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Treatments with thousands therapeutic doses of meldonium failed to alter the Drosophila's circadian clocks but negatively affected the germination of Pisum's seeds

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Treatments with thousands therapeutic doses of meldonium failed to alter the *Drosophila*'s circadian clocks but negatively affected the germination of *Pisum*'s seeds

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ABSTRACT

Meldonium is used clinically as an anti-ischaemic drug to treat myocardial infarction and chronic heart failure, and it had become a news making substance after the banning several elite athletes including Maria Sharapova from competitions. Despite this, the majority of possible biological effects of meldonium remained unexplored. We tested two effects, I) on the circadian clocks of fruit fly (*Drosophila melanogaster*) and II) on the process of pea seed germination (*Pisum sativum*). I. Comparison of the effects of photoperiod and meldomium on the circadian rhythm of locomotor activity during the 2nd and 3rd days in constant darkness revealed the expected effect of the previous photoperiod whereas meldonium in concentrations ranged from 1 (5 mg/kg) to 10,000 therapeutic doses failed to alter the mechanism of the fly's circadian clocks. II. A treatment with meldonium in very high concentration (2000 therapeutic doses) negatively affected the process of germination of pea seeds.

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Introduction

Meldonium is an anti-ischaemic drug used clinically to treat myocardial infarction and chronic heart failure. It is manufactured in Latvia under the commercial name of Mildronate. In Russia, it has been classified as one of "vital therapeutic chemicals" and, therefore, Mildronate is released for free for recipients social assistance. In contrast, on 16 September 2015 the World Anti-Doping Agency (WADA) had included meldonium in the banned list because of "evidence of its use by athletes with the intention of enhancing performance". In other words, the logic is: if several athletes are suspected to use it to enhance performance, it's a performance enhancer. The ban came into effect on 1 January 2016. Soon after this, meldonium has been put under the spotlight due to the banning from tennis Maria Sharapova who claimed that she had been legally taking it for 10 years. Despite all this noise around meldonium, there exists a lack of studies performed with highly trained athletes and published

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in peer reviewed journals that prove that it actually improves exercise performance (Schobersberger et al. 2017). Even more, insufficient scientific evidence leaves room for different speculations, such as, due to consuming meldonium, Ms Sharapóva and other ill-advised athletes may not have attained their true athletic potential (Arduini and Zammit 2016).

The analysis of literature indicated that the vast majority of research into the effects of this making news substance has been only published in Russian. For instance, using "meldonium" and "Mildronate" as key words we found approximately 200 publications in PubMed and much more, around 600, at e-library.ru, the major Russian database of scientific literature. The review and analysis of this literature are beyond the scope of our report, but, in overall, this search for publications on meldonium allowed us to make a conclusion that, so far, several of its possible biological effects have never been examined in scientific research. Therefore, these effects remain to be elucidated in future studies on plant and animal models as well as in further treatment trials with human participants.

Meldonium is a structural analogue of the carnitine precursor, gamma-butyrobetaine (Simkhovich et al. 1988). Since carnitine is required to transport fatty acids into the mitochondria for fatty acid breakdown via beta-oxidation, it is a key regulator of fat metabolism. Carnitine metabolism is affected by meldonium via inhibition of its biosynthesis and transport (Makrecka et al. 2014). This protects mitochondria from an overload of fatty acid metabolites (Zammit et al. 2009). It has been demonstrated that meldonium decreases the beta-oxidation of fatty acids and shifts cellular metabolism towards the oxidation of carbohydrates that requires far less oxygen per ATP molecule than beta-oxidation of fatty acids. Under hypoxic conditions this sparing of oxygen could be of huge benefit (Görgens et al. 2015). Besides, meldonium also increases gene expression related to glucose metabolism and thereby stimulates the aerobic oxidation of glucose (Liepinsh et al. 2008; Dambrova et al. 2016). The most obvious benefits have been seen in the treatment of ischaemic heart disease (Sjakste et al. 2005). Moreover, there exists evidence allowing the expectation that meldonium could be equally beneficial under the low oxygen conditions induced by intense endurance exercise (Dzintare and Kalvins 2012).

The effects of long-term treatment with meldonium on the nervous system were mostly evaluated in experiments on laboratory and domesticated mammals, and such a treatment was shown to induce a profound decrease in tissue concentrations of L-carnitine (Dambrova et al. 2002; Liepinsh et al. 2006, 2009). In one of few animal studies of other possible long-term effects of meldonium an improvement of sexual performance and sperm motility in boars was reported (Bruveris et al. 2012).

