Arq. Bras. Med. Vet. Zootec., v.76, n.5, e13192, 2024 http://dx.doi.org/10.1590/1678-4162-13192
Original Article - Veterinary Medicine

Isoflurane and CO_2 anesthetics used in brain tissue collection and electrolytic impacts

[*Anestésicos isoflurano e CO_2 utilizados na coleta de tecido cerebral e impactos eletrolíticos*]

J.G. *Longo*[1], J.E.G. *Azevedo*[1], K.C. *Oliveira*[2], R.N. *Castro*[2], I.P. *Andrade Júnior*[1], Y. *Oshima-Franco*[2*]

[1]Universidade de Sorocaba (Uniso), Sorocaba, SP, Brasil
[2]Graduate, Universidade de Sorocaba (Uniso), Sorocaba, SP, Brasil

ABSTRACT

The use of gaseous inhalation agents for animal euthanasia offers rapid action due to easy access to the arterial circulation. The neuroprotective property of *postmortem* brain tissue collection was investigated using isoflurane and carbon dioxide (CO_2) on the serum bioindicators of sodium and potassium. Serum samples were collected from 3 groups of animals (n=8) and donated to this study, previously approved by the Ethics Committee on the Use of Animals (CEUA 173/2020). The serum groups analyzed were named isoflurane, CO_2 and cannabidiol (CBD/CO_2). The results demonstrated that the three groups had elevated potassium levels compared to the control group (*, $p<0.05$), indicating hyperkalemia, while no difference was observed in serum sodium. Furthermore, the CO_2 and CBD/CO_2 groups differed significantly from the isoflurane group (#, $p<0.05$), which had the highest level of hyperkalemia. These findings contribute to our understanding of the physiological effects of different euthanasia methods on the biochemical profiles of animals. In conclusion, the use of CO_2 is recommended as a euthanasia method for collecting brain tissue due to the lowest impact on potassium levels.

Keywords: brain collection, CO_2, euthanasia, isoflurane

RESUMO

O uso de agentes inalatórios gasosos para a eutanásia de animais oferece ação rápida devido ao fácil acesso à circulação arterial. A propriedade neuroprotetora para a coleta de tecido cerebral post mortem *foi investigada utilizando-se isoflurano e dióxido de carbono (CO_2) sobre os bioindicadores séricos de sódio e potássio. Amostras de soro foram coletadas de três grupos de animais (n=8) e doadas para este estudo, previamente aprovado pelo Comitê de Ética no Uso de Animais (CEUA 173/2020). Os grupos de soro analisados foram denominados isoflurano, CO_2 e canabidiol (CBD/CO_2). Os resultados demonstraram que os três grupos apresentaram níveis elevados de potássio em comparação ao grupo controle (*, $P<0,05$), indicando hipercalemia, enquanto nenhuma diferença foi observada no sódio sérico. Além disso, os grupos CO_2 e CBD/CO_2 diferiram significativamente do grupo isoflurano (#, $P<0,05$), que apresentou o maior nível de hipercalemia. Esses achados contribuem para a compreensão dos efeitos fisiológicos dos diferentes métodos de eutanásia sobre os perfis bioquímicos dos animais. Como conclusão, recomenda-se o uso de CO_2 como método de eutanásia para coleta de tecido cerebral devido ao menor impacto sobre os níveis de potássio.*

Palavras-chave: coleta de cérebros, CO_2; euthanasia, isoflurano.

INTRODUCTION

Euthanasia method development requires ongoing research in laboratory animal settings, emphasizing practicality, speed, effectiveness, and humane treatment (Seymor and Nagamine, 2016). Prioritizing human safety and addressing public perception is also crucial (Guidelines…, 2013).

Neurochemical evaluation, including the assessment of neurotransmitters released by neurons in brain signaling pathways, presents a challenge due to rapid changes *post*-collection,

*Corresponding author: yoko.franco@prof.uniso.br
Submitted: November 24, 2023. Accepted: February 20, 2024.

particularly with neuropeptides (Fridjonsdottir *et al*., 2018). Following the sacrifice of the animal and tissue extraction, proteases quickly break down larger proteins into smaller fragments, frequently within the mass range of neuropeptides (Sturm *et al*., 2010).

