Structural and ultrastructural evaluation of the rabbit testes after 10 and 30 days of subchronic peroral exposure to bendiocarbamate insecticide

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Introduction

A great deal of reproductive studies dealing with carbamate or other insecticides focused mainly on relatively high single oral doses that acted as direct testicular toxicants causing functional and morphological injuries (Correa and Miller, 2001; Mahgoub and Medany, 2000; Markelewicz et al., 2004). Several studies analysed directly the impact of low doses of carbamates on reproduction and fertility (Hayes and Laws, 1990; Kalla and Chochan, 1980; Worthing, 1983) but largely from the physiological aspect. A thorough literature survey, as of now, showed that not much has been elucidated from the morphological point of view. Therefore, the aim of the present study was to describe structural, ultrastructural and functional changes in the rabbit's testes after peroral administration of bendiocarb at a dose of 5 mg/kg body weight per day. Testes were consistently analysed on days 10 and 30 of the experiment.

Materials and methods

The rabbits used in the experiment were divided to four groups, 10 animals in each. Rabbits from the first control (C1) and first experimental (E1) group were euthanised on day 10 and those from the second control (C2) and second experimental (E2) group on day 30 of the experiment. Tissue samples of size 1 mm^3 were excised from both poles as well as from the central portion of the right testis of each animal. The samples were fixed by immersion in 3% glutaraldehyde and postfixed in 1% osmium tetraoxide (both in 0.15 M cacodylate buffer, pH 7.2–7.4). After dehydration in acetone they were transferred to propylene oxide and embedded in Durcupan ACM. Ultrathin sections were cut using an

ultramicrotome LKB Nova, double contrasted with uranyl acetate and lead citrate and examined under Tesla BS 500 electron microscope at 3200× and 4800× magnification. Semi-thin sections of specimens processed for transmission electron microscopy (TEM) were stained with toluidine blue and examined under a light microscope Jenamed at 1000× magnification. Morphometric parameters of seminiferous tubules were acquired using a light microscope Olympus BX51 with computer support (camera system DP50) and evaluated at 40× magnification in 90 round tubular cross-sections per testis. The relevant data were obtained using Image J 1.45j and processed statistically by means of t-test to determine significance of differences from the control ($p<0.001$). The relative testicular weight was calculated as follows: testicular weight in grams × 100% /body weight in grams. The care and use of animals complied with the current laws and regulations of the Slovak Republic (Korim et al., 2003) and were approved by the Ethical Committee of the Institute of Animal Biochemistry and Genetics (No. 1827/09–221/3).

Results

The mean testicular parameters

We observed significant differences in absolute as well as relative testis weight between the control and both experimental groups (Tab. 1). Diameters of seminiferous tubules and height of the seminiferous epithelium were significantly lower in the treated rabbits after 10 and 30 days of exposure, compared to the controls (Tab. 1). The diameters of the tubular lumina increased in both time periods, but mainly after 30 days of the experiment (Tab. 1).

Tab. 1. Mean levels of testicular parameters in control and treated animals. All parameters evaluated in treated animals differed significantly from the control ($p<0.001$)

Post-exposure time	Absolute weight of testes (g)	Relative weight of testes (%)	Seminiferous tubular diameter (μm)	Seminiferous epithelial height (μm)	Luminal diameter (μm)
Control 10	1.87±0.1	0.093	247	109	29
Control 30	1.85±0.1	0.092	249	110	29
Bendiocarb 10	1.42±0.1	0.071	185	75	35
Bendiocarb 30	1.22±0.1	0.061	164	33	98

