Efficacy of novel albendazole salt formulations against secondary cystic echinococcosis in experimentally infected mice

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Abstract

In this study, we evaluated the efficacy, expressed as a mean weight decrease of the whole echinococcal cyst mass, of novel benzimidazole salt formulations in a murine \textit{Echinococcus granulosus} infection model. BALB/c mice were intraperitoneally infected with protoscoleces of \textit{E. granulosus} (genotype G1). At 9 months post-infection, treatment with albendazole (ABZ), ricobendazole (RBZ) salt formulations, and RBZ enantiomer salts \((R)-{(\pm)}-RBZ-Na\) and \((S)-{(\pm)}-RBZ-Na\) formulations were initiated. Drugs were orally applied by gavage at 10 mg kg\textsuperscript{-1} body weight per day during 30 days. Experimental treatments with benzimidazole sodium salts resulted in a significant reduction of the weight of cysts compared to conventional ABZ treatment, except for the \((S)-{(\pm)}-RBZ-Na\) enantiomer formulation. Scanning electron microscopy and histological inspection revealed that treatments impacted not only the structural integrity of the parasite tissue in the germinal layer, but also induced alterations in the laminated layer. Overall, these results demonstrate the improved efficacy of benzimidazole salt formulations compared to conventional ABZ treatment in experimental murine cystic echinococcosis.

Introduction

\textit{Echinococcus granulosus sensu lato} (Eg) is a cestode parasite with a life cycle involving two mammalian hosts. The adult worm develops in the small intestine of dogs and wild canids, shedding infective eggs through feces into the environment, whereas the larval form (metacestode), acquired through oral ingestion of eggs, persists mainly in the liver and lungs of several species of ungulates, in particular livestock intermediate hosts including sheep, cattle, swine and camel. Humans may act as accidental dead-end intermediate hosts, developing cystic echinococcosis (CE) (Casulli \textit{et al.}, 2019).

CE is a complex disease and the clinical presentation depends on the location of the cyst within the body, the size and number of cysts, the interaction with surrounding structures and possibly the parasite genotype (Kern \textit{et al.}, 2017). The liver is affected in approximately 70\% of cases, followed by the lungs (20\%). Other organs can be affected such as the abdominal or pleural cavities, kidney, spleen, bone, heart, central nervous system and other sites (Hizem \textit{et al.}, 2016). Rare immune-mediated reactions such as urticaria, asthma, membranous nephropathy and anaphylaxis may also occur (Agudelo \textit{et al.}, 2016). Clinical signs, if they ever develop, may arise after a highly variable incubation period of several months to years following infection. CE represents a considerable public health problem in many countries of the world, especially in pastoral regions and rural settings (Possenti \textit{et al.}, 2016; Tamarozzi \textit{et al.}, 2018; Casulli, 2020).

Among the management options for human CE (Brunetti \textit{et al.}, 2010), drug treatment is currently based on the use of poorly soluble benzimidazoles (BMZ), i.e. albendazole (ABZ) or mebendazole (MBZ). To date, the only BMZ licensed for human use are Albendazole, Triclabendazole, Flubendazole, Mebendazole and Thiabendazole. Before the introduction of
BMZs, surgery was the only option for the management of human CE. Since then, BMZs have clearly improved the prognosis for infected patients, and decreased the risk of secondary echinococcosis in patients undergoing surgery (Gollackner et al., 2000; Arif et al., 2008). However, due to the lack of parasiticidal activity of these compounds (Siles-Lucas et al., 2018), and the requirement for long-term treatment, which often lasts for years or even lifelong, there is ample room for improvement in terms of drug efficacy. Alternative formulations of BMZ, nano-compounds of BMZ and different drugs have been tested mainly *in vitro*; however, new formulations and compounds have consistently shown largely similar or lower efficacy than ABZ and only a few candidates reached clinical use (Li et al., 2016; Siles-Lucas et al., 2018; Bakhtiar et al., 2019).

One drawback of the currently used ABZ, and its metabolite ricobendazole (RBZ and ABZ sulphoxide), is the poor intestinal absorption. Thus, the drugs do not reach the desired concentrations to be efficacious against CE or other parasitic diseases against which they are active. Therefore, high dosages and repeated and/or long-term treatments are needed, which expose patients to side-effects, and decrease their quality of life (Horton, 1997).

