# MORPHOLOGICAL CHANGES IN THE SPERM STORAGE TUBULES OF THE JAPANESE QUAIL EXPOSED TO METHY-2-BENZIMIDAZOLE CARBAMATE

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## ABSTRACT

The current investigation was an attempt to establish the effect of various doses of methyl-2-benzimidazole carbamate (carbendazim®) on the morphology of the Sperm Storage Tubules (SST) in the Japanese quail (*Coturnix coturnix japonica*). Carbendazim® in sunflower oil base was administered orally at doses of 0mg/kg (control), 25mg/kg, 100mg/kg, 400mg/kg and 800mg/kg body weight. Tissue samples from Uterovaginal junction were processed for both light (LM) and Transmission electron (TEM) microscopic study following standard procedures. The result showed that, at LM level, no histopathological changes were observed at a dose of 25mg/kg b.w.t. A significant decrease in SST width and luminal diameters was observed at doses of 100mg/kg and 400mg/kg b.w.t ($p < 0.05$). In addition, doses of 400mg/kg and 800mg/kg b.w.t caused leukocytic infiltration and hyperaemia in the *lamina propria-submucosa*. At these doses SST were devoid of spermatozoa. TEM results showed pyknosis, swollen mitochondria, vacuolation and increased number of lysosomes in degenerating SST. The observed morphological changes indicate the ability of carbendazim to disrupt structural integrity of SST as well as its storage capacity. This poses a great threat to the fertility of exposed birds and thus care must be taken to reduce environmental contamination.
**Keywords: Carbendazim, histopathology, ultrastructure, Sperm storage tubules, Japanese quail**

## INTRODUCTION

The structure of sperm storage tubules has been extensively studied in the domestic fowl (Fujii, 1963; Tingari and Lake, 1973; Das, 2003), turkey (Ogasawara & Fuqua 1972; Bakst 1981; Schuppin et al., 1984), Japanese quail (Frieβ et al., 1978; Birkhead and Fletcher, 1994) and Bengalese finch (Birkhead, 1992). The SST are tubular invaginations of the luminal epithelium which are lined by simple columnar epithelium. The number of SST varies between species (Birkhead and Møller, 1992). Up to 3467 SST have been recorded in the Japanese quail (Birkhead and Fletcher, 1994).

The release of spermatozoa from SST occurs intermittently during fertilization and has been suggested to be controlled by ciliary movement on the apical tubular section or flushing action by gland secretions as well as the presence of myoepithelial cells (Schupping et al., 1984;

Birkhead, 1992; Das, 2003). Studies of ciliary movement have shown the involvement of cytoskeletal elements such as; microtubules, intermediate filaments and microfilaments (Sandoz et al., 1983; Chailley et al., 1989). Based on the fact that fertilization in avian species depends on the release of spermatozoa from SST, active ciliary movement as well as structural integrity of glandular cells are important. Methyl-2-benzimidazole carbamate (carbendazim®) disrupt microtubule by binding to the β tubulin subunit of the microtubule (Burland and Gull, 1984; Cruz and Edlind, 1997). Disruption of the microtubule will affect cellular skeleton as well as ciliary movement, thus impaired fertility in exposed birds. To-date little is known on the possible morphological changes in the SST following carbendazim exposure. This study therefore, investigated the histopathological and

ultrastructural changes in the SST of Japanese quail exposed to carbendazim.

## MATERIALS AND METHODS

A total of 35 sexually mature female Japanese quail purchased from Irene Improvement Research Farm, Pretoria were used. The birds were divided into two groups, the control and treatment groups. The treatment group was further divided into four groups of 7 birds each according to the administered dose. Carbendazim (97% Sigma Aldrich) was dissolved in sunflower oil and administered *per os* to treatment birds at dosages of 25mg/kg, 100mg/kg, 400mg/kg and 800mg/kg bodyweight. The doses were selected based on the previous experiment in the Japanese quails (Kimaro et al. 2013). The control birds were given the sunflower oil base orally. During the experiment, food (growers mash, containing maize grain) and water were provided *ad libitum*. Light was controlled at a ratio of 14: 10 hours (light and darkness) throughout the experiment.

