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RESEARCH ARTICLE

Establishing an anesthetic protocol for refinement of intracerebral inoculation procedure

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Abstract

Mouse inoculation test (MIT) is a technique widely used for rabies diagnosis and must be liable to refinement due to animal welfare. The present study aims to compare five different anesthetic associations to stablish a protocol to improve the MIT procedure suitable for animal welfare and safe for a routine of viral isolation in newly weaned mice (3 weeks of age). 80 Swiss-Webster mice (*Mus musculus*) - 40 females and 40 males, 3-week-old, weight ranging from 11 to 14 grams – were used to conduct all procedures. Five anesthetic associations were tested: KX (Ketamine 100 mg/kg and Xylazine 10 mg/kg), KXA (Ketamine 80 mg/kg, Xylazine 5 mg/kg, and Acepromazine 1 mg/kg), KXT (Ketamine 80 mg/kg, Xylazine 5 mg/kg, and Tramadol 5 mg/kg), KXAT (Ketamine 100 mg/kg, Xylazine 10 mg/kg, Acepromazine 2 mg/kg and Tramadol 5 mg/kg) and ATI (Acepromazine 1 mg/kg + Tramadol 5 mg/kg + Isoflurane 5% - 0.5 L/min for induction and 2.5% - 0.5L/min for maintenance). Injectable anesthesia was administered intraperitoneally. We monitored the respiratory rate and body temperature. Response to anesthesia was evaluated according to the induction, surgical anesthesia, and recovery periods. The KXAT and ATI protocols induced surgical anesthesia, with the ATI protocol being the most appropriate and safe to perform the MIT procedure with 100% efficiency, absence of mortality, and rapid recovery of respiratory rate and temperature in the period after the procedure.

Keywords: anesthesia, isoflurane, refinement, tramadol, rabies diagnosis.

INTRODUCTION

Laboratory tests for rabies diagnosis must have high sensitivity and specificity. World Health Organization recommends direct fluorescent antibody as the gold-standard test and viral isolation techniques to confirm the results[1,2]. Replacing animals used in diagnosis for cell culture is expected, which requires a specific laboratory structure and appropriate conservation of samples. On the other hand, it has the advantage of being a faster test compared to the mouse inoculation test (MIT). However, due to its robustness and high sensitivity, the MIT remains the confirmatory technique in most rabies diagnostic laboratories in developing countries[3-6].

MIT by the intracerebral inoculation route is used to diagnose rabies[6]. Brazilian legislation (National Council on the Control of Animal Experiments - CONCEA) classifies it as category 3 of invasiveness (experiments which cause moderate to severe distress or discomfort), having to occur under anesthesia[7]. In the literature, adult animals are frequently used to evaluate anesthetic protocols

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for invasive procedures in mice[8,9], but 3-week-old mice are used to perform MIT, which requires an adjustment on the anesthetic dose to reach surgical anesthesia with safety. However, there is little information about anesthetic protocols in young mice.

Mice are commonly anesthetized using the ketamine–xylazine (KX) association, but these protocols are found in the literature in adult mice. This combination is considered relatively safe in mice and effectively produces analgesia, muscle tone relaxation, and sedation. However, injectable anesthetic protocols for experimentation are not still standardized for doses, route of administration, and anesthetic drugs for young mice[8,10-13].

The use of tramadol as an opioid analgesic and of acepromazine as a tranquilizer associated with anesthetics has also been described in adults mice with benefits[14]. Balanced anesthesia with two or more drugs provides an adequate level of anesthesia and minimizes the risks of anesthetic overdose while enhancing the desired effects[12]. However, dosages must be determined correctly, as acepromazine causes hypotension and a drop in body temperature and tramadol can cause side effects on the cardiovascular system[11].

Inhalants (mainly isoflurane and sevoflurane) are becoming increasingly popular in laboratory animal anesthesia[15]. Compared to injectable techniques, inhalation anesthesia is safer, promotes rapid recovery, and allows quick adjustments and easy maintenance of a steady anesthetic depth. Still, inhalation anesthetics require complex and expensive equipment such as precision vaporizers and flowmeters, specific breathing systems, and efficient scavenging systems to prevent pollution[13].

We highlight the challenge to establish an adequate anesthetic protocol for laboratory rodents due to the problems associated with their small body size: hypothermia, high metabolic rate, and lack of reliable clinical signs of respiratory and cardiovascular functions[13]. Therefore, a proper evaluation of protocols is needed to avoid side effects resulting from inappropriate doses. Thus, the present study aimed to establish and compare different anesthetic protocols for MIT refinement in 3-week-old mice to ensure higher safety and animal welfare.

