Interpreting Neuroendocrine Hormones, Corticosterone, and Blood Glucose to Assess the Wellbeing of Anesthetized Rats during Euthanasia

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Current recommendations for assessing animal wellbeing during euthanasia suggest that measuring neuroendocrine hormones—such as ACTH, noradrenaline, and adrenaline—is preferable to measuring corticosterone and blood glucose because of the sensitivity of neuroendocrine hormones to the acute stress associated with rapid methods of euthanasia. However, these neuroendocrine hormones can be stimulated in ways that confound interpretation of welfare assessment in euthanasia studies. Although this property does not negate the usefulness of neuroendocrine hormones as tools of assessment, it is important to differentiate the stress associated with the induction of anesthesia before the loss of consciousness (an animal wellbeing concern) with the physiologic responses that occur after the loss of consciousness (not an animal wellbeing concern). In this study, rats were anesthetized by using a ketamine–xylazine combination. Once the rats achieved a surgical plane of anesthesia, they were exposed to O_2, CO_2, or isoflurane, followed by terminal blood collection to assess concentrations of ACTH, noradrenaline, corticosterone, and blood glucose. Compared with animals exposed to O_2 or isoflurane, rats exposed to CO_2 had significant increases in their serum concentrations of ACTH and noradrenaline, but blood glucose and corticosterone did not differ between groups. These findings indicate that noradrenaline and ACTH should be used with caution to assess animal wellbeing when the method of euthanasia might confound that assessment.

Assessment of animal wellbeing during euthanasia is of critical importance as clinicians and scientists strive to ensure that methods of euthanasia induce a rapid loss of consciousness and minimize the potential pain or distress experienced by the animal. The physiologic response to acute stressors is moderated through 2 pathways: the sympathetic–adrenal–medullary system and the hypothalamic–pituitary–adrenal axis. In response to an acute stress, the sympathetic–adrenal–medullary system releases noradrenaline in the brain and adrenaline in the adrenal medulla. Concurrently, the acute stressor triggers the hypothalamic–pituitary–adrenal response, initiated in the amygdala and resulting in corticotrophin releasing factor secretion from the hypothalamus. This hormone stimulates the pituitary to release ACTH, which then acts on the adrenal cortex to stimulate the release of corticosterone or cortisol (depending on the species). These neuroendocrine hormones act in concert to increase the blood pressure and heart rate and stimulate the release of glucose into the bloodstream as part of the sympathetic response.[4]

Combinations of behavioral and physiologic measures are used to assess animal wellbeing during euthanasia, with different combinations of each used for various methods, depending on the restrictions inherent in the method of euthanasia.[4,6] For example, behavioral scoring cannot be used for the assessment of the physical methods of euthanasia, such as decapitation or cervical dislocation, because the animal is unable to move.

Likewise, measurement of serum corticosterone levels is inappropriate for evaluating the wellbeing of rodents euthanized with physical methods of euthanasia because corticosterone can take as long as 4 min to rise after a stressful trigger in rats.[7,19,20] In humans, peak levels of cortisol did not occur until 15 min after a stressful stimulus.[17] In contrast, acute CO_2 exposure in mice results in elevated noradrenaline and ACTH levels within 5 s and 30 s, respectively.[5,14,19,20] Given these observations, ACTH, noradrenaline, and adrenaline have been suggested as more sensitive indicators of differences in stress during euthanasia.[4]

However, the method of general anesthesia used for euthanasia can affect these parameters—either directly as an indicator of stress to the animal or as a secondary effect of the general anesthetic. For example, isoflurane typically is not recommended for research studies that monitor blood glucose, because significant decreases have been reported with the use of this anesthetic.[3,9] In addition, the amygdala triggers the hypothalamic–pituitary–adrenal axis in response to alterations in brain pH, which is an inherent part of the mechanism of action of CO_2 narcosis.[8,25] However, it is difficult to separate the induction from the actual exposure of the general anesthetic to determine whether the changes in these parameters are due to the induction of general anesthesia and its associated stressors or are responses to the anesthetic agents themselves. Because samples in these studies are collected after the animal has achieved a surgical plane of anesthesia, these assessments may reflect the physiologic response to anesthesia, independent of the stress of induction.

