Effect of bendiocarb on rabbit testicular structure and spermatozoa motility

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Introduction

Exogenous contaminants with different effects on living organisms include also agrochemicals. These chemicals are used in agriculture with a wide range of products for plant nutrition, crop and animal protection. Such substance is also bendiocarb (2.2-dimethyl-1.3-benzodioxol-4-yl-N-methylcarb) (Sirotakova et al., 2001). No human and animal data was found on the ability of bendiocarb to cause male reproductive problems. Spermatozoa motility is essential for their distribution in the female sexual system and the penetration into the egg. Its monitoring is one of the most important criteria for assessing the quality of semen in practice (Massanyi et al., 2002). The aim of our study was to analyze the *in vivo* effects of different bendiocarb concentrations on rabbit testicular structure and *in vitro* effect on the spermatozoa kinetic parameters.

Materials and methods

In vivo experiment

Animals

Male rabbits (*Oryctolagus cuniculus*, hybrid Hyla-27, n=9) aged 54 days with an average weight of 1250 g were used. Bendiocarb (2,2-dimethyl-1,3-benzodiox-

ol-4-yl-N-methylcarb, Bendiokarb Tech, 98,9%, Bayer, Germany) tablets were administered daily to rabbits at a dose of 5 mg/kg of body weight for 13 days. The reaction of animals to bendiocarb was relatively strong, accompanied by dehydration, diarrhea and alopecia. Hence, it was the 13th day when the doses were limited to 48-hour intervals. The control group did not receive bendiocarb and was fed with a standard granulated compound for rabbits. The killing of the experimental animals was carried out painlessly by ether anesthesia at the 3rd day (1st sampling) and the 60th day (2nd sampling) after the last dose of bendiocarb.

Samples collection

For microscopic analysis samples were taken from the rabbit testis. The samples were fixed in 10% formol, dehydrated in a graded series of ethanol and embedded into paraffin wax. The whole testes were sectioned on a microtome. The serial 7–12 μm thick sections were stained with haematoxylin and eosin (Massanyi et al., 2007; Lukac et al., 2009; Petrovova et al., 2011).

Morphometry

At least five sections from each animal were measured under a light microscope (Carl Zeiss NU-2) with photomachine (Olympus Provis AX) and the Image ProPlus software (Media Cybernetic). The relative volume of the germinal epithelium, interstitium and lumen were measured with respect to each testis, based on micromorphological criteria (Massanyi et al., 2007; Toman et al., 2002).

In vitro experiment

Animals

Male rabbits (n=5) from the New Zealand White – breed (APRC, Nitra, Slovak Republic) were selected on the basis of age normally associated with reproduction (12–14 months) and used in the experiment. The animals were housed in a partially air-conditioned rabbit house (APRC, Nitra) under a photoperiod 16L:8D (minimum light intensity of 80 lux). They were in individual cages fed with a commercial diet and were provided water *ad libitum*. Air temperature of 17±2°C and relative humidity of 70±5% were maintained in the rabbit house.

Semen collection and *in vitro* culture

Semen was collected on a single day (early in the morning) with the help of an artificial vagina. Immediately after collection the individual doses of semen exhibiting a white color without presence of any gel and artificial particles, were

mixed together so as to acquire a pooled sample. The samples were stored in the laboratory at room temperature (20°C) and diluted with saline solution (sodium chloride, 0.9% w/v, Bieffe Medital, Italy). For the *in vitro* culture, bendiocarb (2,2-dimethyl-1,3-benzodioxol-4-yl-N-methylcarb, Bendiokarb Tech, 98,9%, Bayer, Germany) was added to semen samples in various concentrations (experimental groups: B1 – 0.268 mg/ml, B2 – 0.214 mg/ml, B3 – 0.161 mg/ml, B4 – 0.107 mg/ml and B5 – 0.054 mg/ml, control group – BC – with no bendiocarb addition) in the form of saline solutions (sodium chloride, 0.9% w/v, Bieffe Medital, Italy), which were prepared immediately before use (Tab. 1).

