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

Musa Zakariah[a,b *], Mohammed I. A. Ibrahim[a,c], Reneilwe A. Molele[a], Lyndy J. McGaw[d]

[a]*Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, Pretoria 0110, South Africa.*

[b]*Department of Veterinary Anatomy, Faculty of Veterinary Medicine, PMB 1069 University of Maiduguri, Maiduguri, Nigeria.*

[c]*Department of Basic Science, University of West Kordofan, West Kordofan State, Sudan.*

[d]*Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 Pretoria, South Africa.*

[*]Corresponding author. Tel.: +2773015515; +2347036155225.

Email address: mzakariah@unimaid.edu.ng (M. Zakariah)

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, hematoxylin, and eosin, TEM, transmission electron microscope.

Highlights

- Apoptosis of germ cells in the normal testis of Japanese quails was observed for the first time.
- High frequency of apoptosis of germ cells in pre-pubertal and aged birds were detected.
- Electron microscopy is one of the most sensitive techniques in confirming the TUNEL essay,
- An imbalance between degeneration and regeneration of germ cells can lead to infertility.

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 quails, as shown by hematoxylin & 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 quails; TUNEL

1. 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 programed 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, Sharma et al., 2014, Tapia and Pena, 2009, Dadhich et al., 2010, Massoud et al., 2018, Shaha, 2008, Jiménez et al., 2015), 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 favors ease of housing and reduces the cost of feeding, and they also have a short life cycle. They adapt favorably to laboratory manipulations, and their physiological maturing and aging processes are fast due to their short life span.

2. Materials and methods

2.1. Animals used for the study

A total of 28 birds were used for the study, comprising 7 birds in each of the 4 reproductive cycles, namely pre-pubertal (4 weeks old), pubertal (6 weeks old), adult (12 weeks old), and aged (52 weeks old) according to previously classified reproductive cycles (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).

2.2. 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 hematoxylin and eosin (H&E) for light microscopy.

2.3. 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.

2.4. 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 cycles (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).

2.5. 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).

2.6 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.

