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Effects of meldonium on sexual performance, sperm motility, testes morphology and blood biochemical markers in boars

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ABSTRACT

The objective of this study was to evaluate effects of long term (90-day) administration of meldonium [3-(2,2,2-trimethylhydrazinium) propionate] (mildronate, quaterin, MET-88) on sexual performance, sperm motility, testes morphology and biochemical blood markers in boars. Boars were treated with 2.0 g of meldonium daily for 90 days. Administration of meldonium improved sexual performance and sperm motility. Thus, the reaction time (time from exposure to the dummy to the start of ejaculation) was reduced and the progressive motility of spermatozoa was significantly increased in the meldonium-treated boars compared to that of the boars of control group. In addition, the spermatogenic epithelium was thicker and proliferation of interstitial endocrine cells (Leydig cells) was observed in meldonium-treated boars. The concentration of blood serum testosterone was higher in the meldonium-treatment group than in the control group. Meldonium did not affect the concentration of creatinine, total bilirubin, total cholesterol, glucose and aspartate aminotransferase/AST, alanine aminotransferase/ALT activity in blood plasma. In conclusion, 90-day administration of meldonium improved sexual performance and sperm motility of boars and it also increased concentration of testosterone in blood serum. Further studies are necessary to substantiate the potential use of meldonium as a sperm motility and/or sperm quality-enhancing agent in livestock.

1. Introduction

The sperm quality and sexual behavior are the key factors determining male fertility and thus, success of fertilization (Rodríguez-Martínez, 2007; Morrell et al., 2008). Therefore, drug treatments enhancing sperm quality and/or sexual performance could be one of the possible strategies to optimize fertilization in livestock (Louis, 1994; Jacino et al., 2007). Thus, it is important to provide a solid experimental evidence for efficacy and safety of such treatments in livestock.

Meldonium [3-(2,2,2-trimethylhydrazinium) propionate] (quaterin, mildronate, MET-88) is widely used in medicine as an anti-ischemic and cellular energy metabolism regulating drug for treatment of cardiovascular and central nervous system disorders (Simkovich et al., 1988; Kuwajima et al., 1999; Dambrova et al., 2002; Sjakste et al., 2005; Dzerve et al., 2010). These effects of meldonium are considered to be mainly related to its carnitine-lowering effects through inhibition of carnitine biosynthesis (Simkovich et al., 1988), as demonstrated by a number of animals studies (Simkovich et al., 1988; Dambrova et al., 2002, 2008; Liepiņš et al., 2006). Along with inhibition of the carnitine biosynthesis, meldonium has been reported to elevate concentrations of γ-butyrobetaine (GBB) hydroxylase in blood plasma and various tissues including testes (Liepiņš et al., 2006;

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Dambrova et al., 2008). GBB, in its turn has been reported to stimulate the sexual activity and potency (Kalvinsh et al., 2003). Therefore, meldonium could be expected to improve sexual performance. However, 14-day administration of meldonium (100 mg/kg) failed to affect the sexual performance and sperm quality in rats (Dambrova et al., 2008). At the same time, evidence from both animal and human studies suggests that increased levels of L-carnitine could have a positive effect on spermatogenesis and sperm quality (Matalliotakis et al., 2000; Lenzi et al., 2004; Stradaioli et al., 2004; Isidori et al., 2006; Li et al., 2007; Yeste et al., 2010). There are also studies showing that semen quality was not enhanced by carnitine supplementation (Kozink et al., 2004).

So far, effects of meldonium in farm animals have been little studied. In these studies, meldonium has been mainly tested for its effects on digestive and metabolic function (Kurashvili et al., 1983; Klinskaya and Romanov, 1985; Romanov, 1987). It has been also reported to reduce weaning stress in piglets (Tauritis et al., 1984). Furthermore, there are few studies on physiological effects of long-term (more than 14 days) administration of meldonium and these studies have been mainly restricted to its cardiovascular and metabolic effects in rodents and man (Liepinsh et al., 2009; Dzerve et al., 2010). In view of above, it appeared important to study effects of long-term administration of meldonium on mammalian reproductive function.

The objective of our study was to examine effects of 90-day meldonium administration on sperm motility and sexual performance (libido) in boars. In addition, effects of meldonium on morphology of testes and several biochemical markers of blood were studied.