We failed to identify any scientific publications in either English or Russian on the effect of meldonium in plant species, but the web-page with description of the patent on this substance (US4481218 A) contains a claim that it possesses "the ability of controlling plant growth", and that "this compound ensures a 15% increase in the stem diameter of crops such as oats, barley, rye, wheat" (http://patft.uspto.gov/netacgi/nph-Parser?Sect2=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/search-bool.html&r=1&f=G&l=50&d=PALL&RefSrch=yes&Query=PN/4481218).

To the best of our knowledge, the effects of meldonium on the circadian rhythms and sleep have not been tested so far. The only reported finding indicated that, in mice, meldonium dose-dependently inhibited the sleeping time in ethanol-induced loss of righting reflex test (Zvejniece et al. 2010).

Drosophila melanogaster has been used as an animal model of human diseases in a huge number of experimental studies, and this small fly plays a very important role in therapeutic drug discoveries (Pandey and Nichols 2011). Moreover, it seems that, in this species, the circadian rhythms and their underlying molecular-genetic mechanisms were studied more intensively than in any other living creature. Recently, the extraordinary progress in this area of biological rhythm research has been acknowledged by the Nobel Prize committee: the year 2017 Nobel Prize in Physiology/Medicine went to three Drosophila researchers for "their discoveries of molecular mechanisms controlling the circadian rhythm" (Putilov 2018).

Therefore, we recorded the circadian rhythm of locomotor activity in this fly in order to compare two effects on this rhythm, one is the well-established photoperiodic effect (Majercak et al. 1999; Peschel and Helfrich-Förster 2011; Tataroglu and Emery 2014; Schlichting et al. 2016; Dubowy and Sehgal 2017) and another is the effect of meldonium that is hard to predict due to the absence of relevant publications.

Additionally, we tried to replicate a beneficial effect of meldonium on plant development mentioned in the patent US4481218 A. However, we experimented with germinating peas (*Pisum sativum*) instead growing stems of such domestic plants as oats, barley, rye and wheat.

Methods

The experiments with the circadian rhythm in flies

Adult male fruit flies (*Drosophila melanogaster*, 2–3 days old) from Harwich strain maintained in the Institute of Cytology and Genetics (Novosibirsk, Russia) were used in two slightly different experiments aimed on examination of the effects of photoperiod and meldonium on the circadian rhythmicity. The 1st and 2nd experiments were started on April 19th and December 25th, respectively. The times of sunrise vs. sunset at the previous day were 6:16 and 9:52 vs. 20:40 and 17:03, respectively. Prior to these experiments, the flies were kept under the typical room condition. In particular, during their reproducing prior to the experiment the room was naturally illuminated by the outdoor light through a window in building's wall, and the room temperature always remained in the range between 18 and 22°C.

Flies were individually placed for 4 days in glass tubes containing the standard cornmeal-agar media and they were kept under constant darkness at 20 °C. We used the Drosophila Activity Monitoring system ("Trikinetics", Waltham, MA, U.S.A.) to record the number of beam crosses made by a fly in 1 min intervals. The DAMSystem data acquisition software package was downloaded from the TriKinetics web site (www.trikinetics.com). In the 1st experiment, a sterile solution of meldonium delivered by Grindex for intravenous injections (100 mg/ml) was added to food in concentrations corresponding to 1–100 human therapeutic doses (one therapeutic dose is equal to 5 mg per 1 kg of body weight). In the 2nd experiment, we tested 100, 1000 and 10,000 doses of meldonium powder mixed with food. Fifteen flies were randomly included in each of four (three treatment and one control) groups of each experiment (Figures 1–3).

The experiment with germination of pea seeds

The seeds of pea (*Pisum sativum*) from the strain named "Marrowfat First Early" were maintained by K.K. Sidorova in the Institute of Cytology and Genetics (Novosibirsk, Russia) were used in the experiment aimed on examination of the impact of meldonium administration on the germination process. Fifteen randomly chosen seeds were distributed in each of 6 Petri dishes including the control dish. Meldonium was added to water in each of 5 treatment dishes in concentration of 0.001, 0.01, 0.1, 1 and 10 mg/ml (from 0.2 to 2000 therapeutic doses). The germinated seeds were counted starting from the 3rd day (Table 1) and the 8th day was the final day of the experiment (Figure 4).