Tab. 1. Scheme of experimental groups for the spermatozoa motility analysis

Experimental group	Ejaculate (ml)	Physiological solution (ml)	Bendiocarb solution (ml)	Bendiocarb (mg/ml) concentration (mg/ml)
BC	0.1	0.5	-	0
B1	0.1	-	0.5	0.268
B2	0.1	0.1	0.4	0.214
B3	0.1	0.2	0.3	0.161
B4	0.1	0.3	0.2	0.107
B5	0.1	0.4	0.1	0.054

Spermatozoa motility

Each of thus prepared samples was evaluated using a Computer Assisted Semen Analyzer (CASA) system – Sperm Vision (Minitüb, Tiefenbach, Germany) equipped with a microscope (Olympus BX 51, Japan) to assess the spermatozoa motility. Each sample (10 µl drop) was placed into Makler Counting Chamber (depth 10 µm, Sefi-Medical Instruments, Germany). The accuracy of CASA for evaluation of rabbit spermatozoa motility, as well as Makler chamber manual examination has been already described (Roychoudhury et al., 2009; Massanyi et al., 2008; Lukac et al., 2011). A heated stage (37°C) was used during the entire analysis and multiple fields (in less than 2) were analyzed for each sample. Specific CASA software for rabbit spermatozoa motility analysis was used. Using the rabbit specific set up the following parameters were evaluated – total motile spermatozoa (MOT, %, motility > 5 µm/s), progressively motile spermatozoa (PRO, %, motility > 20 µm/s), average path distance (DAP, µm), curved line distance (DCL, µm), straight line distance (DSL, µm), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), straightness index (STR, the average value of the ratio VSL:VAP), linearity index (LIN, the average value of the ratio VSL:VCL), wobble (WOB = VAP:VCL), ampli-

tude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz) in different time periods (Times 0, 30, 60, 90, 120, 150 and 180 min) of culture.

Statistical analyses

Obtained data (expressed in means) were statistically analyzed with the help of the PC program Excel and a statistics package SAS 9.1 (SAS Institute Inc., USA) using the Student's t-test. Statistical significance was indicated by p values of less than 0.05.

Results

In the control animals each seminiferous tubule had a central lumen lined by an actively replicating germinal epithelium mixed with a population of supporting Sertoli cells. Between seminiferous tubules the interstitium containing interstitial cells and blood vessels was present. The largest relative volume in the germinal epithelium was observed in the control group. In both experimental groups a decrease in comparison with the control group was detected but the differences were not significant. The lowest volume in germinal epithelium was in the group from the 1st sampling. The relative volume in the interstitium was increased in both experimental groups in comparison to the control group. The highest volume in the interstitium was in the group from the 1st sampling. An increase in the relative volume of the lumen was registered in both experimental groups in comparison with the control group, but the highest volume in lumen was also in the group from the 1st sampling. Relative volumes in lumen, germinal epithelium and interstitium are shown in Figures 1–3. Qualitative analysis detected a dilatation of blood vessels in the interstitium, undulation of the basal membrane and some empty spaces in the germinal epithelium.

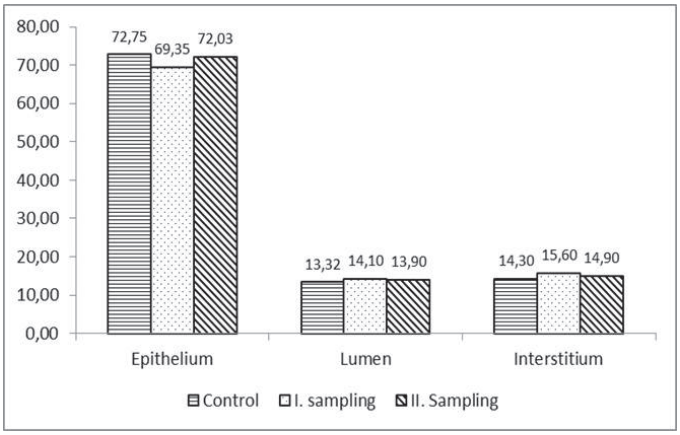

Fig. 1. Relative volume of germinal epithelium, lumen and interstitium in rabbit testes (%)

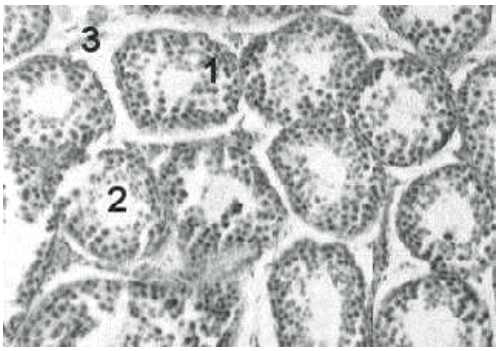

Fig. 2. Testicular structure in the control group. Seminiferous tubules, with high layer of germ cells – the seminiferous epithelium (1) are visible. In the centre of the tubules is the lumen (2). Between the tubules is the interstitium (3) with Leydig cells