2. Materials and methods

2.1. Subjects

A total of 12 Yorkshire boars were randomly selected during weaning of piglets from mothers born as littermates. Before beginning administration of meldonium, the boars were randomly assigned to two experimental groups of 6 animals each: the control group and the meldonium-treatment group.

The boars were individually housed under natural daylight (the length of daylight decreased during study from 15 h 20 min to 8 h 13 min), a constant room temperature of $20 \pm 1\,^{\circ}C$ and intensive rearing conditions. Boars received dry granulated standard feed (Ltd. Mūsa, Latvia) and water *ad libitum*.

All experimental procedures were performed in accordance with guidelines of the Council of the European Communities Directive of 24 November 1986 (86/609/EEC) and were approved by Ethics Council of Animal Protection at the Veterinary and Food Service, Riga, Latvia (Nr. 22; 04.15.2010).

2.2. Study design

The study consisted of two phases – training and experimental. The training for ejaculation started at the age of five months when boars had reached sexual maturity and lasted until they began to ejaculate on the dummy. At this point, the boars were 248–279 days old, with live weight of 126–156 kg and the drug-treatment was initiated. Meldonium (3-(2,2,2-trimethylhydrazinium)propionate) is obtained from JSC Grindeks, Latvia. Daily for 90 days (hereafter, the first and last day of administration referred to as Day 1 and Day 90, respectively) the boars were treated with 2.0 g of meldonium substance diluted in 2–3 g tap water, mixed with a handful of the concentrated feed and placed into each boar's feed bunk. Shortly after, consumption of the entire feed was confirmed and documented. Boars of the control group received the same amount of feed without meldonium.

At the end of the experiment, 10 boars (5 of each group) were euthanized by intravenous injection of T61TM solution (Intervet International B.V., EU) at the dose of 0.1 ml/kg. Two remaining boars (one of each group) were spared for post-experimental observations and they were euthanized one month later.

2.3. Sexual performance

To evaluate the sexual performance (libido) of boars, the reaction time (RT), defined as time elapsed from exposure to the dummy to the start of ejaculation, and the duration of ejaculation (DE) was measured (Estienne and Harper, 2000).

2.4. Quantity of ejaculate and motility of sperm

The semen was collected on the dummy using manual fixation of penis before initiation of drug-treatment (Day 0) and thereafter, once every 10 days (Wolf and Smital, 2009). Ten ejaculates in total were collected from each boar during the experiment. Immediately after collection, the ejaculates were weighed, filtered (US BAG^{TM}; Minitube, Germany) to remove gel and the sperm concentration in gel-free fraction was determined with photometer (SpermaCue; Minitube, Germany). The motility of spermatozoa was determined by taking a drop of semen on a warmed glass slide (37 °C) and covered with a cover slip. At least five fields of vision were looked through, and in each field, ten spermatozoa were evaluated at magnification of 100×, 200× and 400× under the light microscope. The same procedure was repeated four times in each ejaculate as a blind evaluation done by the same person. The proportion of progressive motility of spermatozoa was estimated by a ten-point score system. If all (100%) spermatozoa were progressively motile, then 10 points were assigned, if 90% spermatozoa were progressive motile, then 9 points, and so on.

The prepared smears were stained with Dip Quick Stain (Jorgensen Laboratories, Inc., USA) and evaluated for morphological abnormalities (cytoplasmic droplets, abnormal head shapes, tail defects and total number of abnormalities). Sperm abnormalities were recorded as percentages of the total number of 200 counted spermatozoa. The semen quality parameters were calculated before the administration of meldonium and further as the average values from

all semen collections during the following 40 days periods that corresponds to the total length of spermatogenesis in boars: Days 1–40; Days 41–80 and Days 81–92 (the end of study). If collecting of a semen specimen failed at more than one time point, the respective boar was excluded from any analysis involving quantitative and qualitative parameters of semen. Exact number of boars are shown in Section 3.

2.5. Weight and volume of testes

Immediately after euthanasia, the testes were removed and washed with saline (NaCl 0.9%). The weight of testes and epididymides and the volume of testes were determined.

