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Short communication

Brief anesthesia by isoflurane alters plasma corticosterone levels distinctly in male and female rats: Implications for tissue collection methods

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HIGHLIGHTS

- Rapid anesthesia by isoflurane may impact stress-related markers.
- Plasma corticosterone and hippocampal gene expression were examined in rats.
- Brief exposure to isoflurane elevated corticosterone in female, but not male, rats.
- Expression of the glucocorticoid receptor and its regulators were unchanged.
- Possible interactions of sex and anesthesia should be evaluated in study designs.

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ABSTRACT

Euthanasia by anesthetic agents is commonly performed prior to tissue collection in order to minimize pain and distress to the animal. However, depending on their mechanism of action as well as administration regimen, different methods of anesthesia may trigger an acute stress response through engaging the hypothalamic-pituitary-adrenal (HPA) axis, which can impact numerous other physiological processes that the researcher may wish to examine as endpoints. We investigated the effects of the commonly used anesthetic agent isoflurane on two different endpoints related to the stress response: plasma corticosterone levels and gene expression of the glucocorticoid receptor (GR) as well as several of its regulators including FK506-binding protein 51 (*Fkbp5*) in the hippocampus of male and female rats. Our results indicate that brief exposure to anesthesia by isoflurane prior to decapitation can alter plasma corticosterone levels differentially in male and female rats within minutes without impacting gene expression in the hippocampus. We conclude that collection methods can influence stress-related physiological endpoints in female rats and the potential influence of even brief anesthesia as well as sex differences in response to anesthesia should be evaluated during the experimental design process and data interpretation. This finding is particularly important in light of new NIH standards regarding sex and reproducibility, and care should be taken to be certain that sex differences in endpoints of interest are not an artifact of sex differences in response to collection paradigms.

1. Introduction

The stress response can profoundly impact functional outcomes due to the regulatory role of stress-related systems in other physiological processes in the body. In studies examining terminal endpoints in particular, the effects of the stress response triggered by tissue collection methods must be distinguished from those of the experimental manipulations. In many areas of biomedical

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research utilizing animals, sedation and/or euthanasia by anesthetic agents is commonly performed prior to tissue collection in order to minimize pain and distress to the animal. However, depending on their particular mechanisms of action as well as administration regimen, different methods of anesthesia may induce undetected alterations in physiology through engaging the stress endocrine system [1,2]. These subtle changes may subsequently impact numerous other physiological processes that may include the endpoints of focus, thus producing a confounding effect on the metrics of interest. Therefore, when designing a study, the effects of anesthetic agents and tissue collection methods should be considered regarding not only the desired experimental endpoints but also stress-related outcomes. While anesthetics like isoflurane are often used in the performance of euthanasia in laboratory settings, certain anesthetic agents have reported neurotoxic effects [3,4]. Even if present in the system for a brief period before euthanasia, anesthetic agents may cause persistent effects in tissue and potentially affect cellular processes [5]. Many of the common anesthetic agents modulate ion channels such as the *N*-methyl D-aspartate (NMDA) and the γ-aminobutyric acid A ($GABA_A$) receptors and likely downstream signaling molecules important in neuronal survival and cell death. Therefore, humane methods of euthanasia that do not require anesthesia, such as rapid decapitation, have been used whenever possible in neurochemical studies.

Despite the potential effects of anesthetic agents on the experimental outcome of interest, one must strive to minimize animal pain and distress. The American Veterinary Medical Association makes the following statement regarding physical means of euthanasia: "When properly used by skilled personnel with well-maintained equipment, physical methods of euthanasia may result in less fear and anxiety and be more rapid, painless, humane, and practical than other forms of euthanasia" [6]. The physical means of euthanasia most applicable to laboratory research are cervical dislocation, decapitation, and microwave irradiation [5]. However, cervical dislocation is not suitable for large rats due to the need for increased handling as well as reports that it induces more stress than decapitation in 6-month-old rats [7]. Considering the fact that microwave irradiation requires the use of specially designed equipment, rapid decapitation remains a commonly used physical method of euthanasia without the use of anesthesia.