CE is a neglected disease (Brunetti et al., 2011). In this context, no new drugs or few new formulations of already existing lead drugs for its treatment have been specifically patented in the last 40 years (Patentscope database, 2020). Within the framework of HERACLES European funded project (Casulli et al., 2020), new formulations of antiparasitic soluble drugs (‘Salts of compounds having a benzimidazolic structure’; PCT/IT2016/000191) have recently been patented for the European (EP16788256) and US (US20190016686A1) markets (Cirilli et al., 2020). Among the new BMZ formulations included in this patent, Cirilli and colleagues reported on novel salt formulations of ABZ (ABZ-Na), and of RBZ (RBZ-Na) as well as its enantiomers [(R)-(+)−RBZ-Na and (S)-(−)−RBZ-Na]. These formulations allow improved intestinal absorption. In fact, the poor solubility in water of RBZ (0.06 mg mL$^{-1}$) has been increased at physiological pH to 14.49 mg mL$^{-1}$ for RBZ-Na (Cirilli et al., 2017).

Here we provide a preliminary assessment of these newly described salt formulations with respect to their efficacy against *E. granulosus* metacestodes in terms of mean weight of parasite cyst reduction and the parasite damage they induce in a mouse model of secondary infection. We define secondary CE infection as newly formed hydatid cysts in the peritoneum, pleura or meningeal space, induced by the intraperitoneal injection of protoscoleces in such areas.

**Materials and methods**

**Parasites**

Hydatid cysts were collected from naturally infected sheep in slaughterhouses in Tekirdag (Turkey). Protoscoleces were aspirated from the cysts under aseptic conditions and washed four times with sterile phosphate-buffered saline (PBS) (pH 7.2) supplemented with gentamycin (50 μg mL$^{-1}$; LE Ulagay Pharmaceutical Industry, Istanbul, Turkey). Viability of protoscoleces was determined by methylene blue exclusion test and flame cell activity (Casado et al., 1986). *Echinococcus granulosus* genotype was determined by sequencing the mitochondrial cytochrome c oxidase subunit 1 (cox1) gene, as previously described (Vural et al., 2008). Protoscoleces preparations used for infections showed more than 95% viability and were of parasite genotype G1 (Cucher et al., 2013).

**Benzimidazole preparation**

Salts of the enantiomers of RBZ were prepared according to the procedure previously described (Cirilli et al., 2017). Synthesis of enantiopure and racemic forms of RBZ-Na was carried out by adding a weighed amount of sodium hydroxide (1.1 eq) (solvabilized in ethanol) to the ethanol solution of the non-salified form (1.0 eq). The reaction mixture was stirred for 20 min at room temperature, filtered and the solvent evaporated under reduced pressure. The drugs assessed in this study were: (1) ABZ; (2) ABZ-Na (ABZ sodium salt); (3) RBZ-Na (RBZ sodium salt); (4) (R)-(−)−RBZ-Na enantiomer and (5) (S)-(−)−RBZ-Na enantiomer.

**Experimental infection and drug treatment**

Seventy-two female BALB/c mice, 10 weeks of age, were housed with free access to food and water. After 1 week of quarantine period, they were marked by individual ear tag numbers. Each mouse was infected by intraperitoneal injection of 1500 viable protoscoleces suspended in 500 μL sterile PBS. After infection and before treatment, only abdominal enlargement was observed due to the growth of the cyst. Nine months post-infection, the mice were randomly assigned to six distinct treatment groups of 12 mice each: (1) ABZ, (2) ABZ-Na, (3) RBZ-Na, (4) (R)-(−)−RBZ-Na, (5) (S)-(−)−RBZ-Na and (6) placebo control. Since ABZ and ABZ-Na are poorly water soluble, they were formulated in dimethyl sulphoxide (DMSO) similarly to other published studies (Siles-Lucas et al., 2018). Since RBZ-Na, (R)-(−)−RBZ-Na and (S)-(−)−RBZ-Na are highly soluble, they were formulated in PBS for human delivery. Drugs were administered at a concentration of 10 mg kg$^{-1}$ body weight in a volume of 100 μL by oral gavage on a daily basis for a period of 30 days. The control group was daily dosed with a corresponding amount of DMSO. All formulations were prepared freshly every day.