2.6. Histology of testes

The sample from one testis of each boar ($n = 5$ boars in each group) sized 5 mm × 10 mm × 7 mm was obtained from the middle part of *margo liber* and fixed in 10% of neutral formalin, embedded in paraffin and cut in 5 μm thick sections. Slides were stained with hematoxylin–eosin, Van Giezon and Mallory tri-chrome according to the method previously described (Kiernan, 2008). Five histological slides from each boar were processed using an optical microscope (Leica DM500B) with the software programme Image-Plus 6.1. The thickness of spermatogenic epithelium for each slide was recorded in the form of average value measured and calculated by software from five fields of vision.

2.7. Blood biochemical markers

The blood for biochemical analyses was collected from *vena saphena medialis* at the beginning and end of the study (Day 0 and Day 92, respectively). The blood samples were taken at 10.00 AM. Levels of glucose and total bilirubin were measured in blood plasma by colorimetric test; creatinine, total cholesterol, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were measured in blood plasma by special chemistry tests. The blood samples were analyzed with Vitros DT 60 ll system and relevant kits following the standard test protocol by the producers (Ortho Clinical Diagnostics, Johnson & Johnson, USA). The serum concentrations of testosterone were measured by Testosterone EIA Kit (Cayman Chemical).

2.8. Statistical analysis

All parameters of ejaculates, sperm motility, testes and blood biochemical markers were checked for normality and expressed as means ± SEM. Between group differences were analyzed by a two-tailed unpaired Student's *t*-test (Stata-9 and Microsoft Excel standard program set). Percentages of abnormal sperm between groups were compared by two sample Wilcoxon rank-sum test. The parameters of sexual performance – reaction time (RT) and duration of ejaculation (DE) were analyzed as percentages of completed reaction and ejaculation, respectively, from all the semen collections throughout the study using the Kaplan–Meier graph and differences between the meldonium-treatment group and the control group were assessed by Wilcoxon (Breslow) test (Dohoo et al., 2010). A significance level of $P < 0.05$ was accepted throughout the study.

3. Results

3.1. Effects of meldonium on sexual performance

To evaluate the effects of meldonium on sexual performance of boars, the reaction time and duration of ejaculation were measured. Percentage of completed cases over the time from all semen collections throughout the study is shown in Fig. 1. During first 60 s after exposure to the dummy, reaction was completed and ejaculation started in 42% of boars treated with meldonium while in the control group ejaculation started only in 6% of boars. During the first 120 s, these numbers were 83% and 55%, respectively and in the first 180 s – 93% and 79%, respectively. All these between-group differences were statistically significant (Wilcoxon (Breslow) test, $P < 0.05$). No statistically

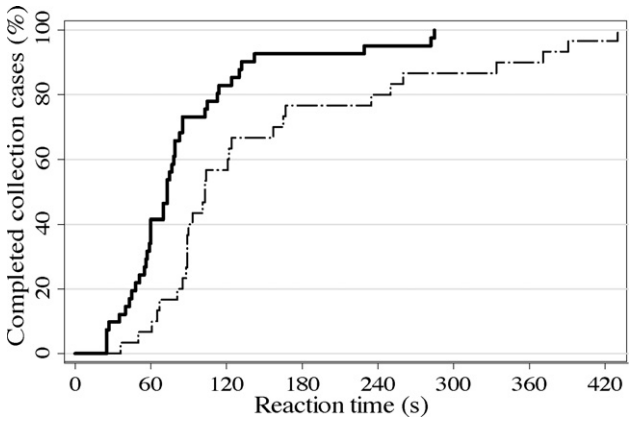

Fig. 1. Effect of meldonium on percentage of the completed sperm collection cases as a function of reaction time (time elapsed from exposure to the dummy to start of ejaculation) in boars. The boars were treated with 2.0 g of meldonium daily for 90 days. The control group was fed the same feed with no meldonium added. Solid line (–) represents meldonium-treatment group ($n = 6$); dashed line (– · – · –)–control group ($n = 4$).

Table 1
Effects of meldonium on ejaculate and spermatozoa (mean ± SEM).

Time of the measurement (study days)	Experimental group (number of boars)	Number of ejaculates	Weight of ejaculate (g)	Concentration of spermatozoa ($\times 10^6$ cells/ml)	Motility of spermatozoa (points)
0	Control (6)	4	165.5 ± 19.6	352.5 ± 14.6	4.6 ± 0.6
	Meldonium (6)	5	111.2 ± 11.6	385.0 ± 30.3	4.8 ± 1.0
1–40	Control (4)	11	157.3 ± 21.5	403.0 ± 31.2	5.3 ± 0.5
	Meldonium (6)	18	198.0 ± 16.6	408.2 ± 15.0	6.6 ± 0.3[a]
41–80	Control (4)	16	204.0 ± 23.1	416.3 ± 27.0	5.6 ± 0.5
	Meldonium (6)	23	198.7 ± 13.9	407.7 ± 12.5	7.3 ± 0.3[a]
81–92	Control (4)	6	199.5 ± 20.3	442.3 ± 41.4	6.0 ± 0.6
	Meldonium (6)	11	208.9 ± 15.4	471.0 ± 18.6	7.9 ± 0.2[a]

[a] $P < 0.05$ Meldonium *vs* control group at the same time of the measurement

significant differences were observed for duration of ejaculation between the meldonium-treatment and the control groups (data not shown).

3.2. Effects of meldonium on quantity of ejaculate and quality of sperm

To determine effects of meldonium on quantity of ejaculate and quality of sperm, the weight of ejaculate and the concentration, progressive motility and morphology of spermatozoa were evaluated. The initial values before beginning of drug-treatment (Day 0) and the average values for the 3 consecutive periods of spermatogenesis – Days 1–40, Days 41–80 and Days 81–92 (the end of study), are presented in Table 1. The weight of ejaculate and concentration of spermatozoa did not differ between the two experimental groups at any of the defined time points (Table 1). On Day 0, there were no differences in the progressive motility of spermatozoa between the meldonium-treatment and the control groups ($P > 0.05$). A gradual increase in the progressive motility of spermatozoa was observed by each consecutive period of spermatogenesis. During the first period of spermatogenesis (Days 1–40), the progressive motility of spermatozoa was 6.6 ± 0.3 points the meldonium-treatment group and 5.3 ± 0.5 points in the control group. In the second period of spermatogenesis (Days 41–80) motility reached 7.3 ± 0.3 points in the meldonium-treatment group meeting the accepted standards for sperm dilution and artificial insemination in swine (Foxcroft et al., 2008). At the end of study (Days 81–92) a further increase in motility up to 7.9 ± 0.2 points was found in the meldonium-treatment group which was significantly higher than the maximal value of 6.0 ± 0.6 points in the control group (Table 1). It is noteworthy that starting from Day 41 to the end of experiment, 36% of the ejaculate specimens collected from the boars of control group met the requirements for the artificial insemination (Foxcroft et al., 2008) while in the meldonium-treatment group the number of such specimens was more than double (73%). Percentages of abnormal spermatozoa in the meldonium *vs* control group were as follows: cytoplasmic droplets 0.1% *vs* 0.4%, abnormal head shapes 0.1% *vs* 0.1%, tail defects 0.4% *vs* 0.5%. Total abnormal forms of sperm were estimated as 0.6% and 1.0% in the Meldonium and control groups, respectively. Differences between groups were not significant ($P > 0.05$).

3.3. Effects of meldonium on volume and weight of testes

No significant differences between the meldonium-treatment group and the control group were found either for the weight and volume of testes or the weight of epididymides (Table 2).

3.4. Effects of meldonium on histology of testes

Histological analysis at the end of experiment showed a normal structure of testes in the control and the meldonium-teated boars (Fig. 2A and B, respectively). Seminiferous tubules were lined with sustentacular cells (Sertoli's cells) perpendicular to the basal membrane and spermatozoa were in various stages of development. Spaces between seminiferous tubules were filled with well-developed interstitial tissue rich in interstitial endocrine cells. In the meldonium-treatment group, a proliferation of interstitial endocrine cells was observed (Fig. 2B). In both experimental groups, there were many small blood vessels, blood and lymph capillaries within interstitial tissue.

To determine the effects of meldonium on the process of spermatogenesis, thickness of spermatogenic epithelium was measured. The spermatogenic epithelium

Table 2
Effects of meldonium on weight and volume of testes and epididymides (mean ± SEM).