MATERIALS AND METHODS

Animals

Eighty Swiss-Webster mice (*Mus musculus*), 40 females and 40 males, with 3 weeks of age, from a breeding colony housed in a conventional facility in the Pasteur Institute, São Paulo, Brazil, were used to conducting the present study. The inclusion criterion was body weight: between 11 and 14 grams at weaning (around 21 days of age). Animals from different litters were selected randomly after weaning, preventing the influence of littermates on results. The animals were housed in individually ventilated polycarbonate cages (width: 29 cm; height: 12 cm and depth: 17 cm) in a ventilated system (Alesco®, São Paulo, Brazil) with corncob bedding (Bioflakes, SP, Brazil). We controlled environmental conditions: room temperature from 22 °C to 26 °C, humidity range from 40% to 60%, and light/dark cycle of 12/12h (lights on at 07:00 AM). We provided a commercial irradiated rodent diet (Nuvilab CR-1, Quimtia®, PR, Brazil) and filtered and autoclaved water *ad libitum*. All experimental procedures were approved by the Ethics Committee on the Use of Animals of the Pasteur Institute, Brazil (protocol 02.2019) and experimental procedures report followed the ARRIVE guidelines[16].

Anesthetic procedure

Anesthetic agents used in this study were ketamine (Dopalen® - Ceva, 100 mg/mL, SP, Brazil), xylazine (Xilazina 10%® - Venco Saúde Animal, 100mg/mL, PR, Brazil), acepromazine (Apromazin 2mg/ml - Syntec do Brazil, 20 mg/mL, SP, Brazil), tramadol (Cloridrato de Tramadol® - União Química, 50 mg/mL, SP, Brazil), and isoflurane (Isoflurano® - Instituto Biochimico, 100 mL, RJ, Brazil).

Animals from the same litter were distributed in a way that they were not part of the same group. We formed five groups with eight females and eight males each to test five different protocols[8,10-13,17].

1. KX: Ketamine 100 mg/kg + Xylazine 10 mg/kg
2. KXA: Ketamine 80 mg/kg + Xylazine 5 mg/kg + Acepromazine 1 mg/kg

3. KXT: Ketamine 80 mg/kg + Xylazine 5 mg/kg + Tramadol 5 mg/kg
4. KXAT: Ketamine 100 mg/kg + Xylazine 10 mg/kg + Acepromazine 2mg/kg + Tramadol 5 mg/kg
5. ATI: Acepromazine 1 mg/kg + Tramadol 5 mg/kg + Isoflurane 5% - 0.5 L/min for induction and 2.5% - 0.5L/min for maintenance.

We weighed each animal on an analytical balance to calculate the drug dilution immediately before application by intraperitoneal route as previously described[18]. Injectable drugs were mixed in a single application in a total volume between 150 to 250uL.

For the ATI protocol, a mixture of acepromazine and tramadol was applied intraperitoneally 10-15 minutes before the anesthetic procedures. Digital inhalation anesthesia equipment (Bonther®, São Paulo, Brazil) was used, containing a digital flow meter, universal vaporizer, and breathing circuit with nose cone and induction box. The animals were placed individually in the induction chamber and anesthesia induced with 5% isoflurane delivered with 21% oxygen (room air) at a flow rate of 0.5 L/min until loss of righting reflex. After anesthesia induction, the mice were transferred to a procedure table and anesthesia maintained with 2.5% isoflurane supplied by a nose cone.

Monitoring started immediately after drug administration. After the loss of the righting reflex, animals were placed in a dorsal recumbent position on a procedure table to evaluate reflexes and other parameters. We evaluated the pedal withdrawal reflex by pressing the hind limbs interdigital space with atraumatic forceps, and the tail pinch reflex by a compression of the tail with atraumatic forceps[19]. From this moment on, to identify the anesthetic periods, the following procedures were performed and recorded for all protocols:

1. Induction time: the period between anesthetic administration and loss of pedal and tail reflexes;
2. Surgical anesthesia time: the period between losing all reflexes and the return of at least one of them;
3. Recovery time: the period between the return of at least one reflex and the return of righting reflex.

The efficiency rate of each protocol was calculated by dividing the number of animals that underwent surgical anesthesia by the total number of animals submitted to the protocol.

The respiratory rate (RR) was measured by counting the respiratory movements per minute (RM/m). Body temperature was determined in the abdomen region using the infrared digital laser thermometer G-tech® (basal temperature of 34.0 °C). During anesthesia, the mice were kept in a heated environment by an electric heater to prevent hypothermia, and lubricating eye drops Vidisic® (Bausch & Lomb, Rochester, USA) were used to avoid cornea dryness.