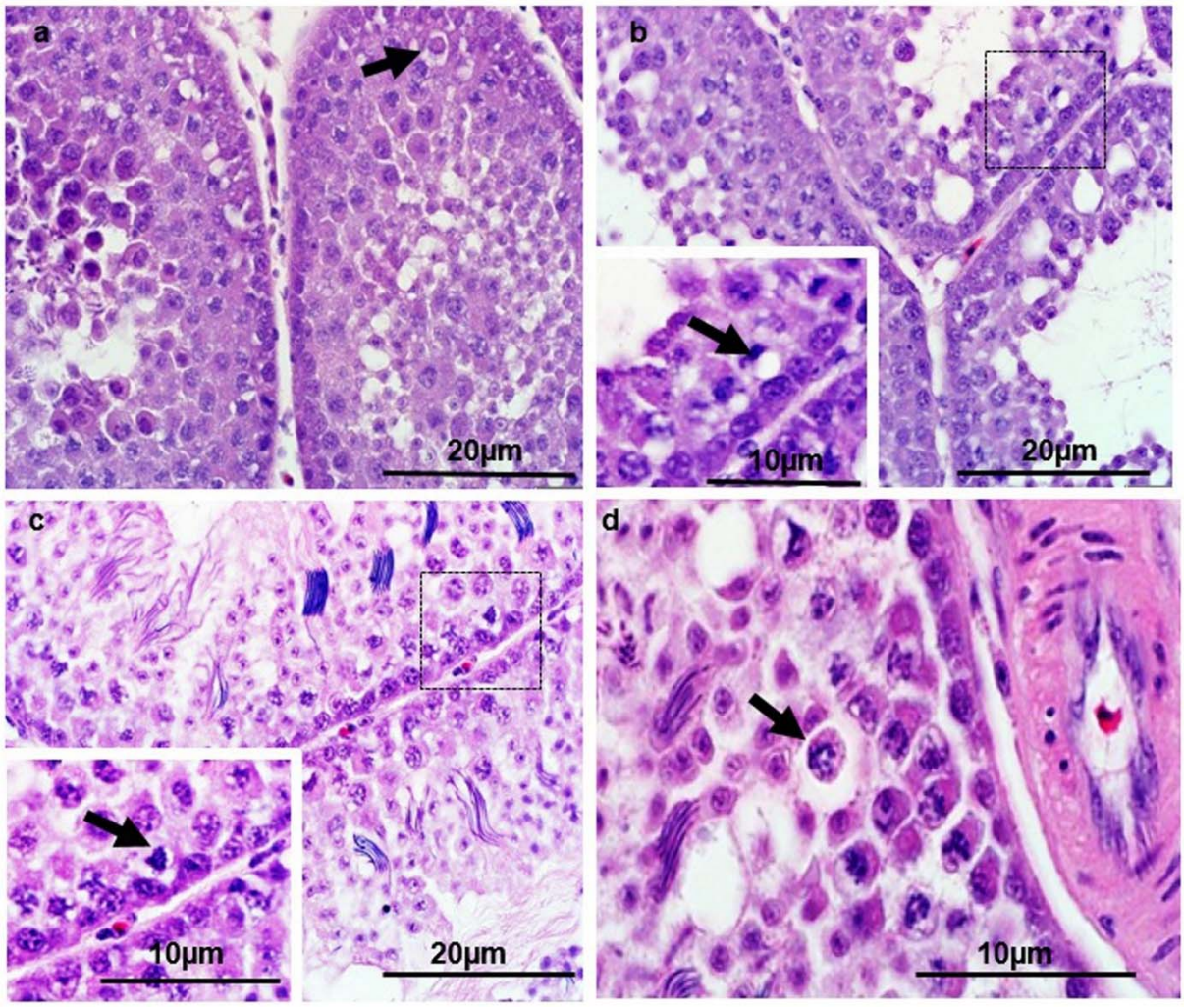

Fig. 1. Transverse sections of seminiferous tubules of the Japanese quail (a) pre-pubertal, (b) pubertal, (c) adult, and (d) aged. Arrows showing apoptotic germ cells.

3. Results

3.1 Hematoxylin & 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 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 2).

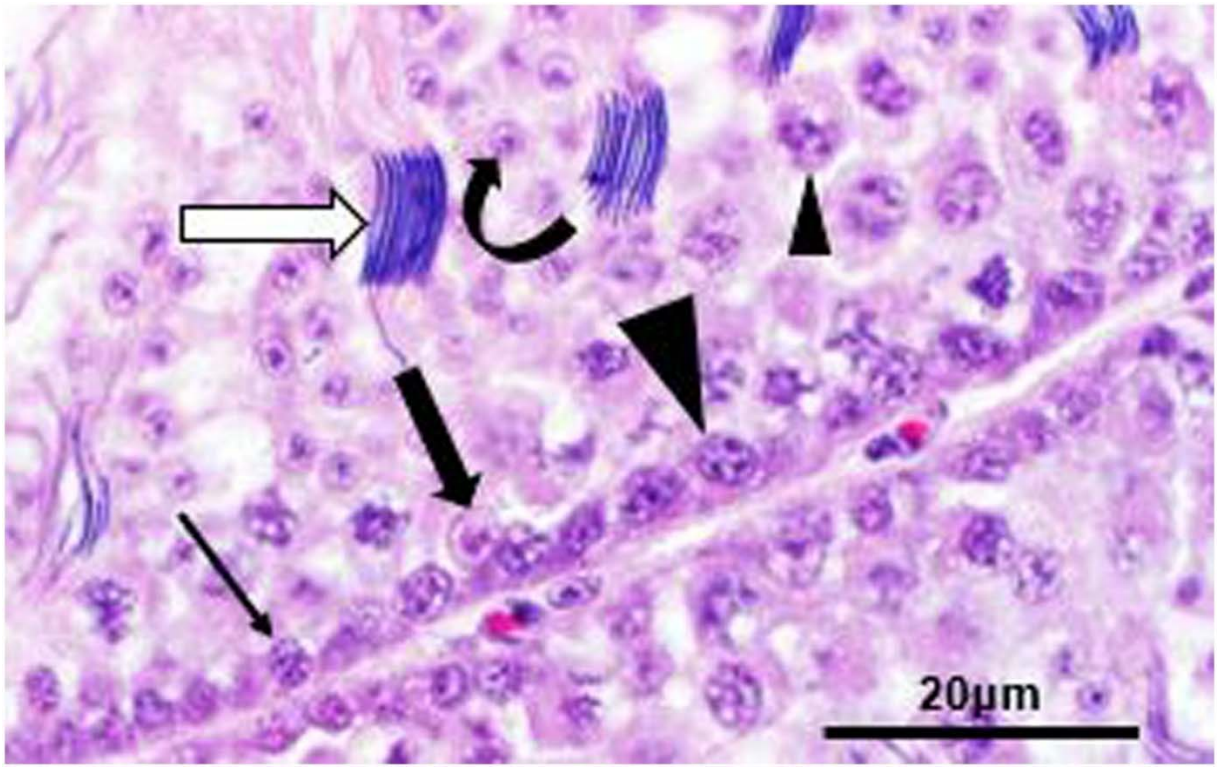

Fig. 2. Transverse sections of seminiferous tubules of an adult Japanese quail. Thin black arrow = dark spermatogonia, thick black arrow = pale spermatogonia, thick arrowhead = type B spermatogonia, thin arrowhead = spermatocyte, curve arrow = round spermatid, white arrow = elongating spermatids.