Forty-eight hours post-exposure to carbendazim, birds were sacrificed by inhalation anaesthesia using carbon dioxide ($CO_2$). Following the death of a bird, pleuroperitoneal cavity was opened and the reproductive tract was dissected immediately.

### Light microscopy

Tissue samples from the Utero-vaginal junction (UVJ) were immersion fixed in 10% buffered formalin for 48 hours. Tissue samples were then processed routinely for light microscopy using automated tissue processor (Shandon excelsior®, Thermo Electron Corporation, Germany) and stained with Haematoxylin and eosin (H&E). Tissue morphometry such as SST width and luminal diameters were evaluated using an image analyzer ((AnalySIS®; Olympus BX 50, Optical Company LTD, Japan). The SST width was measured by drawing a perpendicular line across the gland. Glandular luminal diameter was determined by measuring the perpendicular distance between two opposing gland cells. Morphometrical data were analysed using analysis of variance (ANOVA); SPSS version 17. A probability of 5% was considered significant.

### *Ultrastructure*

Tissue samples from UVJ were immersion fixed in 2.5% glutaraldehyde in 0.1M Millonig's buffer (pH 7.3) for 24 hours. Thereafter, the tissue samples were post-fixed in 2% osmium tetroxide. Following post-fixation, samples were processed for transmission electron microscopy (TEM) using standard techniques.

## RESULTS

### Histomorphometrical observations
### *Control birds*
The mucosal layer in the utero-vaginal junction

### *Carbendazim-treated birds*

### *Histomorphometry*
Table 1 summarises the measured morphometrical parameters of the SST in the control and carbendazim-treated birds. There was a general decrease in the width of the SST following administration of low doses of carbendazim. When compared to the control, a significant decrease in the width of SST was observed at 100 mg/kg and 400 mg/kg bodyweight. A significant decrease was also observed between 25 mg/kg and 100 mg/kg,

as well as, between 100 mg/kg and 800 mg/kg bodyweight (p < 0.05). At a dose of 800 mg/kg bodyweight, carbendazim increased the width of the SST. However, this increase was not statistically significant when compared to the control (p < 0.05). The increase was statistically significant between 400 mg/kg and 800 mg/kg bodyweight (p < 0.05). Consistent with glandular width, the glandular lumena diameters were also decreased by carbendazim treatment. A significant decrease in the glandular lumena diameters was recorded at 100 mg/kg when compared to the control and 25 mg/kg bodyweight groups (p < 0.05).
was lined by simple ciliated columnar epithelium. In the *lamina propria-submucosa*, sperm storage tubules (SST) were observed

(Fig. 1a). The SST were tubular glands formed by luminal epithelial invagination (Fig. 1b). A simple columnar epithelium lined the SST (Fig. 1c). At the proximal extremity (neck) of the gland, the lining epithelium was simple ciliated columnar (Fig. 1b). Numerous spermatozoa were observed in these glands (Fig. 1a&c). The glands measured between 22.3 and 35.45 µm (27.22 ± 0.98) in width. The glandular lumena were averagely 5.26 µm in diameter.
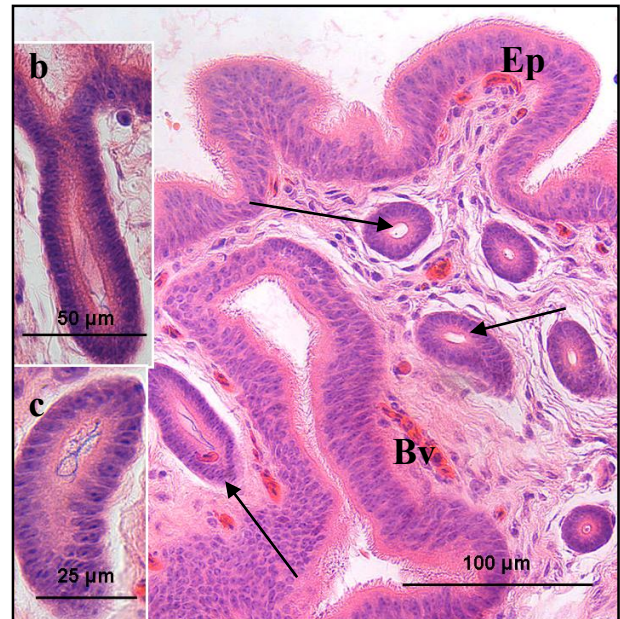