All parameters (reflexes, respiratory rate and temperature) were measured every 5 minutes until recovery from anesthesia.

We simulated the intracerebral inoculation procedure in mice injecting a sterile 0.9% saline solution using a 26G ½ hypodermic needle. However, only if they achieved surgical anesthesia. The site of injection was approximately halfway between the eye and ear and just off the midline[18]. The inoculated volume was 0.03 mL which is the same volume used in the mouse inoculation test.

The application of medications and monitoring of anesthesia were performed by two veterinary researchers (JFSC, KAM) without blinding.

Until the complete recovery, animals were maintained in polycarbonate cages with corncob bedding (Bioflackes, SP, Brazil), with sterilized water and irradiated rodent diet (Nuvilab CR-1, Quimtia®, PR, Brazil) *ad libitum* in a heated environment. All animals were weighed 24 hours after the anesthetic procedure to assess whether anesthesia causes weight loss. Thereafter, we euthanized the mice using carbon dioxide[9].

Statistical analysis

Sample size was defined according to other similar studies, being larger than what is commonly described in the literature[20].

For each group, we demonstrated the mean ± standard deviation (SD). The data were analyzed using the GraphPad Prism 9 program. We performed, when applied, one-way ANOVA with Tukey's

post hoc or two-way ANOVA with Tukey's *post hoc* or nonparametric Student's t-test. We considered $p < 0.05$ as statistically significant.

The ANOVA results were presented by the configuration: F (DFn, DFd) = F ratio (p value), since the F ratio is due to the variability between the means of the groups and variability within the groups associated with a number of degrees of freedom in the numerator and denominator (DFn, DFd). The p-value is calculated from the F ratio. We look for a high F ratio number, which means that the means of the groups are not the same and it is possible to reject the null hypothesis.

Data were normalized to assess the influence of the anesthetic procedure on weight. For that, the weight percent change was calculated to help address different starting weights.

RESULTS

Table 1 summarizes the results obtained from the records performed during anesthesia. At the end of the experiment, 44/80 (55%) mice achieved surgical anesthesia as follows: 8/16 (50%) in the KX protocol, 3/16 (18.75%) in the KXA protocol, 3/16 (18.75%) in the KXT protocol, 14/14 (100%) in the KXAT protocol and 16/16 (100%) in the ATI protocol.

All male and female mice lost the tail reflex in the KX protocol, but only four males and four females also lost the pedal reflexes. Thus, the intracerebral inoculation simulation was performed exclusively in those animals (50%) that achieved surgical anesthesia.

The KXA and KXT protocols obtained the lowest efficiency rate, both with 18.75%, resulting in surgical anesthesia of 3/16 animals. The mice of the KXT protocol were the ones that had most unwanted complications during the process, such as breathing difficulty and abundant salivation. The KXAT protocol also caused the death of two male animals: one during anesthetic induction and the other in surgical anesthetic.

Table 1. Results of anesthetic efficiency, deaths, loss of reflexes, observations, and anesthetic time (minutes) in 3-week-old mice according to the anesthetic protocol used

Evaluated parameters	Anesthetic Protocols				
	KX	KXA	KXT	KXAT	ATI
Efficiency rate (%)	50 (8/16)	18,7 (3/16)	18,7 (3/16)	100 (14/14)	100 (16/16)
Death rate (%)	0 (0/16)	0 (0/16)	0 (0/16)	12,5 (2/16)	0 (0/16)
Loss of Reflexes					
Loss of righting reflex	16/16	16/16	16/16	16/16	16/16
Loss of pedal reflex	8/16	3/16	3/16	15/16	16/16
Loss of tail reflex	16/16	10/16	4/16	16/16	16/16
Observations					
Salivation	1/16	0/16	3/16	2/16	0/16
Breathing difficulty	2/16	0/16	3/16	3/16	0/16
Urination	2/16	0/16	1/16	2/16	0/16
Cyanosis	0/16	0/16	1/16	2/16	0/16
Vocalization	0/16	0/16	2/16	0/16	0/16
Anesthetic times (min±CI)					
Induction	20.2±8.5	9.6 ± 5.5	14.3± 2.0	16.7±5.9	2.1±1.3
Surgical anesthesia	14.2±7.9	10.0±1.0	13.6±14.1	35.0±18.5	7.3±1.9
Recovery	50.8±15.9	39.3±15.0	32.6±25.8	29.0±13.6	12.2±5.8
Total time (min)	76.6±10.8	52.8 ±8.2	46.6±12.3	79.3±15.1	36.1± 6.4

