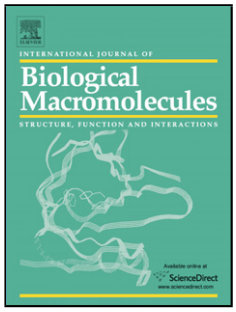

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Efficacy of albendazole:β-cyclodextrin citrate in the parenteral stage of *Trichinella spiralis* infection

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24

Abstract

Albendazole-β-cyclodextrin citrate (ABZ:C-β-CD) inclusion complex *in vivo* antiparasitic activity was evaluated in the parenteral phase of *Trichinella spiralis* infection in mice. An equimolar complex of ABZ:C-β-CD was prepared by spray-drying and tested in CBi-IGE male mice orally infected with L1 infective larvae. Infected animals were treated with 50 or 30 mg/kg Albendazole, (ABZ) equivalent amounts of the ABZ:C-β-CD complex and non treated (controls). Mice received a daily dose on days 28, 29 and 30 post-infection. A week later, larval burden and percentage of encysted dead larvae were assessed in the host by counting viable and non-viable larvae in the tongue. Complexation of ABZ with C-β-CD increased the drug dissolution efficiency nearly eightfold. At 37 days p-i, the reduction percentage in muscle larval load was 35% in mice treated with 50 mg/kg/day ABZ and 68% in those given the complex. Treatment with the lower dose showed a similar decrease in parasite burden. Treated animals showed a high percentage of nonviable larvae, the proportion being significantly higher in mice receiving the complex than in control animals (72-88% *vs.* 11%, P=0.0032). These data indicate that ABZ:C-β-CD increases bioavailability and effectiveness of ABZ against encapsulated *Trichinella* larvae, thus allowing the use of small doses.

Keywords: Albendazole:β-cyclodextrin citrate complex; *T. spiralis* parenteral stages; antiparasitic activity

1. Introduction

Trichinellosis is a parasitic infection produced by a nematode of the genus *Trichinella*. This parasitic disease is a zoonosis emerging and/or re-emerging in several regions of the world. In Argentina, trichinellosis is mainly caused by *Trichinella spiralis* and is endemic in pigs, the major source of human infection [1]. Transmission occurs after ingesting raw or undercooked meat containing viable L1 infective larvae from pork and pork products manufactured without the appropriate sanitary controls [2]. Larvae are released from the nurse cell (due to the gastric digestive fluid pH) and migrate to the intestine; where they burrow into the intestinal mucosa, mature and reproduce in about 30 hours. Newborn larvae yielded by the female parasites migrate through blood or lymphatic vessels and reach striated muscle fibres where they encyst and mature to L1 infective larvae at approximately 30 days post-infection. The signs and symptoms of the disease are directly related to the number of ingested larvae. Since its discovery in 1835 by Owen and Piaget [3], this parasitosis could not be eradicated. There are many factors involved: *T. spiralis* complex life cycle, its lack of host specificity, different structural forms and diverse ecological niches throughout the cycle. Additionally, the acute stage of infection often has no pathognomonic signs and in most infected people diagnosis is made long after being infected, when larvae have invaded the skeletal muscle cells.

Currently, the drugs most commonly used for treating trichinellosis are benzimidazole derivatives such as albendazole, flubendazole, mebendazole, and thiabendazole [4]. However, when included in conventional pharmaceutical forms, these drugs fail to be effective to kill muscle encysted larvae [5-7]. Albendazole (ABZ), a benzimidazole carbamate, is an anthelmintic compound widely used in the treatment of systemic nematode infections [8]. Nevertheless, its effectiveness is limited by its poor water solubility (1 µg/mL

at 25 °C) and the consequent low bioavailability, producing in some cases an unpredictable therapeutic response.

Several strategies may be employed in order to increase solubility, dissolution rate and oral bioavailability of poorly water soluble drugs, including the formation of complexes with cyclodextrins (CDs) [9]. These carriers have the ability to form inclusion complexes with various compounds of low polarity, increasing their apparent solubility. β-cyclodextrin (β-CD) consists of seven glucopyranose units and, owing to its crystalline structure, it shows a deficient water solubility. The substitution of β-CD hydroxyl groups, the most reactive being C_6-OH and C_2-OH, produce very heterogeneous and non crystallizable products. In a previous work, a non-toxic, water soluble carrier β-CD derivative with citric acid, β-CD citrate (C-β-CD), was synthesized and physicochemically characterized [10]. C-β-CD presents acidic groups in its structure that interact strongly with basic drugs such as ABZ, generating extremely stable inclusion complexes.