E1 group – light microscopy

After 10-day exposure to Bendiocarb the structural changes within the testicular parenchyma were apparent. About 8% of the scored seminiferous tubules – T1 (the first type of tubules) were mostly regular in shape and lined with relatively high and typically arranged seminiferous epithelium. They contained typical developing spermatogenic cells from spermatogonia to late elongated spermatids. At higher magnification, these tubules comprised small irregular empty spaces among the developing cells from the level of the basement membrane up to the luminal space. Another unusual feature was the presence of small vacuoles within the cytoplasm of Sertoli as well as inside all types of spermatogenic cells (Fig. 1). Seminiferous tubules of the second type – T2, (about 25% of the volume) were similar to those described above but they additionally contained sloughed, early round or late elongated spermatids (Fig. 2). Approximately 32% of the investigated tubules – T3 (the third type of tubules) were noticeably degenerate with missing adluminal compartment. They showed reduced height of germinal epithelium devoid of spermatids and shortened Sertoli cells. The Sertoli cells possessed basally located round to oval shaped nuclei with typical deep indentations and relatively pale and foamy cytoplasm. Some spermatogonia appeared to be characteristic, other were more oval-shaped than usual. Only occasionally the stellate-shaped dying spermatogonia with dark round nuclei and darker cytoplasm were seen. Some spermatocytes were typical, other were rather swollen but still with typically granular nuclear chromatin. Several spermatocytes were released from the Sertoli cells connection and were either closely associated with Sertoli cells or were present as free cells inside the tubular lumina (Fig. 3). We also noted some tubules with extremely low seminiferous epithelium – T4 (fourth type of tubules), consisting of extremely short Sertoli cells and solitary round to oval spermatogonia or even spermatocytes. The proportion of these tubules reached about 35% (Fig. 4).

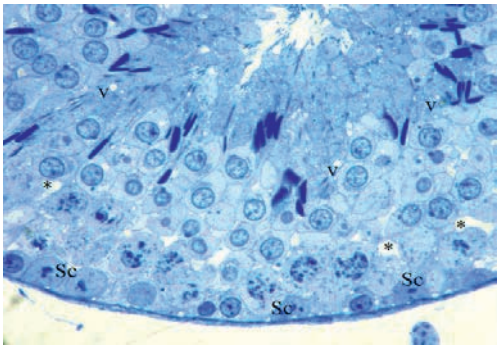

Fig. 1. T1 seminiferous tubule after 10-day exposure to bendiocarb (semi-thin section, toluidine blue), magnification 1000×; Sc – Sertoli cell, v – vacuoles, * – empty spaces

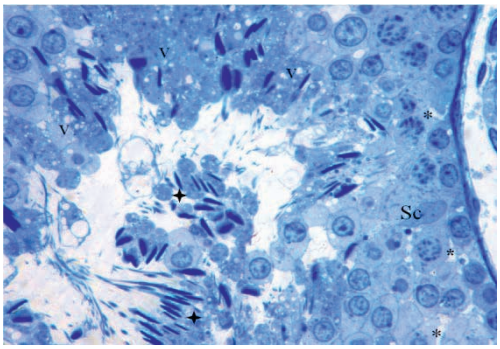

Fig. 2. T2 seminiferous tubule after 10-day exposure to bendiocarb (semi-thin section, toluidine blue), magnification 1000×; Sc – Sertoli cell, v – vacuoles, * – empty spaces, ✦ – sloughed cells

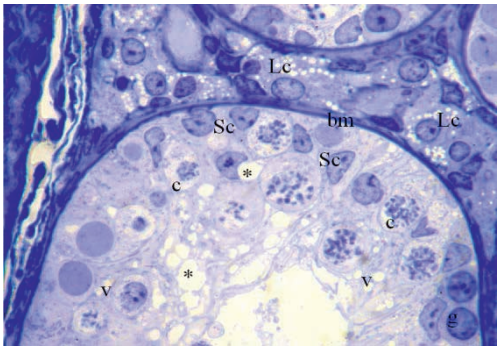

Fig. 3. T3 seminiferous tubule after 10-day exposure to bendiocarb (semi-thin section, toluidine blue), magnification 1000×; Sc – Sertoli cell, v – vacuoles, * – empty spaces, g – spermatogonium, c – spermatocyte, Lc – Leydig cell, bm – basement membrane

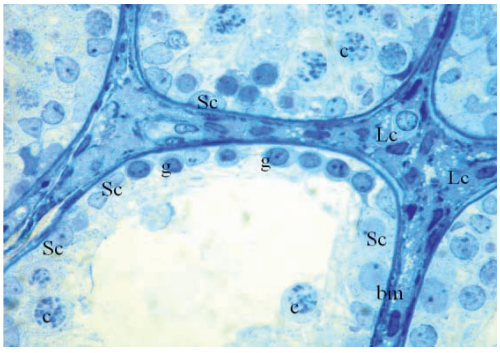

Fig. 4. T4 seminiferous tubule with extremely low germinal epithelium after 10-day exposure to bendiocarb (semi-thin section, toluidine blue), magnification 1000×; Sc – Sertoli cell, g – spermatogonium, c – spermatocyte, Lc – Leydig cell, bm – basement membrane