KX (Ketamine 100 mg/kg and Xylazine 10 mg/kg); KXA (Ketamine 80 mg/kg, Xylazine 5 mg/kg and Acepromazine 1 mg/kg); KXT (Ketamine 80 mg/kg, Xylazine 5 mg/kg and Tramadol 5 mg/kg); KXAT (Ketamine 100 mg/kg, Xylazine 10 mg/kg, Acepromazine 2 mg/kg and Tramadol 5 mg/kg); ATI (Acepromazine 1 mg/kg and Tramadol 5 mg/kg and Isoflurane 5% - 0.5 L/min for induction/2.5% - 0.5L/min for maintenance); CI - confidence interval.

Concerning induction period (F (4,40) = 21.59; p = 0.0001), the ATI protocol presented the shortest induction time (2.18 min ±1.37), and showed significant differences compared to KX (p=0.0001), KXA (p = 0.03), KXT (p=0.005) and KXAT (p=0.0001) protocols (Figure 1A).

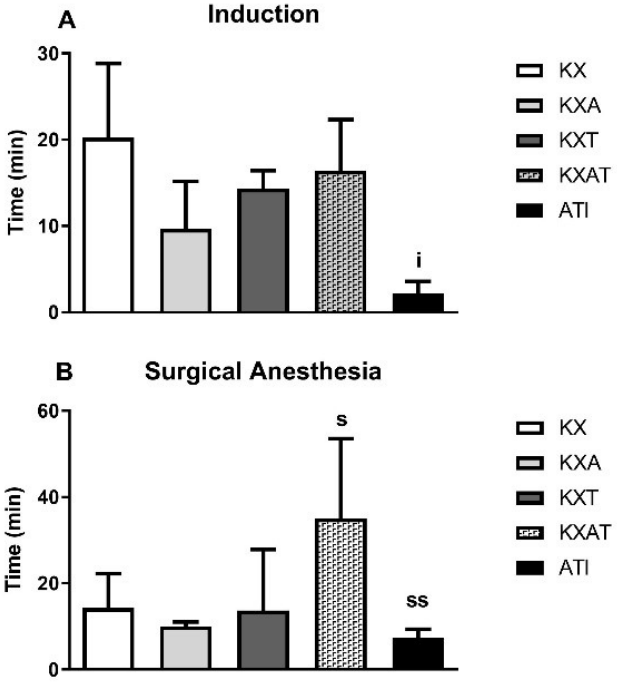

Figure 1. Representation of the average time with standard deviation in minutes for the times of induction (A), surgical anesthesia (B). The protocols and number of animals used in each protocol (those that reached surgical anesthesia) were: KX = Ketamine 100 mg/kg + Xylazine 10 mg/kg – 8 mice; KXA = Ketamine 80 mg/kg + Xylazine 5 mg/kg + Acepromazine 1 mg/kg - 3 mice; KXT = Ketamine 80 mg/kg + Xylazine 5 mg/kg + Tramadol 5 mg/kg - 3 mice; KXAT = Ketamine 100 mg/kg + Xylazine 10 mg/kg + Acepromazine 2mg/kg + Tramadol 5 mg/kg - 14 mice; ATI = Acepromazine 1 mg/kg + Tramadol 5 mg/kg + Isoflurane 5% - 0.5 L/min for induction and 2.5% - 0.5L/min for maintenance, 16 mice. Note: *i* P <0.005 in relation to KX, KXT and KXAT; *s* P <0.05 in relation to KX, KXA and KXT; *ss* P <0.005 in relation to KXAT.

Regarding to surgical anesthesia period (F (4, 39) = 11.31; p = 0.0001), the KXAT protocol showed the longest mean duration (35.00 min ± 18.50 min), compared with the other injectable anesthetic protocols – KX (p = 0.002); KXA (p = 0.01), KXT (p = 0.04) and AIT (p<0.0001) (Figure 1B). The shortest time obtained from KXAT in the surgical anesthesia period was 8 minutes, the longest being 59 minutes. The AIT protocol has the shortest surgical anesthesia period observed with 5 to 11 minutes only.

