Journal of the American Association for Laboratory Animal Science
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Vol 61, No 2
March 2022
Pages 195–200

Evaluation of Carbon Dioxide Euthanasia of Female Sprague Dawley Rats Alone or With Unfamiliar Conspecifics

Debra L Hickman

Most studies evaluating methods of euthanasia to date have focused on the euthanasia of individual animals. However, larger chambers are commonly used to euthanize multiple cages of animals at once. This study evaluated the use of a commercially available system for euthanasia of 1, 2, or 4 cages containing an individual female Sprague–Dawley rat using volume per minute displacement rates (VDR/min) of either 25% or 50% of 100% carbon dioxide. Animal wellbeing was assessed based on physiologic changes (serum noradrenaline and corticosterone) and behavioral assessments (relative frequency of rearing, line crossing, and grooming). The 25% VDR/min was associated with a significantly longer time to loss of consciousness, but this was not associated with significant physiologic or behavioral changes. The 50% VDR/min treatment group was associated with significant increases in the relative frequency of movement from 1 side of the cage to the other. Increases in the relative frequency of rears were detected in the 25% VDR/min treatment group when 2 or 4 rats were in the chamber as compared with a single rat in the chamber. The absence of significant physiologic changes suggest that the behavioral changes may have been associated with the novelty of the euthanasia experience rather than with distress. The location of the cage within the chamber did not significantly affect any of the measured parameters at either 25% or 50% VDR/min. These data suggest that groups of rats euthanized in these chambers are not experiencing decreases in their welfare.

DOI: 10.30802/AALAS-JAALAS-21-000051

Background and Objectives

All methods of euthanasia are associated with the potential for pain or distress, but multiple international guidance documents state that those performing euthanasia are ethically responsible for ensuring that this potential pain or distress is minimized.[2,6] For rodents euthanized in research laboratories, significant debate has developed around the humane use of anesthetic gases, such as carbon dioxide.[5,9,33,36] To date, the majority of relevant studies have focused on the intrachamber concentrations of these gases and the volume per minute displacement rate (VDR/min) associated with minimal potential for pain or distress.[10,11,14,18,23,25,26]

Assessment of animal welfare requires a multifaceted approach using assessment of the animal's behavioral and physiologic changes. However, multiple factors can influence the individual animal's response to the euthanasia procedure. For example, regulatory documents indicate that the home cage should be used for euthanasia whenever possible,[2,6] based on studies that demonstrated transient stress-associated behavioral and physiologic changes associated with cage changes in rodents.[3,8,31,32,37] However, studies that have specifically focused on the euthanasia procedure suggest that the use of a home cage does not confer any advantage to decrease the potential distress experienced by the rodents.[11,12,29] Likewise, regulatory documents recommend that stable groups should be maintained during euthanasia,[2,6] but social contagion literature suggests that pain and distress may be experienced more profoundly in the presence of bonded cage mates, suggesting that exposure to unfamiliar conspecifics may mitigate stress.[20-22,34,35] In addition, emotional stressors may more acutely affect females as compared with males.[19,28,30]

To date, the majority of the literature evaluating the use of carbon dioxide as an agent of euthanasia by anesthetic overdose have evaluated individual animals, euthanized 1 at a time.[10-12,14,23,25,26,38] These studies suggest that VDR/min of 30% to 70% of 100% carbon dioxide are preferable to lower or higher VDR/min rates when euthanizing individual animals. However, common practices range from euthanasia of a single cage, with gas delivered directly into a cage, to large commercial systems in which as many as 40 cages of mice can be euthanized together in a single large chamber at 1 time. No published studies are available regarding the welfare of animals euthanized in large commercial systems. The welfare of animals euthanized in these systems can be affected by factors that have not yet been identified in the existing literature, including exposure to nonfamiliar animals in adjacent cages the chamber and determination of how gas circulates in the larger chamber, affecting the VDR/min experienced by animals at the cage level.

This study was designed to evaluate the welfare of female laboratory rats euthanized in a commercially available bulk euthanasia chamber at either 25% or 50% VDR/min replacement rates with 100% carbon dioxide with either a single rat euthanized at a time or multiple, unrelated rats euthanized within the chamber. The hypothesis was that parameters of wellbeing would be better in animals exposed to unfamiliar animals, with an advantage in the moderate VDR/min (50%).

