TIME-COURSE EFFECTS OF CARBENDAZIM IN THE INFUNDIBULUM OF THE JAPANESE QUAIL (COTURNIX COTURNIX JAPONICA)

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ABSTRACT
The present study was undertaken to establish the long-term effect of a single dose of carbendazim in the tubular region of the infundibulum of Japanese quail using scanning (SEM) and transmission (TEM) electron microscopy. At a dose of 400mg/kg bodyweight, carbendazim in sunflower oil base was administered orally to mature Japanese quails. The control group received the oil base only. The effects of carbendazim on ultrastructural features of infundibulum were examined in spatial temporal periods post-exposure. At SEM level, loss of cilia were observed from 5 days post-exposure. At TEM level, pyknosis, karyorrhexis, swollen mitochondria, dilated RER cisternae, increased number of lysosomes and vacuoles were observed from 24 hours post-exposure. Compound cilia and loss of striated rootlets were also observed. Thickening and duplication of the basal lamina were identified from 12 days post-exposure. These results suggest oviductal regression due to carbendazim toxicity and signs of epithelial recovery at later stages post-exposure.

Keywords: Carbendazim, infundibulum, degeneration, electron microscopy, Japanese quail

INTRODUCTION
Infundibulum is the proximal segment of female reproductive tract in birds. It is known to play an important role in the process of egg formation, i.e. ovulated oocyte is received by the dilated funnel region and as the oocyte descends distally, fertilization occurs in the tubular region of the infundibulum (Okamura and Nishiyama, 1978). In addition, mucosal secretory cells in the tubular region are known to produce chalazae of the developing egg (Rahman, Iwasawa and Yoshizaki, 2007). Based on the potential of this region, any morphological change will affect the reproduction of female bird.

Carbendazim (Methyl-2-benzimidazole carbamate) is a derivative of the benzimidazole group of fungicides. It is widely used in the field crops for the control of fungus (Carter and Laskey, 1982). The fungicidal effect of carbendazim is due to its ability to inhibit mitosis microtubules and consequently interrupt mitotic spindle formation (Davidse and Flach, 1977; Burlad and Gull 1984).

Morphological studies have shown the effect of carbendazim in the reproductive system of birds (Carter and Laskey, 1982; Aire, 2005; Hess et al., 1991; Lim and Miller, 1997). In the previous studies, we reported the effect of various doses of carbendazim in the ovary and oviduct in the Japanese quail within 48 hours post-exposure (Kimaro, 2014; 2015; Kimaro and Kipanyula, 2014; Kimaro et al., 2013). Information on the long-term status of the oviduct following single exposure of carbendazim is still perplexing. This investigation therefore, reports the long-term effects of carbendazim on the morphological features of the tubular region of the infundibulum using scanning and electron microscopy.

MATERIALS AND METHODS

A total of 49 mature Japanese quails were used in the present study. The chicks were purchased from Irene Improvement Research farm, Pretoria and raised in the poultry facility of the Department of Anatomy and Physiology, University of Pretoria. During this period, food (growers mash containing maize grain) and water were provided ad libitum. Light was controlled at a ratio of 14 hours light to 8 hours darkness. At laying period, a single dose of 400 mg/kg bodyweight carbendazim diluted in sunflower oil was administered per os. A dose of 400 mg/kg has been determined to be the minimum toxic dose which causes gross and histological lesions in the quail’s oviduct\textsuperscript{12}. Control birds received an equivalent amount of oil base orally. Samples were collected in spatial temporal periods, such as: 5 hours, 24 hours, 5 days, 8 days, 12 days and 32 days. At each sampling period, 7 birds were sacrificed by inhalation anaesthesia using carbon dioxide (CO\textsubscript{2}). Following the death of a bird, pleuro-peritoneal cavity was opened and tissue samples from the tubular region of the infundibulum were collected immediately. Thereafter, the samples were immersion-fixed in 2.5% glutaraldehyde in 0.1M Millonig’s buffer (pH 7.3) for 24 hours and processed for scanning (SEM) and transmission (TEM) electron microscopy using standard techniques.

RESULTS

Scanning electron microscopy

Control birds

The mucosal surface of the infundibulum was arranged in longitudinally-oriented folds (Fig. 1). Both primary and secondary folds were identified. The surface epithelium consisted of ciliated and non-ciliated cells. Ciliated cells, exhibited long cilia, which partially obscured adjacent non-ciliated cells (Fig. 1). The non-ciliated cells exhibited dome-shaped apical regions, which were covered by short microvilli. Round to oval-shaped glandular openings were observed between the epithelial cells.