To evaluate these responses, we anesthetized Sprague–Dawley rats by using a combination of ketamine and xylazine so that all rats would have the same induction experience,

with exposure to the same stressors. Once the rats achieved a surgical plane of anesthesia, they were placed in a chamber containing 100% O_2, 30%/min volume displacement of 100% CO_2, or 5% isoflurane. The rats were exposed to the treatment for approximately 2 min, after which terminal blood collection was performed for measurement of blood glucose, serum corticosterone, serum noradrenaline, and serum ACTH. By anesthetizing all rats prior to the introduction of the inhaled gases, the objective of this study was to determine whether the gases themselves initiated a physiologic response that was independent of the effects of anesthetic induction.

Materials and Methods

Ethics statement. All work described in this study was approved by the Indiana University School of Medicine IACUC prior to initiation of the study. This program is AAALAC-accredited and is compliant with all federal regulations overseeing the use of animals in research in the United States.

Animals. Adult male ($n = 12$) and female ($n = 12$) Sprague–Dawley rats (HSD:Sprague–Dawley; age: range, 167 to 279 d; mean, 230 d) were used in this study. The rats were first-generation offspring from a breeding study where the parents had been obtained from Envigo (Indianapolis, IN). All rats were pair-housed prior to euthanasia and had not been exposed to any other experimental manipulation. Rats were housed in IVC (Alt Design, Siloam Springs, AR) with hardwood bedding (Sani-Chip, PJ Murphy, Montville, NJ) and paper toweling for nesting materials. Food (Teklad 2018SX, Envigo) was provided without restriction. Reverse-osmosis–treated water was freely available through an automatic watering system. Cages were changed at least weekly in a laminar flow workstation (Nuaire, Plymouth, MN) and autoclaved prior to reuse. Hands and implements were disinfected (MB10, Quip Labs, Wilmington, DE) between cages. The macroenvironment was on a 12:12-h light:dark cycle (lights on, 1900); the temperature was maintained at approximately 72 °F (22 °C), with humidity between 30% and 70%. The colony was screened quarterly by using indirect sentinels. At the time of the study, the colony was free of coronavirus (sialodacryoadenitis virus), parvoviruses (NS1, rat parvovirus, Kilham rat virus, H1 virus, rat minute virus), theliovirus, *Clostridium piliforme*, *Mycoplasma pulmonis*, pinworms (*Aspicularis tetraptera*, *Syphacia* spp.), and fur mites (*Radfordia ensifer*, *Ornithonyssus bacoti*).

Experimental design. All data were collected during a single, 4-h period. The rats were fasted for 12 to 18 h prior to euthanasia for collection of fasting blood glucose values. Each pair was anesthetized at the same time by using a cocktail of ketamine (80 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (4 mg/mL; Bayer, Whippany, NJ) administered at a dosage of 1 ml/kg IP, after which the rats returned to their home cage. Rats were observed until they achieved lateral recumbency. Once they were nonresponsive to a toe pinch (indicating a surgical plane of anesthesia), each rat was randomly assigned to a treatment group and exposed to the treatment gas, as described later. Random assignment was accomplished by creating 2 drawing boxes—one for male rats and one for female—each containing slips of paper indicating treatment group. A paper was removed, and the rat was handled according to the selected treatment group, with 4 male and 4 female rats assigned to each of the 3 treatment groups. Four induction chambers were created by using bedding-free standard rat shoebox cages (Lab Products, Seaford, DE), with solid lids that had been modified with a gas inlet in the center for gas exposure. The chamber volume was approximately 17.5 L. Each rat was placed alone in the induction chamber for gas exposure, which lasted approximately

2 min. For the O_2 (control) group, 100% O_2 was delivered into the chamber at a rate of 3 L/min by using a flowmeter. For the CO_2 group, 100% CO_2 was delivered into the chamber at a volume displacement rate of 30%/min by using a flowmeter. For the isoflurane group, 5% isoflurane was delivered to the chamber by using a precision vaporizer, with an O_2 rate of 3 L/min.