Materials and Methods

Study design. The behavior and physiologic responses of adult female Sprague–Dawley rats euthanized with either 25%

Received: 11 May 2021. Revision requested: 7 July 2021. Accepted: 9 Oct 2021.
School of Medicine, Indiana University, Indianapolis, Indiana
Corresponding Author: Email: hickmand@purdue.edu

or 50% VDR/min of 100% carbon dioxide were recorded. The rats were euthanized individually in cages, but 1, 2, or 4 cages were placed in the chamber. The chamber was considered the experimental unit when comparing the VDR/min, but each cage was considered an experimental unit when comparing location of the cage relative to gas inlets and exhaust ports in the chamber. All work was reviewed and approved by the Indiana University School of Medicine IACUC prior to initiation of the project. The animal care and use program is AAALAC accredited and compliant with all applicable federal regulations.

Animals. One hundred and 12 adult female Sprague–Dawley rats were used for this study. These rats were first generation offspring produced by breeder pairs of Sprague–Dawley rats (Crl:CD; Charles River Laboratories, Wilmington, MA). The breeder pairs and their offspring were raised under 1 of 2 light intensities (either 25 or 200 lx) and in 1 of 2 cage styles (either red or clear plastic) from birth to weaning for a separate study. Pups were removed from the variable light conditions upon weaning (approximately 21 to 25 d of age) and were pair-housed continuously with no further experimental manipulation until commencement of this study. Weight and age data were not collected for this study, but all rats were at least 120 d of age at time of euthanasia. Because social housing with familiar conspecifics can minimize potential stress responses,[4,13,15,16,24] rats were individually housed for at least 1 wk prior to euthanasia.

Experimental design. We calculated that a group size of 8 was needed to detect significant differences in noradrenaline concentrations with an α of 0.05 and a power of 0.80. This calculation was based on the use of mean outcomes of 100 compared with 145 with a standard deviation of 22. Data were analyzed by chamber, rather than by cage, to allow evaluation of the chamber. The original configuration of the large commercial euthanasia chamber delivered gas at a 25% VDR/min of 100% carbon dioxide. With the revision to the AVMA Guidelines on Euthanasia[2] that recommended the use of 30% to 70% VDR/min, the first aim of this study was to compare 25% to 50% VDR/min to determine if a significant difference in the welfare of the rats was associated with these 2 VDR/min of carbon dioxide.

The euthanasia chamber (M1-SBFF-1FM Chamber System with Flow Meter, Euthanex Systems, Palmer, PA) measured 32 in (81 cm) wide by 30.5 in (77 cm) deep by 12.5 in (32 cm) high. The cages used to euthanize the rats measured 17 in (43 cm) wide by 13 in (34 cm) deep by 8 in (20 cm) high. Therefore, a maximum number of 4 rat cages could be placed in the euthanasia chamber at the same time (Figure 1). Three configurations of cages in the chamber (1, 2, or 4 cages) were assessed to determine whether the presence or absence of caged conspecifics affected the welfare response of rats euthanized in this chamber. Therefore, rats were euthanized with: 1) 25% VDR/min of carbon dioxide (1 cage, $n = 8$; 2 cages, $n = 8$; 4 cages, $n = 8$), or 2) 50% VDR/min carbon dioxide (1 cage, $n = 8$; 2 cages, $n = 8$; 4 cages, $n = 8$).

All rats were assessed daily for general health. Rats that developed overt clinical signs of disease (for example, dermatitis) were excluded from the study to minimize the potential confounding effect of immune stimulation on the physiologic stress response; however, none of the rats were removed from the study due to clinical signs of disease. The colony was screened quarterly by using indirect sentinels. At the time of the study, the colony was free of the following pathogens: coronavirus (sialodacryoadenitis virus), parvoviruses (NS1, rat pneumonia virus, 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*).