**Fig. 1**: **a**. Photomicrograph of the *mucosa* in the UVJ region of control bird. SST (arrows) are observed in the *lamina propria-submucosa*. Ep: luminal epithelium; Bv: blood vessels. **b**. A SST is seen formed by epithelial invagination. **c**. A simple columnar epithelium lining the SST.

## Table 1: Mean ± SE histomorphometrical parameters of the SST in the control and carbendazim treatment groups

| Carbendazim dose (mg/kg) | Glandular width (µm) | Glandular lumena diameters (µm) |
|---|---|---|
| 0 | 27.22 ± 0.98 | 5.26 ± 0.31 |
| 25 | 24.48 ± 0.45[b] | 4.61 ± 0.21 [b] |
| 100 | 11.77 ± 3.36[abc] | 2.13 ± 0.21 [ab] |
| 400 | 17.87 ± 3.36[ad] | 3.97 ± 0.92 |
| 800 | 30.41 ± 1.3[cd] | 3.99 ± 0.33 |

[a] Differs significantly from the control
[bcd] Significant difference between treatment groups

### Histopathology

No histological degenerative changes were observed in the SST at doses of 25 mg/kg and 100 mg/kg bodyweight, 48 hours' post-exposure to carbendazim. At a dose of 400 mg/kg bodyweight carbendazim, a few spermatozoa were observed in the lumena of the SST (Fig. 2a). In some instances, the gland lumena were empty. The SST contained a few cells with pallor cytoplasm and pyknotic nuclei (Fig. 2b). At a dose of 800 mg/kg, hyperaemia, perivascular cuffin and cells with pyknotic nuclei were observed in the *lamina propria-submucosa* (Fig. 3a-c). The lumena of SSTs contained no spermatozoa.

### Ultrastructural observations
### Control birds

The SST cells contained round nucleus located basally (Fig. 4a). A cytoplasm which appeared electron lucent, contained a few mitochondria and stacks of Golgi complexes (Fig. 4b). A conspicuous lipid body was observed either in the apical or basal cytoplasmic regions. Desmosomes and occasional tight junctions were observed on the apical plasma membrane (Fig. 4c).
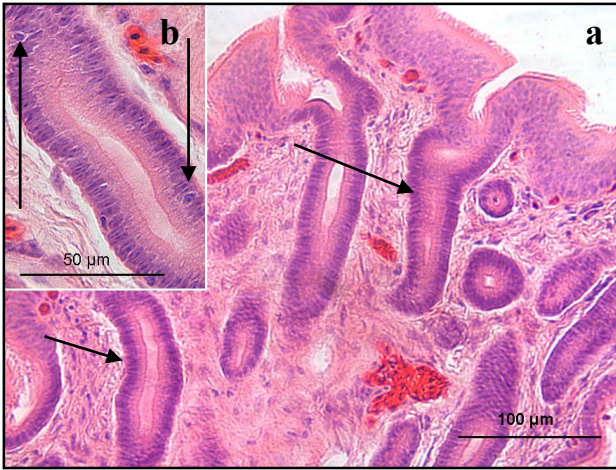
**Fig. 2a**. Photomicrograph of the *mucosa* in the UVJ region of a bird treated with 400 mg/kg bodyweight. Arrows: empty SST; Bv: blood vessels. **b**. SST higher magnification. Arrow: pyknotic nucleus.