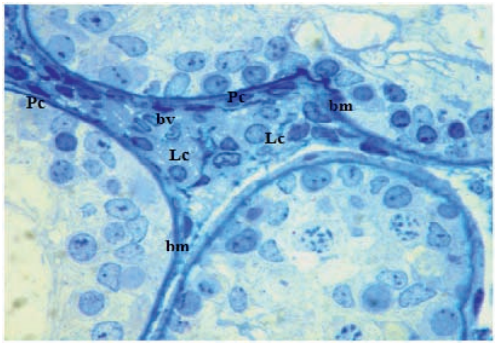

Fig. 5. Interstitial components of the testis after 10-day exposure to bendiocarb (semi-thin section, toluidine blue), magnification 1000×; Lc – Leydig cell, Pc – peritubular cells, bv – blood vessels, bm – basement membrane

Interstitial Leydig cells appeared to be reduced in size and their cytoplasm was dark with higher content of lipid droplets. The nuclei of the Leydig cells were either similar to those in the control or were sometimes rather shrivelled. Peritubular cells, blood vessels and fibrocytes appeared unchanged (Fig. 4 and 5). The basement membrane of each tubule type in experimental animals was thicker (Fig. 1–5) compared to the controls and sporadically irregular or even wavy (Fig. 5).

E2 group – light microscopy

The alteration of testis was more prominent after 30 days exposure to bendiocarb. In 59% of tubules we observed intensively reduced height of seminiferous epithelium consisting of short Sertoli cells and occasional spermatogonia – T4. Many Sertoli cells had notably dark and vacuolated cytoplasm but some had paler cytoplasm that formed ruffled projections towards the luminal space. All spermatogonia were shrivelled and irregularly shaped. Their nucleus was shrivelled as well, and the cytoplasm was dark and vacuolated. The spermatocytes were rather swollen and nuclei had typical granular appearance (Fig. 6).

Other degenerative tubules – T3 (41% of volume) did not contain the adluminal compartment. They comprised Sertoli cells, spermatogonia and spermatocytes, all of them with abnormal structure. No spermatids were found in these degenerative tubules. The Sertoli cells were generally shortened and had deeply indentated and more basally located nuclei than usual. Their cytoplasm was paler and foamy due to the presence of large and numerous vacuoles. Most spermatogonia were rather oval-shaped and relatively normal in appearance, but some were quite shrivelled and dark. The spermatocytes incorporated in the seminiferous epithelium were often irregular in shape and swollen. Their nuclei were either

round or irregular in shape, but typically granular. Other spermatocytes were found as free cells within the wide lumen of the tubule after their sloughing (Fig. 7). The basement membrane delimiting both types of degenerative tubules was extremely thick and in some locations acquired wavy appearance (Fig. 6 and 7).

Leydig cells were grouped into smaller clumps compared to the controls and were reduced in size. Their nuclei were shrivelled and often irregular in shape, the cytoplasm was strongly vacuolar. Other interstitial components appeared unchanged (Fig. 8).

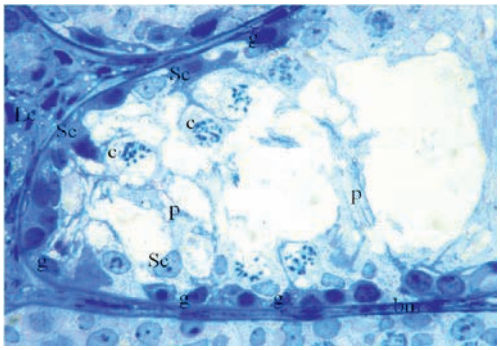

Fig. 6. T4 seminiferous tubule after 30-day exposure to bendiocarb (semi-thin section, toluidine blue), magnification 1000×; Sc – Sertoli cell, p – Sertoli cell cytoplasmic projections, g – spermatogonium, c – spermatocyte, Lc – Leydig cell, bm – basement membrane