Enhanced solubility translates in better bioavailability and should improve the therapeutic efficacy of orally administered drugs. Thus, the aim of this research work was to evaluate *in vivo* the antiparasitic effectiveness of the novel ABZ:β-CD citrate complex (ABZ:C-β-CD) during the parenteral phase in *Trichinella spiralis* infected mice.

2. Materials and methods

2.1. Drug

ABZ (1µg/mL aqueous solubility), β-CD, hydroxypropyl β-CD, and methyl β-CD were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were of analytical grade.

93

94 *2.2. Drug formulation and physicochemical characterization*

95 The synthesis of C-β-CD was performed as described by Garcia et al. (2014) [10]. Briefly,
96 the inclusion complexes were prepared by the spray drying method (SD). ABZ (0.56 mol)
97 was dissolved in acetic acid (10 mL) and then C-β-CD (0.56 mol) and water (20 mL) were
98 added to the solution. The resulting solution was spray-dried in a Mini Spray Dryer Buchi B-
99 290 (Flawil, Switzerland) under the following conditions: inlet temperature: 130^{o}C, outlet
100 temperature: 70^{o}C, air flow: 38 m^{3}/h, feed rate 5 mL/min, and aspirator set 100 %.

101 The dissolution profiles were performed according to the U.S. Pharmacopeia (USP)
102 conditions, in an USP Standard Dissolution Apparatus Hanson Research SR8 Plus
103 (Chatsworth, CA), equipped with a rotational paddle (50 rpm). The dissolution medium
104 (900mL of 0.1N HCl) was maintained at 37 °C. Dissolution efficiency (DE) of the
105 pharmaceutical dosage form, a concept proposed by Khan and Rookes in 1975 [11] and
106 defined as the area under a dissolution curve between specified time points, was calculated
107 using the following equation:

108 $$\text{Dissolution efficiency \% (DE)} = \frac{\int_0^t y \times dt}{y_{100} \times t} \times 100$$

109 where y is the percentage of dissolved product at time t.

110

111 *2.3. Animal model*

112 Adult CBi mice (90-100 days old) of the CBi colony from the Animal Facilities of the
113 Instituto de Genética Experimental, Facultad de Ciencias Médicas, Universidad Nacional de

Rosario (from here on CBi-IGE), were used. The CBi-IGE stock comprises five genetically distinct lines; four of the lines resulted from selection experiments for body conformation while the fifth (CBi) was the unselected control [12]. The lines are currently in their 128th generation of selective breeding and have a theoretical inbreeding coefficient greater than 0.99. All mice were kept in the same room under identical breeding conditions (23 ± 2 ºC, on a 12-hour-on/12-hour-off light cycle) and received food (Cargill Laboratory Chow, pelletized) and water *ad libitum*. Animals were treated in accordance with the institutional regulations (National University of Rosario School of Medicine permit number 4978/2013) which comply with the guidelines issued by the Institute for Laboratory Animal Resources, National Research Council, USA.

2.4. Parasite

Trichinella spiralis L1 infective larvae were obtained from donor CBi infected mice. Briefly, the muscle mass was subjected to artificial digestion at 37ºC in a 1% w/v pepsin - 0.1 N HCl solution to release the encysted larvae [13]. These were washed and resuspended in sterile saline solution; from this suspension the infection dose for each animal was prepared by counting individual larvae.