Statistical analyses

The SPSS statistical software package (IBM, Armonk, NY, U.S.A., version 22.0) was used for the statistical analyses. Analysis was performed on two full 24-h cycles of locomotor activity obtained by averaging over 1-h intervals from 21:00 of the 1st day to 21:00 of the 3rd day. Three-way and four-way repeated measure ANOVAs (rANOVAs) were applied to test significance of the effects of two repeated measures ("Day" and "Clock Hour") and one or two independent factors ("Dose" and/or "Experiment") on the number of beam crosses (Figures 1–3). Degrees of freedom were corrected using Greenhouse-Geisser correction controlling for type 1 error associated with violation of the sphericity assumption, but the original degrees of freedom are reported in Results.

We used χ^2-test for performing pairwise comparisons (df = 1) of the proportion of germinated seeds obtained in the control dish with a proportion in each of 5 other (treatment) dishes. Bonferroni corrections were applied to account for the number of tests.

Results

Comparison of the photoperiodic effect with the effects of treatments with meldonium

None of rANOVAs yielded a significant main effect of an independent factor (either "Dose" or "Experiment") on the number of beam crosses. In contrast, the effect of "Clock Hour" was always highly significant ($p < 0.001$) indicating the persistence of a robust circadian rhythm in any of treated groups throughout an experiment (Figure 1). The main effect of "Day" was mostly non-significant.

Any rANOVA yielded a very reliable interaction between "Experiment" and "Clock Hour" ($p < 0.001$) whereas an interaction between "Dose" and "Clock Hour" was found to be non-significant. Such results are illustrated by lower and upper plots in Figure 2, respectively. Four-way rANOVA of the illustrated subset of activity data yielded a highly significant interaction between the independent factor "Experiment" and "Clock Hour" ($F_{23/1288} = 20.87$, $p < 0.001$) and non-significant interactions between the independent factor "Dose" and "Clock Hour" ($F_{23/1288} = 1.32$, $p = 0.238$). These and other results suggested the significant effect on the circadian clocks underlying the fly's rhythm caused by seasonal change in photoperiod and the absence of the treatment effect on these clocks irrespective of its dose.

The effects of treatments of the flies with meldonium in different doses

Among other interactions with independent factor "Dose", the cases of significant triple interaction of "Dose" with "Day" and "Clock Hour" were most notable. However, the analysis

