthalate (DBP) on the reproductive function of adult male Japanese quails *(Coturnix coturnix japonica) (UM Bello)*

The application dated 29 August 2012 and the amendment dated 13 August 2012 was ethically approved by the Animal Use and Committee at its meeting held on 17 September 2012.

Kind regards

E Mostert

Elmarie Mostert

AUCC Coordinator

Copy Dr UM Bello

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**The effects of di(n-butyl) phthalate (DBP) on the reproductive function of adult male Japanese quails *(Coturnix coturnix japonica)***
PROJECT NUMBER	**V058-12 (Amend 2)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr UM Bello**

STUDENT NUMBER (where applicable)	**UP_10252445**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Japanese quail**	
NUMBER OF ANIMALS	**To be reported**	
Approval period to use animals for research/testing purposes		**November 2015-Novemer 2016**
SUPERVISOR	**Prof. H Groenewald**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	23 November 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

5 March 2020

Approval Certificate
Annual Renewal (Ext 1)

AEC Reference No.:	V034-18
Title:	Changes in the testicular capsule and peritubular boundary tissue in prepubertal, pubertal and adult Japanese quail (Coturnix coturnix Japonica)
Researcher:	Mr LI Khumalo
Student's Supervisor:	Prof MN Madekurozwa

Dear Mr LI Khumalo,
The **Annual Renewal** as supported by documents received between 2019-06-07 and 2020-02-24 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-02-24.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number Available
Japenese quail (*Coturnix coturnix Japonica*)	21

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-03-05.
3. Please remember to use your protocol number (V034-18) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa