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Research article

Development of an improved anesthesia protocol to increase CF1 mice survival in a portal vein infection with *Echinococcus granulosus* sensu lato protoscoleces

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ABSTRACT

In order to optimize the survival rate of animals, the purpose of this study was to evaluate an injectable anesthesia protocol for the development of a murine model of hepatic cystic echinococcosis in female CF-1 mice. Three protocols of injectable anesthesia were evaluated during the infection of mice with *Echinococcus granulosus* sensu lato protoscoleces via the portal vein. The use or not of pre-anesthesia [atropine (0.4 mg/kg) and tramadol (2 mg/kg)] and the incorporation or not of yohimbine (0.5 mg/kg) (a reverser of xylazine) in mice anesthetized with ketamine/xylazine 80/8 mg/kg were evaluated. Most mice treated only with ketamine/xylazine 80/8 mg/kg did not achieve a deep surgical anesthetic plane. All mice treated with pre-anesthetic drugs achieved a deep surgical anesthetic plane after the administration of the anesthetic cocktail. Pre-anesthetic drugs application significantly reduced time induction of animals compared with those that received only anesthetic cocktail. Recovery time was significantly faster in the group that received yohimbine. Mice underwent laparotomy that did not receive yohimbine after surgery had a survival rate of 67%, whereas in the group treated with yohimbine the survival was 100 %. We recommend the protocol that applied pre-anesthetic drugs + ketamine/xylazine 80/8 mg/kg + yohimbine, as safe and reliable for the portal vein infection of mice with protoscoleces of *E. granulosus* sensu lato.

1. Introduction

Human cystic echinococcosis, a zoonosis caused by the larval stage of *Echinococcus granulosus* sensu lato (s. l.), is characterized by long term growth of hydatid cysts most commonly in the liver and lungs. This parasitic infection is a chronic, complex, and still neglected disease (Wen et al., 2019).

Animal models play an important role in the study of novel drugs, surgical approaches, and vaccine development (Zhang et al., 2017b). In order to establish animal models for human cystic echinococcosis, many rodent species have been experimentally infected by oral inoculation of eggs or intraperitoneal injection of protoscoleces (Romig and Bilger, 1999; Mourglia-Ettlin et al., 2011). Recently, Zhang et al. (2017b) established a murine model by injection of protoscoleces of *E. granulosus* s. l. in the portal vein of C57B/6 mice.

Portal vein injection is also used in murine models for the development of pathologies such as liver metastasis (Goddard et al., 2016; Orci et al., 2018) and parasitic diseases (Nakaya et al., 1997; Jarillo-Luna et al., 2002; Chen et al., 2017; Zhang et al., 2017a, 2017b; Cortes et al., 2019; Abulizi et al., 2019). These procedures require laparotomy and therefore must be performed under anesthesia and analgesia to prevent or minimize the discomfort, pain, and/or distress of mice (Flecknell, 2009).

There are two anesthesia induction and maintenance procedures, by inhalation or by injection. The inhalation has several advantages such as control of the depth of anesthesia, less cardiopulmonary depression, and faster recovery (Erickson et al., 2019). However, the use of these agents

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requires additional specific devices and is expensive (Buitrago et al., 2008; Davis, 2008; He et al., 2010). In contrast, injectable anesthesia is inexpensive, easier to perform, and researchers are not exposed to the potential adverse effects of vapors. On the other hand, the use of injectable anesthesia may be associated with cardiovascular and respiratory depression, prolonged recoveries, lower margin of safety, and hard to control anesthesia depth (Tammam et al., 2019).

Several researchers have reported the use of different anesthesia protocols (inhalational or injectable) to perform laparotomy and subsequent intraportal injection of an agent in mice (Arnheiter et al., 1976; Nakaya et al., 1997; Jarillo-Luna et al., 2002; Chen et al., 2017; Kawaguchi et al., 2017; Zhang et al., 2017a, 2017b; Sherman et al., 2014; Goddard et al., 2016; Orci et al., 2018; Abulizi et al., 2019; Cortes et al., 2019). However, only a few studies have described the use of analgesic for pain management and it was applied at the end of the surgical procedure (Jarillo-Luna et al., 2002; Sherman et al., 2014; Goddard et al., 2016; Orci et al., 2018). On the other hand, the use of pre-anesthetic drugs (atropine) was reported by Chen et al. (2017). In addition, when xylazine is used for sedation, none α2 antagonist was applied (Kawaguchi et al., 2017). Furthermore, the information on the survival and health condition of animals after the surgical is missing.

Chloral hydrate is one of the injectable drugs most used to induce anesthesia in this type of procedure (Chen et al., 2017; Zhang et al., 2017a, 2017b; Abulizi et al., 2019). However, it can cause adynamic ileus (Fleischman et al., 1977) and it often produces only a light plane of anesthesia in rodents. Therefore, chloral hydrate should be replaced by more effective and safer agents (Baxter et al., 2009). Ketamine in combination with other drugs (droperidol, xylazine, acepromazine maleate) was also used as an injectable anesthesia protocol to performed portal vein infection (Jarillo-Luna et al., 2002; Kawaguchi et al., 2017; Cortes et al., 2019).

Recently, we have established a murine model of hepatic cystic echinococcosis in female CF-1 mice by intraportal infection with protoscoleces of *E. granulosus* s. l. (Manuscript in progress). This surgical procedure was performed by a veterinary doctor together with personnel trained in the handling of laboratory animals. Female CF-1 mice were previously anesthetized intraperitoneally (IP) with a mixture of 100 mg/kg of ketamine (sedative, analgesic, and anesthetic drug) and 10 mg/kg of xylazine (sedative α2 agonist) (Flecknell 2009; Schuetze et al., 2019). After confirming the surgical plane, laparotomy and infection were performed via the portal vein (Goddard et al., 2016; Zhang et al., 2017b). At the end of the surgery, 2 mg/kg of tramadol hydrochloride was injected subcutaneously (SC) as an analgesic drug. Mice were kept on the heating pad for the entire duration of the surgery and postsurgical recovery to avoid hypothermia. The post-surgery survival rate was 57%. These animals received analgesic treatment during the following 48 h post-surgery. The remaining 43% of the animals died after prolonged sleep following surgery.

A proper experimental design not only reduces the number of animals exposed but also ensures the refinement of the procedures to obtain reliable and replicable results (National Research Council US, 2011). After our preliminary experience and in order to optimize the survival rate of animals, the aim of the current study was to evaluate an injectable anesthesia protocol for the development of a murine model of hepatic cystic echinococcosis in female CF-1 mice.

2. Materials and methods

2.1. Experimental animals

Animal procedures and management protocols were approved by the Institutional Animal Care and Use Committee (RDs 468/17 and 211/2018) of the Faculty of Exact and Natural Sciences, National University of Mar del Plata, Argentina and carried out in accordance with the revised form of The Guide for the Care and Use of Laboratory Animals (National Research Council US, 2011). Unnecessary animal suffering was avoided throughout the study.

All mice were kindly provided by Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA, Mar del Plata, Argentina). Female CF-1 mice (*Mus musculus*; 6–7 weeks of age, body weight 26 ± 2 g, n = 22) were used for this study. The animals were housed in polycarbonate cages (3–5 mice per cage) containing disposable bedding. Cages were enriched with shelter for nesting (paper huts). The housing room was maintained at 22 ± 1 °C temperature, relative humidity of 45–65%, and a 12:12-h light: cycle. Food (Cooperación, Asociación de Cooperativas Argentinas) and water were given ad libitum. Feed was supplied in an overhead feeder. Mice were allowed at least 1 week to acclimate to the housing facility and cage environment prior to the start of the study.

2.2. Protoscolex collection

Protoscoleces of *E. granulosus* s. l. were collected aseptically from liver and lung hydatid cysts of infected cattle slaughtered in an abattoir located in Buenos Aires province, Argentina. Viability was assessed by the methylene blue exclusion test (Elissondo et al., 2006).