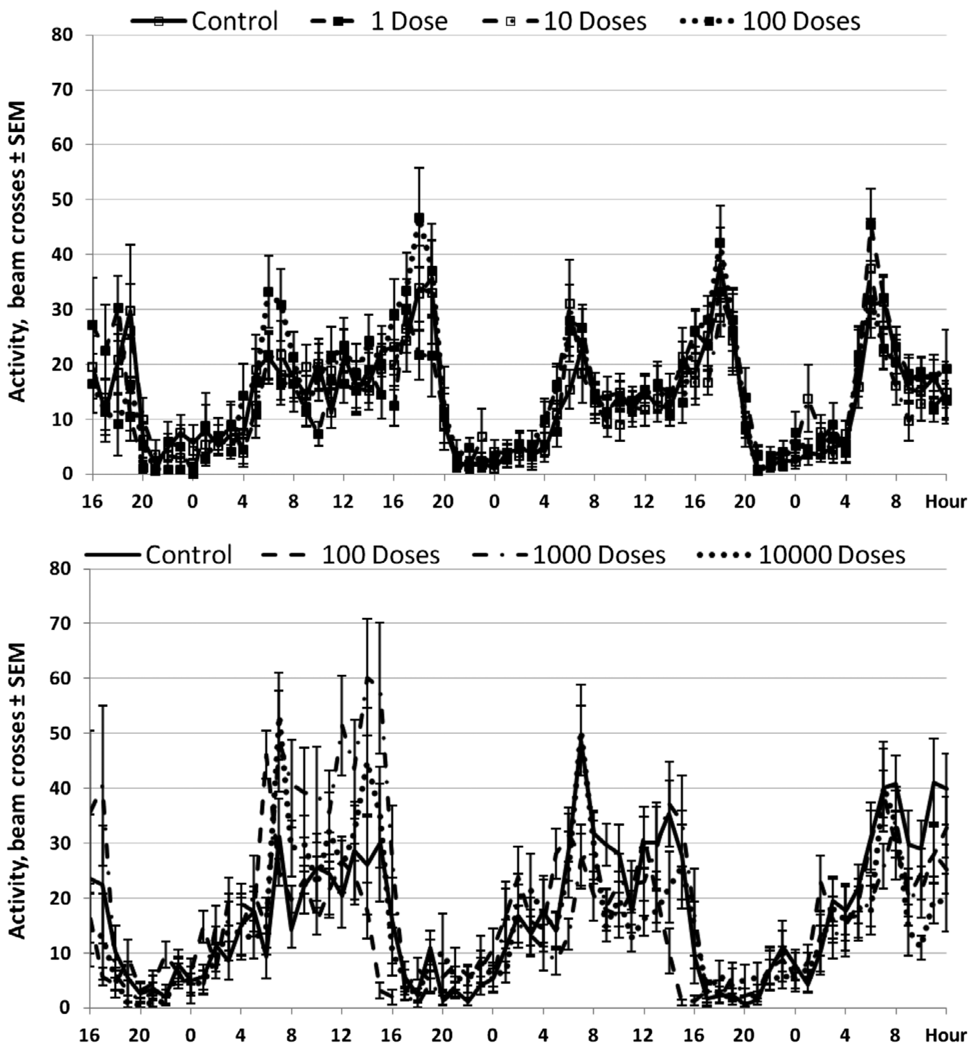

Figure 1. Time courses of locomotor activity in the 1st and 2nd experiments with 4 groups of flies.
Notes: Activity: Locomotor Activity; SEM: Standard Error of Mean; Dose: Concentration of meldonium expressed in therapeutic Doses; Hour: Clock Hour. Data for the 1st day were excluded.

did not reveal that this interaction is dose-dependent. Three-way rANOVAs of the subsets of activity data illustrated by upper and lower plots of Figure 3 yielded significant triple interactions of "Dose" with "Day" and "Clock Hour" in comparison of control with 1000 and 10 000 therapeutic doses ($F_{23/664} = 3.60$, $p = 0.001$ and $F_{23/664} = 2.22$, $p = 0.031$, respectively). This interaction was also significant in comparison with 100 therapeutic doses in data of the 2nd experiment ($F_{23/664} = 2.06$, $p = 0.042$). Dual interaction of "Dose" with "Day" was significant exclusively in comparison with 1000 therapeutic doses ($F_{1/28} = 6.78$, $p = 0.014$). It seems that the treated flies were more alert on the 2nd day whereas the control flies were more alert on the 3rd day of the winter experiment (Figure 3). Therefore, such results, in general, suggested that very high doses of meldonium tended to increase locomotor activity of flies on the 2nd day but not on the following days (Figures 1–3).