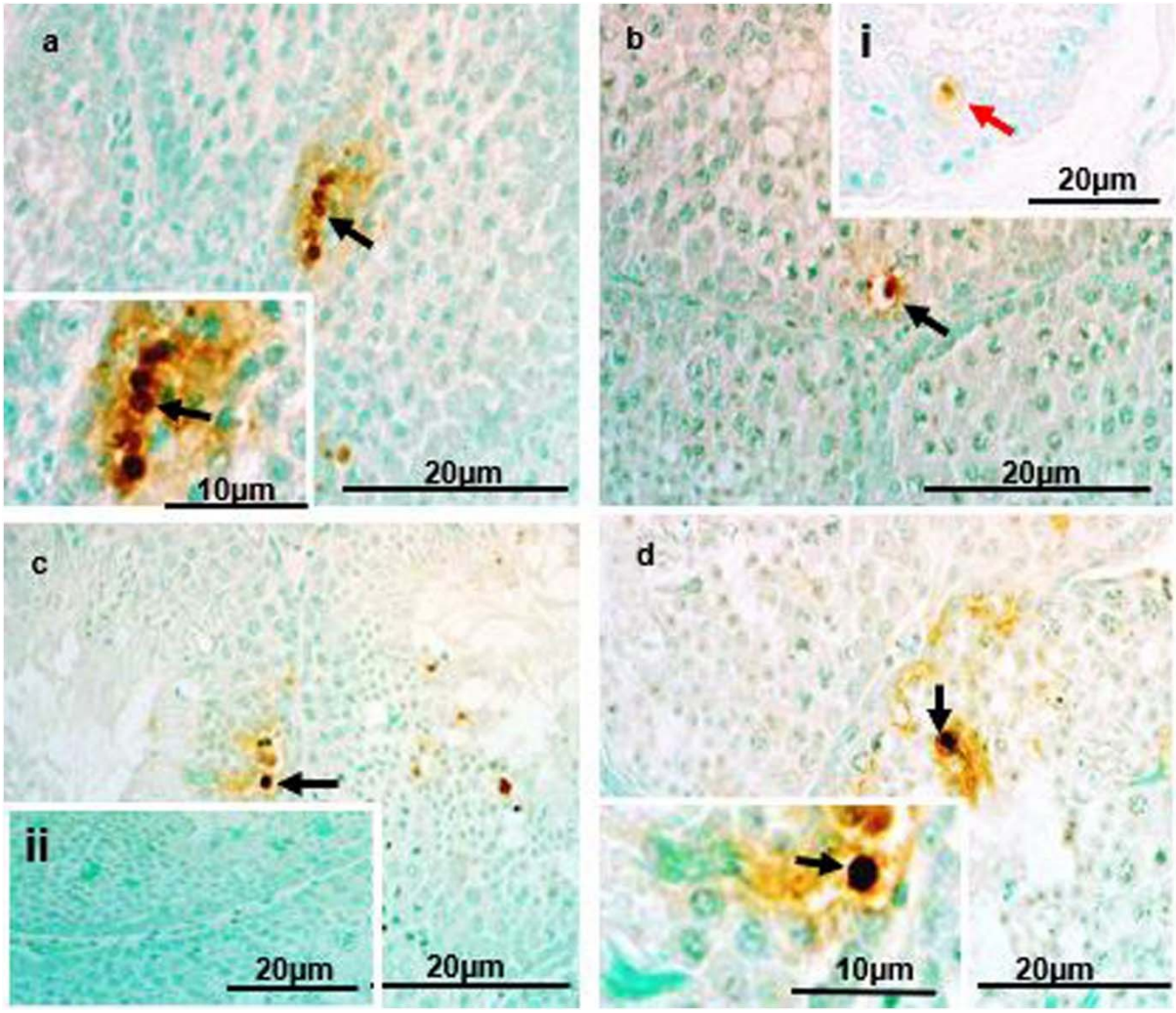

Fig. 3. TUNEL labelling of germ cells of Japanese quails of varying ages; **a** = pre-pubertal, **b** = pubertal, **c** = adult, **d** = aged, **i** = positive control from the tissue of mammary gland, and **ii** = negative control. Black arrows = TUNEL-positive cells from all the age groups; red arrow = TUNEL-positive cell from the positive control (mammary gland tissue).

3.2 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 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 8).

3.3. Transmission electron microscopy

3.3.1. 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).