2.3. Drugs, route of administration and dilution

All components were pharmaceutical grade injectable solutions. The drugs were diluted in sterile saline immediately prior to the administration. The doses of drugs used in the present study were selected according to data published for mice (Carpenter and Marion, 2013; Erickson et al., 2016; Flecknell, 2009).

Atropine sulfate (0.4 mg/kg SC, Jonh Martin) and tramadol hydrochloride (2 mg/kg IP, Algen20, Richmond Vet Pharma) were administered as pre-anesthetic drugs. The anesthetic cocktail contained ketamine HCL (80 mg/kg, Holliday) and xylazine (8 mg/kg, Richmond Vet Pharma) and it was given by IP route at different doses according to the protocol. All IP injections were administered in the lower right quadrant of the abdomen. In two protocols, yohimbine (0.5 mg/kg IP, Río de Janeiro) was applied immediately at the end of the suture. In all protocols, the anti-inflammatory and analgesic drug dexamethasone (2 mg/kg SC, Lamar) was administered at the end of surgery, and tramadol hydrochloride (2 mg/kg SC) was administered every 24 h for two days.

2.4. Anesthetic protocols

Three different protocols were evaluated 1) anesthetic cocktail ketamine + xylazine (n = 8); 2) pre-anesthetic drugs + anesthetic cocktail ketamine + xylazine (n = 6); and 3) pre-anesthetic drugs + anesthetic cocktail ketamine + xylazine. + yohimbine (n = 8).

Following the principles of the 3Rs (Replacement, Reduction, and Refinement), the infected mice that survive were used to characterize the development of the infection (Manuscript in progress).

2.5. Study design and criteria of assessment

Mice were previously weighed to determine the exact dose of each drug. The drugs were given at a maximum injection volume of 100–120 μl. To avoid any interference of circadian rhythm, the experiment was always performed from 8.00 a.m. to 15.00 p.m. In protocols 2 and 3, atropine and tramadol were administered 10 and 15 min before anesthetic cocktail injection, respectively. In all protocols, after administration of the anesthetic cocktail, each mouse was transferred to a plastic box to observe its behavior and vital signs. Respiratory rate was visually monitored. After confirming the loss of reflexes (Table 1), the animal was laid in dorsal recumbency on a heating pad (between 35 and 37 C°) to maintain body temperature. Artificial tears were placed over each eye to avoid excessive drying during the surgical procedure (Goddard et al., 2016).

Table 1. Clinical criteria to evaluate three anesthetic protocols in CF-1 mice.

Criteria	Assessment
Survival	Percentage of animals that survived the surgery.
Loss of reflexes: - Righting reflex (RR) - Pedal withdrawal reflex (PWR)	Percentage of animals that lose the RR and PWR.
Induction time (minutes)	Period from the administration of the anesthetic cocktail until the loss of RR, TPR.
Recovery time (minutes)	Period from the end of surgery to the recovery of all reflexes and the return of the ability to walk.
Duration of action (minutes)	Period between the losses of all reflexes until the recovery of mice.

The abdomen was shaved and aseptically prepared for laparotomy. A 1.5 cm incision was made from the bladder up to the level of the xiphoid. The intestines were carefully moved to one side with sterile gauze to expose the portal vein (Goddard et al., 2016). Protoscoleces (n = 200) of *E. granulosus* s.l. (viability >98%) resuspended in 100 μl of sterile saline were injected in the portal vein using a 30G needle (Zhang et al., 2017b). Then, the intestines were placed back into the abdomen and the abdominal wall and skin were sutured with nylon (0.7 mm). Mouse was maintained on the heating pad for recovery with frequent inspection (Goddard et al., 2016). The postoperative care consisted of pain management and isolation of animals from loud noises and strong light during 24 h post-infection. The treatment of pain included a single injection of dexamethasone at the end of the procedure and the administration of tramadol once a day during the first 48 h after surgery.

The criteria to evaluate the response of mice under the different anesthetic protocols are shown in Table 1.