2.5. Infection and treatment

CBi males (n=30) were orally infected with two *T. spiralis* infective L1 larvae per g of body weight (mean weight 35±3g). After infection, the animals were randomly divided into five groups (n=6 per group) and were treated with a single daily oral dose of ABZ or ABZ:C-β-CD during the chronic phase of the infection, on days 28, 29 and 30 post-infection. ABZ

dosage was based on previous studies [14]. Groups I and II received a dose of 50 or 30 mg/kg ABZ; groups III and IV were given, respectively, a dose of the complex equivalent to 50mg/kg or 30mg/kg ABZ; group V, non-treated given the vehicle alone, was used as control of infection. Treatment efficacy was assessed by comparing muscle larval burden in treated mice with that in non-treated animals. Both the total number of muscle encysted larvae and their viability were determined in the tongue of each animal, since this is a preferred site of encystment in mice [15, 16]. Briefly, mice were sacrificed by CO_2 seven days after administration of the last dose; the tongue was excised, weighed and submitted to artificial digestion following the method already described. Since the tongue digest has a small volume, all recovered larvae were counted and the result was expressed as the number of larvae per g of tissue (relative larval load, RLL). L1 larvae viability in the suspension was evaluated by a methylene blue vital stain based on the finding that dead and moribund larvae show alterations in their chitin layer that facilitates the penetration of the dye [17]. One hour after adding the saline solution, the supernatant was removed and 1mL of 0.5 mg/mL methylene blue solution in distilled water was added to an equal volume of larvae suspension. The mixture was incubated for three hours at 37ºC. L1 larvae were then washed with saline and observed under optical microscopy at 40X magnification. Blue stained *T.spiralis* larvae were counted as dead (Fig. 1); this was corroborated by absence of movement and/or the typical "comma" stance in some larvae.

The reduction percentage in larval burden was calculated for each animal, using the following formula [14]

$$\text{Mouse “A” RLL reduction percentage} = \frac{\textit{control group mean RLL} - \textit{mouse “A” RLL}}{\textit{RLL control group mean}} \times 100$$

2.7. Statistical analysis

The statistical significance of the differences in relative larval load among groups was examined with a one-way analysis of variance, followed by the Bonferroni post-test to compare pairs of groups [18]. Differences in treatment efficacy was assessed by the non parametric Kruskall-Wallis test, using Dunn's test for between groups comparison [18]. Differences were considered significant if $P<0.05$.

3. Results

3.1. Dissolution profiles

Fig. 2 shows the dissolution profiles of ABZ pure drug and ABZ:C-β-CD inclusion complexes. DE, the parameter derived from the dissolution curves clearly showed an improvement in the solubility of the complexes compared with the pure drug. DE increased nearly eightfold when ABZ (11.9 %) was complexed with C-β-CD (95.0 %), demonstrating the high effectiveness of C-β-CD and the spray drying technique to enhance the drug dissolution rate.

3.2. Antiparasitic activity assay in Trichinella spiralis infected mice

Table 1 shows the antiparasitic activity of ABZ:C-β-CD and ABZ pure drug, against encapsulated larvae. The total number of muscle encysted *T. spiralis* larvae recovered (RLL) significantly decreased in treated animals compared with controls ($P=0.0057$). This reduction in worm load was observed both in ABZ and ABZ:C-β-CD treated mice

irrespective of the dose administered, and, though not significantly, was highest in the group receiving ABZ:C-β-CD 50, 68% *vs*. 35% attained by the ABZ 50 treated mice.

Besides the observed decrease in the total number of recovered encysted larvae, the treatment also induced a loss of viability as assessed by the vital stain (Table 1, Fig. 1). Thus, when both number and viability of recovered larvae were considered, ABZ:C-β-CD in either dosage reached a 90 % reduction in muscle burden as compared to 79 % obtained with ABZ.

4. Discussion

As stated by Dupouy-Camet et al. (2002) [19], anthelmintics must act against all forms of the parasite and thus in various locations of the body, to be completely effective in treating trichinellosis, Though usually active during the enteral and migratory stages of the infection, the current drugs fail to be effective to kill muscle encysted larvae.