Commonly used anesthesia/euthanasia methods for brain collection (Ko *et al*., 2019) include ketamine, often in conjunction with xylazine (Réus *et al*., 2014); isoflurane, an alternative associated anesthetic with potential neuroinflammatory effects and increased c-Jun N-terminal kinase (JNK) phosphorylation (Altay *et al*., 2014); CO_2 asphyxiation, inducing a hypoxic state activating mitogen-activated protein kinases (MAPKs) signaling for cell survival (Risbud *et al*., 2005); and rapid decapitation without anesthesia, potentially eliciting a stress response and influencing MAPK signaling in the pre-frontal cortex and hippocampus (Meller *et al*., 2003).

In this study, we employed two inhalation anesthetics, isoflurane and CO_2, for brain collection. Inhaled anesthetics act within the central nervous system by enhancing signals to chloride channels (GABA receptors) and potassium channels while simultaneously depressing neurotransmission pathways (Miller *et al*., 2023). These pathways involve various neurotransmitters, such as acetylcholine affecting both muscarinic and nicotinic receptors, glutamate acting on NMDA receptors, and serotonin influencing 5-HT receptors (Deng *et al*., 2014).

Therefore, the impact of isoflurane and CO_2 on serum sodium and potassium concentrations was assessed to identify a preferable euthanasia anesthetic for brain collection. Electrolytes play a crucial role in various cellular metabolic processes, which can be influenced by anesthesia (Hardman and Hahn, 2017).

MATERIAL AND METHODS

Male Wistar rats, weighing between 290-300 g, were purchased from Anilab Animais para Laboratório (Paulínia, SP, Brazil). The rats were housed in cages (2 rats/cage) at the Lapetox (Toxicological Research Laboratory) vivarium at the University of Sorocaba. The facility was equipped with an environmental micro ventilation system (Alesco®) ensuring proper exhaust and ventilation. Light/dark cycles were maintained every 12 hours with controlled timing. The rats were provided with chemical-free wood shavings for bedding, and food (Neovia Nutrição e Saúde Animal Ltda, Paulínia, São Paulo, SP, Brazil) and water were available *ad libitum*. The study received approval from the Ethics Committee on Animal Use – CEUA of the University of Sorocaba – SP, under protocol 173/2020.

The groups of animals (n=8 each) were: 1) Control isoflurane: serum from animals subjected to chronic daily administration of water via gavage (1 mL) for 90 days, with access to rat food and water *ad libitum*. Subsequently, the rats were anaesthetized with isoflurane (Instituto Biochimico Ind. Farm. Ltda, Rio de Janeiro, RJ, Brazil) within a plastic cylinder and then euthanized by decapitation. 2) Control CO_2: serum from animals subjected to chronic daily administration of water via gavage (1mL) for 90 days, with access to rat food and water *ad libitum*. Subsequently, the rats were anaesthetized with CO_2, within a chamber (Chamber W/Dump Door Med, Harvard Apparatus®), and then euthanized by decapitation. 3) Experimental group: serum from animals subjected to chronic daily administration of 50 µL cannabidiol (200 mg/mL from Prati-Donaduzzi, Paraná, PR, Brazil) completed with water up to 1 mL, administered via gavage (1 mL) for 90 days, with access to rat food and water *ad libitum*. Subsequently, the rats were anaesthetized with CO_2, within a chamber (Chamber W/Dump Door Med, Harvard Apparatus®), and then euthanized by decapitation.

Serum from the above three groups of animals euthanized with either isoflurane or CO_2 (n=8 each) was provided for this research. The samples were obtained from a study led by K.C.O., where brain samples were collected for neurochemical evaluation following chronic exposure to cannabidiol over 90 days (Oliveira *et al*., 2023). Blood samples for sodium and potassium determination were collected during decapitation without anticoagulant, then centrifuged, and stored at -80 °C until use.