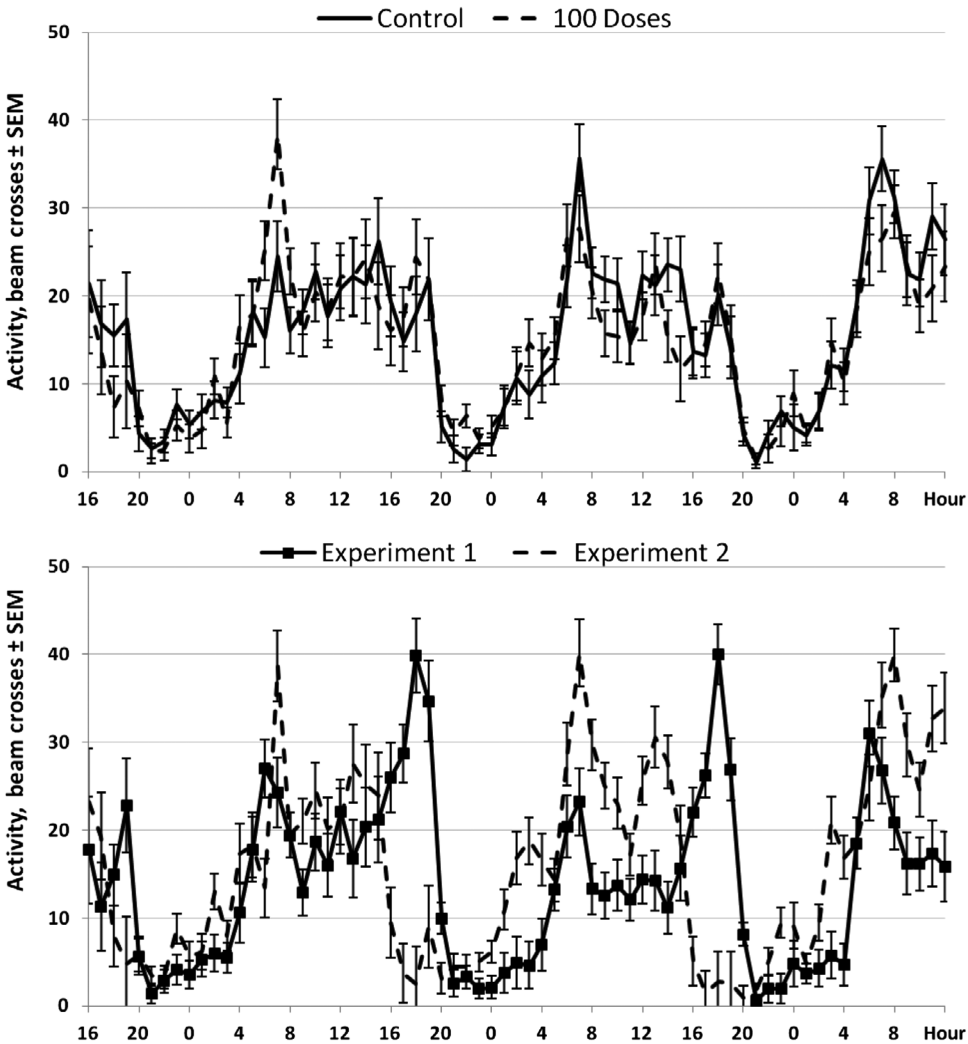

Figure 2. Time courses of locomotor activity avearaged across two groups of flies in two experiments.
Notes: Activity: Locomotor Activity; SEM: Standard Error of Mean; Dose: Concentration of meldonium expressed in therapeutic Doses; Hour: Clock Hour. Data for the 1st day were excluded. For each group of flies the data were averaged over two experimensts (upper plots) and for each of two experiments the same data were averaged over two groups (lower plots).

The effects of treatments of pea seeds with meldonium in different doses

Meldonium in very high dose was found to have a negative effect on sprouting seeds. On the 4th day of experiment this effect measured as an amount of germinated seeds reached a statistically significant level (Table 1). The difference with both control and low doses remained significant until the last day of the experiment (Figure 4).

Discussion

We examined the effects of meldonium on the circadian clocks of fruit fly and on the process of pea seed germination. The effects of photoperiod and meldomium on the circadian