ABZ has been shown to be active against the enteral and parenteral phase of the parasite in experimental trichinellosis but a decline in the parasite drug sensitivity was observed during the invasive and encystment phase [20]. The results described herein confirm that ABZ pure drug has a relatively low antiparasitic activity against encysted larvae. Treatment with ABZ had an efficacy comparable to that reported by López-García et al. (1997) [21] who treated mice with 50 mg/kg/day ABZ from days 34 to 36 p-i. These authors concluded that ABZ is efficacious against *T.spiralis* encysted larvae if given at a dose of 100 mg/kg/day (94.7% reduction). Li et al. (2012) [22] came to a similar conclusion, recommending a dose of 250 mg/kg/day against encapsulated *T. spiralis* larvae. Moreover, the anthelmintic effects of a single treatment did not improve by repeated treatment (50, 75 or 100 mg/kg/day for three days, beginning on day 34 p-i) as the efficacies were always lower or identical to those of a

single treatment at the corresponding doses [23]. At variance with those reports, mice treated with 20 mg/kg/day for longer periods, from days 30 to 60 p-i, achieved a 70% decrease in muscle larval burden [6]. Altogether, these reports indicate the need for high dosage and/or long treatment periods to improve the antiparasitic efficacy of ABZ against encysted *T. spiralis* larvae.

The results of the present study showed that ABZ complexed with the novel derivative of β-cyclodextrin, β-cyclodextrin citrate, is more efficient than ABZ alone against the parenteral stages of *T. spiralis* larvae. Larval burden on day 37, seven days after administering the last dose, was significantly reduced, possibly because larvae cysts were damaged and rapidly destroyed due to a better distribution of the drug in blood and tissues. Similarly, 2-hydroxypropyl β-cyclodextrin inclusion complexes of ABZ [24] or a benzimidazole derivative [25] had a better anthelmintic activity than the pure drug, probably as a consequence of the increased solubility of the drug in the complex.

The finding of a significantly increased proportion of dead worms in the larvae recovered from mice treated with the ABZ:C-β-CD complex also suggests the greater efficacy of the formulation compared with the pure drug. The enhanced effectiveness was probably a consequence of improved drug bioavailability resulting from the increased solubility and dissolution rate, which lead to a better absorption. It had been reported that plasma concentration of the active metabolite ABZ-sulphoxide is significantly increased when an ABZ-β-CD complex is given to mice in a model for *Trichinella* infections [26].

Improving oral absorption of poorly soluble drugs has focused on supersaturating delivery systems to induce its precipitation in the gastrointestinal tract [27, 28] thus enhancing bioavailability and therapeutic efficacy. Super-saturation is a thermodynamically metastable state that enforces the precipitation. Appropriate evaluation of super-saturation, precipitation and possibly precipitation inhibition is concluded for the efficient development of new

pharmaceutical formulations. Moreover, the results described herein, as well as those by others [6, 21, 22] indicate that administration of high doses to enhance ABZ oral absorption does not improve the antiparasitic efficacy of the drug against encysted larvae and has the disadvantage of accentuating its typical side effects.

It is worth mentioning that the results herein described were obtained after a short treatment and using small doses of the ABZ:C-β-CD inclusion complex, similar to those recommended for the treatment of human trichinellosis. This treatment protocol was as effective as the higher doses or longer treatment periods proposed by other authors. Moreover, it has the advantage that should be accompanied with a decrease in serious adverse effects. Thus, the proposed system shows promising results to treat the parenteral stage of *T. spiralis* infection.

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Transparency declarations

None to declare.

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304 **Figure captions**

305 **Fig. 1**. *T. spiralis* L1 larvae micrograph showing a dead larvae (arrow) stained with
306 methylene blue. Magnification 40X.

307

308 **Fig. 2**. Dissolution profile of 100 mg ABZ pure drug and the same dosage of ABZ:C-β-CD,
309 molar ratio 1:1. Test conditions were 0.1N HCl at 37 ºC. Each point represents the mean of
310 three measurements ± one standard deviation. Open circles (ABZ, pure drug) filled circles
311 (ABZ:β-CD).

Table 1. Effect of decreasing doses of ABZ and ABZ:C-β-CD on muscle relative larval load* (RLL) in *T. spiralis* infected CBi mice

Drug	ABZ dosage (mg/kg bw)	RLL§		Drug efficacy#	
		Total	Dead larvae	RLL reduction percentage	Dead larvae percentage
ABZ	50	795 ± 145.7[a]	464± 103.7[a]	35 (0-70)[a]	57 (35-95)[a,b]
	30	634 ± 103.5[a]	381 ± 48.8[a]	52 (17-72)[a]	69 (39-84)[a,b]
ABZ:C-β-CD	50	511 ± 109.7[a]	333± 83.6[a,b]	68 (29-82)[a]	72 (31-100)[b]
	30	766 ± 154.2[a]	552± 46.5[a]	51 (0-66)[a]	88 (54-100)[b]
Control	---	1226 ± 98.6[b]	132 ± 25.9[b]	---	11 (5-21)[a]