Carbendazim treated birds

No degenerative changes were observed in the mucosal surface at 5hr, as well as, 24 hours post-exposure to carbendazim. At days 5 and 8 days, discrete areas of deciliation were observed (Fig. 2a). In these areas of deciliation, short ciliary stems and swollen microvilli were encountered (Fig. 2b). Some degenerating ciliated cells contained ciliary tufts with only a few intact cilia. At days 12 and 32 post-exposure to carbendazim, deciliation continued to be the dominant degenerative change observed. Cilia with swollen tips, as well as, those with nodules along the shaft were identified. Degenerating non-ciliated cells were raised above the surface and lined by short, swollen microvilli.

Figure 1: SEM photomicrograph of the infundibulum from a control bird. Primary folds (PF) are observed separated by cleft (asterisk). Note the presence of ciliated cells obscuring the non ciliated cells.
Transmission electron microscopy

Control birds

A simple columnar epithelium consisting of ciliated and non-ciliated cells lined the tubular region of the infundibulum (Fig. 3). The epithelial cells appeared to be predominantly ciliated. The ciliated cells contained centrally-located, euchromatic, oval nuclei, surrounded by an electron lucent cytoplasm. The cytoplasm of the ciliated cells contained mitochondria, smooth (SER) and rough (RER) endoplasmic reticulum, Golgi complexes, as well as, a few lysosomes (Fig. 3). The apical plasma membrane of the ciliated cells was lined by cilia and microvilli. Supporting the cilia were basal bodies, anchored by rootlets and basal feet (Fig. 3). The basal bodies were hollow structures consisting of circularly-arranged, nine-triplet microtubules. Anchoring the basal bodies were rootlets, which are striated structures, attached to the lateral aspects of the basal bodies. Associated with basal bodies and rootlets were basal feet, which were composed of filaments or microtubules. Basal bodies, basal feet and rootlets were identified in the apical cytoplasmic regions of the ciliated cells. On cross section, each cilium was composed of a complex of nine pairs of microtubules surrounding a central pair of microtubules. A plasma membrane lined the cilium. At the distal end of the cilium, the plasma membrane was modified to form a “cilia necklace”. The apical ends of the cilia were tapered and formed structures termed “cupping plates”.

Non-ciliated cells in the tubular region of the infundibulum contained round, basally-located nuclei. Membrane-bound secretory granules or bodies, occupied most of the cytoplasm (Fig. 3). The secretory granules, which were round to ovoid in shape, contained a homogeneous material of an intermediate electron density. Round to elongated mitochondria were distributed throughout the cytoplasm. A few arrays of SER and RER were also observed. The apical plasma membrane was modified to form numerous, uniform microvilli (Fig. 3). Desmosomes and intermediate junctions united adjacent cells (Fig. 3).

A granular basal lamina separated the surface epithelium from the underlying lamina propria-submucosa. The basal lamina, which was approximately 80 nm thick, was composed of a distinct lamina densa and lamina lucida. Underlying the basal lamina were collagen fibres.

Carbendazim treated birds

Following carbendazim treatment, ultrastructural changes were observed in a few ciliated cells 5 hours post-exposure. The observed changes included: nuclear condensation and margination of nuclear chromatin; blebbing of the nuclear membrane; cytoplasmic vacuolation and swelling...
of mitochondria (Fig. 4a). No degenerative changes were observed in the non-ciliated cells. Cytoplasmic vacuolation was identified in a few gland cells.

Twenty-four hours post-exposure to carbendazim, numerous lysosomes and vacuoles were observed in the cytoplasm of ciliated cells (Fig. 4b). The vacuoles were concentrated perinuclearly, as well as in the basal cytoplasmic regions. Swollen mitochondria, as well as, mitochondria with disintegrating, enclosing membranes were observed in the ciliated cells. Cellular junctions and cilia were structurally intact.

Degenerating non-ciliated cells contained a few swollen mitochondria. Relatively few microvilli lined the luminal surface of the degenerating non-ciliated cell. No structural changes were observed in the secretory granules of the non-ciliated cells. At this stage, cells with a few swollen mitochondria were observed in the tubular gland cells. In addition, nuclei of the degenerating gland cells exhibited condensed and marginalized nuclear chromatin. These gland cells contained a few secretory granules, which were concentrated apically. The lining microvilli and cellular junctions of the gland cells were intact.

TEM observations 5 days post-exposure to carbendazim revealed the presence of pyknotic nuclei, myelin figures and numerous lysosomes in the apical cytoplasmic regions of degenerating ciliated and non-ciliated cells. The cytoplasm of these cells contained swollen mitochondria, as well as dilated cisternae of rough endoplasmic reticulum (RER). At this stage, cellular junctions between the epithelial cells were still intact. Degenerating tubular gland cells contained pyknotic nuclei and numerous vacuoles.