Animals were observed daily for signs of morbidity and these data were recorded on comprehensive health monitoring standard operating procedure which included assessments of body weight loss, change in appetite, and in stool consistency as well as behavioral and appearance alterations (National Research Council US, 2011).

2.6. Statistical analysis

Data are reported as median and interquartile range (IQR). The analysis was carried out using Instat 3.0 software program (GraphPad Software, San Diego, CA, USA). The induction time of mice were compared by Kruskal Wallis Test (nonparametric method) followed by Dunn's Multiple Comparisons Test. The recovery time and duration of action were compared by Mann-Whitney Test. In all cases, P values less than 0.05 ($P < 0.05$) were considered statistically significant.

3. Results

The response of CF-1 mice to three anesthesia regimens to perform portal vein infection with *E. granulosus* s. l. protoscoleces is shown in Table 2. The median of surgery duration was 12 (2) minutes.

In protocol 1 where only ketamine and xylazine were administered, only 37.5% of mice achieved a deep anesthetic plane. On the other hand, all mice treated with pre-anesthetic drugs (protocols 2 and 3) achieved a deep surgical anesthetic plane and profound analgesia after the administration of anesthetic cocktail. Time induction of animals that received only the anesthetic cocktail was significantly longer compared to those treated with pre-anesthetic drugs ($P < 0.05$; Table 2).

Mice underwent laparotomy with anesthetic schemes 1 and 2 (without yohimbine) had a mortality rate of 33%, whereas with scheme 3 (with yohimbine) all animals survived. No statistically significant differences were found in the recovery time between protocols 1 and 2 ($P = 0.55$). On the other hand, a statistically significant reduction of the recovery times of mice under protocol 3 compared to those treated with protocol 2 were observed ($P = 0.0005$; Table 2).

The behavior and appearance of the animals that survived the procedures were normal after 6 months post-infection. The 78.6 % of these animals developed hepatic cysts.

4. Discussion

The role of anesthetics in animal research models is crucial, yet often ignored, and is almost never considered as the main subject during experimental design (Zuurbier et al., 2014). In the present work, we report an anesthesia protocol for the portal vein infection of female CF-1 mice with *E. granulosus* s. l. protoscoleces that allowed 100% survival of the animals post-surgery. In addition, this protocol reduced anesthesia induction and recovery times.

Ketamine and xylazine is the combination of injectable drugs most commonly used as anesthetic regimen in procedures in mice and rats (Herrmann and Flecknell, 2019). Our results clearly show that the single cocktail of ketamine/xylazine 80/8 mg/kg would not be recommendable to perform a laparotomy. In most of the mice the required surgical plane was not achieved, while the animals that reached the anesthetic plane showed the longest induction times. In contrast, Kawai et al. (2011) using the same dose of cocktail achieved an anesthetic action of about 20 min in male ICR mice. Therefore, the

Table 2. Safety, efficacy, and time course of anesthesia after administration three anesthetic protocols to perform portal vein infection with *Echinococcus granulosus* s. l. protoscoleces in female CF-1 mice.

Protocols	(1) K 80/X 8	(2) Pre-anesthetics + K 80/X 8	(3) Pre-anesthetics + K 80/X 8 + Y
Loss of Reflexes[a]	3/8	6/6	8/8
Induction Time (min)	8 (2,5)*	3 (0.75)	3 (0)
Survival[b]	2/3	4/6	8/8
Recovery Time (min)	68.5 (35.5)	54 (14.25)	28.5 (9.75)**
Duration of action (min)	80.5 (36.5)	66 (14.25)	40.5 (11.25)**

K: ketamine, X: xylazine, Pre-anesthetic (tramadol and atropine), Y: yohimbine.
Time course data are given in minutes and present as median (interquartile range).
(*) $P < 0.05$, statistically significant differences between protocol 1 vs. protocols 2 and 3.
(**) $P = 0.0005$, statistically significant differences between protocol 2 vs. protocols 3.
[a] N° of mice that lose reflexes after the administration of the anesthetic cocktail/total n° of mice in the group.
[b] N° of recovered mice after surgery/total n° of operated mice.

effect of anesthetic drugs can vary according to murine strain and sex (Kawai et al., 2011).