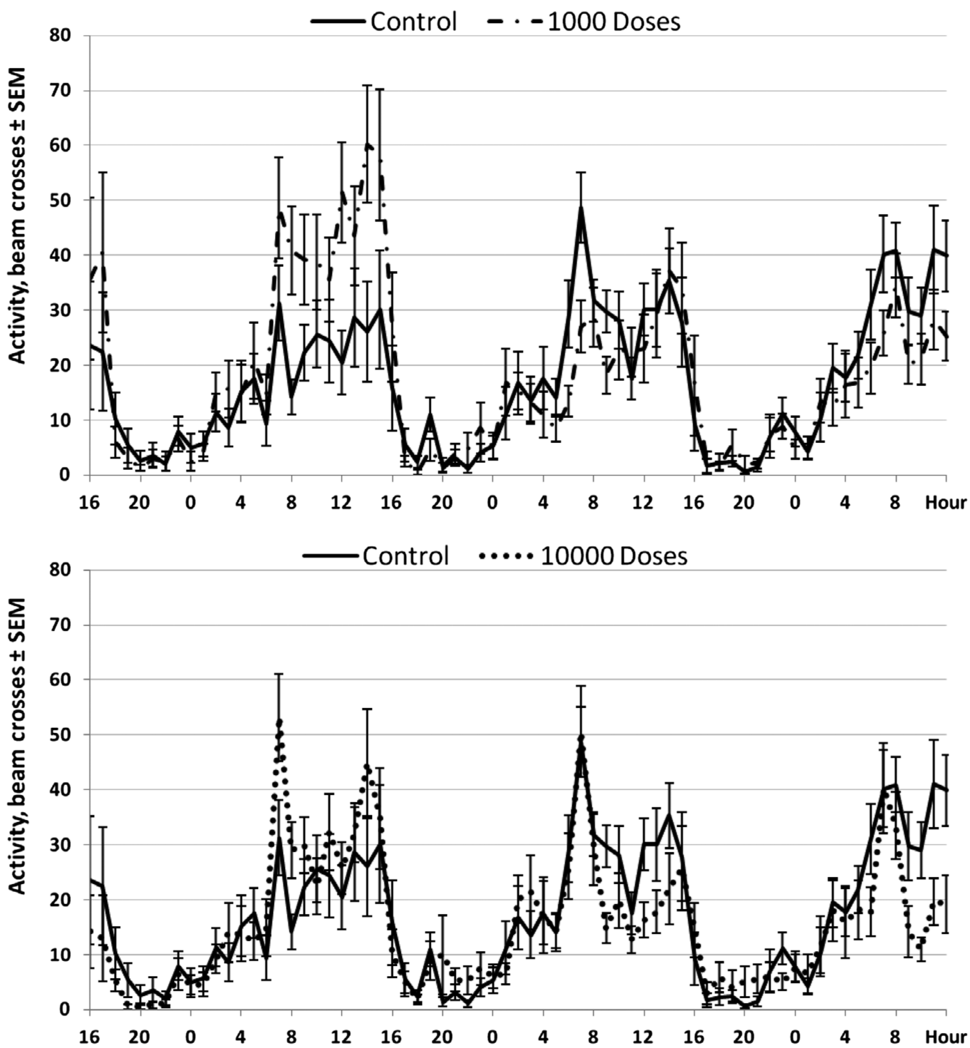

Figure 3. Time courses of locomotor activity in groupes treated with maximal doses.
Notes: Activity: Locomotor Activity; SEM: Standard Error of Mean; Dose: Concentration of meldonium expressed in therapeutic Doses; Hour: Clock Hour. Data for the 1st day were excluded.

Table 1. The effect of meldonium on the process of germination of seeds (the 3rd and 4th days).

Concentration in		Sprouting peas		χ^2-test: 3rd day		χ^2-test: 4th day	
mg/ml	TD	3rd day	4th day	χ^2	p	χ^2	p
Control	0	6	12	–	–	–	–
0.001	0.2	7	12	0.136	0.712	0	1.000
0.01	2	5	10	0.144	0.704	0.682	0.409
0.1	20	4	11	0.600	0.438	0.186	0.666
1	200	3	9	1.429	0.232	1.429	0.232
10	2000	1	2	4.658	0.031	**13.393**	**0.00025**

Notes: Each of 6 groups including Control consisted of 15 pea seeds. Results of χ^2-test in *bold*: Significantly *lower* fraction of germinated seeds ($p < 0.01$after correction for 5 tests) was revealed by pairwise comparison with Control. TD: Therapeutic Dose. See also final results in Figure 4.