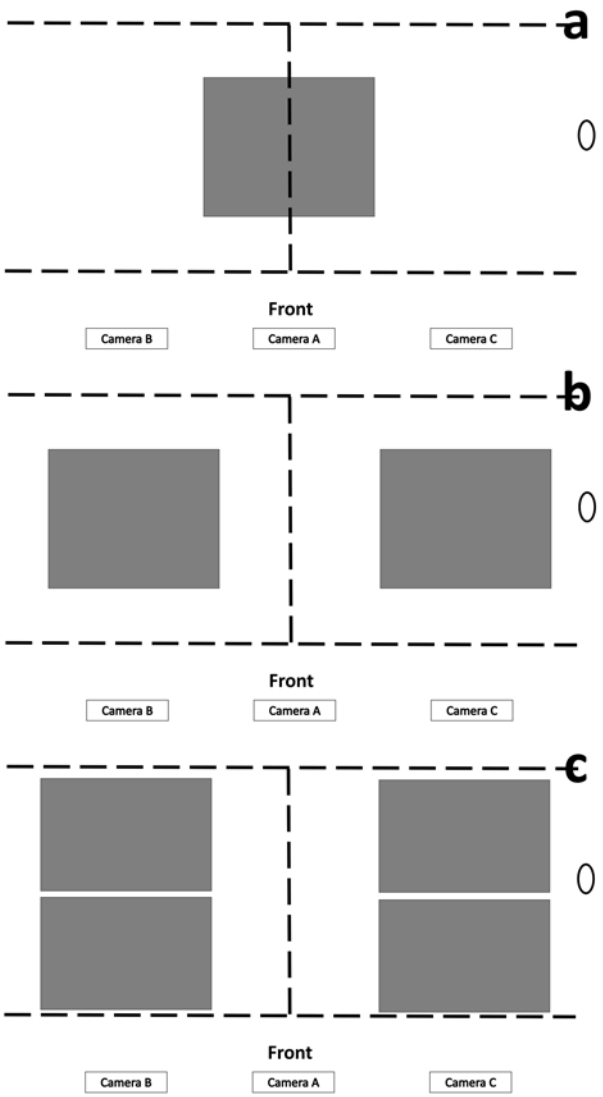

Figure 1. Schematic of gas inlet (dotted lines) and cage location (gray box(es)) in commercial euthanasia chamber. Circle represents exhaust location. (a) Cage location for single cage euthanasia. (b) Cage location for euthanasia of 2 cages. (c) Cage location for euthanasia of 4 cages. Footage from camera A was used to score behaviors of single cage euthanasia. Footage from camera B and camera C was used to score behaviors for the euthanasia of 2 or 4 cages.

Upon enrollment in this study, each rat was individually housed in an opaque polypropylene rat shoebox cage (Ancare, Bellmore, NY) with shaved wood (7093 Teklad, Envigo, Indianapolis, IN) for bedding. The cages were handled conventionally, without filter tops. Feed (Teklad 2018SX, Envigo, Indianapolis, IN), and acidified water were provided without restriction. The light cycle was 12:12 light:dark with lights turning on at 0700. Gnawing materials (for example, gnawing bones or blocks, BioServ, Flemington, NJ) were provided for enrichment. The macroenvironment was maintained at 68 to 72 °F (20 to 22.2 °C), and 30% to 50% relative humidity. Cages were changed at least twice weekly and sanitized in a tunnel washer before reuse, consistent with the facility's standard operating procedures. Personal protective equipment including hair bonnet, isolation gown, mask, and gloves were used when working with the rats.

Randomization was performed by numbering all cages in the room from 1 to 112. The 6 potential treatment and subtreatment combinations (25% compared with 50% VDR/min of

carbon dioxide and number of cages in the chamber [1, 2, or 4]) were assigned numbers 1 to 6 and a random number generator (randomnumbergenerator.org) was used to generate random sets of the numbers 1 to 6. Cages were used in numerical order to fulfill the treatment group assigned by the random number generator (for example, rat 1 might be enrolled in the first treatment group, but rats 2 through 5 were enrolled in the second treatment group). A single observer scored all videos.

Euthanasia. To perform the euthanasia procedure, each rat was removed from its home cage by gently grasping at the base of the tail, then scooping the body up in order to place it in a transparent rat cage (Innovive, San Diego, CA) to facilitate behavioral scoring during euthanasia. Each rat had a range of 1 to 10 min (typically 5) in the novel cage before being placed in the euthanasia chamber due to the variation in times required to prepare 1, 2 or 4 cages for placement in the euthanasia chamber (SmartBox, EZ Systems, Palmer, PA). The cages were placed in the euthanasia chamber in a consistent location depending on the number of cages in that treatment group (Figure 1). The location of each cage was documented for future analysis. When all cages were in the chamber, it was closed and activated according to the manufacturer's instructions for rat euthanasia. The euthanasia process was digitally recorded using a security camera system (Lorex, Linthicum, MD) for behavioral scoring later. Upon completion of the euthanasia cycle (programmed to have at least 10 min of dwell time exposed to carbon dioxide to result in irreversible euthanasia), the cages were removed from the chamber and a blood sample was collected by cardiac puncture. All procedures were performed between 1300 and 1700 to minimize the potentially confounding effect of variations in physiologic and behavioral responses at different times during the day.