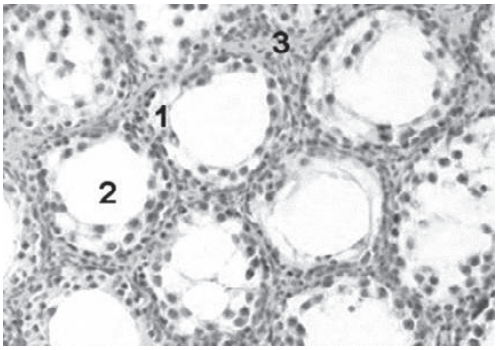

Fig. 3. Testicular structure after bendiocarb administration. Seminiferous tubules with a relatively low layer of germ cells – a decreased seminiferous epithelium (1) with some empty spaces is detected. The lumen (2) in the tubules has larger diameter than in the control group. In the interstitium (3) also dilatation of blood vessels and an undulation of the basal membrane after bendiocarb administration was detected

Spermatozoa motility analysis at Time 0 detected a slight increase in comparison with the control group. However, after 30 min of incubation the motility was decreased, and the highest motility was observed in the control group BC (95.64±2.26%). At Time 0, the highest motility was detected in group B3 ($p<0.05$), attaining 87.33±9.02% which received bendiocarb administration of 0.161 mg/ml. The lowest spermatozoa motility was detected in group B1 (30.15±22.20%) with the highest bendiocarb administration (0.268 mg/ml) in Time 180. The motility decreased with increasing the bendiocarb administration and with extending the period of incubation. However, in Time 90 and 120

the highest motility was in group B5 with the lowest administration of bendiocarb (0.054 mg/ml).

The progressive motility showed a decreasing tendency with experimental bendiocarb administration. At Time 0 a higher progressive motility was detected in each experimental group in comparison to the control group except group B1 with the lowest progressive motility (66.91±13.16%). But after 30 min of incubation the progressive motility decreased in all groups in comparison with the control group (91.07±3.96%). Increased doses of bendiocarb and the period extending of incubation decreased the progressive motility as well. However, in Time 90 and 120 the highest progressive motility was in group B5 with the lowest administration of bendiocarb (0.054 mg/ml). The lowest progressive motility was also in group B1 (9.01±9.00%) after 180 min of incubation with the highest bendiocarb administration (0.268 mg/ml). Data of motility and progressive motility parameters in different time periods are illustrated in Figures 4–10.

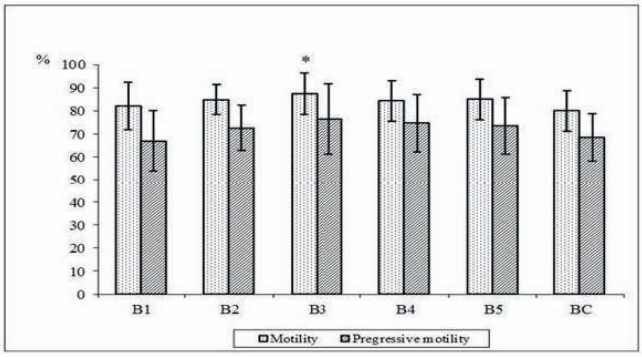

Fig. 4. Motility and progressive motility of the rabbit spermatozoa at Time 0; *p<0.05

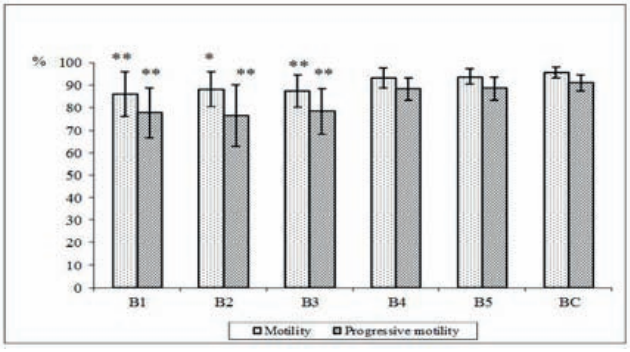

Fig. 5. Motility and progressive motility of the rabbit spermatozoa in Time 3; *p<0.01, **p<0.001

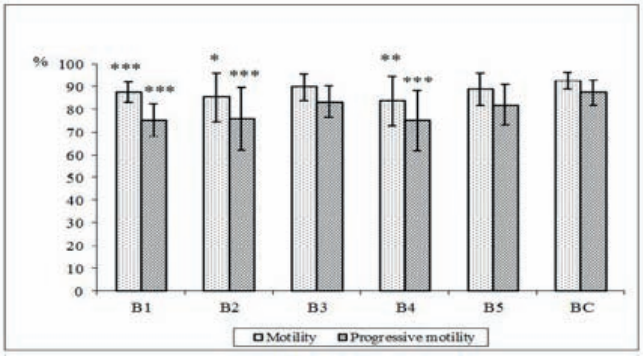

Fig. 6. Motility and progressive motility of the rabbit spermatozoa in Time 60; *p<0.05, **p<0.01, ***p<0.001

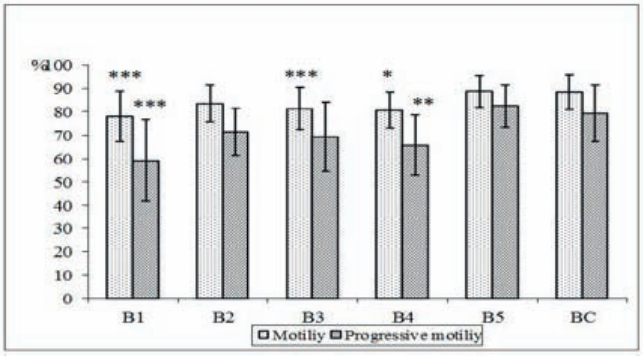

Fig. 7. Motility and progressive motility of the rabbit spermatozoa in Time 90; *p<0.05, **p<0.01, ***p<0.001

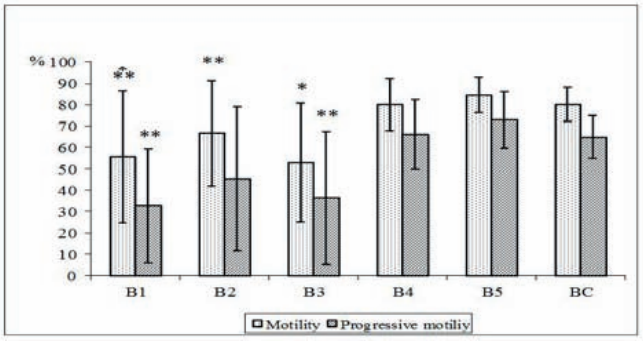

Fig. 8. Motility and progressive motility of the rabbit spermatozoa in Time 120; *p<0.01, **p<0.001

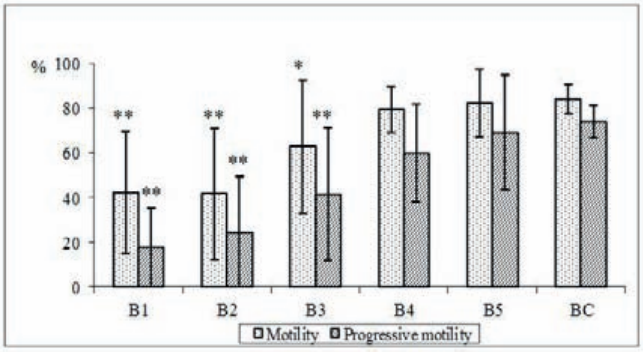

Fig. 9. Motility and progressive motility of the rabbit spermatozoa in Time 150; *p<0.01, **p<0.001

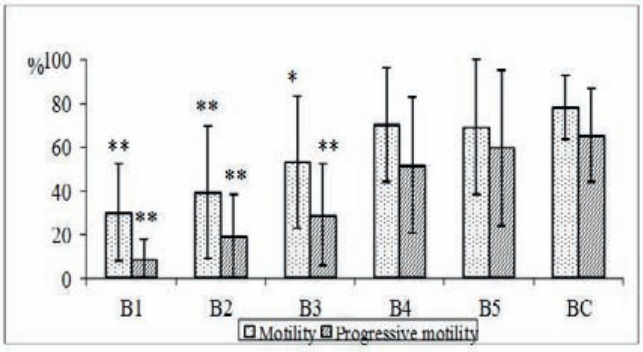

Fig. 10. Motility and progressive motility of the rabbit spermatozoa in Time 180; *p<0.01, **p<0.001

Detailed evaluation of spermatozoa distance (DAP, DCL, and DSL) and velocity (VAP, VCL, and VSL) parameters detected the decrease depending on the Time of incubation and on the bendiocarb concentration almost in all experimental groups in comparison to the control group (Tab. 2 and 3). A significant decrease (p<0.05–0.001) was detected in groups B1, B2 and B3 with the highest bendiocarb concentrations (0.268–0.161 mg/ml) at the beginning of the cultivation. Extending the period of incubation a significant decrease was observed in all experimental groups except for group B5 with the lowest bendiocarb concentration (0.054 mg/ml).