Referring to the recovery period (F (4, 39) = 12,95; p < 0.0001), the KX protocol had the highest mean duration (50.88 min ± 15.91 min), followed by the KXA (39.33 min ± 15.04 min), KXT (32.67 min ± 25.89 min), KXAT (29.00 min ± 13.63 min), and ATI (12.25 min ± 5.83 min) protocols (Figure 2C). In the multiple comparisons test, KXAT had a statistically significant decrease compared with KX (P = 0.004) and AIT (p =0.008). Also, ATI had a statistically significant reduction compared with KX (P<0.001) and KXA (p = 0.01).

Comparing the total time length (F (4, 75) = 16.74) among the protocols, we found that the ATI (36.19 min ± 6.45 min) was significantly shorter than the KX (76.69 ±10.85, P<0.0001), KXA (52.8 min ± 8.2 min, p = 0.0005) and KXAT (79.36 min ± 15.11 min, P < 0.0001) protocols, but not in comparison with KXT (46.6min ± 12.3 min) (Figure 2D).

No significant difference was observed according to the nonparametric t-test between males and females in the surgical anesthesia period (t(42) = 0.90; p = 0.37) and in the total procedure time for each protocol – KX (t (14) = 0.12; p = 0.91); KXA (t(14) = 0.02; p = 0.98); KXT (t (14) = 1.52; p = 0.15); KXAT (t (12) = 0.85; p = 0.41) and ATI (t (14) = 0.64; p = 0.52).

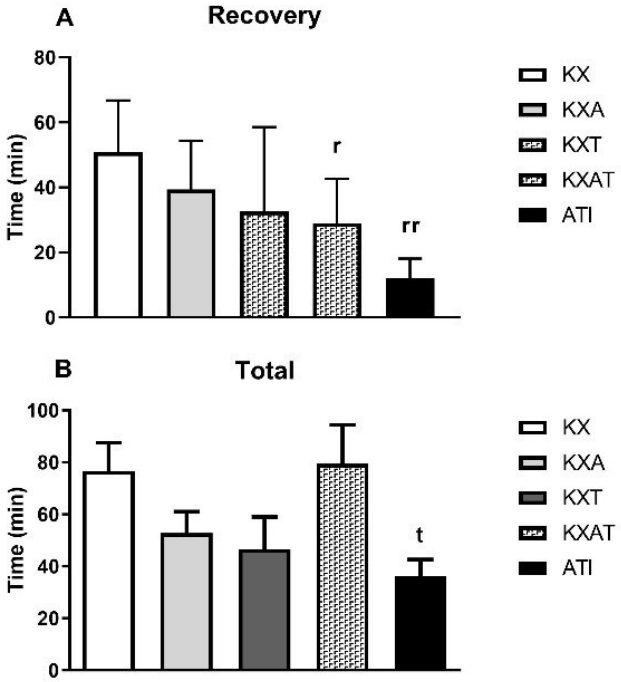

Figure 2. Representation of the average time with standard deviation in minutes for the times of induction recovery (A) and total (B). The protocols and number of animals used in each protocol (those that reached surgical anesthesia) were: KX = Ketamine 100 mg/kg + Xylazine 10 mg/kg – 8 mice; KXA = Ketamine 80 mg/kg + Xylazine 5 mg/kg + Acepromazine 1 mg/kg - 3 mice; KXT = Ketamine 80 mg/kg + Xylazine 5 mg/kg + Tramadol 5 mg/kg - 3 mice; KXAT = Ketamine 100 mg/kg + Xylazine 10 mg/kg + Acepromazine 2mg/kg + Tramadol 5 mg/kg - 14 mice; ATI = Acepromazine 1 mg/kg + Tramadol 5 mg/kg + Isoflurane 5% - 0.5 L/min for induction and 2.5% - 0.5L/min for maintenance, 16 mice. Note: *r* P <0.05 in relation to KX; *rr* P <0.005 in relation to KX and KXAT and *t* P <0.05 in relation to KX, KXT and KXAT.

Figure 3 shows the mean body temperature and respiratory rate over 1 hour of anesthetic procedures. It is possible to note a drop in the means temperature (°C) values throughout the execution of all protocols, not returning to the basal levels during the recovery period (Figure 3A). Likewise, all protocols showed a slope in RR (Figure 3B). However, we observe a significantly more pronounced decrease in RR at 5 and 10 minutes of anesthesia in the ATI protocol when compared to the KX, KXA, KXT, and KXAT protocols.

There was no significant difference in RR and temperature between males and females within the same period by two-way ANOVA (P>0.05).

Regarding the evaluation of weight ($F(4, 73) = 6,260$) by one-way ANOVA with *post hoc* Tukey the KXAT protocol was the only protocol that did not present weight gain and was statically different from KX ($p = 0.0011$) and ATI ($p = 0.0002$) protocols.