At the end of the 2-min period, the rat was removed from the induction chamber, and terminal cardiac exsanguination was performed. When additional anesthesia was required to complete euthanasia of the anesthetized animal after cardiac exsanguination, pentobarbital (390 mg/500 g delivered at a dose of 1 mg/kg; Euthasol, Virbac Animal Health, Fort Worth, TX) was administered intrathoracically. Death was assured by performing a bilateral pneumothorax. Blood glucose was measured immediately, and the remainder of the blood sample was split between an EDTA-treated tube and a serum-separator tube. Both samples were centrifuged; the resulting plasma (EDTA tube) and serum (serum-separator tube) were transferred into microfuge tubes and stored at –80 °C until use.

Blood glucose. Blood glucose was measured from a whole-blood sample by using a glucometer (FreeStyle Lite, Abbott, Abbott Park, IL).

Serum corticosterone. Serum corticosterone was measured by using a rat–mouse corticosterone ELISA kit (catalog no. 07DE-9922, MP Biomedicals, Santa Ana, CA). Serum samples were undiluted and run in duplicate. The plates were read on an ELISA plate reader set to 450 nm by using SoftMax Pro 7.0 (Molecular Devices, Sunnyvale, CA). Concentrations were calculated by using the 4-parameter logistic curve assay on MyAssays.com.

Serum noradrenaline. Serum noradrenaline was measured by using a noradrenaline research ELISA kit (catalog no. BA E5200, LDN Immunoassays and Services, Nordhorn, Germany). Serum samples were diluted 1:10 in 0.01 M HCl prior to processing and run in duplicate according to the manufacturer's recommendation. The plates were read on an ELISA plate reader set to 450 nm by using SoftMax Pro 7.0 (Molecular Devices). Concentrations were calculated by using the 4-parameter logistic curve assay on MyAssays.com.

Plasma ACTH. Plasma ACTCH was measured nu using an ACTH ELISA (catalog no. MO46006, MD Bioproducts, Oakdale, MN). Plasma samples were undiluted. The plates were read on an ELISA plate reader set to 405 and 450 nm by using SoftMax Pro 7.0 (Molecular Devices). Concentrations were calculated by using the MD Bioproducts ACTH ELISA (405 nm) and MD Bioproducts ACTH ELISA (450 nm) assays on MyAssays.com.

Statistics. For statistical analysis, the raw data were assessed for normal distribution, after which means were compared between treatments by using 2-way ANOVA. Interactions between sex and treatment were also evaluated. Only differences with a *P* value of less than 0.05 were considered to be significant. All statistical analyses were conducted by using JMP 8.0 (SAS Institute, Cary, NC). Corticosterone was undetectable in 2 serum samples (1 female in the O_2 group and 1 female in the isoflurane group).

Results

Blood glucose did not differ between treatment groups ($F_{2,33} = 0.4246$, $P = 0.6576$) or show significant effects of sex ($P = 0.2521$) or interaction between treatment and sex ($P = 0.8404$; Table 1). Likewise, serum corticosterone concentration did not differ between treatment groups ($F_{2,31} = 0.6594$, $P = 0.5242$) or show significant effects of sex ($P = 0.7144$) or interaction between treatment and sex ($P = 0.1159$). Serum noradrenaline

Table 1. Effects of various inhalants on serum biochemical markers in anesthetized rats

	O_2	Isoflurane	CO_2
Blood glucose (mg/dL)	213.69 ± 9.61	226.64 ± 10.45	221.25 ± 10.00
Corticosterone (ng/mL)	147.83 ± 25.40	120.71 ± 27.83	107.27 ± 25.40
Noradrenaline (pg/mL)	59.79 ± 25.39	54.92 ± 26.51	207.04 ± 25.38[a]
ACTH (pg/mL)	8.26 ± 10.20	24.03 ± 12.49	63.97 ± 10.20[a]