Observing the straightness a similar decrease, depending on the time of incubation and on the bendiocarb concentration almost in all experimental groups in comparison to the control group was detected. At Time 0 only in the groups with the highest bendiocarb concentrations (B1 and B3) was a significant (p<0.05–0.01) decrease in comparison to the control group. After 180 min in-

cubation a significant decrease ($p<0.001$) was also in groups with the highest bendiocarb concentrations (B1 and B2) in comparison to the control group.

Tab. 2. Values of spermatozoa motility parameters in each experimental group at the Time 0, 30, 60 and 90; DAP – average path distance (μm); DCL – curved line distance (μm); DSL – straight line distance (μm); VAP – average path velocity (μm/s); VCL – curvilinear velocity (μm/s); VSL – straight line velocity (μm/s)

TIME 0						
Group	**DAP**	**DCL**	**DSL**	**VAP**	**VCL**	**VSL**
B1	23.93±6.36	52.02±10.73	19.51±6.08	54.09±14.51	116.77±23.91	44.18±13.76
B2	26.27±3.55	54.64±4.06	21.72±4.36	58.93±8.65	122.05±9.57	48.89±10.31
B3	26.29±4.38	53.14±6.66	20.95±4.02	60.55±11.17	121.35±15.00	48.54±10.27
B4	29.03±6.83	55.08±10.18	23.98±7.14	66.85±15.44	125.99±21.78	55.52±16.32
B5	30.56±5.64	58.01±7.20	25.09±5.64	69.53±12.18	131.13±14.68	57.37±12.27
BC	30.14±8.48	54.60±7.90	26.20±8.88	69.04±19.59	124.53±18.48	60.17±20.54
TIME 30						
B1	27.66±4.74	56.14±6.91	22.54±4.67	62.03±10.47	125.74±15.46	50.60±10.31
B2	28.03±2.19	53.60±3.63	20.99±2.71	65.97±5.27	125.70±8.96	49.60±6.60
B3	32.21±2.15	61.91±5.69	24.28±3.21	74.78±4.55	143.23±10.77	56.54±6.98
B4	32.41±3.27	59.19±6.22	24.23±3.47	76.29±6.62	139.03±12.69	57.12±7.12
B5	32.67±4.37	59.11±8.77	24.39±4.55	76.32±9.28	137.50±18.85	57.21±9.84
BC	32.51±2.99	53.94±4.36	26.14±3.44	76.03±6.05	125.75±8.70	61.35±7.11
TIME 60						
B1	25.24±4.51	51.78±7.68	19.52±3.84	57.91±10.67	118.36±17.95	44.81±8.95
B2	30.80±6.02	61.52±13.68	23.29±4.74	70.74±13.01	140.93±29.77	53.37±9.98
B3	27.71±1.38	53.75±5.66	21.49±1.61	64.60±3.37	125.03±11.42	50.26±3.83
B4	31.13±4.97	59.53±11.52	24.26±4.53	72.14±10.66	137.47±24.82	56.33±9.87
B5	32.36±3.72	57.86±9.00	25.70±2.62	75.20±8.91	134.28±21.36	60.02±6.19
BC	33.21±4.44	57.11±11.02	25.93±4.22	77.47±9.91	132.90±24.93	60.70±9.50
TIME 90						
B1	20.80±5.37	46.37±8.69	16.77±5.18	45.97±11.94	101.96±18.77	37.10±11.44
B2	21.42±4.17	47.00±6.74	17.45±3.86	48.44±9.56	105.94±14.58	39.48±8.78
B3	25.01±6.43	50.67±9.01	21.05±6.34	56.26±15.46	113.35±21.13	47.48±15.05
B4	25.46±4.69	50.00±8.02	20.61±3.58	58.56±10.66	114.69±17.68	47.44±7.66
B5	29.66±1.48	54.40±3.54	24.23±1.86	68.93±3.34	125.96±6.12	56.51±3.88
BC	30.14±3.82	52.43±4.21	24.25±4.34	71.13±8.75	123.34±9.01	57.42±10.01

