Isoflurane as euthanasia method: mice mitochondrial bioenergetics impact

Isoflurano como método de eutanásia: impactos na bioenergética mitocondrial de camundongos

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ABSTRACT
The mouse euthanasia method can impact some experiment types, which could add undesirable artifacts to the measurements. Hence the importance of investigating euthanasia methods that use anesthetics, which are generally used for this purpose as they imply minimal stress to the animal and to the researcher. This study proposes to investigate changes in mitochondrial bioenergetics that the use of isoflurane may imply in the euthanasia of female mice compared to the cervical displacement method that does not use drugs. To this end, a high-resolution respirometer, Oroboros®, was used in physiological conditions at 37 °C. Our results indicate that the use of isoflurane in high quantities and for a long time can significantly inhibit the phosphorylation state when complex I substrates are used, which can directly impact ATP production when compared to animals euthanized with cervical dislocation.

Keywords: euthanasia, isoflurane, isolated mitochondria, bioenergetics.

RESUMO
O método de eutanásia de camundongos pode impactar alguns tipos de experimentos, o que pode adicionar artefatos indesejáveis às medições. Daí a importância de se investigar métodos de eutanásia que utilizem anestésicos, que geralmente são utilizados para esse fim por implicarem mínimo estresse ao animal e ao pesquisador. Este estudo se propõe a investigar alterações na bioenergética mitocondrial que o uso do isoflurano pode implicar na eutanásia de camundongos fêmeas em comparação ao método de deslocamento cervical que não utiliza anestésicos. Para tanto, foi utilizado um respirômetro de alta resolução, Oroboros®, em condições fisiológicas a 37 °C. Nossos resultados indicam que o uso de isoflurano em grandes quantidades e por longo tempo pode

inibir significativamente o estado de fosforilação quando são utilizados substratos do complexo I, o que pode impactar diretamente na produção de ATP quando comparado a animais sacrificados com luxação cervical.

Palavras-chave: eutanásia, isoflurano, mitocôndrias isoladas, bioenergética.

1 INTRODUCTION

The criteria for choosing the method of euthanasia for laboratory animals must permeate the objective of the study. However, it depends on several factors, including applicable guidelines and laws, the minimization of animal pain and distress, the ability and proficiency of the researcher, and the safety and emotional needs of the personnel performing the euthanasia (DE MOURA et al., 2022; SHOMER et al., 2020). In addition, studies have revealed that mitochondria participate in the onset of cardiac protection elicited by volatile anesthetics such as isoflurane before ischemia (PRAVDIC et al., 2012). The isoflurane-induced formation of reactive oxygen species, in turn, may further inhibit mitochondrial respiration (ZAUGG et al., 2002).

Numerous studies point out that the use of isoflurane as a euthanasia method increases the stress level of mice and rats, due to the increase of corticosterone in the bloodstream (MCKINNEY et al., 2022). Some studies indicate that euthanasia with low doses inhalation of isoflurane can reduce mitochondrial injury at reperfusion in the heart of Wistar rats. In the brain the mechanisms of isoflurane neurotoxicity are unknown. Wei et. al (2008) study (WEI et al., 2008) has demonstrated that isoflurane activates the endoplasmic reticulum membrane IP3 receptor, producing excessive calcium release and triggering apoptosis. Neurons with enhanced IP3 receptor activity, as in certain cases of familial Alzheimer's or Huntington's disease, may be especially vulnerable to isoflurane cytotoxicity (PARRY et al., 2021).

These effects should be considered when selecting the best method of euthanasia for each case. The present study proposes to investigate the effects of inhaled isoflurane as a euthanasia method in isolated liver mitochondrial respiration in physiological conditions with complex I substrates: glutamate and malate. Since mouse liver mitochondria are widely used in studies of mitochondrial bioenergetics, which organ has a high concentration of mitochondria and an easy extraction protocol.

2 METHODS

Sample size

To determine the optimum sample size, we used calculation of sample size by power analysis described in (FESTING; ALTMAN, 2002). From this and considering the optimization of the experiment whit the smaller number of animals, we arrived at 12 animals.