Data are given as mean ± SE ($n = 24$).
[a]Within the parameter, value differs significantly ($P \leq 0.05$) from those for other inhalants.

was higher in anesthetized rats exposed to CO_2 compared with O_2 or isoflurane ($F_{2,33} = 11.5712$, $P = 0.0002$), but there were no significant differences between sexes ($P = 0.5554$) or interaction between treatment group and sex ($P = 0.9153$). Mean serum ACTH concentration was higher in anesthetized rats exposed to CO_2 compared with O_2 or isoflurane ($F_{2,30} = 7.3265$, $P = 0.0026$). Male rats had higher serum ACTH concentrations ($43.71 ± 8.33$ pg/mL) than did females ($19.74 ± 9.62$ pg/mL; $P = 0.0418$), but there was no significant interaction between treatment group and sex ($P = 0.6989$).

Discussion

In anesthetized rats, serum noradrenaline and ACTH were higher after exposure to CO_2 as compared with exposure to oxygen or isoflurane, but no other significant differences were noted. This finding suggests that caution should be used when using serum noradrenaline or ACTH as a marker of wellbeing when comparing CO_2 euthanasia with isoflurane euthanasia, even though others have reported that these hormones may be useful for comparing concentrations of CO_2 or changes relative to baseline.[24] The lack of significant differences between the groups with regard to blood glucose and serum corticosterone is expected, given the short exposure time of 2 min relative to the time needed for significant changes in these parameters to emerge.[18,24] However, these markers were included in this study to evaluate whether the use of these 2 measures was confounded in studies using prolonged euthanasia, such as those using very low volume displacement of CO_2 or isoflurane.[1,7,19,20]

Blood glucose levels of rats exposed to isoflurane did not differ from those of rats exposed to O_2 or CO_2. This result may have implications for studies sensitive to the confounding effect of isoflurane on blood glucose in fasted rats, because it suggests that none of these inhalants has significant effects after short exposures. Isoflurane's mechanism of action has not yet been characterized, although the compound is understood to interact with GABA, glutamate, and glycine receptors, thus inhibiting motor function and receptor activity.[11,12,15,16,21]

In contrast, the mechanism of action for CO_2 overdose is well-characterized. As blood gas concentrations of CO_2 increase (hypercapnia and hypercarbia), serum pH levels are driven down, initiating respiratory acidosis. The amygdala detects this acidosis and triggers the physiologic responses to engage the sympathetic response, managed by the hypothalamic–pituitary–adrenal axis and sympathetic–adrenal–medullary system. For example, acid-sensing ion channel 1a (ASIC1a) is recognized to contribute to the development of fear behaviors.[22,23,25] Therefore, the observed increases in the rats' serum ACTH and noradrenaline levels were expected. However, the significant increase in the ACTH concentration of male rats compared with females was surprising, given that previous work suggested that females generally demonstrate higher increases in response to stress.[2,13]

The current study draws into question whether noradrenaline and ACTH actually measure a fear response when they are used in welfare studies of anesthesia and euthanasia. The data obtained here suggest that the response is independent of the sensation of a hypothetical 'loss of control' experienced by the animal during the induction of anesthesia. Given that all of these rats were anesthetized prior to gas exposure and because the alterations in these neuroendocrine hormones were only present in anesthetized rats exposed to CO_2, the rats would not consciously be experiencing fear, pain, or distress.[10] Therefore, when samples are collected after the animal has lost consciousness, the current results argue that the measured response may reflect stimulation of the amygdala in response to the respiratory acidosis induced by CO_2 and thus not be indicative of potential distress experienced by the animals during the induction phase.

The current findings suggest that noradrenaline and ACTH should be interpreted with caution to assess animal wellbeing between methods of euthanasia, particularly when the method's mechanism of action might confound that assessment. In addition, these data strengthen the role of including behavioral analysis as a key component of experimental design when assessing animal wellbeing.

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