Tab. 3. Values of spermatozoa motility parameters in each experimental group at the Time 120, 150 and 180; DAP – average path distance (μm); DCL – curved line distance (μm); DSL – straight line distance (μm); VAP – average path velocity (μm/s); VCL – curvilinear velocity (μm/s); VSL – straight line velocity (μm/s)

TIME 120						
Group	**DAP**	**DCL**	**DSL**	**VAP**	**VCL**	**VSL**
B1	11.01±4.68	28.63±12.80	7.56±3.43	24.03±10.44	61.86±27.54	16.58±7.73
B2	15.57±5.36	38.00±9.13	11.22±5.20	34.97±12.91	83.98±21.12	25.42±12.40
B3	17.39±8.60	38.11±18.32	13.16±7.37	38.51±19.95	83.90±41.33	29.25±17.10
B4	21.56±6.14	45.38±6.75	17.00±5.31	49.47±15.37	103.13±18.33	39.13±13.15
B5	26.20±8.68	51.33±10.76	21.06±7.39	60.16±20.90	117.05±27.14	48.50±17.78
BC	27.83±2.91	51.73±5.31	22.52±3.23	64.73±6.44	119.86±11.44	52.54±6.97
TIME 150						
B1	8.09±5.77	22.21±16.25	5.61±4.14	17.51±12.59	47.55±34.52	12.18±9.13
B2	8.87±8.03	21.19±18.64	6.98±6.43	19.61±17.90	46.47±41.20	15.44±14.37
B3	14.02±8.94	31.17±18.92	11.33±7.63	31.49±20.39	69.59±42.53	25.46±17.47
B4	19.46±6.48	43.41±9.19	15.01±5.84	43.57±15.21	96.62±21.57	33.63±13.32
B5	24.94±9.43	47.86±12.37	19.91±8.14	57.77±22.55	110.10±30.68	46.46±19.58
BC	31.05±5.60	57.74±9.77	25.06±5.33	71.94±12.24	133.53±21.48	58.18±11.74
TIME 180						
B1	7.29±5.65	17.78±13.62	4.95±3.78	15.93±12.48	38.34±29.32	10.86±8.43
B2	7.75±6.68	19.90±18.05	5.71±4.90	17.26±14.67	43.52±38.29	12.91±11.18
B3	9.48±6.01	25.01±15.66	6.91±4.78	20.47±13.07	53.74±33.72	14.98±10.46
B4	15.26±8.02	32.05±15.06	12.36±6.97	34.50±18.75	71.84±34.42	28.06±16.37
B5	23.46±12.43	45.42±23.18	19.05±10.60	53.91±28.76	103.85±53.22	43.94±24.50
BC	29.02±5.14	55.41±7.51	23.00±4.57	67.64±12.39	128.44±18.10	53.77±10.60

Linearity was decreased in all experimental groups in comparison to the control group. In Time 0 a significant decrease was in groups B1 ($p<0.001$), B2 ($p<0.01$) and B3 ($p<0.01$) with the highest bendiocarb concentrations. In Time 30 to 180 we observed a significant ($p<0.001$) decrease in all experimental groups in comparison to the control group. In Time 180 the lowest values were in all experimental groups in comparison to the control group.

In wobble observation at Time 0 a significant decrease was detected only in groups B1 and B2 with the highest bendiocarb concentrations in comparison to the control group. With extending incubation the wobble decreased and in Time 180 we observed a significant ($p<0.001$) decrease also in groups with the

highest bendiocarb concentrations (B1, B2 and B3) in comparison to the control group.

In the amplitude of lateral head displacement observation (Fig. 11) we detected a significant ($p<0.001$) decrease in the experimental group with the highest bendiocarb concentration (B1) (3.83±0.44 μm) and a significant ($p<0.001$) increase in groups with the lowest bendiocarb concentrations – B4 (5.33±0.39 μm) and B5 (5.40±0.75 μm) in comparison to the control group (4.62±0.52 μm) in Time 30. After 90 to 180 min incubation a decrease in all experimental groups in comparison to the control group was found.