**Blood and tissue sampling**

Blood and parasite cyst samples were collected on day 30 at different time points after the last drug application, namely 0, 15, 30 and 180 min after dosing. Three mice per time point and group were sampled, with the exception of group ABZ-Na (two mice sampled at 30 min), and groups RBZ-Na (two mice sampled at 15 min), and (S)-(−)−RBZ-Na (two mice sampled at 15 min), since three mice died at different days during daily check in the 9-month infection period, prior to the treatment. Autopsy was performed on these three animals and revealed that some cysts bursted and there was fluid in the abdominal cavity. Therefore, we think that the cause of death may be due anaphylaxis consequent to the rupture of cysts. We did not find any unusual findings other than cyst burst at autopsy.

Mice were anaesthetized and euthanasia was conducted by exsanguination by cardiac puncture. Blood was collected in EDTA-containing tubes and stored at 4°C for 8 h. Blood samples from each time point within groups were pooled and centrifuged for 10 min at 1300 g at 4°C. Plasma was separated in 500 μL aliquots and stored at −20°C until use. After death by exsanguination, mice were dissected by opening the peritoneal cavity, and the hydatid cysts were removed, separated from host tissue and the total cystic mass was weighed. From each mouse, cyst fluid samples were collected from five randomly selected cysts using a sterile syringe and samples were stored at −20°C until use. One large (diameter ranging from 10 to 25 mm) and one medium-sized (diameter ranging from 5 to 9 mm) cyst from six animals per group were randomly selected and processed for microscopical evaluation.
**Histochoromical evaluation of parasite cysts**

Methanol-carnoy (methacarn) fixative (Puchter et al., 1970) was injected into the cysts immediately following cyst fluid aspiration. After 1 h at room temperature, cysts were cut into two pieces and fixation resumed for another 4 h. Tissue samples were then sequentially dehydrated in absolute ethanol processed in methyl benzoate and benzole and then embedded in paraffin (Heym and Forssmann, 1981).

Consecutive 5–6 μm thick sections were cut and placed onto glass slides, and were stained using trichrome staining for histological structures (Gray, 1954), and PAS-Alcian blue-(PAS/AB) for neutral and acid mucopolysaccharides (Bancroft et al., 1990). Specimens were assessed microscopically using an Olympus BX51 microscope (Tokyo, Japan).

**Scanning electron microscopy (SEM)**

Following cyst fluid aspiration, cysts were injected with 4% glutaraldehyde in 0.2 M Sorensen’s buffer at pH 7.2 (Murtey and Ramasamy, 2016). After 1 h, cysts were cut in half and were further fixed for 4 h. For SEM examination, cyst samples were rinsed in Sorensen’s buffer and were dehydrated in graded series of ethanol, and then air-dried. The cysts were placed onto SEM stubs with the germinal layer (GL) exposed upwards, and the samples were inspected without coating in a QUANTA 250 FEI (Oregon, USA) at 100 Pa according to the protocol recommended by Murtey and Ramasamy (2016).

**HPLC analysis**

**Reagents and instruments**

High performance liquid chromatography (HPLC) analyses were performed by using a stainless-steel Chiralpak IA (250 mm × 4.6 mm i.d., 3 μm) column (Daicel, Tokyo, Japan). HPLC-grade solvents were supplied by Sigma-Aldrich (Milan, Italy). The HPLC system consisted of a Dionex PS80 LPG pump (Dionex, Sunnyvale, CA, USA), an ASI-100T autosampler, a STH 585 column oven and a PDA-100 UV detector. The data were acquired and processed by using a Chromeleon Datasytem ( Dionex).

**Preparation of the calibration curves**

Solutions for calibration curves were prepared with pooled plasma samples from non-infected and non-treated mice (400 μL) spiked with known amounts of ABZ (5.140, 2.570, 1.028 and 0.206 μg), RBZ (5.340, 2.670, 1.068 and 0.214 μg), (ABZ-SO2) (5.180, 2.590, 1.036 and 0.207 μg) and albendazole sulphone (ABZ-Na) (69.0%), followed by (R)-(+)RBZ-Na (56.2%), RBZ-Na (41.0%) and ABZ (36.4%). (S)-(−)-RBZ-Na was the least effective (13.3%). (Fig. 1 and Table 1). Of note, treatment with the salt of ABZ (ABZ-Na) resulted in significantly lower cystic weight compared to treatment with conventional ABZ formulation diluted in DMSO (P = 0.037). Overall, differences were also found among the hydro-soluble drugs (P = 0.001). In particular, mean cyst weights were significantly lower in the group treated with RBZ-Na compared to the (S)-(−)-RBZ-Na group (P = 0.039). In terms of cyst weights, (R)-(+)RBZ-Na treatment was also significantly more effective compared to treatment with (S)-(−)-RBZ-Na (P = 0.001). No significant differences were found between groups treated with RBZ-Na and (R)-(+)RBZ-Na.