Fig. 3**a**. Photomicrograph of the *mucosa* in the UVJ region of a bird treated with 800 mg/kg b.w.t. Thick arrows: congested blood vessels. **b**. Asterisk: aggregation of inflammatory cells. Bv: blood vessel. **c**. Arrow: pyknotic nucleus.

Along the lateral plasma membrane, infolding and formation of lateral process were observed. The length of the lateral processes was increasing toward the basal cellular region.
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The cells rested on a basal lamina (approximately 55nm thick). The basal lamina displayed both the lamina densa and lamina lucida (Fig. 4d)
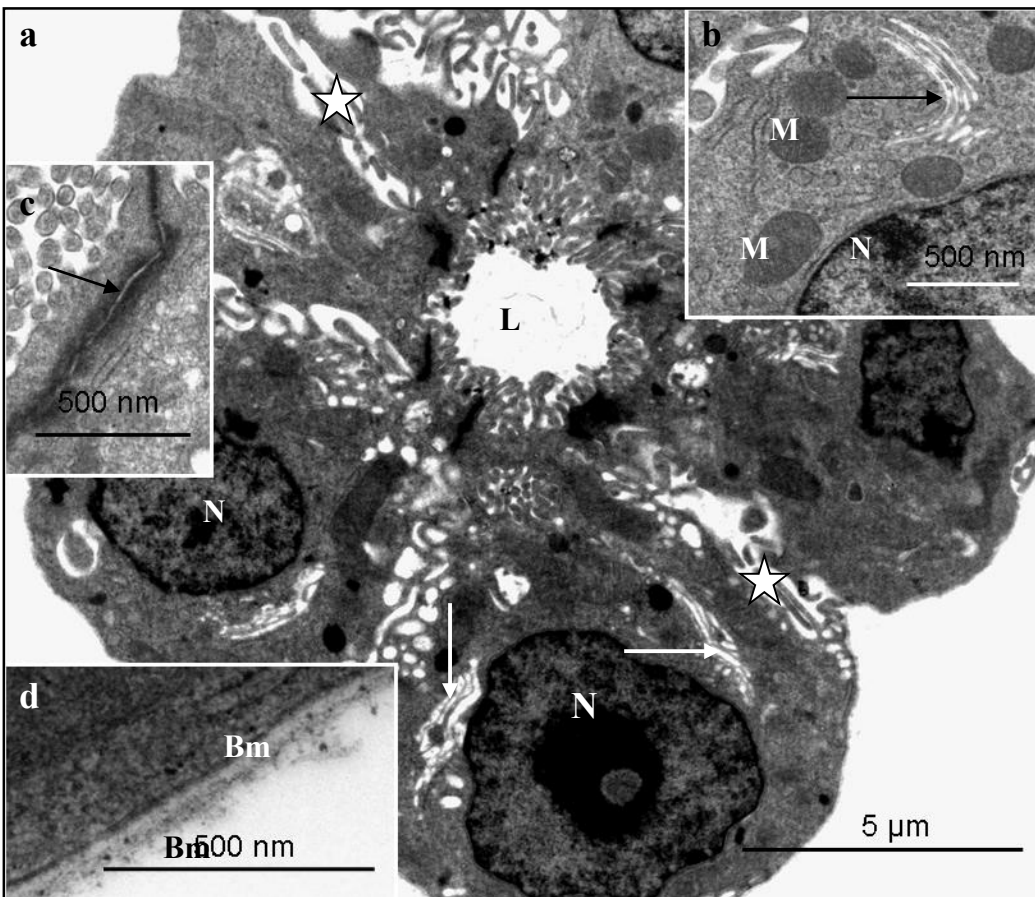


**Fig. 4a**. TEM photomicrograph of a SST from control bird. N: nucleus; arrows: golgi complexes; asterisks: processes along the lateral plasma membranes; L: glandular lumen. **b**. Golgi complex (arrow) and mitochondria (M) adjacent nucleus (N). **c**. Arrow: Desmosome. **d**. Bm: basal lamina supporting SST.