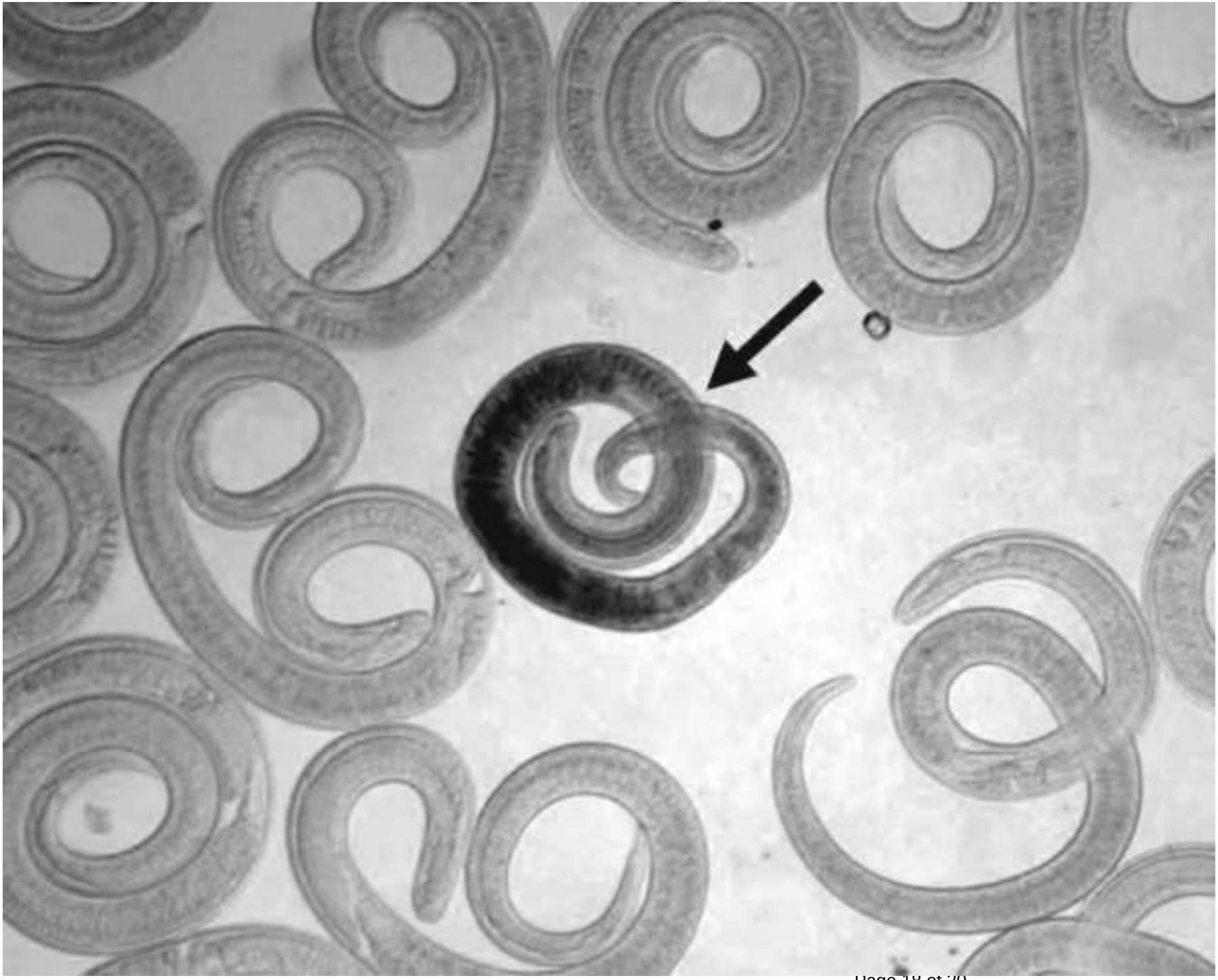
§ Mean ± SEM #Median (range)

Differences among groups were evaluated by a one-way ANOVA, using Bonferroni's post-test for comparisons between groups (**Relative larval load**), or by the nonparametric Kruskall–Wallis test, and Dunn's test for between groups comparison (**Larval load reduction percentage** and **Dead larvae percentage**).