At 8 days post-exposure to carbendazim, degenerating ciliated cells containing pyknotic nuclei, swollen mitochondria and numerous vacuoles (Fig. 5a). In a few ciliated cells, blebbing of the nuclear membrane, nucleolar margination, as well as, condensation and margination of nuclear chromatin were observed. The cytoplasm in these cells was electron lucent. Very few morphologically normal non-ciliated cells were encountered in the luminal epithelium at this stage. The degenerating non-ciliated cells exhibited relatively few microvilli (Fig. 5a). Swollen mitochondria and myelin figures were observed in the cytoplasm (Fig. 5b). Secretory granules in these cells were structurally normal. The basal lamina underlying the epithelial cells was approximately 125 nm thick. At this stage, invagination and occasional duplication of the basal lamina were observed (Fig. 5c). In addition, the basal lamina lacked a distinct lamina lucida. The lamina densa of the basal lamina contained electron dense particles, which obliterated the lamina lucida.
Figure 5: TEM photomicrographs of epithelium in the tubular region of the infundibulum, 8 days post-exposure to carbendazim. a. Asterisk: lysosomes; v: vacuoles; Arrow: pyknotic nucleus; arrowhead: microvilli. N: nucleus. Cc: degenerating ciliated cells; Nc: non-ciliated cell b. Arrow: myelin figure c. Bm: basal lamina, note the invaginations and increased thickness.

Degenerating tubular gland cells contained pyknotic nuclei, swollen mitochondria, vacuoles, as well as, degenerating secretory granules (Fig. 6a). Numerous lysosomes were identified in the supranuclear regions of the degenerating gland cells (Fig. 6a). In the initial stages of degeneration, the degenerating secretory granules displayed three distinctive zones. A small central zone, which contained an electron lucent material. An intermediate zone, which contained thick electron dense band, surrounding the central zone. An outer or peripheral zone, which was larger than the intermediate zone, contained particles of an intermediate electron density (Fig. 6b). In the advanced stages of degeneration, the entire secretory granule contained an electron lucent material circumscribed by a narrow electron dense band (Fig. 6c). At this stage, cellular junctions along the lateral plasma membranes of the gland cells were intact. However, the basal plasma membrane was discontinuous and contained electron dense deposits (Fig. 6d). Duplication of the basal lamina underlying the glandular epithelium was also observed (Fig. 6d).

At 12 days post-exposure to carbendazim, the presence of myelin figures in ciliated epithelial cells was a common feature. Swollen mitochondria and pyknotic nuclei were observed in both ciliated and non-ciliated cells. Degenerating nuclei displayed nucleolar and chromatin margination. In these nuclei, chromatin condensation was also evident. Numerous vacuoles were seen in the cytoplasm of degenerating ciliated cells. Coalesced secretory granules were observed in the non-ciliated cells. The degenerating secretory granules contained a homogeneous electron lucent material. Cells with swollen mitochondria and pyknotic nuclei were observed in the tubular glands. Margination of nuclear chromatin was also observed in a few of the degenerating glandular cells. The basal lamina underlying both luminal and tubular epithelia displayed similar changes as seen in 8 days post-exposure to carbendazim.

At 32 days post-exposure to carbendazim, compound (multi-tubular) cilia were frequently observed (Fig. 7a). Degenerating ciliated cells contained pyknotic nuclei, swollen mitochondria
and numerous vacuoles. Myelin figures and nuclear membrane blebbing were also observed in degenerating ciliated cells. In addition, aggregations of granular material and filamentous bundles were evident in the apical cytoplasmic regions (Fig. 7b, c). The degenerating non-ciliated cells displayed protrusions of the apical regions (Fig. 7d). Underlying the epithelial cells was a thick basal lamina (approximately 200 nm). In some areas, duplication of the basal lamina was observed. At this stage the gland cells were lined by a few short microvilli.

**Figure 7:** TEM photomicrographs of epithelium in the tubular region of the infundibulum, 32 days post-exposure to carbendazim. a asterisk: compound cilia; arrow: myelin figure. b. Asterisk: Granular aggregations c. IF: filamentous aggregations. d. Asterisk: A cytoplasmic protrusion. Nc: non-ciliated cell. Cc: ciliated cell, note indistinct rootlets striations.

Degenerating gland cells contained irregular-shaped nuclei, which displayed nucleolar and chromatin margination, as well as, nuclear membrane blebbing (Fig. 8a, b). Occasional pyknotic nuclei and aggregation of lysosomes were also observed in degenerating gland cells. Numerous vacuoles were observed in the apical and basal cytoplasmic regions of the cells. A few electron lucent secretory granules were identified in the perinuclear regions of the degenerating gland cells. Although apical cellular junctions were occasionally intact (Fig. 8a), no cellular junctions were observed along the lateral plasma membranes. The basal lamina underlying the glandular epithelium was similar to that observed in the 8 and 12 days post-exposure to carbendazim.