Experimental group (number of boars)	Weight of testes without epididymides (g)		Volume of testes without epididymides (ml)		Weight of epididymides (g)	
	Right testis	Left testis	Right testis	Left testis	Right testis	Left testis
Control (5)	406.8 ± 35.9	422.0 ± 27.2	378.0 ± 33.8	384.0 ± 29.9	123.0 ± 9.7	127.2 ± 8.7
Meldonium (5)	432.6 ± 18.8	440.8 ± 22.3	414.0 ± 20.9	420.0 ± 21.0	129.8 ± 4.8	127.4 ± 3.4

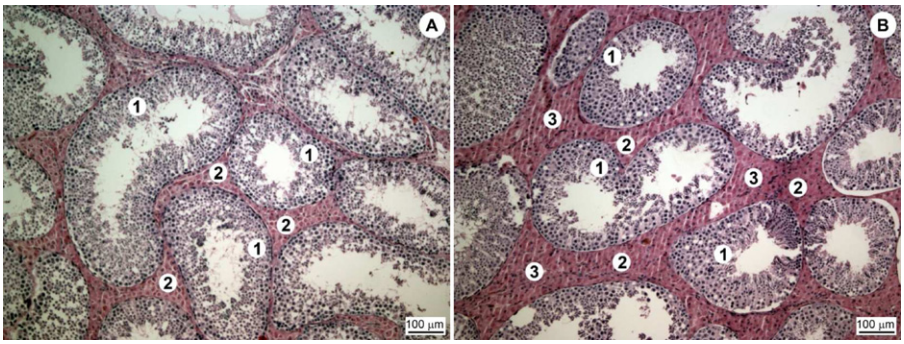

Fig. 2. Microphotographs showing representative sections of boar testes. The boars of the meldonium-treatment group (B) were treated with 2.0 g of meldonium substance daily for 90 days. The control group (A) received no meldonium. 1, Convoluted seminiferous tubules; 2, interlobular tissues; 3, proliferation of interstitial glandulocytes (Leydig cells). Hematoxylin–eosin staining 100×; bar = 100 μm.

Table 3
Effects of meldonium on blood biochemical markers (mean ± SEM).

Time of the measurement	Before treament (Day 0)		After treatment (Day 92)	
Experimental group (number of boars)	Control (6)	Meldonium (6)	Control (6)	Meldonium (6)
Biochemical markers:				
Creatinine (μmol/l)	213.3 ± 22.2	223.1 ± 13.1	164.7 ± 12.2	164.5 ± 17.0
Total bilirubin (μmol/l)	2.45 ± 0.25	2.9 ± 0.18	1.72 ± 0.19	2.25 ± 0.17
Cholesterol (mmol/l)	1.75 ± 0.11	1.71 ± 0.08	1.39 ± 0.12	1.34 ± 0.12
Glucose (mmol/l)	4.25 ± 0.14	4.38 ± 0.20	4.53 ± 0.16	4.38 ± 0.2
ASAT (IU/l)	44.3 ± 4.10	38.3 ± 3.90	34.3 ± 2.8	30.2 ± 2.3
ALAT (IU/l)	56.7 ± 2.60	48.7 ± 2.60	63.5 ± 3.7	57 ± 1.9
Testosterone (ng/ml)	1.5 ± 0.6	2 ± 0.6	1.2 ± 0.5	3.4 ± 0.7[a]

[a] $P < 0.05$ Meldonium *vs* control group at the same time of the measurement.

was significantly thicker ($P < 0.05$) in the meldonium-treatment group (90.4 ± 2.23 mkm) than in the control group (76.2 ± 2.49 mkm).

3.5. Effects of meldonium on blood biochemical markers

The blood biochemical markers are presented in Table 3. There were no differences in concentrations of creatinine, total cholesterol, total bilirubin, glucose and activity of aspartate aminotransferase/AST, alanine aminotransferae/ALT between the meldonium-treatment group the control group. All these markers were in the normal range. At the beginning of study, there were no differences in serum concentration of testosterone between the meldonium-treated and the control boars. In contrast, concentration of testosterone in blood serum at the end of study was significantly higher ($P < 0.05$) in meldonium-treated boars than in the control boars (Table 3).

4. Discussion

To our knowledge, this is the first study showing that 90-day administration of meldonium improved sexual performance and sperm motility in boars. Furthermore, this prolonged administration of meldonium was not associated with unwanted side effects such as pathological changes in testes or alterations in blood biochemical markers of liver and kidney function. The reaction time (time elapsed from exposure to the dummy to the start of ejaculation) in meldonium-treatment group was shorter and ejaculation started earlier than in the control group. In addition, the percentage of completed sperm collection cases was also increased in meldonium-treatment group. The exact mechanism underlying improved sexual performance in meldonium-treated boars remains to be elucidated. However, this improved performance could be linked to higher blood serum concentrations of testosterone in meldonium-treated boars.