For each variable, differences between groups not sharing the same superscript are significant at the 0.05 level.

*Larval load was measured in the tongue, a preferred site of encystment in mice.

Figure(s)



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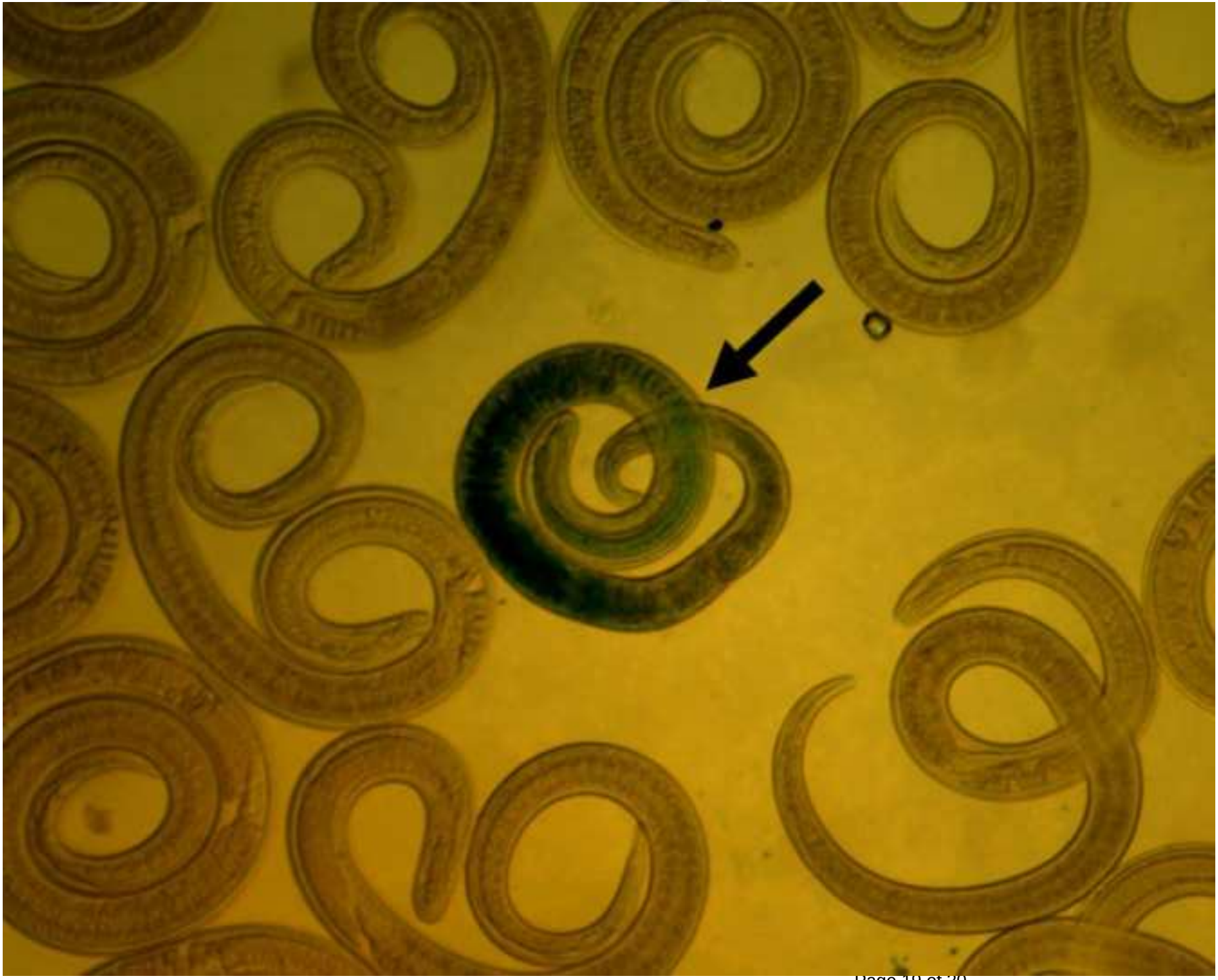