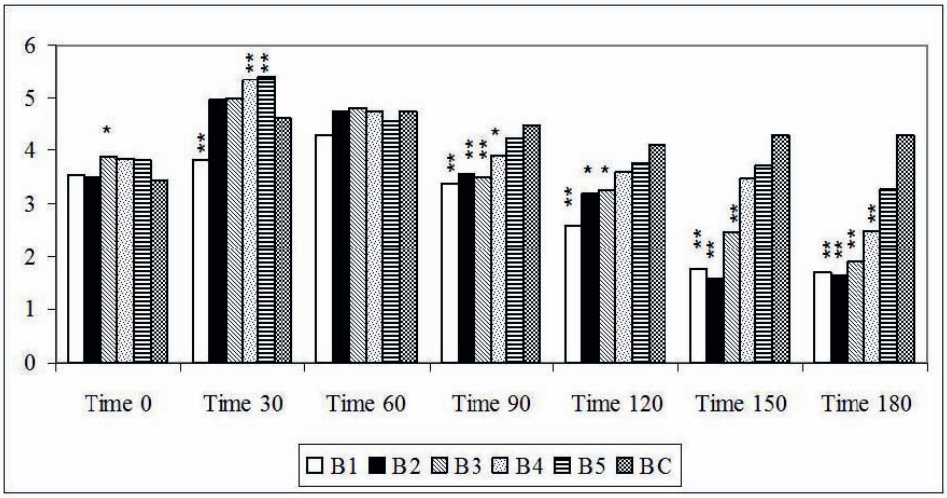

Fig. 11. Amplitude of lateral head (ALH, μm/s) in each experimental group and at different incubation time; *p<0.05, **p<0.001

The biggest differences were at Time 180, where a significant ($p<0.001$) decrease was observed in groups B1 (1.72±1.48 μm), B2 (1.63±1.41 μm), B3 (1.90±1.17 μm) and B4 (2.50±1.20 μm) in comparison to the control group (4.30±0.68 μm).

Measurement of the beat cross frequency (Fig. 12) showed a significant ($p<0.05$) increase in group B3 (33.46±3.09 Hz) at Time 0 and in B1 (36.27±3.31 Hz) at Time 30 in comparison to the control group (37.15±3.65 Hz/Time 0, 33.00±3.25 Hz/Time 30). A significant decrease was observed after 120 and more min of incubation. The highest decrease was also in the groups with the highest bendiocarb concentrations – B1 (12.45±9.58 Hz), B2 (14.58±13.41 Hz) and B3 (19.01±12.57 Hz) in comparison to the control group (32.42±2.34 Hz).

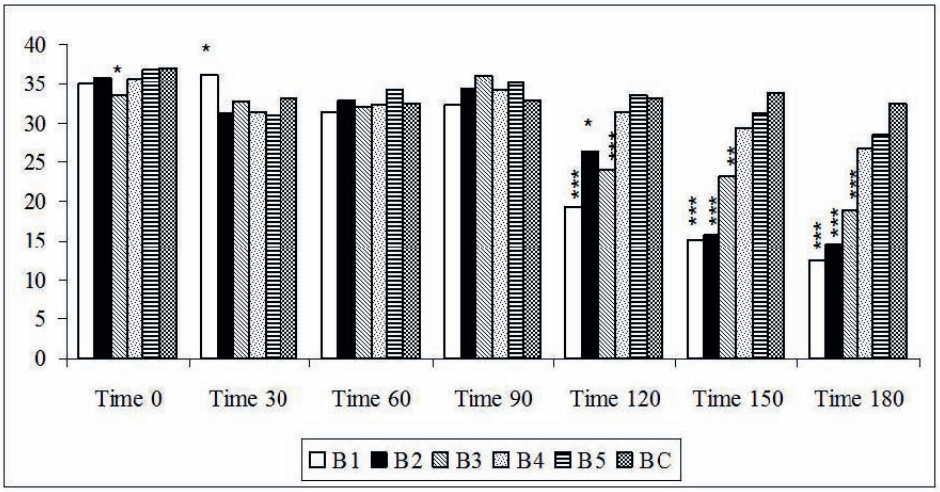

Fig. 12. Beat cross frequency (BCF, Hz) in each experimental group and at different incubation times; *p<0.05, **p<0.01, ***p<0.001

Discussion

In this work we studied the effect of different bendiocarb concentrations on structural and functional changes in the rabbit testes, as well as the kinetic parameters of rabbit spermatozoa.