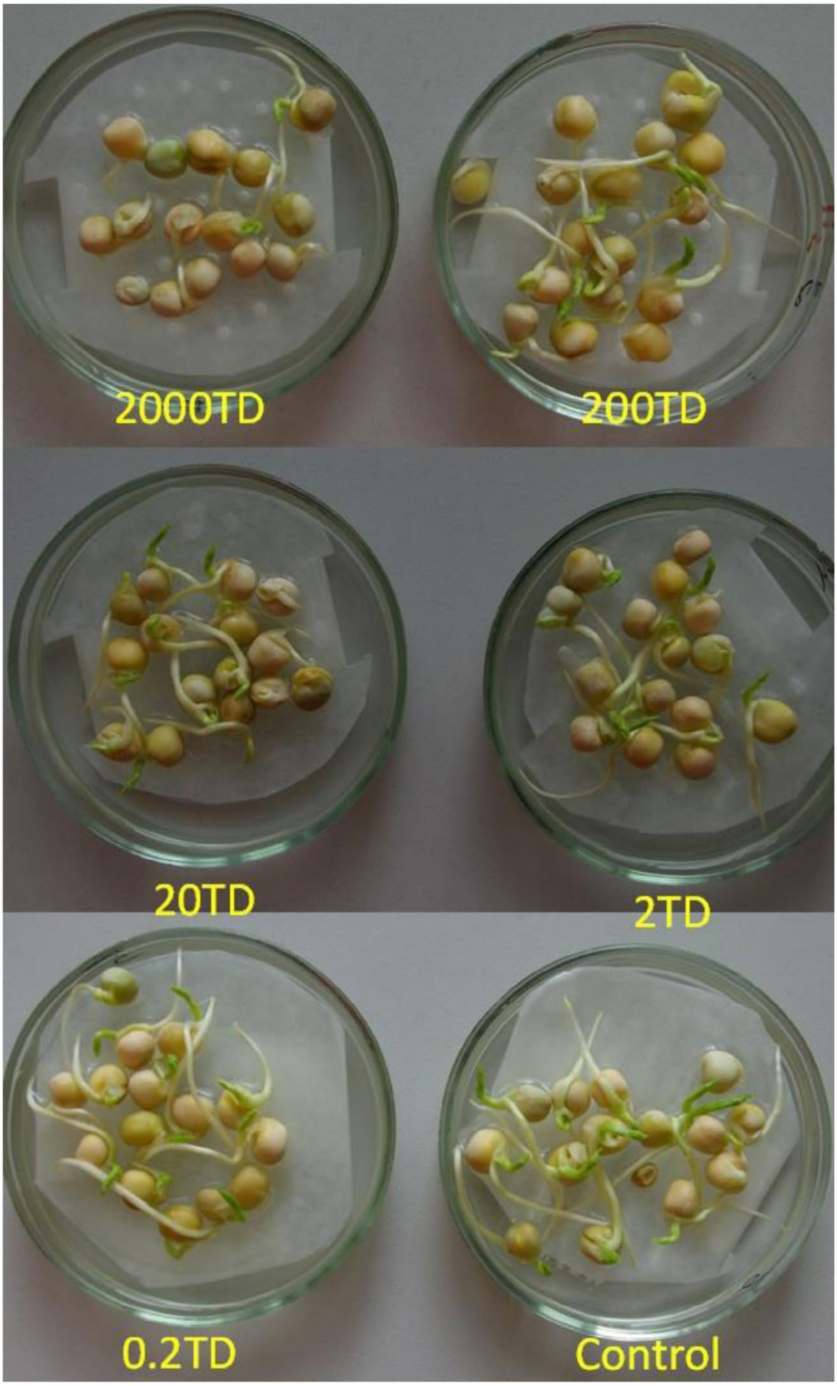

Figure 4. The final effect of meldonium (the 8th day) on the process of germination of seeds.
Note: TD: Concentration of meldonium expressed in Therapeutic Doses.

rhythm of locomotor activity were compared in the fruit fly. The expected effect of the previous photoperiod was found. In contrast, any treatment with meldonium (in concentrations ranged from 1 to 10 000 therapeutic doses) failed to alter the mechanism of the fly's circadian clocks. We also showed that there remained a possibility of influence of very high doses of meldonium (e.g. 100 therapeutic doses or more) on the level of locomotor activity but, anyway, this influence seems not to be long-lasting and dose-dependent. Moreover, we compared the effects of different doses of meldonium on the process of pea seed germination and found that a treatment with a very high concentration (2000 therapeutic doses) has a negative impact on this process.

More specifically, the studies of the mechanism underlying the effect of photoperiod on the circadian rhythm of locomotor activity in the fruit fly indicated that this rhythm is adjusted to seasonal change in day length by a phase shift of the evening oscillator leading to change in timing of the evening peak of activity and duration of daytime "siesta" (Schlichting et al. 2016; Dubowy and Sehgal 2017). This is exactly what was observed in our experiments when the data collected in spring and winter were compared (Figure 2, lower part). This mechanism was preserved under the treatment with meldonium in high concentration (Figure 2, upper part).

On the other hand, the comparison of the circadian cycles observed on the 2nd and 3rd days of the winter experiment revealed significant differences between these two cycles in control group as compared to the groups treated with high doses of meldonium (Figure 3). Therefore, such results suggested that the overdosing can increase locomotor activity of flies and that this effect is neither long-lasting nor dose-dependent. At least, it disappeared despite prolongation of the treatment on the 3rd day and it contrasted with a tendency of increase of locomotor activity on this day in control flies of the winter experiment (Figure 3). It can be concluded that replication and more detailed examination of the alerting effect of very high doses of meldonium are required in future experimental studies.

The possibility of a very high dose of meldonium to alter a biological mechanism was demonstrated in the experiment with germination of seeds. However, we found that the effect on a plant was negative in contrast to information about the experiments with growth of other species of domesticated plants provided by the description of the patent on this substance (US4481218 A). Therefore, it can be concluded that the effects of meldonium in therapeutic doses on plant development desire further scientific investigations.

Disclosure statement

No potential conflict of interest was reported by the authors.

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