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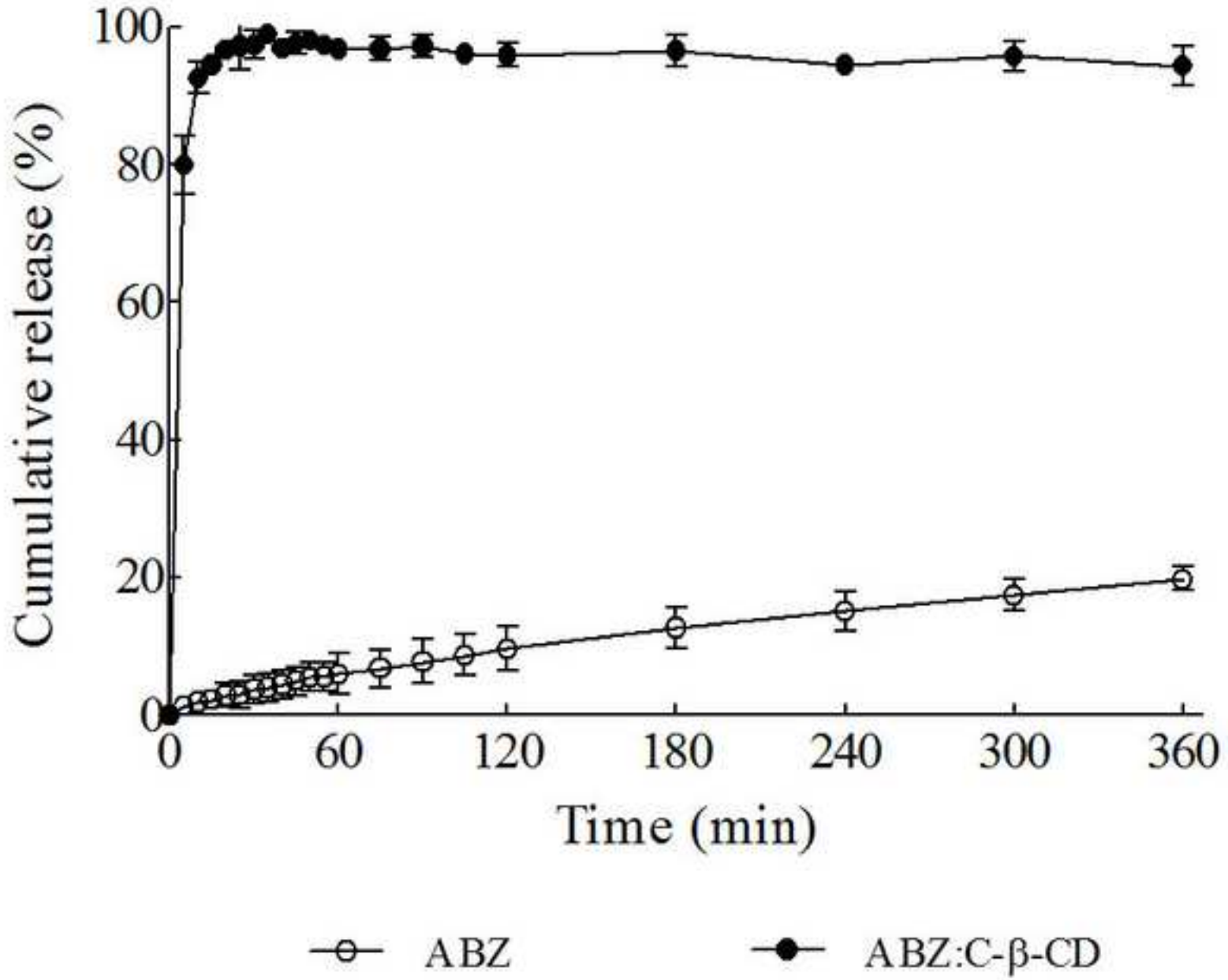
Cumulative release (%)
100
80
60
40
20
0
0
60
120
180
240
300
360
Time (min)
ABZ
ABZ:C-β-CD