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Research Article

THE USE OF SELECTED ANAESTHETIC DRUGS IN SEARCH OF A METHOD FOR IMPROVING EARTHWORMS' WELFARE

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ABSTRACT

This paper describes selected effects of body contact of earthworms *Dendrobaena veneta* Rosa with local anaesthetic (LA) drugs used for human anesthesia (lidocaine and prilocaine) and anaesthetics for aquatic animals (MS-222). The findings showed safe and effective immobilization of earthworms with prilocaine at a concentration of 0.25-1%. At the applied concentrations lidocaine was safe, but less effective. On the other hand, MS-222, at the applied concentrations had a strongly irritating effect for earthworms and induced convulsive body movements connected with a discharge of coelomic fluid. The results may be relevant both for improving the welfare of earthworms during experiments and for the organization of research involving testing drugs on invertebrates. In this case, by using earthworms as an experimental model and by applying the method for measuring their mobility after contact with anaesthetics, which has been described in this article, it might be possible to replace experiments on guinea pigs, rabbits, rats and mice, which are expensive and require an approval of an ethics committee, with laboratory tests on earthworms.

Keywords: local anaesthetics (LA), pharmacological screening, MS-222, earthworms, *Dendrobaena veneta* Rosa, welfare of laboratory animals.

INTRODUCTION

Earthworms (Lumbricidae) have been used in a variety of field research [Lukkari *et al.* 2004] and laboratory experiments [Plytycz *et al.* 2011] for a long time, and due to their high sensitivity to environmental stress they are a good model for identifying pollution in ecosystems [Kostecka *et al.* 2012]. To meet the requirements of numerous experiments, it is necessary to temporarily immobilize specimens while keeping them alive. To ensure humanitarian method of such a treatment it should be performed "under anasthesia", because annelids (Annelida), just like any living organisms, show sensitivity to external stimuli (ladder-like nervous system [Jura 2007]).

The use of animals in research procedures should comply with Directive [Directive 2010/63/EU] of the European Parliament and of the Council, because of the new available scientific knowledge on factors impacting animals' welfare. Recent studies have provided evidence for the capability of all animals to respond to stress, pain, suffering or distress. Therefore, currently the problem of laboratory animals' welfare is frequently discussed, what leads to raising minimum standards for handling these animals in conformity with the latest scientific accomplishments. Similarly, the public opinion expresses concern for the use of animals in various procedures, therefore, they should always be treated as feeling creatures and their use should be limited to the areas which can benefit the well-being of people, animals and the environment. It should be emphasized that animals should be used for research or educational purposes only if there is no other means to investigate specific problems. The choice of adequate research methods is also of key importance as they will allow for obtaining most satisfying results while causing the lowest levelof suffering. Other important aspects of researchers' work include protection of biological diversity that is why the use of endangered species in research should be limited to the unavoidable minimum.

With regards to the implementation of the Directive [Directive 2010/63/EU] on the protection of animals used for scientific purposes, it is important to introduce methods and means improving their condition during experiments (in accordance with the principles of replacing them, limiting their participation or enhancing the procedures of animal research). To ensure proper treatment of animals used in experiments it is necessary to keep them in conditions meeting their living requirements [Annex III 2010/63/EU] and to introduce such organizational innovations which allow for improving welfare of laboratory animals and for reducing their exposure to stress.

Importantly, considerations related to the concept of "animal welfare" must be interdisciplinary, since they involve a combination of domains: scientific, ethical and even economic. While developing measures focusing on animal welfare it is also necessary to recognize a significant function of broadly understood social sciences because of the need of better understanding of the man-animal relationships and to establish effective education which not only practically affects the treatment of animals in laboratories but also impacts the conditions of livestock and will contribute to the development of sustainable agriculture as such [Keeling 2005; Lund *et al*. 2006].