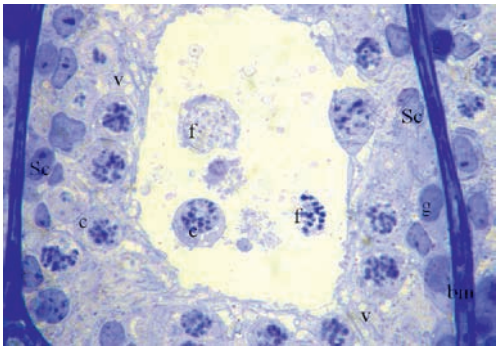

Fig. 7. T3 seminiferous tubule after 30-day exposure to bendicarb (semi-thin section, toluidine blue), magnification 1000×; Sc – Sertoli cell, v – vacuoles, g – spermatogonium, c – spermatocyte, f – sloughed spermatocyte, Lc – Leydig cell, bm – basement membrane

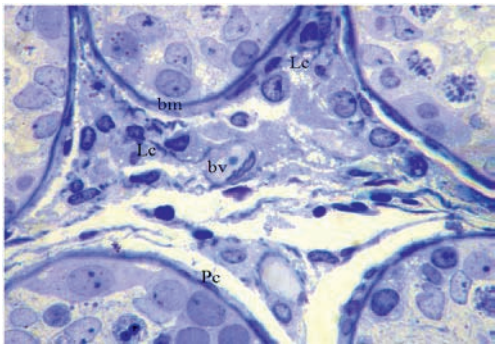

Fig. 8. Interstitial components of the testis after 30-day exposure to bendiocarb (semi-thin section, toluidine blue), magnification 1000×; Lc – Leydig cell, Pc – peritubular cells, bv – blood vessels, bm – basement membrane

E1 group – electron microscopy

Ten-day action of bendiocarb induced considerable changes in cellular ultrastructure which were tubule type dependent. The tubules which seemed to be unchanged under the LM – T1 contained typicaly high Sertoli cells with round to oval shaped, deeply indented, basally located nuclei with characteristic chromatin organization. Their cytoplasm contained characteristic organelles, except for increased amount of phagocytized material and electronlucent vacuoles of different size and shape. The spermatogonia, spermatocytes and spermatids had normal ultrastructure except for occasional electronlucent vacuoles inside the cytoplasm. Between the adjacent spermatogenic cells or between spermatogenic and Sertoli cells one could observe round or elliptic empty spaces throughout the height of the germinal epithelium (Fig. 9).

More pronounced ultrastructural changes in spermatogenic and Sertoli cells were detected inside the degenerative tubules – T3, with missing adluminal compartment. Most Sertoli cells were generally shortened and possessed either slightly or more seriously swollen mitochondria, electronlucent vacuoles, and higher amount of phagocytised material within their cytoplasm compared to the controls. Most spermatogonia were unchanged, but some possessed shrivelled nuclei, more electrondense cytoplasm and swollen mitochondria. All spermatocytes were swollen. Their nuclei were unchanged and contained granular chromatin, but the cytoplasm was more electronlucent in comparison with the controls and had swollen mitochondria. We also registered occasional necrotizing cells with strongly damaged organelles and markedly peripherally condensed nuclear chromatin. Because these cells were strongly ultrastructurally changed, we estimated their type only on the basis of their location. The majority of them were probably either spermatogonia or spermatocytes. No spermatids were noted in these tubules (Fig. 10).

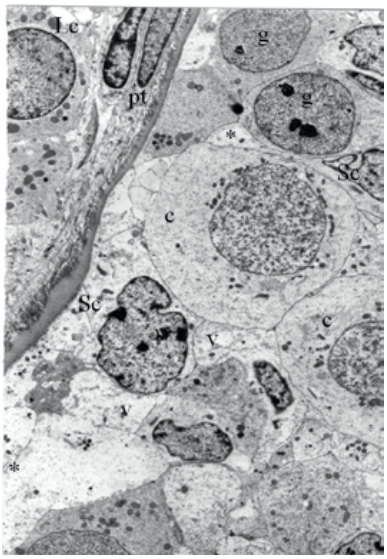

Fig. 9. Detail of T1 seminiferous tubule after 10-day exposure to bendiocarb (electron micrograph), magnification 3200×; Sc – Sertoli cell, v – vacuoles, * – empty spaces, g – spermatogonium, c – spermatocyte, Lc – Leydig cell, pt – peritubular cell