The results were expressed as the mean ± Standard Error of the Mean (SEM) and

statistically analyzed using Anova One-way, followed by Tukey's test. The significance level was 5% for all experiments ($p<0.05$), analyzed using Origin 9.5 statistical software (OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION

Dysfunctions associated with sodium (Na^+) and potassium (K^+) are common metabolic abnormalities frequently encountered by anesthesiologists. These issues can stem from various pathological conditions and, if left untreated, can swiftly escalate into life-threatening situations. Despite the straightforward fundamentals of evaluation and therapy, the underlying physiology is occasionally inadequately understood. Errors in managing these conditions are prevalent and can exacerbate the underlying issues (Freshwater-Turner *et al*., 2008).

Na^+ and K^+ distribution in the body is inverse, with sodium predominantly present in the extracellular fluid (ECF), while potassium is the primary intracellular cation. Sodium typically has an ECF concentration of 140 mmol/L, contrasting with its intracellular concentration of only 10 mmol/L. Conversely, potassium exhibits a high intracellular concentration of approximately 150 mmol/L, with only about 1% found in the plasma, maintained between 3.5 to 4.5 mmol/L (Freshwater-Turner *et al*., 2008).

The sodium concentration gradient between extracellular and intracellular fluid is maintained by the sodium-potassium ATPase pump. Total body sodium levels are regulated through renal excretion (Bradshaw and Smith, 2008). Conversely, even slight increases in serum potassium levels can quickly become life-threatening. Rapid expulsion of excess potassium from oral intake by the kidneys is not feasible, highlighting the importance of intracellular buffering for balance. As the kidneys eliminate surplus potassium and serum levels decrease, potassium is again released from the cells (Freshwater-Turner *et al*., 2008).

In this study, we measured sodium and potassium electrolyte levels in response to the administration of inhalation anesthetics during the collection of rat brain tissue samples. As depicted in Fig. 1, there was no statistically significant difference in Na^+ level (in mmol/L) among the groups exposed to isoflurane (154.7 ± 6.4) or CO_2 (148 ± 8.2 alone or 142.6 ± 1.1 CBD/CO_2) anesthesia compared to the control group (138.8 ± 0.2).

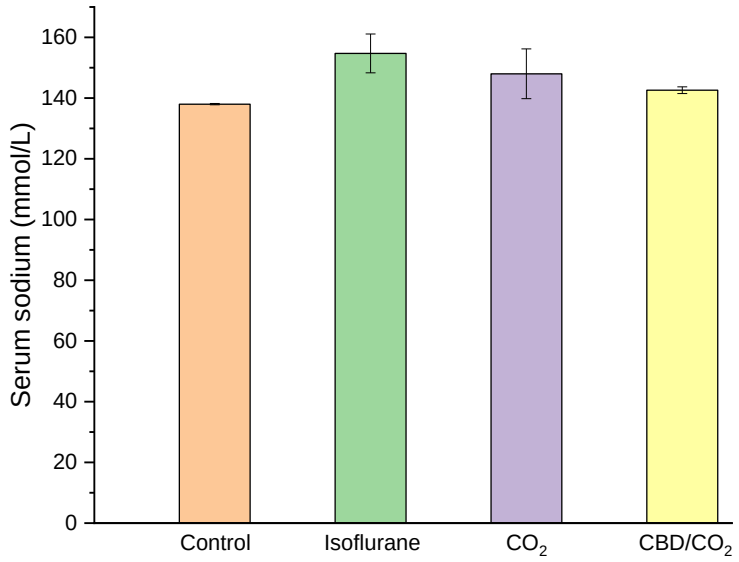

Figure 1. Serum Na^+ level in rats. Reference values for rats typically range between 133-144 mmol/L (Houtmeyers *et al*., 2016). It's noteworthy that the isoflurane group exhibits a higher value than animals anaesthetized with CO_2 although the data did not show statistical significance.