The blood was placed in a serum separator microtube and allowed to clot for at least 5 min. The blood sample was spun in a microcentrifuge, and the serum was removed and stored at −80 °F until the study was completed and all samples were ready for processing. Blood samples were identified with a numerical designation, and the technician who performed the ELISA assessment was blind to treatment group.

ELISA. Serum noradrenaline was measured using a noradrenaline ELISA kit (BA E-5200; LDN Immunoassays and Services, Nordhorn, Germany). Serum samples were diluted 1:10 in 0.01 N HCl prior to processing, according to manufacturer recommendations. 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 using the 4-parameter logistic curve assay on MyAssays.com.

Serum corticosterone was measured using a corticosterone mouse ELISA kit (07DE-9922; MP Biomedical, Santa Ana, CA). Serum samples were undiluted. The plates were read on an ELISA plate reader set to 450 nm using SoftMax Pro 7.0 (Molecular Devices, Sunnyvale, CA). Concentrations were calculated using the 4-parameter logistic curve assay on MyAssays.com.

Behavioral scoring. Three video cameras that were affixed to a table kept a consistent distance from the front of the euthanasia chamber were used to collect behavioral data for each euthanasia (Figure 1). Each camera location was selected to obtain clear and consistent recordings of each cage, regardless of configuration within the chamber, so that a single digital feed was collected for each cage. Each rat was scored from the time that the gas started until the rat engaged in the "nose touch" behavior. Nose touch was defined as the behavior that is characterized by cessation of ataxic movement followed by the dropping of the head until the nose touches the bottom of the cage. This behavior is generally followed shortly by lateral recumbency and is used to approximate the loss of consciousness, plus or minus 5 s.[23] The observer scoring the behavioral video was blind to the VDR/min treatment group that they were observing, but could not be completely blind to the number of cages in the chamber due to the potential to see other cages in the chamber (behind or to the side of the cage of interest). Behaviors assessed included rears (defined as standing on rear feet), line crossing (defined as moving shoulders across the midline of the cage, where the midline of the cage was demarcated by the placement of the lowest point on the wire feeder), and grooming events (defined as vigorous rubbing of the face with the forepaws). The relative frequency of each behavior was calculated by dividing the total number of events by the seconds from gas initiation to nose touch. The relative frequencies were compared between groups. If the chamber contained 4 cages, scoring rats in the cages located behind the first 2 cages was difficult, so only the front 2 cages were behaviorally scored.

Statistical analysis. For the overall comparison between the VDR/min rates, the values for all rats in a trial were averaged prior to analysis. To determine if the location of the cage affected the behavioral and physiologic parameters, data from individual rats were compared across VDR/min and the location of the cage in the chamber. The data were tested for normality using the Kolmogorov-Smirnov test of normality (https://www.socscistatistics.com/tests/kolmogorov/default.aspx). All descriptive data are expressed as mean ± SEM. All data were normally distributed with the exception of the corticosterone concentrations, which were therefore log-transformed prior to analysis. For statistical analysis, a 2-way ANOVA was performed to compare means between treatments and between subtreaments within a treatment. Interactions between treatment were also evaluated. Only differences with a probability less than 0.05 were considered to be significant. All statistical analyses were conducted using JMP 8.0 (SAS Institute, Cary, NC).

Results

The first statistical tests compared the VDR/min rates and evaluated interactions between the VDR/min and the number of rats in the chamber during the euthanasia process. Rats euthanized with the 25% VDR/min had significantly higher the time to "nose touch" than did rats in with 50% VDR/min, but the 50% VDR/min treatment group had a greater relative frequency of line crosses as compared with the 25% VDR/min treatment group. All data are presented in Table 1. A significant

Table 1. Comparison of mean parameters for volume per minute displacement rates.