The results of studies investigating the activity of local anaesthetics may contribute to improved welfare of laboratory animals [Librowski *et al.* 2001]. Drugs of this type include prilocaine and lidocaine, which reversibly hinder conduction in nervous fibers. These substances have become widely used in anesthetizing specific parts of the human body and they neutralize central pain syndromes [Librowski *et al*. 2004]. The mechanism of activity in this group of drugs is based on blocking voltage-gated sodium channels [Bujak-Giżycka *et al.* 2009] and leads to reversible elimination of pain stimuli conduction with no loss in consciousness [Calvey 1995], contrary to general anaesthetics which lead to analgesia, loss of consciousness, muscle relaxation and loss of reflexes. Importantly, depending on the chemical structure and pharmacodynamic properties of local anaesthetics (LA) their half-life, and consequently their activity period is different [Bujak-Giżycka *et al.* 2009].

In the case of earthworms the previously applied anaesthetics, e.g. chloroform or contact with cold surfaces, exert an irritating effect, and induce convulsive body movements linked with excretion of coelomic fluid through the pores in the body surface, and discharged along with it are coelomocytes, i.e. cells responsible for the immunity of earthworms. Reduction in the pool of coelomocytes may impact a number of body functions. Coelomocytes discharge through the pores in the body surface are collected in a non-invasive way for research related to immunology. These are obtained by exciting earthworms in a controlled manner, by immersing them in 5% ethanol [Cooper *et al.* 1995], by ultrasound stimulation [Hendawi *et al*. 2004] or by stimulation with weak electric current [Roch 1979], which induces violent movements of the body (wiggling) and a discharge of coelomic fluid with coelomocytes suspended in it. In many earthworm species, including *Dendrobaena veneta*, coelomocytes include a high rate of eleocytes (chloragocytes) separated from chloragogen tissue; riboflavin (i.e. vitamin B2) accumulated in their granules tints, the discharged fluid with yellow colour and its contents can be measured with spectrofluorimeter [Plytycz and Morgan 2011].

The present study was an attempt to apply local anaesthetics (LA) used in humans (prilocaine and lidocaine) and an agent administered to aquatic animals (MS-222), to anesthetize earthworms, which could contribute to improving their welfare during experiments. The measure of anesthesia was their response to stimulation with weak electric current.

MATERIAL AND METHODS

The experiment was designed to test the activity of local anaesthetics: lidocaine (Fischer Chemicals) and prilocaine (Sigma Aldrich) as well as MS-222 (Tricaine methanesulfonate; Ethyl 3-aminobenzoate methanosulfonate salt;

Sigma Aldrich; Fluca). Various concentrations of the aforementioned solutions were used to anesthetize adult (*clitellate*) specimens of the earthworm species *Dendrobaena veneta* Rosa. A series of experiments was conducted in 2012.

Earthworms, in groups of six specimens, were placed in Petri dishes filled with PBS–solution (0.9% saline solution) in the case of controls (0%), or filled with solutions of lidocaine, prilocaine or MS-222 (with concentrations of 0.125% – 2%) in PBS – in the case of respective experimental groups (Table 1).

Observation of earthworms' response to the solution of a specific substance was conducted for various durations of exposition to the anaesthetic (from 5 to 30 min). After the designated time the earthworms were individually taken to a 6-well plate (Nunc), specially adjusted for connecting with electricity [Podolak-Machowska *et al.* 2012] and filled with PBS–solution. The plate was covered with a lid whose surface contained a grid of fields, facilitating the observation of changes in the earthworm's position at one-second intervals. Earthworm's behaviour was recorded for 15 s after connecting electric current (4.5V); then their induced mobility was analyzed individually. After the observation was completed, the earthworms were carried over to a tissue soaked with PBS–solution in order to check whether they regained their original mobility. Mobility of specific specimens was described taking into account recorded films, which allowed calculation of the differences in the location of their body with respect to the grid designed specifically for the experiment. The assumed measurement of mobility in earthworm specimens was the sum of the changes in their position with respect to the elements of the grid, occurring at one-second intervals. This procedure was modelled in the earlier study designed to specify the method for measuring earthworm mobility, yet the grid for the field of observation was modified [Podolak-Machowska *et al.* 2012].