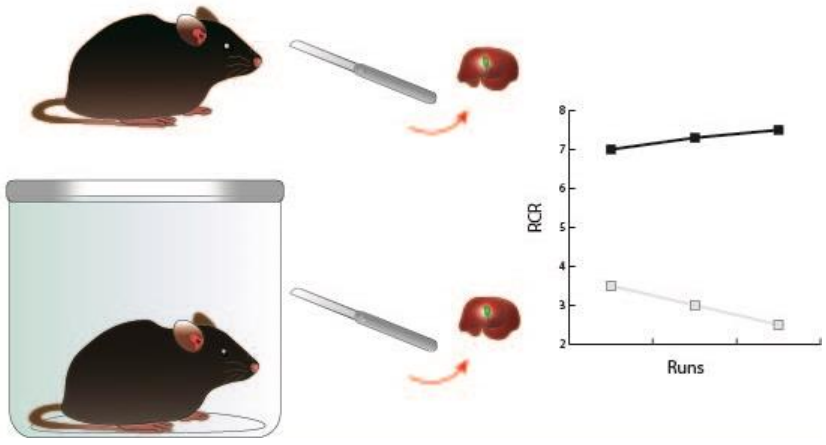
Animals

12 C57BL/6 female mice (2–5 months old) were housed at 22 ± 2°C on a 12 h–12 h light–dark cycle with free access to a standard laboratory rodent chow diet (Nuvital, Curitiba, PR, Brazil) and filtrated water offered ad libitum. The animals were acclimatized for at least 4 weeks before the experiments. The use of mice and the experimental protocols were approved by the local Committee for Ethics in Animal Research (CEUA-IFSC, approval number 8604100921).

Mice Euthanasia

12 C57BL/6 mice were randomly assigned to the euthanasia or control group. Six mice randomized to the euthanasia group inhaled 3.0 L/m3 of isoflurane for 60 seconds. Briefly, mice breathed spontaneously, and anesthetic and oxygen concentrations were constant inside the closed recipient, where 1 ml of isoflurane was added to the top of the recipient (with a volume of 0,00035 m^3). The animal was gently placed into the box and left for 60 seconds. Immediately after this time, the animal no longer has a heartbeat and has whitish eyes. It is removed, and cervical dislocation is performed to ensure euthanasia. The control group was individually maintained in a similar recipient with no anesthetic during the same time, and then cervical dislocation was performed.

Figure 1: Study schematization. Two mice are randomly selected; one will be euthanized only by cervical dislocation, while the other will be exposed to isoflurane inhalational anesthetic for 60 seconds. After euthanasia, both organs will follow the liver mitochondria isolation protocol. The sample is then submitted, under conditions close to physiological, to the respirometer, where data on respiratory control, states 3 and 4, and the uncoupled state are collected. Source: Figure created by the authors.



Mitochondria isolation

Fresh mitochondria were isolated from the livers of female young adult mice by differential centrifugation, as described previously (CHWEIH; CASTILHO; FIGUEIRA, 2015). Briefly, animals were euthanized as described previously and the organ was removed, minced and washed with 4°C isolation medium 1 (250 mM sucrose, 10 mM Hepes, 1 mM EGTA (pH 7.2, KOH)). Next, the liver was homogenized, and the suspension was centrifuged at 800 g for 10 min, at 4 °C. The supernatant was transferred to a clean tube and centrifuged at 7.700 g for 10 min, at 4 °C. The pellet was suspended one more time in medium 2 (250 mM sucrose, 10 mM HEPES and 0,3 mM EGTA (pH 7.2, KOH) and centrifuged at 7.700 g for 10 min, at 4 °C. The final pellet was washed and suspended in 100 µl of isolation medium 3 (250 mM sucrose and 10 mM HEPES (pH 7.2, KOH), and the samples were maintained on ice. All samples had a useful lifetime of 4 hours, in that time interval the samples were viable and energetically stable, maintaining the RCR around (6 ± 1) the obtained immediately after isolation.

Mitochondria concentration isolation

Protein concentrations were determined using the Bradford method modified by adding 1% cholate (HOMANDBERG, 2009), in which the absorbance (at 595 nm) is considered directly proportionalto the concentration of protein in the solution analyzed. BSA solutions in five different concentrations (0.125, 0.25, 0.5, 1.0 and 2 mg/ml) were used as standards at room temperature.

Respirometry

Oxygen consumption was measured at 37 °C using OROBOROS Oxygraph-2k (Innsbruck, Austria) in 2 ml of reaction medium (125 mM sucrose, 65 nmM KCl, 10 mM HEPES, 2 mM K_2HPO_4 and 1 mM $MgCl_2$). After measurements of the basal O_2 consumption (state 2 respiration) with addiction of glutamate (5 mM) and malate (2,5 mM), stimulated respiration by oxidative phosphorylation (state 3 respiration, S3) was elicited by the addition of ADP (500 mM), then 1 μg/mL of oligomycin was added to inhibit the oxidative phosphorylation (OXPHOS) by ATP synthase and obtain the resting leak respiration (state 4, S4). Finally, after oligomycin, the uncoupler carbonil cyanide m-chlorophenyl hydrazone (CCCP) was added to obtain uncoupled state (U). The final concentrations of mitochondrial proteins in the experiment were of 0.5 mg/ml. The parameters adopted in this study were calculated from the respiratory states, and are: respiratory control ratio (RCR) which is calculated as state 3/state 4, indicating the mitochondria viability

preservation (in raw data, RCR for all controls,in this study, are at least 5); uncoupling control ratio, UCR, which can indicate energy losses resulting from an impartial or imperfect coupling, so that the smaller the UCR the greater the energy loss, this parameter can vary from 6 to 20, a dimensionless number, however it depends directly on the conditions of the sample (GNAIGER, 2014).