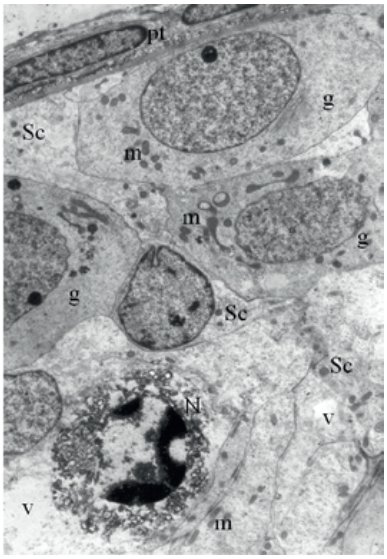

Fig. 10. Detail of T3 seminiferous tubule after 10-day exposure to bendiocarb (electron micrograph), magnification 3200×; Sc – Sertoli cell, m – mitochondria, v – vacuoles, N – necrotising cell, g – spermatogonium, pt – peritubular cell

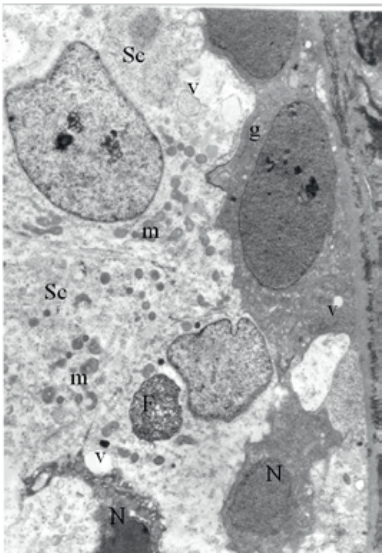

Fig. 11. Detail of T4 seminiferous tubule after 10-day exposure to bendiocarb (electron micrograph), magnification 3200×; Sc – Sertoli cell, v – vacuoles, m – mitochondria, g – spermatogonium, N – necrotising cell, F – fagocytised material

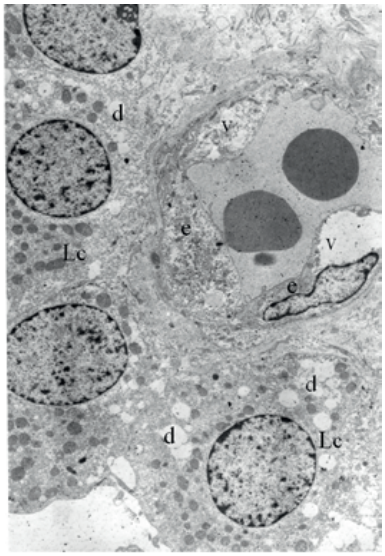

Fig. 12. Interstitial space within the testis after 10-day exposure to bendiocarb (electron micrograph), magnification 3200×; Lc – Leydig cell, d – lipid droplets, e – endothelial cell, v – vacuoles

The tubules with extremely low seminiferous epithelium, T4, possessed Sertoli cells highly reduced in size with normally shaped nuclei but vacuolated cytoplasm and somewhat swollen mitochondria. There were also various phagocytised particles present in many profiles within the cytoplasm of Sertoli cells. The majority of spermatogonia were shrivelled, with extremely electrondense cytoplasm and completely broken organelles. Some had characteristic nuclei but in other the nucleus was distinctly shrivelled. If spermatocytes were present, they possessed typical nuclei but more electronlucent cytoplasm, which contained damaged organelles. The tubules contained also some necrotising cells with shrivelled nuclei and deeply electrondense cytoplasm with damaged organelles (Fig. 11).

The Leydig cells residing in the interstitial spaces resembled those in the controls, with regular nuclei and typical organelles except for slightly higher number of lipid droplets within their cytoplasm. Endothelial cells comprising blood capillaries within the interstitium were mostly normal, but sometimes contained different vacuoles inside their cytoplasm (Fig. 12).