The obtained recordings were analyzed using BEST player v. 2.106, and the results were presented as mean values and standard errors (Mean + SE) obtained using Microsoft Excel v. 2010. Statistically significant differences were determined using a student's t-test ($p<0.05$).

RESULTS AND DISCUSSION

The study showed earthworms' different responses resulting from the contact with the test substances. In the control groups, during the experiments none of the *D. veneta* specimens showed unusual behaviours or discharged coelomic fluid with immunocompetent cells, which is one of the symptoms characteristic for a lack of stressful factors.

The experiments also showed that only at the highest applied concentration (2%) prilocaine and lidocaine induced convulsive body movements linked with discharge of the yellow coloured coelomic fluid, and at lower concentrations these substances did not have irritating impact; this suggests these substances can safely be used for anesthetizing earthworms.

On the other hand, MS-222 was found to have an irritating effect for these animals; negative response was observed even in the first seconds of contact with their bodies. This was manifested in wiggling and convulsive movements leading to a discharge of immunocompetent cells, followed by extravasation and narrowing of the body. That morphological deformity occurred at the highest concentrations used during the tests. MS-222 is an approved anesthetizing agent for fish [Weber *et al*. 2009; Vera *et al*. 2010], yet it was reported by Molinero and Gonzales [1995] that long-term contact with as well as high concentrations of MS-222 may induce stress response in fish, revealed by higher concentration of cortisol in blood. Importantly, when earthworms were exposed, the

Table 1. The outline of the conducted experiments

Study groups	Tested substance concentration [%]			Laboratory animals (earthworms)	Exposure time [min]
	Lidocaine	Prilocaine	MS-222		
Control	0	0	0	*Dendrobaena veneta* Rosa 6 mature specimens per each variant of the concentration	5 – 30
Experimental groups	–	0.125	0.125		
	0.25	0.25	0.25		
	0.5	0.5	0.5		
	1	1	1		
	2	2	2		

Table 2. Discharge of coelomic fluid by *D. veneta* earthworms in contact with the test substance at 5-minute exposure

Substance \ Concentration	0% (control)	0.125%	0.25%	0.5%	1%	2%
Prilocaine	– – –	– – –	– – –	– – –	– – –	+ – –
Lidocaine	– – –	no data	– – –	– – –	– – –	+ – –
MS-222	– – –	+ – –	+ + –	+ + –	+ + +	+ + +

Comments: The intensity of coelomocyte-containing fluid discharge according to the scale designed specifically for the experiment : + + + means the most intense of discharge; – – – means no discharge of the coelomocyte-containing fluid.

toxic effect of MS-222 was less pronounced with a decrease in the administered concentration, yet even at the concentration of 0.125% it continued to induce a discharge of coelomic fluid (Table 2).

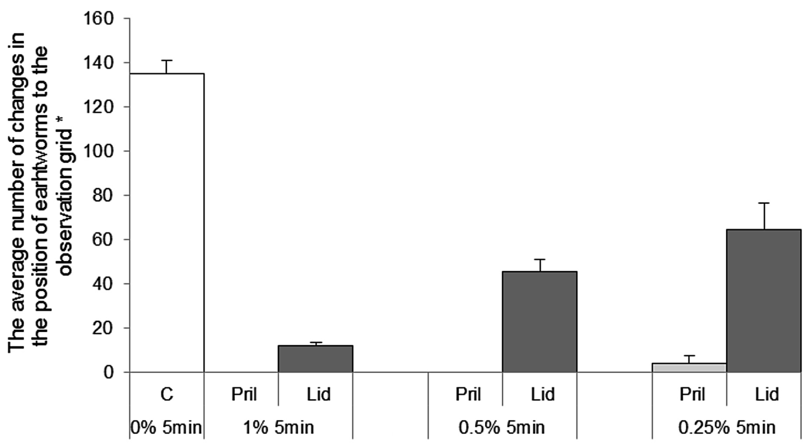
The study also showed varying anaesthetic effects following contact with the specific substances (Figure 1). Since the irritating impact of MS-222 could be seen during the first seconds of its contact with earthworms' bodies, further analysis related only to lidocaine and prilocaine. The presentation of results for different times of exposition to the aforementioned agents was also restricted to the effects of activity for 5 minutes, as it was the shortest of all effective periods under examination, therefore the most convenient to anesthetize earthworms.