The combination of anesthetic drugs has better results compared to an individual drug since one substance cannot generate all the effects that are desirable in anesthesia (Aleman-Laporte et al., 2019). Moreover, the combination avoids the unwanted effects produced by high dosages of a single component. For this reason, we used atropine and tramadol as pre-anesthetic drugs.

The anesthetic protocol should have an adequate balance of muscle relaxation, analgesia, and unconsciousness (Donati, 2003) that is achieved with the administration of sedatives, analgesics, and anesthetics drugs, respectively. This balance allows a smooth induction, increases safety margin, and minimizes postoperative pain (Flecknell, 1996; Ilkiw, 1999). Tramadol is an analgesic drug (opioid) used in mice (Herrmann and Flecknell, 2019). The analgesic should be preoperatively administered because most analgesics reach the full onset of action between 15 and 45 min after its application (Miller and Richardson, 2011). The use of tramadol as a pre-anesthetic drug allows reducing the doses of the anesthesia cocktail (Aleman-Laporte et al., 2019). Besides, this drug enhances the intraoperative patient stability, reducing the noxious stimuli and peripheral inflammation, and reduces postoperative pain (Buitrago et al., 2008; Miller and Richardson, 2011; Aleman-Laporte et al., 2019). In our study, the administration of tramadol allowed us to reduce the ketamine and xylazine dose that we used in a preliminary study (100/10). All mice treated with tramadol reached the anesthetic plane. Moreover, the induction time was 3 times faster than in animals without pre-anesthetic treatment.

Atropine sulfate, an anticholinergic drug, is frequently applied subcutaneously or intraperitoneally in small animals 10 min before anesthesia induction to prevent the bradycardia induced by xylazine. In addition, atropine reduces bronchial secretions and protects the heart from the vagal stimulation which may occur during surgical procedures (Davis, 2008; Jiron et al., 2019). For this reason, we consider that the administration of atropine could stabilize the ketamine–xylazine treated mice.

Due to their small body size, rodents are prone to hypothermia, respiratory, and cardiovascular depression caused by xylazine, and mortality increases with prolonged postsurgical sleep time. This risk can be reduced by using agents such as the α2 antagonists (yohimbine or atipamezole) that reverse the effect of xylazine (Herrmann and Flecknell, 2019). The α2 antagonists allow a rapid normalization of physiological function. As a result, it could improve anesthetic results and overall animal health. On the other hand, decreasing unnecessary anesthesia time may also decrease the time that the bioterium staff spends observing sleeping mice (Janssen et al., 2017). Despite this, most researchers continue using ketamine and xylazine without the application of an agent to reverse xylazine sedation (Albrecht et al., 2014).

In our study, at the end of the surgery, animals belonging to protocol 3 were treated with yohimbine. The recovery time and the duration of action were significantly shorter when yohimbine was applied. Moreover, survival of operated mice was 100%. This result is in accordance with those reported by Janssen et al. (2017). Their results revealed that mice anesthetized with xylazine and ketamine became ambulatory much earlier when the effects of xylazine were reversed using an α2 antagonist (Janssen et al., 2017).

In conclusion, the anesthetic protocol that applied pre-anesthetic drugs (tramadol and atropine) + ketamine/xylazine + yohimbine was the most appropriated for the infection via the portal vein of female CF1 mice with *E. granulosus* s. l. protoscoleces. Our results could be useful for other authors working with murine models of diseases; however, it would be important to evaluate the anesthetic protocol under the new conditions (mice strain, sex, age, infectious agent, etc). This protocol ensures animal welfare and follows the recommended anesthetic balance for this invasive procedure. In addition, it is a feasible and economically viable method that reduces recovery time and avoids adverse effects in mice.

Declarations

Author contribution statement

Nathalia P Scioscia, Patricia E Pensel: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Guillermo M Denegri: Contributed reagents, materials, analysis tools or data.

María Celina Elissondo: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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