E2 group – electron microscopy

After 30-day exposure to bendiocarbamate we registered more severe ultrastructural disturbances among the cells within all tubules. In the degenerative tubules T3 (without adluminal compartment), there were some relatively slightly affected Sertoli cells, partly reduced in size, but many Sertoli cells were damaged considerably. They possessed strongly vacuolated cytoplasm with broken organelles and a higher quantity of the phagocytised material than usual. There were also shrivelled and necrotizing Sertoli cells with electrondense cytoplasm and damaged organelles were rarely seen within the epithelium. Many spermatogonia were extremely shrivelled, having electrondense cytoplasm with damaged organelles, but few of them seemed to be relatively intact. All spermatocytes were rather swollen and their electronlucent cytoplasm contained destroyed organelles, namely swollen mitochondria. Some necrotising cells were occasionally visible within the degenerative tubules (Fig. 13). The tubules with extremely low seminiferous epithelium, T4, comprised much shortened Sertoli cells with slightly affected nuclei but vacuolated cytoplasm and reduced number of organelles, which were damaged. There were only defective spermatogonia with irregular shape and electrondense cytoplasm with broken organelles (Fig. 14).

The Leydig cells within the interstitium were noticeably reduced in size. Their nuclei were mostly normal, but in some instances they were highly irregular in shape. The cytoplasm of Leydig cells contained many large lipid droplets, and their cellular organelles were partially swollen. The endothelial cells contained

various vacuoles within the cytoplasm. Most peritubular cells had characteristic ultrastructure but some revealed signs of cellular swelling (Fig. 15 and 16).

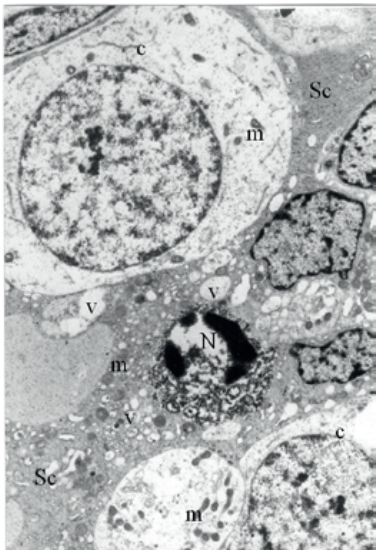

Fig. 13. Detail of seminiferous tubule T3 after 30-day exposure to bendiocarb (electron micrograph), magnification 3200×; Sc – Sertoli cell, v – vacuoles, m – mitochondria, c – spermatocyte, N – necrotising cell

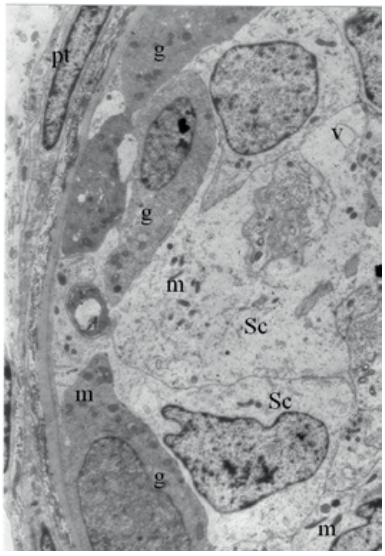

Fig. 14. Detail of seminiferous tubule T4 after 30-day exposure to bendiocarb (electron micrograph), magnification 3200×; Sc – Sertoli cell, v – vacuoles, g – spermatogonium, m – mitochondria, pt – peritubular cell

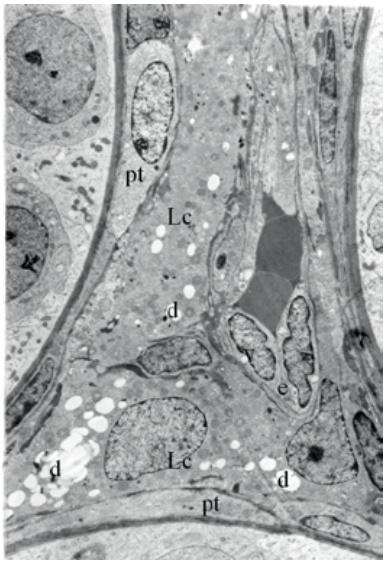

Fig. 15. Interstitial space within the testis after 30-day exposure to bendiocarb (electron micrograph), magnification 3200×; Lc – Leydig cell, d – lipid droplets, v – vacuoles, e – endothelial cell, pt – peritubular cell