Sodium, a crucial nutrient, serves as the primary extracellular fluid cation, playing a vital role in maintaining extracellular volume, osmolality, membrane potentials, and facilitating transmembrane transport processes (Gomes *et al*., 2017). Excessive dietary sodium intake is associated with elevated blood pressure (Farquhar *et al*., 2015). Of relevance to anesthesiologists are calcium's impacts on the myocardium, vascular smooth muscle, and blood coagulation (Aguilera and Vaughan, 2000). However, the interaction with anesthesia in cases of mild to moderate hypertension does not warrant surgery postponement (Hanada *et al*., 2006). Our results indicate that anesthesia does not interfere with serum sodium levels.

Figure 2 presents a statistically significant difference in K^+ levels (in mmol/L) among the groups exposed to isoflurane (6.9 ± 0.3) or CO_2 (5.5 ± 0.3 alone or 5.6 ± 0.1 CBD/CO_2) anesthesia compared to the control group (3.2 ± 0.05).

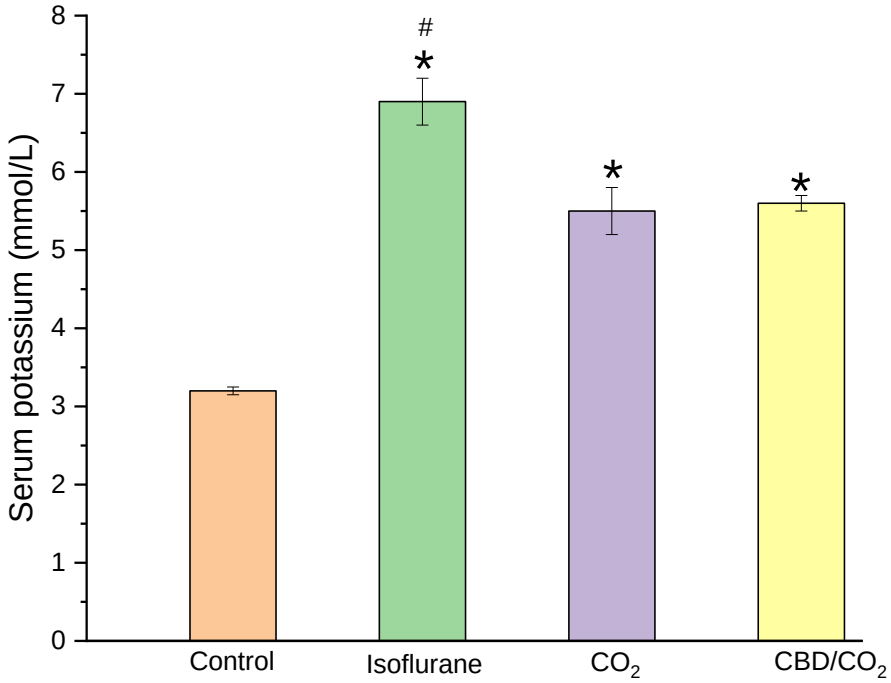

Figure 2. Serum K^+ levels in rats. Reference values for rats ranged between 3.6 to 5.3 mmol/L (Houtmeyers *et al*., 2016). Notably, the isoflurane group exhibits a higher value than animals anaesthetized with CO_2. *, $p<0.05$ compared to control. #, $p<0.05$ compared to CO_2 groups.

Maintaining high intracellular and low extracellular potassium levels is crucial for cellular functions and the stability of the cell membrane electrical gradient. Recently, there has been an increasing number of reports documenting unanticipated hyperkalemia during anesthesia in dogs, leading to consequences ranging from brady-dysrhythmias to cardiac arrest (Taylor *et al*., 2018; Louro *et al*., 2020; Pye and Ward, 2023).

Despite this, CO_2 inhalation remains the predominant method of euthanasia for laboratory rats and mice. Furthermore, it is commonly used for subsequent terminal blood sampling in the context of clinical biochemical assays (Khokhlova *et al*., 2022) or brain tissue collection (Ko *et al*., 2019), as performed in the CBD/CO_2 group.