The series of experiments obtained promising results for anesthetizing *D. veneta* earthworms with prilocaine. In comparison to the controls (0%), both 1% and 0.5% solution of prilocaine resulted in a complete lack of response of the examined specimens to the stimulation with electricity (4.5 V).

Anesthetizing *D. veneta* earthworms with lidocaine proved to be less effective ($p<0.002$) (Figure 1), even though both prilocaine and lidocaine are amino amides of medium strength and medium time of activity due to the presence of amide bond [Bujak-Giżycka *et al*. 2009]. A five minute exposition to prilocaine at lower concentration (0.25%) did not result in calming completely the response of earthworms to the activity of electricity (Figure 1).

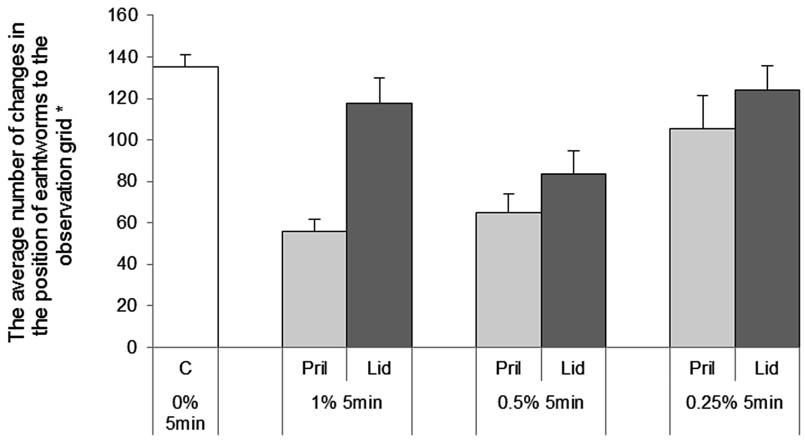
All earthworms anesthetized with prilocaine and lidocaine regained mobility after 2.5 hours (all were found alive and in good condition 24 hours later), what suggests that the aforementioned drugs are safe to use (Figure 2).

Summing up, we can say that the results provide a start regarding the possibilities and methods for building overall welfare of Lumbricidae during various experiments carried out using this group of invertebrates. The initial evidence seems promising, yet a number of questions need to be asked, most importantly the following. Does complete immobilization of earthworms following the application of prilocaine mean their perception of



* A large sum of differences in positions indicates the full mobility of tested specimens

Fig. 1. The effect of prilocaine and lidocaine on the mobility of earthworms *D. veneta* induced by electric current (4.5 V) measured for 15 seconds after 5 min exposure



* See Fig. 1.

Fig. 2. Recovery of mobility by earthworms *D. veneta* 2.5 hours after treatment with prilocaine or lidocaine (5 min)

stimuli has been decreased? Is the response to the applied substances differentiated by species diversity, body mass, maturity stage and living conditions of earthworm specimens, and in what way? Do earthworms' responses to anaesthetics depend on the season of research (their activity level), and in what way? How do temperature and other abiotic factors impact the effectiveness of the substances under consideration?

It should also be emphasized that the measurement of earthworms' mobility used in the experiment is a good method of testing the effectiveness of various local anaesthetic agents for these animals.

CONCLUSIONS

1. The present study initiates the search for agents improving earthworms' welfare during experiments. The results show that prilocaine can be recognized as an effective and safe anaesthetic for those animals and can be used for instance during activities at the stages of designing and organizing experiments with those invertebrates.
2. The obtained results can also be treated as an initial stage for organizing research aimed at testing drugs on vertebrates. Using earthworms as an experimental model and applying the described method for measuring their mobility after contact with anaesthetics it may be possible to replace experiments on mice, rats, hamsters, guinea pigs, rabbits, dogs, cats, quails or primates, which are expensive and require an approval of ethics committee, with tests on earthworms.

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