DISCUSSION

Data comparison from the five anesthetic protocols demonstrated that the KX, KXA, and KXT are less efficient in achieving surgical anesthesia. Although efficient, we considered the KXAT protocol as unsafe because its mortality rate was 12.5%. The only protocol that showed efficacy and security was the ATI.

The KX protocol showed an efficiency of 50% since 8 of the 16 mice tested achieved surgical anesthesia. Buitrago et al.[10] tested the same KX protocol - ketamine (100 mg/kg) and xylazine (10 mg/kg) in 6-month-old BALB/c mice and none of these animals reached the surgical anesthesia. Arras et al.[8] using a protocol with the same dose of ketamine (100 mg/kg) and twice as much xylazine (20 mg/kg) obtained success for 20-30 minutes with 20 HanIbm:NMR mice between 12 to 16 weeks of age.

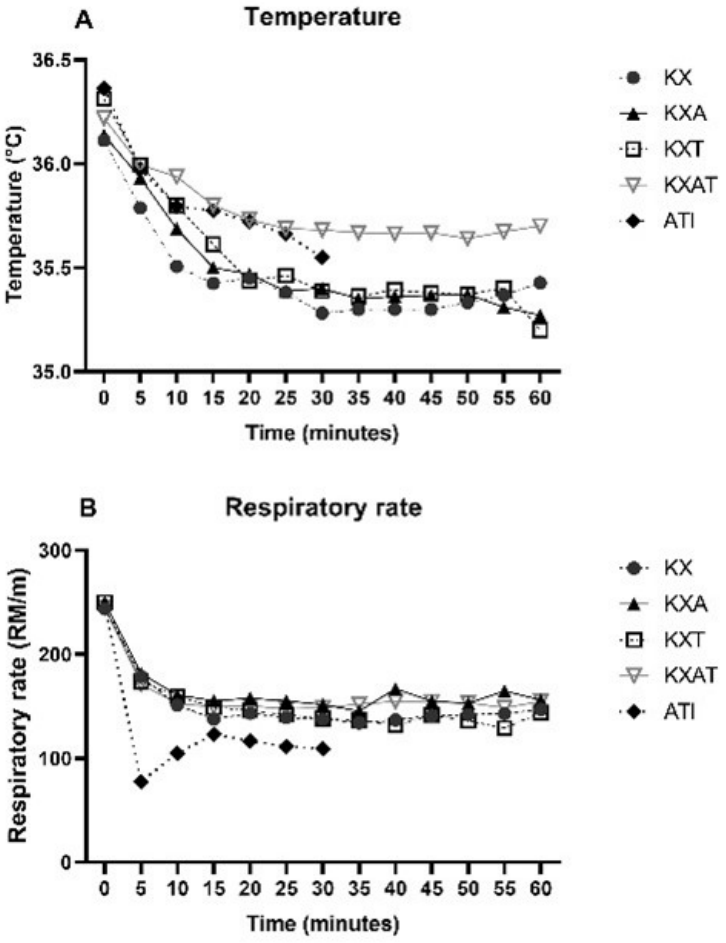

Figure 3. Respiratory rate (RM/m) and body temperature (°C) over an hour of procedure. The protocols used were: KX - Ketamine 100 mg/kg + Xylazine 10 mg/kg; KXA - Ketamine 80 mg/kg + Xylazine 5 mg/kg + Acepromazine 1 mg/kg; KXT - Ketamine 80 mg/kg + Xylazine 5 mg/kg + Tramadol 5 mg/kg; KXAT - Ketamine 100 mg/kg + Xylazine 10 mg/kg + Acepromazine 2mg/kg + Tramadol 5 mg/kg; ATI - Acepromazine 1 mg/kg + Tramadol 5 mg/kg + Isoflurane 5% - 0.5 L/min for induction and 2.5% - 0.5L/min for maintenance.

These differences showed the variability of responses that can occur using mice of different ages and strains, demonstrating the importance of evaluating anesthetic protocols for laboratory animals, small rodents especially. Age influences anesthesia because mice younger than 8 weeks have an immature liver enzymatic system and reduced homeostatic response. Thus, they metabolize anesthetics less efficiently than adults. With the enzymatic system in animals with more days of life, the result may be different, but the risk of hypoglycemia, hypothermia, and hydro electrolyte and acid-base imbalances is higher in these animals because they are smaller in size and their thermoregulatory centers are immature[13].