After *in vivo* bendiocarb administration was likely to decline spermatogenesis in the testis as a result of ultrastructure disruption in place of spermatozoa production. Observations of bleeding in the interstitium are likely to reduce the testosterone production in Leydig cells, which decreases male fertility (Dzugan et al., 2011; Dzugan and Ksiazkiewicz, 2009). This damage, however, was only temporary and disappeared after some time due to bendiocarb excretion from the body and testes recovering. This confirms the claim of Mojzisova and Sulla (2005), which states that carbamates leave residues only slightly. Elimination from the body usually takes place up to eight days after the contamination, which depends mainly on the chemical nature of the derivative (Lukas and Zak, 2007). Challis and Aacock (2006) state that the bendiocarb elimination takes place in the body excretion via urine. To avoid further bendiocarb action, the symptoms disappeared after 24 hours. Petrovova et al. (2010a) monitored the bendiocarb effects on the development of chicken embryos. Bendiocarb was injected through a paper membrane at 200 µl. The authors analyzed the embryo lethality and weight loss in hatched chicks. However, they indicate that bendiocarb did not cause significant changes in the embryos compared with the control group.

Petrovova et al. (2010b) also observed changes in rabbit lymphatic tissue after bendiocarb administration. The authors argue that bendiocarb did not cause any significant changes in structure and function of the rabbit small intestine. They also state that long-term bendiocarb administration to rabbits at a dose of 5.0 mg/kg of body weight resulted in a suppression of functional lymphocytes and phagocytes activity, and relative lymphocytosis. Flesarova et al. (2007) report the bendiocarb action on the rabbit thymus histological structure. Bendiocarb was administered *per os* for 90 days. Quantitative evaluation demonstrated a significantly increased relative cortex abundance and lower representation of thymus medulla. By detailed morphometric analysis they found that the number and diameter of thymocytes was reduced after bendiocarb administration compared with the control group without bendiocarb addition. Capcarova et al. (2010) determined the bendiocarb effect on selected parameters of rabbit homeostasis. Animals from experimental groups received bendiocarb *per os* in a dose 5 mg/kg of body weight per day. A significant increase in the creatinine content, increase of aspartate aminotransferase and gamma glutamyl transferase and a decrease of glutamate dehydrogenase inform about possible failure of liver and/or kidney caused by bendiocarb. They claim that the decrease of haemoglobin, mean corpuscular haemoglobin concentration, platelet count can signify a defection in haemoplastic system, functionally (activity) similar to testis.

In the *in vitro* experiment the highest bendiocarb dosages and prolonging time of incubation decreased the motility, progressive motility and all kinetic parameters of rabbit spermatozoa. Ostro and Urdzik (2005) indicate that estrogenic and antiandrogenic effects of xenobiotics reduce the number and spermatozoa motility and thus adversely affect the fertility in men. They indicate that xenobiotics may influence the development of male germ cells at different stages from proliferating spermatogonia to mature spermatozoa. Their toxic effects may be manifested by cell death, sublethal damage of the cells or by changing its genetic makeup. Sharpe and Skakkebaek (1994) found that the decline in spermatozoa number by the action of xenobiotics is also due to an increasing number of other diseases, male reproductive organs (testicular cancer). The cause of the rise in the incidence of these diseases may be an increased concentration of xenoestrogens in maternal blood during intrauterine development. Maracek and Antal (2005) state the effect of phenoxycarb (ethyl (2–4 fenoxyfenoxyethyl)) on the reproductive system. Feeding the sheep, this pesticide has induced a series of changes in the clinical and biochemical profile of blood serum, spermatozoa motility and morphology, the quality of the oestrus cycle and fertility levels according to reproductive parameters. They also observed patho-morphological changes in the reproductive organs of the experimental animals. The authors also describe the effect of another pesticide carbendazim (methyl 2-benzimidazole carbamate), which was tested in male rats. They observed effects on testes

similar to those described in this study. At the same time a reduced and disappeared fertilizing ability of males in the fifth post-expiration week has been found. Seminiferous tubule atrophy, histologically detected in the range of 85% of testes in rats after exposure to 245 days, is the result of synergistic effect of 2.5-hexandion and carbendazim on the molecular level of microtubules in ducts with subsequent disruption of spermatogenesis.

Conclusions

Our findings clearly suggest a negative effect of bendiocarb on the structure of seminiferous epithelium in the place of spermatozoa production as well as rabbit spermatozoa motility. These findings suggest that the bendiocarb reduces male fertilization.

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