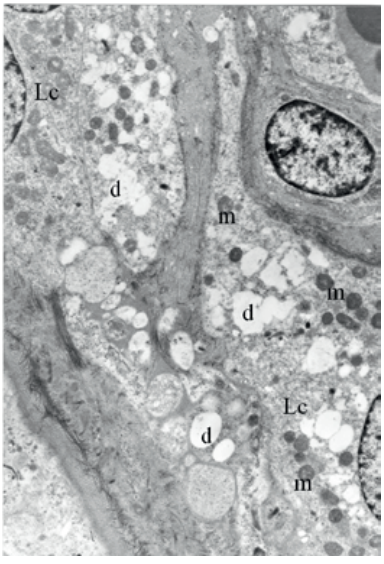

Fig. 16. More detailed view of the interstitial space within the testis after 30-day exposure to bendiocarb (electron micrograph), magnification 4800×; Lc – Leydig cell, d – lipid droplets, m – mitochondria

Discussion

The impact of several insecticides of the carbamate group, such as bendiocarb, propoxur, methomyl and benomyl, or their metabolites, such as carbendazim, on male reproductive system has been studied also from the morphological aspect, but these studies involved mostly relatively high single doses of these agents (Blanchard et al., 1996; Hess and Nakai, 2000). For example benomyl, perorally administered at a dose of 400 mg/kg, caused complete atrophy of seminiferous tubules in rat testes on day 70 post exposure with subsequent 58% reduction of the testicular weight (Hess and Nakai, 2000). Other authors evaluated also fertility of the tested rats and recorded 50% decrease in the examined parameters (Blanchard et al., 1996). Next study on rats, which dealt with 10-day exposure to the same dose of benomyl, showed less pronounced effect, as the total seminiferous tubular atrophy reached only 21% on day 70 and 50% on day 245 (Carter et al., 1987). The seminiferous tubular atrophy is often associated with a thicker basement membrane surrounding the tubules (Aire, 2005; Nisbal and Paniagua, 1984) and this characteristic seems to be quite important because, reportedly, once the basement membrane of the tubule has thickened, that portion of the tubule may no longer be available for normal spermatogenesis (Carter et al., 1987). One of the most serious features recorded in the present study was atrophy of seminiferous tubules with typical thickening of the basement membrane which afflicted 35% of seminiferous tubules after 10-day exposure and almost 60% of seminiferous tubules after 30-day exposure. Within the frame of confirmation of the prognosis of the testicular atrophy is the association with occluded efferent ductules and epididymal ducts by sloughed cells coming from the testes. Although we did not observe any ex-current ducts system in our experiment, another similar study, which produced the same morphological findings, allowed us to assume its partial occlusion (Gotoh et al., 1999).

Other histopathological signs observed in our study were the occurrence of degenerative tubules that missed their adluminal compartment and the disorganization of the remaining epithelium. This is in agreement with many studies conducted on different animal species. Peroral administration of carbendazim at a single oral dose of 400 mg/kg caused severe testicular changes in Japanese quails (Aire, 2005) very similar to those detected in our study, however, there was not obvious sloughing of the seminiferous epithelium in the quails. The seminiferous epithelial sloughing might be the major degenerative change in rats (Hess et al., 1991; Nakai et al., 1992). The mechanism by which the carbamate metabolites (similar to colchicine) cause the sloughing of germ cells may be associated with the disruption of microtubules and other cytoskeletal components within germ and Sertoli cells (Nakai, 1998; Nakai et al., 1995; Nakai et al., 2002). From the temporal aspect it was proved that sloughing of premature spermatogenic cells from the seminiferous tubules occurred as early as 2 h after

a single 400 mg/kg dose of carbendazim (Hess et al., 1991). Evidently, the adverse consequence of intensive sloughing of premature spermatogenic cells within the degenerative tubules is the ductal occlusion, which can subsequently multiply the destruction of seminiferous epithelium resulting in seminiferous tubular atrophy (de Kretser, 1977).