Experimental Outcome	25% VDR/min of 100% CO_2	50% VDR/min of 100% CO_2	Comments (**denotes significance*)
Latency to "Nose touch" (seconds)	88 ± 3	62 ± 3	F(1,46) = 50.4298 *P < 0.0001**
Log concentration of corticosterone	1.63 ± 0.08	1.47 ± 0.08	F(1,46) = 2.1458 *P* = 0.1498
Serum noradrenaline (pg/mL)	0.04 ± 0.01	0.04 ± 0.01	F(1,46) = 0.5191 *P* = 0.4749
Rearing (relative frequency)	0.093 ± 0.008	0.102 ± 0.008	F(1,46) = 0.6037 *P* = 0.4411
Line crossing (relative frequency)	0.069 ± 0.004	0.081 ± 0.004	F(1,46) = 4.3673 *P = 0.0422**

Data presented as mean ± SEM. Significance set at $P < 0.05$.

interaction was detected between VDR/min and the number of rats in the chamber for the relative frequency of rears per minute ($P = 0.0270$), with significantly more rears in trials with multiple rats as compared with trials having only 1 rat in the chamber (Figure 2); significant interactions were not detected between VDR/min and number of rats in the chamber for any of the other parameters assessed. Grooming behavior was not observed in any of the rats.

Because of the difference in the relative frequency in rears in the trials with multiple rats in the chamber as compared with the trials with a single rat in the chamber, the next analysis excluded the trials with a single rat in the chamber. This allowed comparisons between individual rats based on cage location within the chamber, without confounding the analysis by having a single rat compared with multiple rats within the chamber. No significant differences were detected in the physiology or behavior of the rats based on location in the right or the left of the chamber (Table 2).

When looking for differences between the front and the back of the chamber, we could only compare the physiologic assessments as collecting behavioral data from the rats in the cages in the back was difficult due to the location of cameras used for this study. No significant differences were detected in the physiologic data based on cage location in the front or back of the chamber (Table 3).

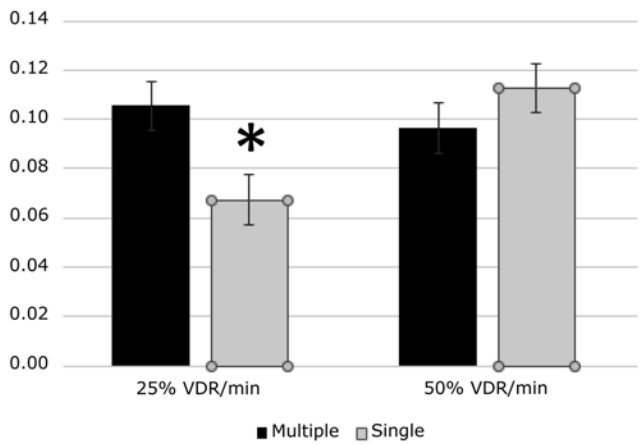

Figure 2. Relative frequency of rears per second exhibited in the 25% and 50% VDR/min treatment groups depending on euthanasia of a single rat within the chamber as compared with multiple rats within the chamber. Significance set at $P < 0.05$ and denoted with asterisk.

Discussion

With the current recommendations of VDR/min of 30% to 70%,[2] we expected to find significant indicators of distress associated with the use of a 25% VDR/min. While the time to surrogate loss of consciousness ("nose touch") was significantly longer in rats euthanized with the VDR/min of 25%, we found no significant differences in noradrenaline or corticosterone between the 2 groups. Other work using individual animals suggests that VDR/min in the 20% to 30% range may be less distressing or painful than VDR/min of 10% or less[10] or 70% or higher,[7,18,27,38] respectively.

The results of the behavioral analysis should be considered when interpreting the apparent lack of physiologic changes associated with the 2 VDR/min. The relative frequency of rearing was significantly lower in rats euthanized alone in the chamber with 25% VDR/min as compared with rats euthanized with unfamiliar conspecifics in adjacent cages in the chamber. This difference did not occur with 50% VDR/min. However, the relative frequency of line crossing was higher with 50% VDR/min as compared with 25% VDR/min, regardless of the number of rats in the chamber. As both behaviors can be

Table 2. Analysis of the outcomes from individual animals with cages in the left of the chamber compared with cages in the right of the chamber.