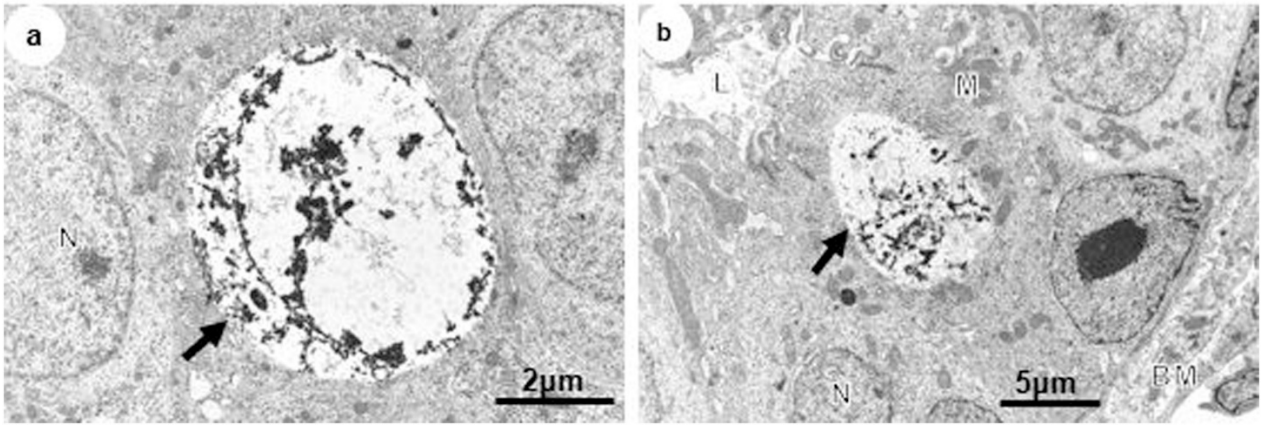

Fig. 4. Electron micrographs of seminiferous tubules of pre-pubertal birds. **(a, b)**, arrows = apoptotic bodies. BM = basement membrane; N = nucleus; L = lumen.

3.3.2. Pubertal birds

In the pubertal birds, the main finding is 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 5).

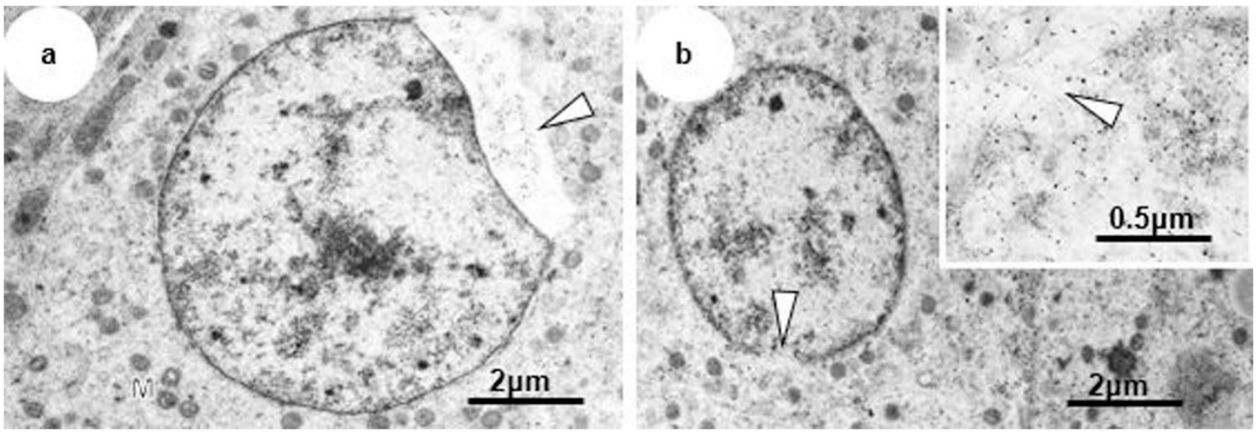

Fig. 5. Electron micrographs of seminiferous tubules of pubertal birds. **(a, b)**, white arrowheads = disruption of nuclear membrane (also in inset). M = mitochondria.

3.3.3. 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 6).