When the specific types of germ cells are destroyed, empty spaces appear in the epithelium where the cells are missing (Hess and Nakai, 2000) – this statement closely corresponds with our observations. The above mentioned changes may be attributed to abnormalities in the germ cells and/or dysfunction of the associated Sertoli cells, although some authors declared that there are various testicular toxicants which target only germ cells or only Sertoli cells in a very specific manner (Lee et al., 1999). For example, radiation exposure primarily targeted the actively dividing germ cells and acutely increased the incidence of germ cell death without causing any detectable damage to Sertoli cells (Hasegawa et al., 1998), while phtalates and 2,5-hexadione selectively caused dysfunction of Sertoli cells resulting in multiple germ cells sloughing (Blanchard et al., 1996; Richburg and Boekelheide, 1996). This confirmed the well-known fact that Sertoli cells play a critical role in normal testicular homeostasis (Markelewicz and Boekelheide, 2004; Boekelheide et al., 2003).

Regarding to steroidogenes, our structural and ultrastructural observations showed slight (shorter exposure) to medium (longer exposure) injury to Leydig cells which is in agreement with the studies dealing with testicular steroidogenesis. Some authors described reduction in plasma testosterone levels in rats treated with herbicide and also structural changes in interstitial endocrine cells, such as pleomorphism, a decrease in size or irregular nuclei (Mahgoub and Medany, 2000; Victor-Costa et al., 2010).

Studies dealing with prolonged or repeated exposure to relatively low doses of carbamates, such as the present study, are less common than those investigating effects of higher single doses. The authors who investigated long lasting or resumed exposure to carbamates generally predicated a not very strong effect, especially in adults. For example, in rats that daily received propoxur at a low dose (7.5 mg/day) for 28 days, no harmful effect was observed on parameters such as growth rate, cholinesterase levels or food consumption (Gosselin et al., 1984; Stellmann, 1998; USDA, 2001). With regard to reproductive parameters, there are two different standpoints about the effect of low doses of carbamates. One involves none or only weak negative effects on parameters such as parental food consumption, growth, lactation, and growth of the pups or fertility (Hayes and Laws, 1990; Worthing, 1983).

It is generally known, that especially birds are very sensitive to various toxicants including carbamates, although the toxicity varies with types of birds, for example oral LD50 in chickens is 47 mg/kg, while in Japanese quails it is about 5 mg/kg (Thomson, 1992).

Specific studies carried out on bird embryos exposed to low doses of bendiocarb failed to detect strong negative impacts (Petrovova et al., 2009a; Petrovova et al., 2009b). This may be explained by very efficient detoxification or transformation of the studied agents to non-poisonous forms, thus making it possible for rats or birds to tolerate daily doses approximately equal to the LD50 of insecticide for long periods, provided that the dose is spread out throughout the day rather than ingested all at once (Hayes and Laws, 1990). On the contrary, several reproductive studies claimed clear adverse effect of perorally applied carbamates at low dosages on newborn rats: propoxur at a dose of 1.6 mg/kg fed to rats between 6th and 15th day of pregnancy, and on the 15th day after birth had evincible negative effect on newborn animals (RTECS, 2001). The offspring of female rats fed 5 mg/kg of propoxur during gestation and weaning exhibited reduced birth weight, retarded development of some reflexes, and evidence of central nervous system impairment (Hayes and Laws, 1990). Although low doses of the studied pesticides were not evaluated as very dangerous, attention should be paid to manipulation with them and ways of their application, especially with regard to young or pregnant individuals, or their protection with vitamins, antioxidants, or other effective agents (Rajeswary et al., 2007).

Despite the fact that the present study investigated a relatively low dose of the tested substance it revealed quite substantial impact on the testes of rats which can indicate some risk related to fertility in humans.

Conclusions

Peroral administration of bendiocarb at a dose of 5 mg/kg per day affected adversely the rabbit testis after 10-day and especially after 30-day exposure. The testicular parameters, such as the absolute and relative weight of the testis, were significantly lower in the treated animals compared to the control, which may be attributed to degenerative changes detected by light and electron microscopy as well as by morphometric analysis. We assumed that the degenerative changes in seminiferous tubules as well as in interstitial Leydig cells were caused by direct impact of bendiocarb and its metabolites.

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