Inhaled anesthetics induce skeletal muscle relaxation and impact sensory nerve conduction (Miller *et al*., 2023). While neurological complications linked to hyperkalemia are relatively rare, they can manifest as muscle weakness and paralysis. Such paralysis is

occasionally observed in individuals with familial periodic paralysis or can occur sporadically in cases of severe hyperkalemia (Ko *et al*., 2019).

Conversely, hyperkalemia's beneficial impact in cardioprotection extends to preventing reperfusion malignant arrhythmias. This preventive action is attributed to factors such as the ability to forestall or reverse myocardium ischemic contracture, restore myocardial contraction diastolic function, and alleviate calcium overload and intracellular potassium loss during reperfusion (Takata *et al*., 2013). By targeting the reduction of calcium overload, hyperkalemia presents a novel avenue for therapeutic intervention in the management of acute cerebral ischemic stroke and subsequent reperfusion (Qin *et al*., 2018).

The current investigation has limitations as it is restricted to the analysis of electrolytes Na^+ and K^+, revealing hyperkalemia in the isoflurane group. The CO_2-euthanized groups did not exceed the upper limit of K^+ levels (5.3mmol/L) (Houtmeyers *et al*., 2016). In line with the findings of Ko *et al*. (2019), we lean towards considering CO_2 as the preferred method for brain tissue collection, especially as these authors provide additional data on minimal naive mitogen-activated protein kinases (MAPK) activation.

CONCLUSION

According to our findings, carbon dioxide asphyxiation emerges as the preferred method for terminal euthanasia when aiming for brain tissue collection compared to isoflurane.

REFERENCES

AGUILERA, I.M.; VAUGHAN, R.S. Calcium and the anaesthetist. *Anaesthesia*, v.55, p.779-790, 2000.

ALTAY, O.; SUZUKI, H.; HASEGAWA, Y. *et al*. Isoflurane on brain inflammation. *Neurobiol. Dis*., v.62, p.365-371, 2014.

BRADSHAW, K.; SMITH, M. Disorders of sodium balance after brain injury, *Cont. Educ. Anaesth. Crit. Care Pain*, v.8, p.129-133, 2008.

DENG, J.; LEI, C.; CHEN, Y. *et al.* Neuroprotective gases--fantasy or reality for clinical use? *Prog. Neurobiol*., v.115, p.210-245, 2014.

FARQUHAR, W.B.; EDWARDS, D.G.; JURKOVITZ, C.T.; WEINTRAUB, W.S. Dietary sodium and health: more than just blood pressure. *J. Am. Coll. Cardiol*., v.65, p.1042-1050, 2015.

FRESHWATER-TURNER, D.; GREEN, R.; MCCORNICK, B. Body fluid compartments, sodium and potassium. *Update Anaesth*., v.24, p.43-51, 2008.

FRIDJONSDOTTIR, E.; NILSSON, A.; WADENSTEN, H. *et al*. Brain tissue sample stabilization and extraction strategies for neuropeptidomics. *Methods Mol. Biol*., v.1719, p.41-49, 2018.

GOMES, P.M.; SÁ, R.W.M.; AGUIAR, G.L. *et al.* Chronic high-sodium diet intake after weaning lead to neurogenic hypertension in adult Wistar rats. *Sci. Rep*., v.7, p.5655, 2017.

GUIDELINES for the euthanasia of animals. Schaumburg: AVMA, 2013. Available in: https://www.avma.org/KB/Policies/Pages/ Euthanasia-Guidelines.aspx. Accessed in 24 Nov. 2023.

HANADA, S.; KAWAKAMI, H.; GOTO, T.; MORITA, S. Hypertension and anesthesia. *Curr. Opin. Anaesthesiol*., v.19, p.315-319, 2006.

HARDMAN, J.G.; HAHN, R.G. Fluid and electrolyte physiology in anaesthetic practice. In: HARDMAN, J.G.; HOPKINS, P.M.; MICHEL, M.R.F. (Eds.). *Oxford textbook of anaesthesia*. Oxford*:* Oxford Academic, 2017.