**Figure 8:** TEM photomicrograph of a tubular gland, 32 days post-exposure to carbendazim. a. N: Irregular-shaped nuclei; nu: nucleoli; thin arrow: pyknotic nucleus; thick arrow: lysosomes; arrowhead: microvilli; L: gland lumen; Bm: basal lamina. b. degenerating nucleus. Arrowhead: blebbing of the nuclear membrane; nu: nucleoli.

**DISCUSSION**

The present study was an attempt to establish the long-term effects of carbendazim on the ultrastructural features of the infundibulum in the Japanese quail (Kimaro, 2015) and domestic fowl (Wyburn, Johnston, Draper and Davidson, 1970). In this region, the mucosal layer was lined by simple columnar epithelium which consisted of ciliated and non-ciliated cells. At scanning electron microscopic level, deciliation was the
predominant degenerative change observed on the mucosal surface. Deciliation could be due to carbendazim toxicity which weakens the axonemal microtubules by binding on-to β-tubulin subunit of microtubule and thus affect its assembly. Two types of β-tubulin genes (TUB1 and TUB2) have been reported (Cruz and Edlind, 1997). The report showed that TUB1 gene serve as a principal target during interaction with benzimidazole fungicides.

At TEM level, in addition to loss of cilia, formation of compound cilia and abnormal basal body attachment were observed from 12 to 32 days post-exposure. Presence of compound cilia was an early sign of cilia loss. This argument is supported by a study in the pecking ducks which showed loss of cilia in group post-exposure to methyl mercury (Balachandran, Bhatnagar and Geinger, 1985). Abnormal basal body attachment suggested failure in coordinated cilia movement. This is due to a positive correlation existing between basal bodies attachment and cilia movement. In the Japanese quail, a correlation between the attachment and orientation of basal bodies and cilia bitting in the oviductal epithelium have been reported (Chailey et al., 1982; Boisvieux-ulrich et al., 1985; Lemullois et al., 1987; Boisvieux-ulrich et al., 1991). Indeed, a strong basal body attachment is required to control egg laying process. Presence of compound cilia and abnormal basal body attachment at this stage suggests a defective cilia biting which may consequently impact negatively on the production and reproduction of exposed birds.

TEM observations also revealed aggregation of intermediate filaments and increased number of lysosomes on the apical cytoplasmic regions of degenerating cells from 5 days post exposure. This was consistent with disappearance of striated rootlets at advanced stages of degeneration. Intermediate filaments have been shown to be associated with striated rootlets (Sandoz et al., 1983). They form a network which connects the cytoplasm to the lateral plasma membrane. The observed filamentous aggregation suggests degeneration of microtubules into filament. Similar findings have also been reported in the fibrocyte and leukocytes exposed to microtubule disrupting agents, vinblastine and colchicines (Bensch and malawisa, 1969).

Degeneration of cytoplasmic organelles and nucleus such as presence of swollen mitochondria, increased number of lysosomes and pyknotic nuclei suggested occurrence of apoptosis induced by carbendazim toxicity. Indeed, degeneration of mitochondria activates caspase cascade through intrinsic apoptotic pathway. Therefore, presence of degenerating mitochondria might be early signs of oviductal regression in exposed birds. Further immunohistochemical study is needed to mark the onset of apoptosis following carbendazim exposure.

Degeneration of tubular gland cells was evident from 24 hours post-exposure to carbendazim. Degeneration of these cells at this stage could be attributed to the high rate of absorption and accumulation of carbendazim in body tissues. In the Japanese quail, carbendazim residues have been detected in the liver, pectoral muscles and eggs 8 weeks post-exposure (Reisinger et al., 2006). Based on the functional importance of gland cells in the female bird i.e. secretion of chalazae which controls the axis of embryo development and production of sperm-associated bodies, which supports fertilization, degenerative changes observed suggests reproductive failure in exposed birds.

In this investigation, morphological changes were observed in the basal lamina underlying both the luminal and glandular epithelia. The changes were more pronounced at days 12 and 32 post-exposure to carbendazim. At this stage duplication, breakage and invaginations of the basal lamina were evident. Thickening of the basal lamina has been reported in human glomeruli (Beisswenger and Spiro, 1970), skeletal muscles (Gulati et al., 1983) and blood vessels of diabetic patients (Giamini and Dyck, 1995). In these studies, the increased basal
lamina thickness was associated with epithelial recovery. It is possible that the increased thickness (duplication) of a basal lamina observed in the current study suggests recovery of the epithelium. According to a report by Vracko (Vracko, 1974), epithelial regeneration is normally accompanied by formation of a new basal lamina.

In conclusion, the present study has revealed the morphological changes occurring in the tubular region of the infundibulum following oral administration of a single dose of carbendazim. The observed degenerative changes suggested that single dose of carbendazim causes oviductal regression with signs of recovery from day 32 post-exposure.

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