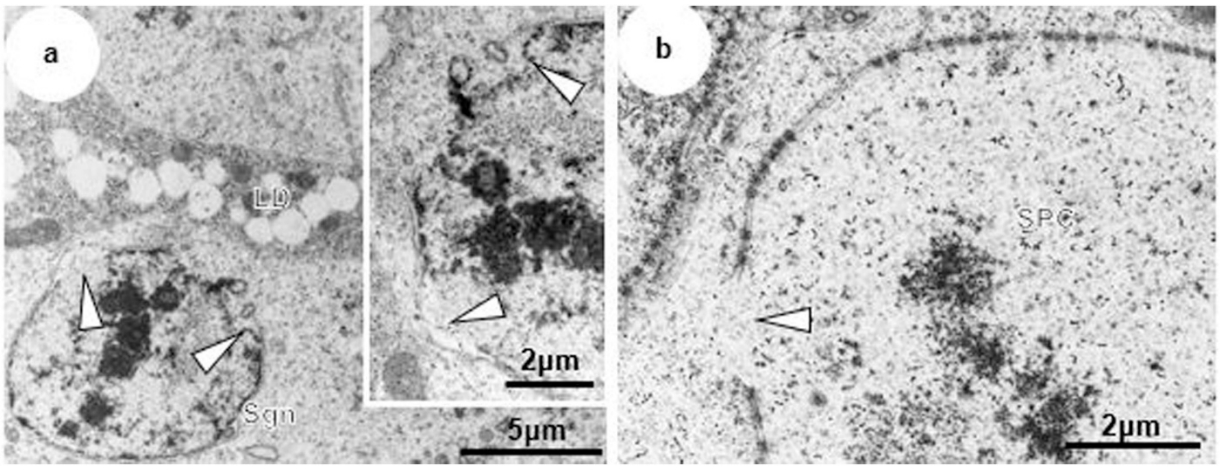

Fig. 6. Electron micrographs of seminiferous tubules of adult birds. **(a, b),** white arrowheads = rupture of nuclear membranes (also in inset). Sgn = spermatogonia; SPC = spermatocyte; LD = lipid droplets.

3.3.4. 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 7a). Some germ cells with intact nuclear membranes, however, displayed dilated intercellular spaces (Figure 7b). Rupture of the nuclear membrane was also observed, as in the other age groups (Figure 7c, d).

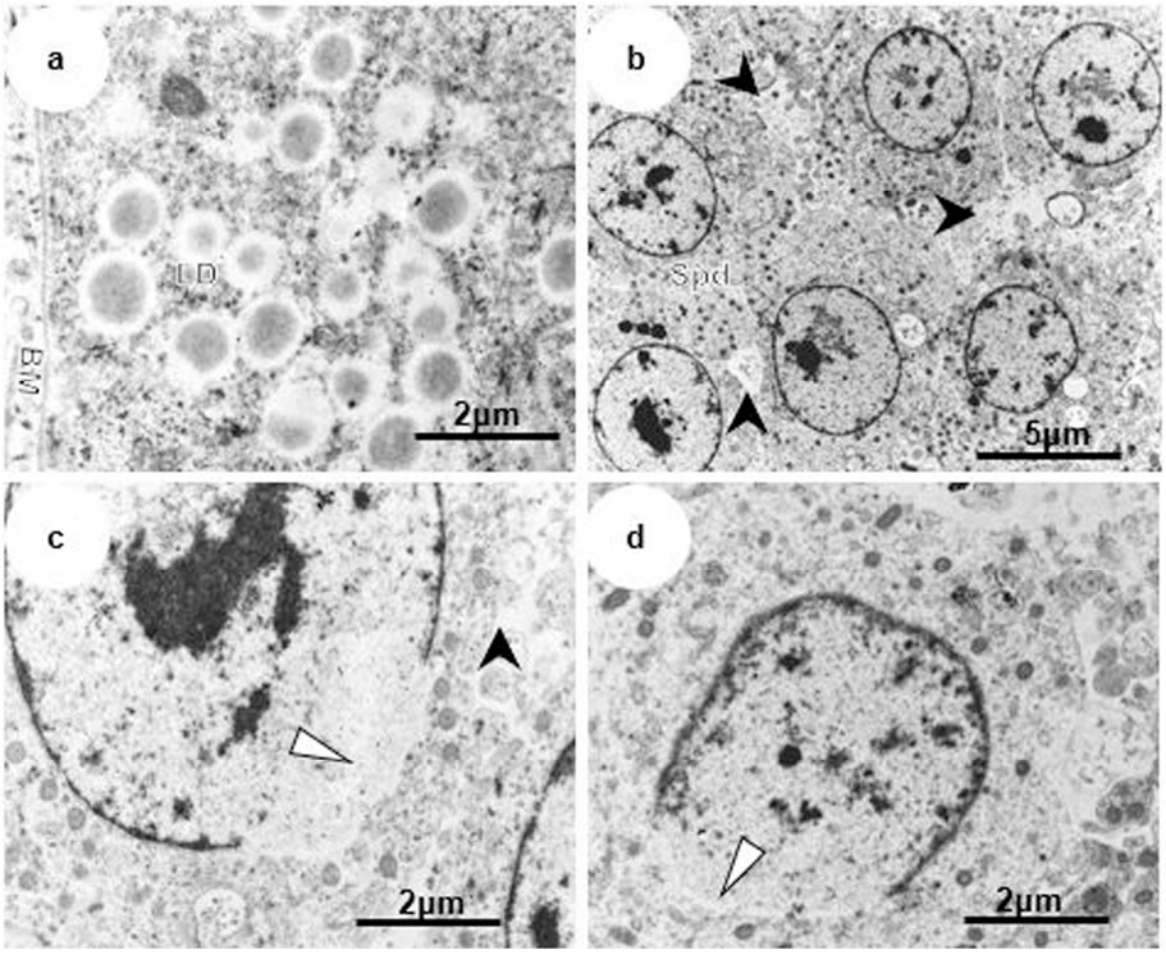

Fig. 7. Electron micrographs of seminiferous tubules of aged birds. **(a),** BM = basement membrane; LD = lipid droplets. **(b),** Spd = round spermatids, black arrowheads = dilation of intercellular spaces. **(c and d),** white arrowheads = rupture of nuclear membrane (also in inset), black arrowhead = dilation of intercellular space.