HOUTMEYERS, A.; DUCHATEAU, L.; GRÜNEWALD, B.; HERMANS, K. Reference intervals for biochemical blood variables, packed cell volume, and body temperature in pet rats (Rattus norvegicus) using point-of-care testing. *Vet. Clin. Pathol*., v.45, p.669-679, 2016.

KHOKHLOVA, O.N.; BOROZDINA, N.A.; SADOVNIKOVA, E.S. *et al*. Comparative study of the aftereffect of CO_2 inhalation or tiletamine-zolazepam-xylazine anesthesia on laboratory outbred rats and mice. *Biomedicines*, v.10, p.512, 2022.

KO, M.J.; MULIA, G.E.; VAN RIJN, R.M. Commonly used anesthesia/euthanasia methods for brain collection differentially impact MAPK activity in male and female C57BL/6 mice. *Front. Cell Neurosci*., v.13, p.96, 2019.

LOURO, F.; RASZPLEWICZ, J.; ALDERSON, B. Hyperkalaemia during general anaesthesia: six cases. *Vet. Rec. Case Rep*., v.8, p.e001075, 2020.

MELLER, E.; SHEN, C.; NIKOLAO, T.A. *et al*. Region-specific effects of acute and repeated restraint stress on the phosphorylation of mitogen-activated protein kinases. *Brain Res*., v.979, p.57-64, 2003.

MILLER, A.L.; THEODORE, D.; WIDRICH, J. Inhalational Anesthetic. [Updated 2023 May 1]. In: *StatPearls* [Internet]. Treasure Island (FL): StatPearls, 2023. Available in: https://www.ncbi.nlm.nih.gov/books/NBK554540/ Accessed in: 24 Nov. 2023.

OLIVEIRA, K.C.; AZEVEDO, J.E.G.; CASTRO-DA-SILVA, M.L.R. *et al*. Impacto da administração crônica de canabidiol em ratos. *Rev. Contrib. Cienc. Soc*., v.16, p.15350-15372, 2023.

PYE, E.; WARD, R. Hyperkalaemia in a greyhound under general anaesthesia. *Vet. Rec. Case Rep*., v.11, p.e585, 2023.

QIN, T.; LI, N.; TAN, X.F. *et al*. Works on heart, how about brain? Effect of hyperkalemia on focal cerebral ischemia/reperfusion injury in rats. *Eur. Rev. Med. Pharmacol. Sci*., v.22, p.2839-2846, 2018.

RÉUS, G.Z.; VIEIRA, F.G.; ABELAIRA, H.M. *et al*. MAPK signaling correlates with the antidepressant effects of ketamine. *J. Psychiatry Res.*, v.55, p.15-21, 2014.

RISBUD, M.V.; GUTTAPALLI, A.; ALBERT, T.J.; SHAPIRO, I.M. Hypoxia activates MAPK activity in rat nucleus pulposus cells: regulation of integrin expression and cell survival. *Spine*, v.30, p.2503-2509, 2005.

SEYMOUR, T.L.; NAGAMINE, C.M. Evaluation of isoflurane overdose for euthanasia of neonatal mice. *J. Am. Assoc. Lab. Anim. Sci*., v.55, p.321-323, 2016.

STURM, R.M.; DOWELL, J.A.; LI, L. Rat brain neuropeptidomics: tissue collection, protease inhibition, neuropeptide extraction, and mass spectrometric analysis. *Methods Mol. Biol*., v.615, p.217-226, 2010.

TAKATA, K.; TOMIYAMA, Y.; TANAKA, K.; OSHITA, S. Cardioprotective effects of hyperkalemia during simulated ischemia/reperfusion in neonatal rat cardiomyocytes-preservation of Na^+/K^+-ATPase activity-. *J. Med. Invest*., v.60, p.66-76, 2013.

TAYLOR, P.; PRYMAK, C.; HIRD, J. *et al*. Unanticipated hyperkalaemia. *Vet. Rec*., v.182, p.84, 2018.