### Carbendazim-treated birds

There were no ultrastructural changes observed in the SST cells at doses of 25 mg/kg and 100 mg/kg b.w.t. At a dose of 400 mg/kg b.w.t, the SST contained a few cells with pyknotic nuclei, numerous vacuoles and multi-granular bodies (Fig. 5a). In addition, there was an increased number of lysosomes in the apical cytoplasmic regions. In some instances, degenerating nuclei displayed chromatin condensation and margination. Desmosomes and microvilli on the apical plasma membrane were structurally normal. The basal lamina measured approximately 60nm thick. Both lamina densa and lamina lucida were clearly differentiated (Fig. 5b).
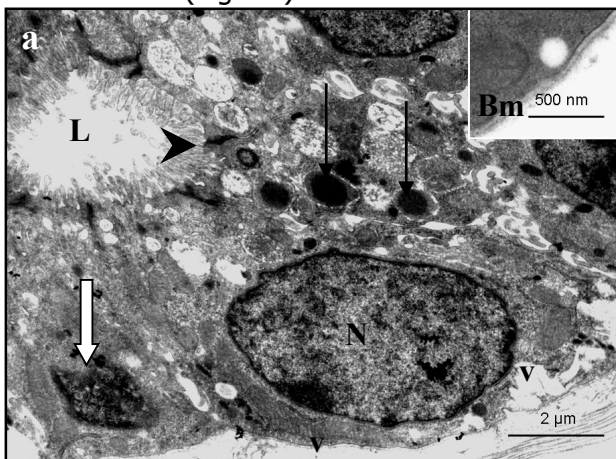


**Fig. 5a**. TEM photomicrograph of a SST from a bird treated with 400 mg/kg b.w.t. Open arrow: pyknotic nucleus; V: vacuoles; thin arrows: lysosomes; N: normal nucleus; arrowheads: desmosome; L: glandular lumen. **b**. basal region of the SST. Bm: basal lamina.

At a dose of 800 mg/kg bodyweight carbendazim, degenerating SST cells contained large vacuoles in the basal cytoplasmic regions (Fig. 6a). Swollen mitochondria and numerous lysosomes were observed (Fig. 6b). A few secondary lysosomes were also identified. Nucleolar margination and condensation of nuclear chromatin characterized degenerating nuclei. At this dose, microvilli lining the apical plasma membrane were structurally normal. Desmosomes in this region were intact. However, there was reduced number and length of the lateral plasma membrane processes. The basal lamina underlying the SST cells was 68nm thick. No degenerative changes were observed in the basal lamina.
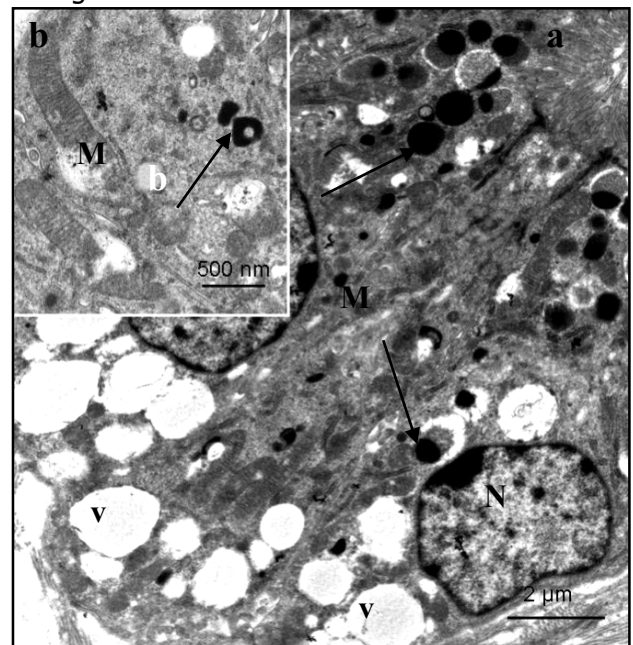


**Fig. 6a**. TEM photomicrograph of a SST from a bird treated with 800 mg/kg b.w.t. v: vacuoles; Arrows: lysosomes; N: nucleus. **b**. M: degenerating mitochondria; Arrow: secondary lysosome adjacent the swollen mitochondria.