Green et al.[21] describes a dose of ketamine of 80 mg/kg IM or IP combined it to xylazine of 16 mg/kg. Flecknell et al.[11] describes an association of ketamine (80-100 mg/kg IP) with xylazine (10 mg/kg IP) with acepromazine (3 mg/kg IP), also in his text that brings together the experience of many authors, the author comments on postoperative analgesia with tramadol (5 mg/ml SC or IP). He et al.[14] describes an association of ketamine (65 mg/kg IP) with xylazine (13 mg/kg IP) and acepromazine (1.5 mg/kg IP). We tested protocols based on these doses of anesthetics and when combined with acepromazine we reduced the tranquilizer dose and the xylazine dose (KXA protocol) or only xylazine dose (KXT protocol) because the mice in the present study were young (21 days of age). However, the

KXA and KXT protocols proved inadequate to promote general anesthesia and are not recommended for this purpose. Green et al.[21] with the doses described above identified that the use of ketamine, even when associated with a high dose of xylazine, is insufficient to perform surgical interventions in mice (age not mentioned). In contrast, the protocol described by He et al.[14] was efficient for surgery.

The KXAT combination has not been reported in the literature yet. This study showed 100% efficiency in promoting hypnosis, muscle relaxation, loss of autonomic reflexes, and analgesia. However, it presented a mortality rate of 12.5% (2/16), both males evaluated at necropsy for the presence of internal bleeding or organ damage, not detected in these animals, caused by intraperitoneal injection. Therefore, this protocol is more applicable in an environment equipped with an oxygen delivery device and intensive monitoring, which does not apply to the present study. Nevertheless, dose readjustment may be necessary to increase the safety of the anesthetic protocol.

Comparing the values of injectable and inhalation anesthetics must be done with caution due to the period of surgical anesthesia since the effect of inhalation anesthesia is present while the animal is under it. The anesthetic elimination from the body occurs in a new respiratory cycle as soon as the inhalation mask is removed. It was required approximately seven minutes after tranquilization and analgesia by preanesthetic medication to perform the procedure (Table 1). The KX, KXA, KXT, and KXAT protocols have prolonged desirable and collateral effects (breathing difficulty, cyanosis, body temperature decrease). They persist even after the end of intracerebral inoculation because most of the substances used have no antagonistic drugs available, except for xylazine and tramadol. Therefore, we must wait for the injectable anesthetic metabolization. Also, the average recovery and total period values reflect the preanesthetic medication administered before induction using isoflurane. Its elimination occurs almost immediately after the removal of the inhalation mask. However, until the complete metabolization of the preanesthetic, the animal remains tranquilized without the righting reflex and low skin temperature[11].

Adult females generally require higher drug doses than males. However, there was no significant difference between male and female mice at 21 days of age in the present analysis, possibly because they have not reached sexual maturity yet (newly weaned animals) - hormones are not an influence on drug metabolism[13].

The decrease of body temperature was observed in all tested protocols disregarding a temperature-controlled environment as described by Arras et al.[8]. This result was expected as a side effect of the drugs, however, the use of an infrared thermometer instead of a rectal thermometer may have measured lower temperatures. An abrupt fall in core temperature is frequent in rodents due to their large surface area relative to their low body mass[11,15]. For this reason, anesthetic procedures are impossible without heating the mice artificially because anesthetic and preanesthetic medications induce hypothermia, aggravated by room temperature[22,23]. The hypothermic condition causes significant physiological effects, including prolonged recovery time from anesthesia, increased potency of inhalant anesthetics, and death[11,13]. Specific equipment like heating pads, preferably devices that adjust the blanket temperature according to the animal body temperature, is recommended to prevent hypothermia. Following anesthesia, animals should be placed in a suitable incubator to maintain body temperature during recovery[15].

All protocols depressed the respiratory rate, one of the main side effects of anesthesia causing severe hypoxia and hypercapnia[8]. Isoflurane showed a prominent decrease in respiratory rate as described by Tsukamoto et al.[24], however side effects such as respiratory distress and cyanosis were observed only in the injectable protocols. This may have occurred because in the inhalation protocol the animals were under the effect of anesthesia for a shorter period compared to the injectable protocols. To improve oxygen saturation, some authors recommend supplying oxygen before and during anesthesia[25,26], but this is still not a frequent procedure when using the combination of injectable anesthesia. Fornari et al.[27] demonstrated that using 30-35% oxygen during the anesthesia in rats with ketamine and dexmedetomidine resulted in an increased survival rate and a significantly reduced post-surgical weight loss. Alemán-Laporte et al.[28] demonstrated that administering 100% oxygen during injectable anesthetic protocols immediately improved oxygen saturation and recovery capability. Blevins et al.[29] demonstrated that the use of supplemental oxygen conferred survival value to mice anesthetized with injectable anesthesia.