Here we compared the effects of the anesthetic agent isoflurane followed by euthanasia to those of rapid decapitation on two different endpoints related to the stress response: plasma corticosterone levels and gene expression of the several genes involved in mediating the stress response. Expression of the glucocorticoid receptor (GR, gene name: *Nr3c1*) – the main effector of the HPA axis-mediated stress response – as well as its negative regulator FK506-binding protein 51 (gene name: *Fkbp5*) and positive regulators FK506-binding protein 52 (gene name: *Fkbp4*) and peptidyl-prolyl isomerase D (gene name: *Ppid*) were examined in the hippocampus, a brain region densely populated with GR and sensitive to the effects of stress [8]. Although isoflurane can alter gene expression patterns in the brain over the course of several hours or days [9,10], it is not clear whether it can induce rapid changes in the expression of genes involved in the stress response in the span of minutes—a question particularly relevant for studies using isoflurane as anesthesia prior to euthanasia. Although the impact of isoflurane on serum hormone and brain cholinesterase levels have been examined in male and female rats [1], to our knowledge, the current study is the first to investigate isoflurane's effects on stress-related gene expression in the male and female rat brain. Our results indicate that anesthesia by isoflurane prior to decapitation can alter stress-related hormone concentrations differentially in male and female rats without impacting gene expression in the brain.

2. Materials and methods

2.1. Animals

Adult Wistar male and female rats (n = 12–13, 300–400 g) were purchased from Charles River Laboratories (Wilmington, MA), housed in an AAALAC-approved facility under standard laboratory conditions, and maintained on a 14:10 reverse light:dark cycle with free access to food and water. The Emory University Institutional Animal Care and Use Committee approved all animal use procedures. Animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Experimental design: rapid decapitation vs. isoflurane anesthesia prior to decapitation

Prior to the start of the experiments, rats were randomly assigned into one of three treatment groups ($n = 4$–5 per group). All animals were allowed to sit undisturbed in a quiet environment for 3 h following removal from the animal facility and prior to euthanasia. Collection took place at least 3 h prior to the end of the animals' light cycle. A separate room was designated for isoflurane induction and decapitation each, and both of these rooms were separated from the rat holding room. One animal was euthanized at a time, and the induction chamber was cleaned with 70% ethanol and water between each animal. Rats were either euthanized by rapid decapitation using a guillotine (RD, no anesthesia) or exposed to one of two different dosing regimens of the anesthetic isoflurane (Slow or Fast induction) prior to decapitation by guillotine. The slow-induction regimen consisted of exposing each individual rat to an induction chamber with 2% isoflurane (in oxygen) for 2 min followed by 5% isoflurane for 3 min. Rats in the fast-induction group were exposed individually to 5% isoflurane (in oxygen) in an induction chamber for 5 min. Animals in the isoflurane groups were euthanized by decapitation after making sure the animal was completely unresponsive to tail pinch. The personnel were kept constant throughout the entirety of the experiment.

2.3. Blood and tissue collection

Following euthanasia by rapid decapitation, trunk blood was immediately collected from animals in all groups and centrifuged at 1800 rcf. Plasma was then transferred into fresh tubes and stored at −80 °C until used for hormone analysis. At the same time as blood collection, brains were rapidly removed, flash frozen on dry ice, and stored at −80 °C until they were used for quantitative RT-PCR analyses.

2.4. Endocrine analyses

Total plasma corticosterone levels were assayed using the ELISA kit purchased from Enzo Life Sciences (sensitivity: 27 pg/ml, Farmingdale, NY, USA) according to the manufacturer's instructions. Samples were run in duplicates, and the coefficient of variance among the duplicates was less than 15%.

2.5. Quantitative RT-PCR

The hippocampus was dissected under RNAse-free conditions, homogenized, and RNA was extracted using an RNeasy Mini