## DISCUSSION

The present investigation has highlighted the morphological changes that occur in the SST of the Japanese quail following exposure to methyl-2-benzimidazole carbamate. In this investigation, the results of morphometrical study show that, carbendazim exposure caused atrophy of SST. The atrophy of the SST observed, might cause reduction of the size, amount and storage period of spermatozoa in carbendazim-exposed birds. This idea is supported by research findings in the domestic fowl (Birkhead and Moller, 1992). According to Birkhead and Moller (1992), there is positive correlation between the volume of SSTs and number of spermatozoa stored. The report further shows that, the size (length) of the SSTs correlates with the length of spermatozoa and duration of storage.

Based on histological results, the morphology of SST observed in the control group was generally consistent with published information in the Japanese quail (Frieβ et al., 1978; Holm and Ridderstrale, 2002), domestic fowl (Tingari and Lake, 1973; Bakst, 1998) and turkey (Schuppin et al., 1984; Bakst, 1987). The results show that SST are tubular invaginations lined by simple columnar epithelium. Contrary to the observation made in the domestic fowl (Tingari and Lake, 1973; Das, 2003), no smooth muscle cells were observed around quail's SST. This observation confirms earlier finding by Frieβ et al. (1978) in the Japanese quail. Following carbendazim exposure, degenerative changes such as pyknosis and cuboidal metaplasia were observed. Degeneration of SST cells may lead to impaired sperm storage potential in exposed birds.

It has been established that SSTs serve as sperm storage sites for the entire length of fertile period in birds (Schupping et al., 1984; Pierson et al., 1988; Birkhead, 1992). Reduced number of spermatozoa and presence of empty SSTs observed in carbendazim-treated groups might impact negatively on the fertility of exposed birds. This is based on the fact that fertility in birds depends on the proportion of SST containing viable spermatozoa (Bilgili and Renden, 1984; McDaniel et al., 1981; Brillard and Antoine, 1990). According to these reports, fertility in the domestic fowl correlated to the number of spermatozoa attached to the perivitelline layer, which were influenced by proportion of SST containing spermatozoa.

In the current study, presence of empty SST could have been caused by sperm autolysis due to epithelial secretion as reported in the domestic fowl by Koyonagi and Nishiyama (1981). However, sperm autolysis was not apparent in the present study. It is likely that empty SST were due to degeneration of spermatozoa as a result of carbendazim toxicity. Carbendazim has been shown to cause degeneration of spermatozoa in mammals (Hess and Nakai, 2000) and birds (Aire, 2005).

At ultrastructural level, the morphology of the SST in the control birds was in accordance to the findings by Frieβ et al. (1978). Following carbendazim exposure, degenerating cells were common in the SST. In these degenerating gland cells, swollen mitochondria were frequently observed. Presence of swollen mitochondria suggests an increased cellular permeability induced by carbendazim through cytoskeletal damage. Heggenesset al. (1978) reported association of cytoskeleton and mitochondria. The report shows that, mitochondria are tightly connected with cytoskeletal elements. Destruction of cytoskeletal elements by colchicine or taxol increased mitochondrial membrane permeability in Ehrlich ascites tumour cells (Evtodienko et al., 1996). In addition, administration of colchicine induced release of *cytochrome c* in cultured cerebellar granule cells (Gorman et al., 1999). *Cytochrome c* is an apoptotic-inducing factor released by damaged mitochondria (Kroemer et al., 1998).

Based on the finding of this study, it is concluded that carbendazim causes pathological changes in the SST at high doses. Thus care must be taken when using this chemical in the field to avoid environmental contamination.

## Acknowledgement

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