**Histopathology: salts of BMZ induced alterations in the GL and laminated layer (LL) of E. granulosus metacestodes**

Trichrome staining (Fig. 2) showed that the GL of cysts in control animals consisted of 3–4 sequential cellular layers, including the tegument, with an average thickness of 12.8 μm. Nuclei of GL cells were clearly visible (Fig. 2A). In cysts from drug-treated mice, the GL was substantially reduced to a thin layer, sometimes only represented by the tegument, with only a few discernible nuclei (Figs 2B and 2E). In cysts recovered from

**Results**

Efficacy of salts of BMZ against E. granulosus metacestodes in the murine model

Following infection of mice with E. granulosus sensu stricto (G1 genotype) protoscoleces, the development of hydatid cysts proceeded over a time span of 9 months, followed by daily treatments by oral gavage during 30 days. Necropsies were performed 10 months after infection. Cysts were found in the abdominal cavity of all mice. The mean weights of the total cyst load in all treatment groups were significantly different from the control group, with the exception of the (S)-(−)-RBZ-Na treatment. The highest efficacy, expressed as a mean weight decrease of the whole echinococcal cyst mass, was obtained by treatment with ABZ-Na (69.0%), followed by (R)-(+)RBZ-Na (56.2%), RBZ-Na (41.0%) and ABZ (36.4%). (S)-(−)-RBZ-Na was the least effective (13.3%). (Fig. 1 and Table 1). Of note, treatment with the salt of ABZ (ABZ-Na) resulted in significantly lower cystic weight compared to treatment with conventional ABZ formulation diluted in DMSO (P = 0.037). Overall, differences were also found among the hydro-soluble drugs (P = 0.001). In particular, mean cyst weights were significantly lower in the group treated with RBZ-Na compared to the (S)-(−)-RBZ-Na group (P = 0.039). In terms of cyst weights, (R)-(+)RBZ-Na treatment was also significantly more effective compared to treatment with (S)-(−)-RBZ-Na (P = 0.001). No significant differences were found between groups treated with RBZ-Na and (R)-(+)RBZ-Na.
In all specimens, larger bud-like formations of various sizes (up to 20 μm in diameter) could be detected (Figs 3B, 3C and 3F), which most likely represent various stages of cellular aggregates that could potentially be in the process of forming brood capsules.

**SEM: salts of BMZ induced alterations in the germinal layer of E. granulosus metacestodes**

SEM was performed to visualize the GL and potential drug-induced alterations (Fig. 3). In control specimens (Fig. 3A), the GL exhibited a distinct surface structure, which was covered with numerous round cells of 3–5 μm in diameter which, based on their size and shape, were most likely undifferentiated cells that form an important part of the GL. In ABZ (Fig. 3B) and ABZ-Na (Fig. 3C) treated cysts, the surface of the GL was more flattened, the presence of these round cells was not as distinct, although structures resembling undifferentiated cells were also visible. In contrast, the GL surface of cysts obtained from RBZ-Na (Fig. 3D) (R)-(+)–RBZ-Na (Fig. 3E) and (S)-(−)–RBZ-Na (Fig. 3F) treated mice exhibited a much more fibrous structure, with small round cells only occasionally visible. In all specimens, larger bud-like formations of various sizes (up to 20 μm in diameter) could be detected (Figs 3B, 3C and 3F), which most likely represent various stages of cellular aggregates that could potentially be in the process of forming brood capsules.