Although the methods section often does not describe the specific carrier, isoflurane frequently is delivered in 100% oxygen. However, Wilding et al.[30] suggested that both 100% and 21% oxygen are acceptable to give isoflurane to mice. We used 21% oxygen (room air) to deliver isoflurane, considering that the intracerebral inoculation procedure requires a short period under anesthesia. Nevertheless, the use of oxygen at higher concentrations should be considered to refine the technique. Therefore, providing oxygen is recommended in both injectable and inhaled anesthesia[30].

Change in body weight 24 hours after the procedure indicates interference in the physiological activities related to the medication and dose administered since the largest number of mice that lost weight was in the KXAT protocol, represented by the inversion in the graph (Figure 4). Post anesthetic distress can lead to loss of body weight in rodents after injectable anesthesia[26,31-33]. Anesthesia increases blood glucose levels in mice[34,35], which can also reduce appetite and food intake. Alteration of the circadian rhythm can also have an influence on animal weight after anesthesia. Ketamine disturbs the circadian rhythm, which may result in reduced food intake[36]. Additionally, post-surgical pain and stress following procedures undertaken during the light phase can suppress feeding and drinking because they occur mainly during the dark phase[15].

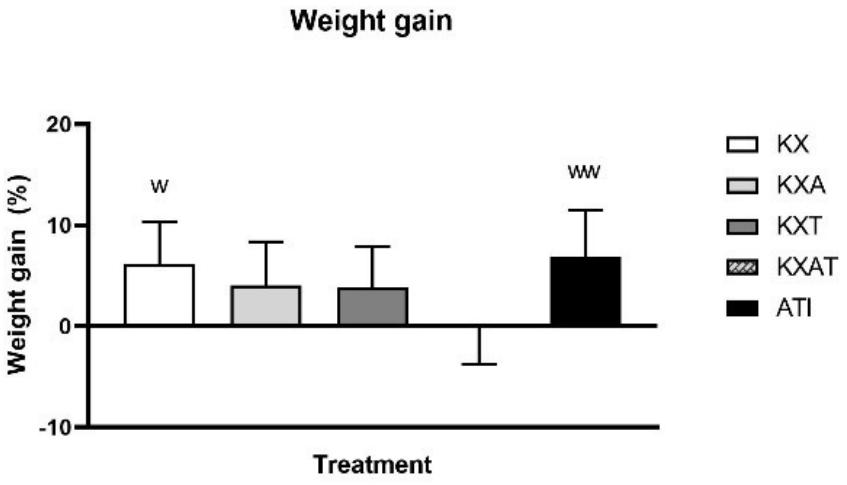

Figure 4. Representation of the average weight gain (percentage) after 24 hours with standard deviation for each anesthetic protocol. The protocols used were: KX - Ketamine 100 mg/kg + Xylazine 10 mg/kg; KXA - Ketamine 80 mg/kg + Xylazine 5 mg/kg + Acepromazine 1 mg/kg; KXT - Ketamine 80 mg/kg + Xylazine 5 mg/kg + Tramadol 5 mg/kg; KXAT - Ketamine 100 mg/kg + Xylazine 10 mg/kg + Acepromazine 2mg/kg + Tramadol 5 mg/kg; ATI - Acepromazine 1 mg/kg + Tramadol 5 mg/kg + Isoflurane 5% - 0.5 L/min for induction and 2.5% - 0.5L/min for maintenance. Note: *w* $P < 0.05$ in relation to KX and ATI.

The refinement in animal experimentation, searching for pain alleviation and distress reduction, is consistent with appropriate ethical conduct, in addition to being a well-established measure in legislation[15,37]. Adopting an effective and safe anesthetic protocol is essential for the welfare of laboratory animals used for scientific and diagnostic purposes[24]. The results suggest that multimodal anesthesia combining acepromazine (1 mg/kg), tramadol (5 mg/kg) and isoflurane (5% induction and 2.5% maintenance, 0.5 L/min), is the most efficient and safe protocol for intracerebral inoculation, showing an absence of mortality, lower drop in temperature and respiratory rate, and faster postanesthetic recovery compared to other tested protocols.

LIMITATIONS

The method constructed was satisfactory for the study, however, we can mention as limitations the lack of monitoring of some parameters, such as heart rate, oxygen saturation and use of a rectal thermometer, which could provide greater details of the influence of drugs on vital parameters.

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Data availability

Data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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