3 STATISTICAL ANALYSES

All raw data had their parameterization tested using the Shapiro-Wilk statistical test. For data an approximately normal distribution, the *One-Way ANOVA* test was applied, in a comparison between the irradiated sample and the control. Data are presented as mean ± standard deviation for parametric data. All statistical calculations were performed using PAST software (HAMMER; HARPER; RYAN, 2001). Values of $p<0.05$ were considered statistically significant. For all results presented, in this study, at least 3 independent experiments in duplicates were performed.

4 RESULTS

To investigate possible effects on isolated liver mitochondria due to euthanasia method with and without isoflurane, respiration experiments were performed. Except for exposure to isoflurane both samples were subjected to the same conditions. Figure 2A indicates changes in RCR. Respiratory control ratio of animals euthanized after exposure to isoflurane (light purple bars) varies around 2.5 and 4.5 ± 0.7, while animals which were not exposure to isoflurane (light green bars) varies around 7.5 and 8.5 ± 0.5.

Figure 2: Respiratory states as a function experiment number for isolated mitochondria in reaction medium; S3, S4, and U were measured in (pmol/s.ml). A) Respiratory control ratio. B) State 3, in the presence of ADP. C) State 4, in the presence of oligomycin. D) CCCP-induced uncoupled state. For each experiment two animals were used (isoflurane as a euthanasia method and no isoflurane euthanasia - control). *significant values with $p < 0.05$. **significant values with $p < 0.001$.

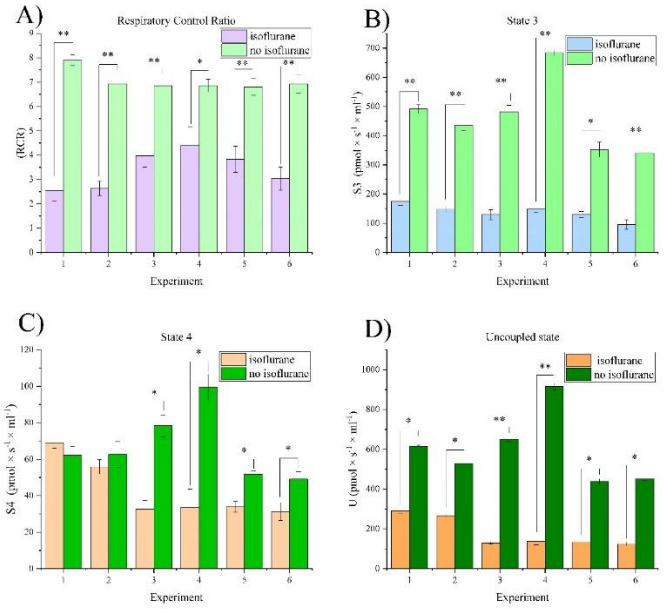


Figure 2B, state 3, shows a significant difference between the control and isoflurane euthanasia methods, varying from 95 and 180 pmol x s^{-1} x ml^{-1} to isoflurane euthanasia method (light blue bars) and 350 and 650 pmol x s^{-1} x ml^{-1} to euthanasia method without isoflurane (green bars). Figure 2C, state 4, present a decreasing tendency in state 4 to isoflurane euthanasia method (salmon color bars) varying from 30 to 65 pmol x s^{-1} x ml^{-1} when compared to control animal (green bars) which varies from 60 to 100 pmol x s^{-1} x ml^{-1}. Figure 2D, uncoupled state, shows significant differences to isoflurane euthanasia method (orange bars) varying from 50 to 250 pmol x s^{-1} x ml^{-1} when compared to control (dark green bars) data, which varies from 500 to 900 pmol x s^{-1} x ml^{-1}.