25% Volume Per Minute Displacement Rate			
Experimental Outcome	Left	Right	Comments (**denotes significance*)
Latency to "Nose touch" (seconds)	87 ± 3	85 ± 3	F(1,30) = 0.2373 *P* = 0.6297
Log concentration of corticosterone	1.66 ± 0.10	1.47 ± 0.10	F(1,30) = 0.9017 *P* = 0.7634
Serum noradrenaline (pg/mL)	0.04 ± 0.01	0.05 ± 0.01	F(1,30) = 0.1356 *P* = 0.7144
Rearing (relative frequency per second)	0.118 ± 0.010	0.093 ± 0.010	F(1,30) = 3.0746 *P* = 0.0897
Line crossing (relative frequency per second)	0.070 ± 0.007	0.064 ± 0.007	F(1,30) = 0.3678 *P* = 0.5487
50% Volume Per Minute Displacement Rate			
Latency to "Nose touch" (seconds)	64 ± 4	62 ± 4	F(1,30) = 0.0835 *P* = 0.7746
Log concentration of corticosterone	1.51 ± 0.10	1.70 ± 0.07	F(1,30) = 0.1441 *P* = 0.7061
Serum noradrenaline (pg/mL)	0.03 ± 0.01	0.05 ± 0.01	F(1,30) = 1.3073 *P* = 0.2588
Rearing (relative frequency per second)	0.103 ± 0.013	0.091 ± 0.013	F(1,30) = 0.4107 *P* = 0.5265
Line crossing (relative frequency per second)	0.083 ± 0.009	0.080 ± 0.009	F(1,30) = 0.0549 *P* = 0.8163

Data presented as mean ± SEM. Significance set at $P < 0.05$.

Table 3. Analysis of the physiologic assessments from individual animals with cages located in the front, center, or back of the chamber.

25% Volume Per Minute Displacement Rate				
Experimental Outcome	Center	Back	Front	Comments (**denotes significance*)
Log concentration of corticosterone	1.57 ± 0.07	1.81 ± 0.08	1.66 ± 0.08	F(2, 53) = 2.6532 *P* = 0.0801
Serum noradrenaline (pg/mL)	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	F(2,53) = 1.9365 *P* = 0.1543
50% Volume Per Minute Displacement Rate				
Log concentration of corticosterone	1.50 ± 0.11	1.53 ± 0.13	1.38 ± 0.13	F(2, 53) = 0.3836 *P* = 0.6833
Serum noradrenaline (pg/mL)	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	F(2,53) = 0.6707 *P* = 0.5156

Data presented as mean ± SEM. Significance set at P < 0.05.

interpreted subjectively as anxiety or exploratory behavior,[1,17] the physiologic data becomes important to consider. In this case, the lack of significant differences in the neuroendocrine responses between the 2 VDR/min suggests that the behaviors are potentially attributable to exploratory behaviors. However, because rats were moved from an opaque cage to the clear cage before euthanasia and their time in the novel cage was variable, the possibility exists that these values were increased by the placement of each rat in the novel environment of a novel cage before placement in a novel environment with a novel experience, although previous work found that the use of an unfamiliar induction chamber did not lead to significant differences in physiologic and behavioral assessments as compared with the use of the home cage.[11,12] We found a greater relative frequency in rearing for the rats exposed to unfamiliar conspecifics during the longer time to unconsciousness that occurred with the 25% VDR/min, suggesting that interactions with the pheromones of the unfamiliar conspecifics in the adjacent cages distracted them from the onset of the effects of the gas, while those euthanized at the 50% VDR/min did not have sufficient time without distraction to engage in these behaviors.

Because the location of the gas inlets inherently creates differences in gas delivery between the cages, depending on where they are located within the chamber, the effect of the location of the cage on the wellbeing of the animals was a critical assessment of this study. However, overall the location of the cage within the chamber did not have a significant effect on the assessed parameters.

In summary, these data suggest that no statistically significant differences in the selected parameters of animal welfare occur when this commercially available euthanasia system is used to euthanize female Sprague–Dawley rats at 25% to 50% VDR/min when up to 4 individually caged rats are euthanized in the chamber at one time. Additional study is needed to assess the use of this chamber with group housed animals, males, and other small rodents, such as mice. Likewise, future studies should assess how the presence or absence of familiar conspecifics affects the wellbeing of rodents euthanized under similar conditions. Although more work is required to determine the best parameters for use in chambers designed to euthanize multiple cages of rodents at a time, this study suggests that these chambers successfully accomplish euthanasia in a manner similar to that reported for individual rodents euthanized with carbon dioxide.

Acknowledgments

Declaration of Interests. Funds to support this study were provided by Euthanex Systems without condition. The author independently designed the study, performed the analysis, and reported the study.

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