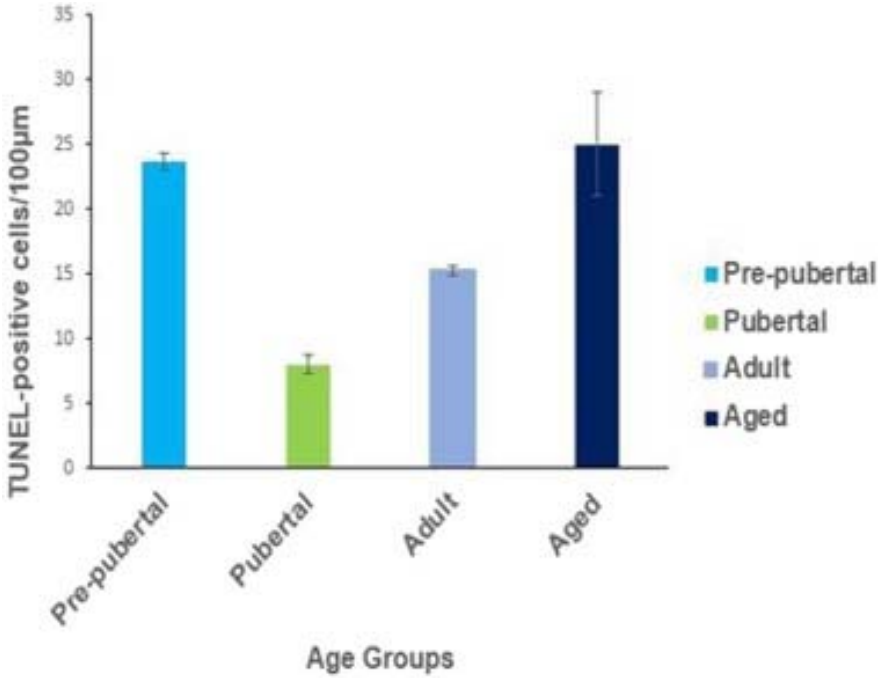

Fig. 8. Bar chart showing the frequency of TUNEL-positive cells between the different age groups. TUNEL-positive cell counts were significantly higher in pre-pubertal and aged than other age groups ($P < 0.05$).

4. Discussion

To our knowledge, this is the first study on apoptosis of germ cells in the normal testis of Japanese quails 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 markers of apoptosis found in matured 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 (Grunewald et al., 2005, Sakkas et al., 2004). 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.

5. 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 seems to correspond to that of mammalian species that have been widely studied. The germ cell degenerations revealed by hematoxylin 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.

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).

Competing interests

The authors declare that there are no competing interests.

Acknowledgment

The authors are grateful to Lizette du Plessis and Antoinette Lensink of the Electron Microscopy Unit, Department of Anatomy and Physiology, University of Pretoria for their technical support. The National Research Foundation, South Africa funded this study.

References

Aire, T.A. 2007. 'Spermatogenesis and Testicular Cycles'. In: Jamieson BGM. (ed.), Reproductive Biology and Phylogeny of Birds, Vol. 1, Phylogeny, morphology, hormones, and fertilization. New Hampshire 03479, USA; Plymouth, UK: Science Publishers, Inc., 279-348.

Aire, T. A., Du Plessis, L., Rennie, E., Gupta, S. K. & Deokar, M. 2019. Spermatid differentiation, with particular reference to acrosomogenesis, in the passeridan bird, Carib grackle (*Quiscalus lugubris*). Tissue and Cell, 61**,** 8-20.

Aitken, R. J., Findlay, J. K., Hutt, K. J. & Kerr, J. B. 2011. Apoptosis in the germ line. Reproduction, 141**,** 139.

Allan, D., Harmon, B. & Roberts, S. A. 1992. Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. Cell Proliferation, 25**,** 241-250.

Ansari, B., Coates, P., Greenstein, B. & Hall, P. 1993. In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. The Journal of Pathology, 170**,** 1-8.

Anzar, M., He, L., Buhr, M. M., Kroetsch, T. G. & Pauls, K. P. 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biology of Reproduction, 66**,** 354-360.

Aoki, K., Satoi, S., Harada, S., Uchida, S., Iwasa, Y. & Ikenouchi, J. 2020. Coordinated changes in cell membrane and cytoplasm during maturation of apoptotic bleb. Molecular Biology of the Cell, 31**,** 833-844.