**Pharmacokinetic properties of BMZ and their salts in E. granulosus-infected mice analysed by HPLC**

After final drug dosing, plasma and hydatid fluid samples were collected at different time points from *E. granulosus*-infected mice, and the concentrations of the metabolites of RBZ (ABZ-SO₂), (R)-(+)–RBZ-Na and (S)-(−)–RBZ were measured by HPLC. Maximum plasma levels occurred after 15 min. The highest metabolite concentration was measured for the enantiomer (S)-(−)–RBZ after ABZ treatment (Fig. 4A), and at a similar level (approximately 50 μg mL⁻¹) after treatment with the enantiomer itself (Fig. 4E). However, (S)-(−)–RBZ was also the major metabolite after treatments with RBZ-Na and ABZ-Na, albeit at considerably lower levels (approximately 20 and 8 μg mL⁻¹, respectively; Figs 4B and 4C). (S)-(−)–RBZ was virtually undetectable in mice treated with (R)-(+)–RBZ-Na (Fig. 4D). ABZ-SO₂ levels were in a similar range (peak of 8–10 μg mL⁻¹) after ABZ, RBZ-Na and (R)-(+)–RBZ-Na treatments. The (R)-(+)–RBZ enantiomer exhibited low levels (5 μg mL⁻¹ or below) after treatment with all compounds, except for after treatment with (R)-(+)–RBZ-Na (Fig. 4D). Latest at 180 min after initiation of drug treatment, none of the metabolites was detectable any more, except for after the application of ABZ, where the (S)-(−)–RBZ level was still elevated (Fig. 4A).

The concentrations of the two enantiomers of RBZ were also measured in the hydatid cyst fluids of the different experimental groups. These data and a comparison with the corresponding plasma levels are shown in Fig. 5. After treatments with ABZ, ABZ-Na, RBZ-Na and (S)-(−)–RBZ, only (S)-(−)–RBZ was...
detectable in cyst fluid (Fig. 5). (R)-(+)RBZ-Na was only detected in cyst fluid upon treatment with that particular enantiomer (Fig. 5). Maximum levels of metabolites in the cyst fluid were reached 30 min after drug applications, thus 15 min after maximum levels in plasma were detected.

Discussion

The currently applied chemotherapeutical treatment of CE relies on the use of benzimidazole carbamate derivatives such as ABZ and mebendazole. In particular, ABZ has been widely used for the treatment of CE, but also for the treatment of alveolar echinococcosis, caused by the closely related cestode E. multilocularis (Siles-Lucas et al., 2018). However, ABZ exhibits a low water solubility, and is therefore not well absorbed, which in turn warrants cumbersome treatments with ABZ-Na and (R)-(+)RBZ-Na formulations, but not of the (S)-(−)-RBZ enantiomer, exhibit promising efficacy in the murine model of secondary CE, as assessed by weight decrease of the total intra-peritoneal cyst mass after 1 month of treatment subsequent to a 9-month old infection. In addition, alterations of the GL and LL structure of cysts from mice treated with these drug formulations were observed at the microscopic level when compared to those recovered from control mice.

The higher efficacy of (R)-(+)RBZ-Na in this in vivo study confirms what has been previously suggested for (R)-(+)RBZ in others in vitro or ex vivo studies on zoonotic helminths such as Taenia solium, Fasciola hepatica, Trichinella spiralis and E. granulosus (Alvarez et al., 2000; Bolás-Fernández et al., 2004; Bao et al., 2008; Paredes et al., 2013).

Pharmacokinetic studies of ABZ application in mice have been reported earlier in a study focusing on E. multilocularis infection (Stettler et al., 2004), but only ABZ-SO2 levels were measured. In this earlier study, ABZ-SO2 levels peaked at 1 h post drug application (no earlier time points were measured), whereas serum levels reached a maximum of 6 μg mL−1, and declined rapidly thereafter. Taking into account the different experimental settings in terms of vehicle, timing of drug application, dosing etc., the results of this study are rather similar. The novelty of the approach reported in this paper is the analysis of the RBZ enantiomer plasma levels. Interestingly, plasma levels of (S)-(−)-RBZ were always higher than those of (R)-(+)RBZ in our model, which is in accordance with the known predominance of (S)-enantiomer formation after treatment with a racemic drug in rats and mice (Delatour et al., 1990, 1991; García et al., 1999, 2003; Capece et al., 2003). Also, intra-cystic levels of (R)-(+)RBZ were not measurable in any group, with the exception of mice directly treated with (R)-(+)RBZ-Na, suggesting that intra-cystic level of (R)-(+)RBZ could be below the detection limit even at the second assessed time point (15 min post-dosage). These results, together with the shape of the pharmacokinetic plasma curves, indicate a rapid metabolization of the compounds, which impairs measurements of the actual C_max in our experiments. However, the presence of measurable plasma levels of the (R)-(+)RBZ-Na enantiomer coincided with the most efficacious treatments with ABZ-Na and (R)-(+)RBZ-Na in terms of mean cyst weight reduction.