As for the semen quantity and quality, meldonium-treatment affected neither the ejaculate weight nor spermatozoa concentration nor percentage of abnormal sperm. Abnormalities of spermatozoa did not exceed the accepted values: cytoplasmic droplets <15%, abnormal head shapes <1%, tail defects <3%, total abnormal forms ≤20% (Flowers, 1998). However, a long-lasting increase in spermatozoa motility was found in meldonium-treated boars. It is noteworthy that in the meldonium-treatment group spermatozoa motility reached 7.0 points already after the first period of spermatogenesis (after Day 40), thus meeting the standard of sperm dilution for artificial insemination (Crabo, 1997; Ruiz-Sanchez et al., 2006), and it remained at this level to the end of experiment. The average spermatozoa progressive motility of ejaculate in boars of control group were below 7 points (Table 1).

Meldonium administration for 90 days did not affect the weight and volume of testes or the weight of epididymides. Histological examination of testes showed that spermatogenic epithelium was thicker in meldonium-treated boars than in the control group. The increase of spermatogenic epithelium suggests that meldonium might affect the

process of spermatogenesis. In addition, administration of meldonium caused a proliferation of interstitial endocrine cells. Further studies at cellular and molecular level are necessary to clarify these effects.

Meldonium caused an increase in concentration of testosterone in blood serum, while concentrations of creatinine, total bilirubin, total cholesterol, glucose and aspartate aminotransferase/AST, alanine aminotransferase/ALT activity were not affected. Biosynthesis of testosterone is genetically controlled (Lubritz et al., 1991). However, various endogenous and exogenous factors such as age (Swiestra and Rahnefeld, 1967; Gay, 1971), circadian rhythms (Edqist, 1980; Zamaratskaia et al., 2004), nutritional factors (primarily, protein content) (Louis, 1994) are known to affect testosterone levels. A number of biologically active substances have been tested for their effects on testosterone production (Kattesh, 1982; Al-Taras, 2005; Ljungvall et al., 2005; Claus et al., 2007). Some of these substances such as letrozole, an aromatase inhibitor (Kattesh et al., 1982), and exogenously administered testosterone (Al-Taras, 2005) stimulate testosterone biosynthesis. On the other hand, substances such as 2-ethylhexyl phthalate, estradiol benzoate (Claus et al., 2007), triphenyltine and tributyline (Ljungvall et al., 2005; Ohno et al., 2005) inhibit testosterone biosynthesis. There is data suggesting that there might be a positive correlation between an increased level of testosterone and the growth of sperm production in boars (Dorst et al., 1976; Bender et al., 2006). However, we observed only sperm motility increase along with the increase of plasma testosterone level, but not significant growth in sperm production (semen volume and concentration), similarly as Walker et al. (2004).

Previous studies showed that 14-day oral administration of meldonium at a dose of 100 mg/kg affected neither sexual activity, nor sperm quality (motility and the concentration of spermatozoa), nor testosterone concentration in rats suggesting that prolonged administration of meldonium might be necessary to achieve its positive effects on these parameters. This emphasizes importance of our present findings in boars and warrants further studies on effects of meldonium on reproductive function in other mammalian species. Although our results indicate sperm motility increasing properties of meldonium, more detailed studies addressing effects of meldonium on reproductive parameters including sperm quality are necessary. Such studies would be of particular importance in view of the potential use of meldonium as a sperm motility and/or sperm quality enhancing agent in livestock.

5. Conclusions

1. The obtained results show that long-term (90-day) administration of meldonium improved sexual performance and increased sperm motility in boars, which might be linked to elevated levels of blood serum testosterone found in meldonium-treated boars.
2. 90-Day administration of meldonium increased also thickness of spermatogenic epithelium and caused a proliferation of interstitial endocrine cells in boars.
3. The exact mechanisms underlying stimulating effects of meldonium on reproductive function in boars remain to be elucidated. More detailed studies addressing effects of meldonium on reproductive parameters including sperm quality are necessary. Such studies are of particular importance in view of the potential use of meldonium as a sperm motility and/or sperm quality enhancing agent in livestock.

Conflict of interest statement

The authors declare that they have no conflicting interests.

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