Berensztein, E. B., Sciara, M. I., Rivarola, M. A. & Belgorosky, A. 2002. Apoptosis and proliferation of human testicular somatic and germ cells during prepuberty: high rate of testicular growth in newborns mediated by decreased apoptosis. The Journal of Clinical Endocrinology & Metabolism, 87**,** 5113-5118.

Billig, H., Furuta, I., Rivier, C., Tapanainen, J., Parvinen, M. & Hsueh, A. 1995. Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. Endocrinology, 136**,** 5-12.

Borges, H. L., Linden, R. & Wang, J. Y. 2008. DNA damage-induced cell death: lessons from the central nervous system. Cell Research, 18**,** 17-26.

Brum, A., Sabeur, K. & Ball, B. 2008. Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. Theriogenology, 69**,** 1041-1055.

Cai, L., Chen, S., Evans, T., Deng, D. X., Mukherjee, K. & Chakrabarti, S. 2000. Apoptotic germ-cell death and testicular damage in experimental diabetes: prevention by endothelin antagonism. Urological Research, 28**,** 342-347.

Dadhich, R. K., Real, F. M., Zurita, F., Barrionuevo, F. J., Burgos, M. & Jiménez, R. 2010. Role of apoptosis and cell proliferation in the testicular dynamics of seasonal breeding mammals: a study in the Iberian mole, Talpa occidentalis. Biology of Reproduction, 83**,** 83-91.

Dutta, D., Park, I. & Mills, N. C. 2012. Fixation temperature affects DNA integrity in the testis as measured by the TUNEL assay. Toxicologic Pathology, 40**,** 667-674.

Ehmcke, J. & Schlatt, S. 2006. A revised model for spermatogonial expansion in man: lessons from non-human primates. Reproduction, 132**,** 673-680.

Bailly, A. P. & Gartner, A. 2013. Germ cell apoptosis and DNA damage responses. Advances in Experimental Medicine and Biology, 757**,** 249-76.

Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. The Journal of Cell Biology, 119**,** 493-501.

Griswold, M. D. 1998. The central role of Sertoli cells in spermatogenesis. Seminars in Cell & Developmental Biology. Elsevier, 9, 411-416.

Grunewald, S., Paasch, U., Said, T. M., Sharma, R. K., Glander, H.-J. & Agarwal, A. 2005. Caspase activation in human spermatozoa in response to physiological and pathological stimuli. Fertility and Sterility, 83**,** 1106-1112.

Heninger, N. L., Staub, C., Blanchard, T. L., Johnson, L., Varner, D. D. & Forrest, D. W. 2004. Germ cell apoptosis in the testes of normal stallions. Theriogenology, 62**,** 283-297.

Hikim, A. S. & Swerdloff, R. S. 1999. Hormonal and genetic control of germ cell apoptosis in the testis. Reviews of Reproduction, 4**,** 38-47.

Huss, D., Poynter, G. & Lansford, R. 2008. Japanese quail (*Coturnix japonica*) as a laboratory animal model. Lab Animal, 37**,** 513-519.

Jara, M., Carballada, R. & Esponda, P. 2004. Age-induced apoptosis in the male genital tract of the mouse. Reproduction, 127**,** 359-366.

Jiménez, R., Burgos, M. & Barrionuevo, F. J. 2015. Circannual testis changes in seasonally breeding mammals. Sexual Development, 9**,** 205-215.

Johnson, L., Thompson Jr, D. L. & Varner, D. D. 2008. Role of Sertoli cell number and function on regulation of spermatogenesis. Animal Reproduction Science, 105**,** 23-51.

Kerr, J., Maddocks, S. & Sharpe, R. 1992. Testosterone and FSH have independent, synergistic and stage-dependent effects upon spermatogenesis in the rat testis. Cell and Tissue Research, 268**,** 179-189.

Levy, S. & Robaire, B. 1999. Segment-specific changes with age in the expression of junctional proteins and the permeability of the blood-epididymis barrier in rats. Biology of Reproduction, 60**,** 1392-1401.

Liu, G., Gong, P., Zhao, H., Wang, Z., Gong, S. & Cai, L. 2006. Effect of low-level radiation on the death of male germ cells. Radiation Research, 165**,** 379-389.

Loo D. T. 2011. In situ detection of apoptosis by the TUNEL assay: an overview of techniques. Methods of Molecular Biology, 682, 3-13.

Lue, Y., Hikim, A. P. S., Wang, C., Bonavera, J. J., Baravarian, S., Leung, A. & Swerdloff, R. S. 1997. Early effects of vasectomy on testicular structure and on germ cell and macrophage apoptosis in the hamster. Journal of Andrology, 18**,** 166-173.

Massoud, D., Lao-Pérez, M., Hurtado, A., Abdo, W., Palomino-Morales, R., Carmona, F. D., Burgos, M., Jiménez, R. & Barrionuevo, F. J. 2018. Germ cell desquamation-based testis regression in a seasonal breeder, the Egyptian long-eared hedgehog, Hemiechinus auritus. PloS One, 13**,** e0204851.