Fig. 2. Sections of paraffin-embedded E. granulosus cysts recovered from non-treated control (A, G) and drug treated (B–F, H) mice. Sections were stained with trichrome (A–F) and PAS (G) and PAS/AB (H), to visualize the GL, the adventitial layer (AL) and the LL. In (B), mice were treated with ABZ; in (C) with ABZ-Na; in (D) with RBZ-Na; in (E) with RBZ; in (F) with (S)-(−)-RBZ-Na; in (G) with PAS; in (H) with PAS/AB. Note the bud-like cellular aggregates marked with arrows.

Fig. 3. SEM of the GL surface of E. granulosus cysts recovered from non-treated control (A) and drug treated (B–F) mice. Treatments in (B) = ABZ; (C) = ABZ-Na; (D) = RBZ-Na; (E) = (R)-(+)RBZ-Na; (F) = (S)-(−)-RBZ-Na. Note the bud-like cellular aggregates marked with arrows.
Our results are an important addition to the potential arsenal of benzimidazole formulations that could be used for the treatment of CE (Siles-Lucas et al., 2018). Drug repurposing or modification of existing drugs are the way forward, since the development of novel compounds for the treatment of CE is hampered by the expected low-level market return, inducing the pharmaceutical industry not to invest in drug discovery for this disease. Benzimidazoles are attractive compounds as they are widely used, and data on their pharmacological properties are widely available. Improving these properties through novel formulations will be an important step in drug development for neglected diseases, including CE. Despite considerable efforts and interesting results obtained in vitro (Siles-Lucas et al., 2018), only a few drugs investigated for repurposing for CE
have been shown to be efficacious in vivo. Combination treatments using FBZ and netobinin, which is a prodrug of ABZ used as a veterinary anthelmintic (Garcia-Llamazares et al., 1997), were shown to be efficacious in a rodent CE model. In E. granulosus-infected sheep and goats, oxfendazole and oxenda- zole combined with nitazoxanide, were shown to be as efficacious as ABZ whereas not requiring daily uptake of the drug due to their prolonged bioavailability (Gavidia et al., 2009; Gonzalez et al., 2019). Other benzimidazoles exhibiting interesting protoscolidal and metacestodicidal activity in vivo are flubendazole (Elissondo et al., 2007; Ceballos et al., 2015) and nodocazole (Shkolar et al., 2014). However, all these compounds have not been licensed for the use in humans to date.

Conclusion and future perspectives

These new formulations of salts of RBZ and its enantiomers exhibit a number of potential advantages such as increased water solubility, a possible parenteral administration for animal and human use, improved bioavailability in plasma, and thus better anthelmintic activity. Moreover, the improved bioavailability helps to decrease adverse reactions of BMZ drugs (namely hepatotoxicity, alopecia, gastrointestinal disturbances, thrombocyto- paenia and severe leucopaenia), through decreasing dosage of racemic and enantiopure formulations. In addition to this, since in vivo racemization was not observed after administration of individual enantiomers, a therapeutic application of enantiopure forms of sulphoxides could be envisaged.

Moreover, because of these encouraging experimental results and the simple, rapid and affordable synthesis of both racemic and enantiopure forms of salts of BMZ, the proposed formulations are suitable for a sustainable and affordable large-scale pharmaceutical development of these alkaline salts for treatment of helminth infectious diseases (Cirilli et al., 2017).

The way forward will be to test the efficacy, pharmacokinetics and more interestingly the toxicity of these potential new drug formulations in different helminth species.

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Conflict of interest. None.

Ethical standards. This study was previously approved by the Namik Kemal University Animal Experiments Local Ethics Committee (NKUHADYEK 2015/12). The experimental test strictly followed all procedures recommended by Regulation on the welfare and protection of animals used for experimental and other scientific purposes, Ministry of Food, Agriculture and Livestock, Turkey (Reg. 2011/28141).

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