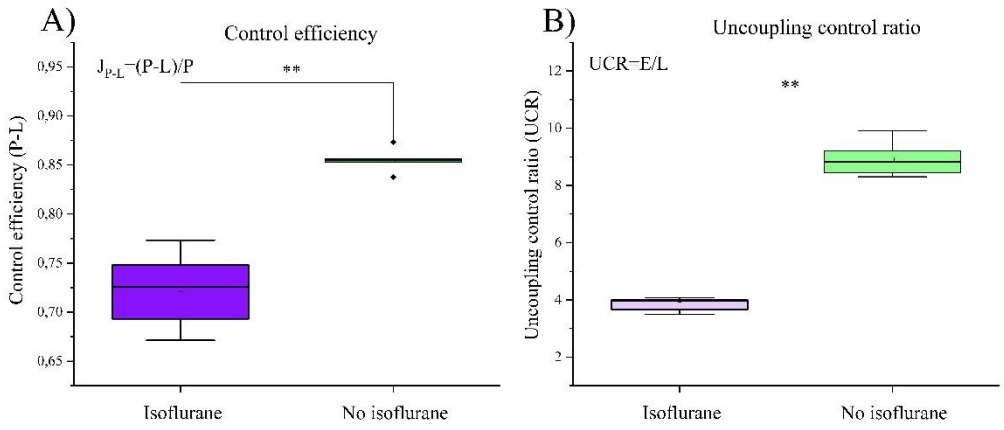
5 DISCUSSION

RCR is an indicator of the quality of isolated mitochondria bioenergetics functions preservation, and thus indicates the viability of the mitochondrial population in the sample. It is equally important to verify the behavior of each respiratory state separately. The decrease in RCR, here presented, may indicate loss of viability of mitochondrial functions due to the euthanasia method. While S3 can indicate the capacity for oxidative phosphorylation in a coupled state, which represents if both ETC and ATPase are active. If S3 is lower compared to the control, it may indicate loss of ATP production. This reduction in S3 may be indicative of a lower capacity for oxidative phosphorylation, which may be due to

uncoupling or other dysfunctions. S4 alterations can indicate membrane permeability changes which leads to loss of mitochondrial function. Uncoupled state, U, indicates the maximum velocity of the electron transport chain, the change in the velocity may be indicative of dysfunction and loss of mitochondrial phosphorylative capacity. There, data are showing loss of the electron transport chain velocity, which can lead to low production of ATP.

The efficiency coefficients, P-L and UCR, are shown in Figure 3, Control efficiency (P-L), Figure 3A, may be indicative of limitations of the phosphorylation system, resulting from dysfunctions in ETC or ATPase.

Figure 3: Control and efficiency coefficients. A) efficiency control (P-L) indicates limitation of the oxidative phosphorylation system; B) uncoupling control (UCR) indicates dissipation in mitochondrial coupling – the higher the value, the strongest is indicates a higher system coupling. Parameters were calculated from the raw data of respiratory states S3, S4 and U. *significant values with $p < 0.05$; **significant values with $p < 0.001$.



UCR, Figure 3B, also shows a decrease in relation to the control for the same three (higher) doses, indicating possible energy dissipation along the phosphorylation system. These dysfunctions are possibly linked to uncoupling of mitochondria under this condition.

In a recent study, Young et al. (2020), presented the RCR for mice (C57BL/6) euthanized in two stages, the first with 5% isoflurane and then cervical dislocation. The RCR, for mitochondria isolated from the liver, varied between 4 and 6, not exceeding the highest value obtained. Although the objective of this work is the investigation to determine if lactate can support mitochondrial bioenergetics, RCR data were presented and demonstrate values 30% lower, on average, than those obtained in this study for animals that did not receive the anesthetic isoflurane (YOUNG; OLDFORD; MAILLOUX, 2020). In contrast, the study by Ronchi et al. presents RCR for mitochondria isolated from the liver of mice (C57BL/6) euthanized only by cervical dislocation, RCR values were greater

than 9. Both studies used glutamate and malate substrates for mitochondrial respiration (RONCHI et al., 2016).

6 CONCLUSION

Respiratory measurements showed that isolated mitochondria from the isoflurane euthanized method (long exposure) inhibits oxidative phosphorylation in comparison to control euthanasia method (no isoflurane). In summary, the observed results indicate that isoflurane method leads to decrease in ATP production and increases energy dissipation in the electron transport chain. Under the conditions investigated in this study, the inhalational anesthetic isoflurane used in the mice euthanasia in long exposure leads to bioenergetics significantly changes in liver mitochondria. With this, we reiterate the importance of choosing the euthanasia method so that artifacts that can alter mitochondrial viability are not added to the results. As work prospects, other types of anesthetics used intravenously, as Cytamine xylazine and Protofol can be tested. Furthermore, it is possible to investigate other aspects of the effects of anesthetics on mitochondria beyond those used here, as the amount of ATP production and structural mitochondrial changes.

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