Madekurozwa, M. C. & Mpango, M. M. 2020. The shell gland in laying and natural moulting commercial egg-type chickens: A histomorphological and ultrastructural study. Anatomia, Histologia, Embryologia, 49, 521-531.

Nihi, F., Gomes, M. L. M., Carvalho, F. A. R., Reis, A. B., Martello, R., Melo, R. C. N., Almeida, F. & Chiarini-Garcia, H. 2017. Revisiting the human seminiferous epithelium cycle. Human Reproduction, 32**,** 1170-1182.

Oatley, J. M. & Brinster, R. L. 2008. Regulation of spermatogonial stem cell self-renewal in mammals. Annual Review of Cell and Developmental Biology, 24**,** 263-286.

Orita, K. N., Junzo Sasaki, Shigeto Kanda, Nobuhiko Kimura, Shigenobu Nomiya, Koji Yuen, Yu Masuda, Yorihisa. 1999. Does TUNEL staining during peri-and post-natal development of the mouse inner ear indicate apoptosis? Acta Oto-Laryngologica, 119**,** 22-26.

Ottinger, M. A., Abdelnabi, M., Li, Q., Chen, K., Thompson, N., Harada, N., VigliettI-Panzica, C. & Panzica, G. C. 2004. The Japanese quail: a model for studying reproductive aging of hypothalamic systems. Experimental Gerontology, 39**,** 1679-1693.

Palacios, N. M., Escobar, M. E. A., Mendoza, M. M., Crispín, R. H., Andrade, O. G., Meléndez, J. H. & Martínez, A. A. 2016. Prepubertal male rats with high rates of germ-cell apoptosis present exacerbated rates of germ-cell apoptosis after serotonin depletion. Reproduction, Fertility and Development, 28**,** 806-814.

Pentikälnen, V., Erkkilä, K. & Dunkel, L. 1999. Fas regulates germ cell apoptosis in the human testis in vitro. American Journal of Physiology-Endocrinology and Metabolism, 276**,** 310-316.

Print, C. G. & Loveland, K. L. 2000. Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays, 22**,** 423-430.

Richburg, J. H. 2000. The relevance of spontaneous-and chemically-induced alterations in testicular germ cell apoptosis to toxicology. Toxicology Letters, 112**,** 79-86.

Royere, D., Guérif, F., Laurent-Cadoret, V. & De Reviers, M. Therese. H. 2004. Apoptosis in testicular germ cells. International Congress Series. Elsevier,1266, 170-176.

Sakkas, D., Seli, E., Manicardi, G. C., Nijs, M., Ombelet, W. & Bizzaro, D. 2004. The presence of abnormal spermatozoa in the ejaculate: did apoptosis fail? Human Fertility, 7**,** 99-103.

Saraste, A. & Pulkki, K. 2000. Morphologic and biochemical hallmarks of apoptosis. Cardiovascular Research, 45**,** 528-537.

Shaha, C. 2008. Germ cell apoptosis: relevance to infertility and contraception. Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Immunology, Endocrine and Metabolic Agents), 8**,** 66-78.

Shanna, R., Chauhan, P. & Fulia, A. 2011. Vitamin E: an antioxidant therapy to protect endosulphan induced follicular toxicity. International Journal of Pharmacology, 7**,** 821-828.

Sharma, R., Bhat, R., Goyal, A. & Bhardwaj, J. 2014. Germ cells apoptosis during spermatogenesis in mammals. International Journal of Pharmacy and Pharmaceutical Sciences, 38, 68-9.

Sinha Hikim, A. P., Rajavashisth, T. B., Hikim, I. S., Lue, Y., Bonavera, J. J., Leung, A., Wang, C. & Swerdloff, R. S. 1997. Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. Biology of Reproduction, 57**,** 1193-1201.

Soldani, C. & Scovassi, A. 2002. Poly (ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis, 7**,** 321-328.

Tapia, J. & Pena, F. 2009. Apoptotic Events in Male Germ Cells and in Mature Mammalian Spermatozoa. In: Salido, G. M. & Rosado, J. A. (eds.) Apoptosis: Involvement of Oxidative Stress and Intracellular Ca2+ Homeostasi. Dordrecht: Springer Netherlands, 8, 165-166.

Tornusciolo, D., Schmidt, R. E. & Roth, K. A. 1995. Simultaneous detection of TDT-mediated dUTP-biotin nick end-labeling (TUNEL)-positive cells and multiple immunohistochemical markers in single tissue sections. Biotechniques, 19**,** 800-805.

Zirkin, B. R. & Chen, H. 2000. Regulation of Leydig cell steroidogenic function during aging. Biology of Reproduction, 63**,** 977-981.