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Wellbeing of Alcohol-preferring Rats Euthanized with Carbon Dioxide at Very Low and Low Volume Displacement Rates

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The 2013 *AVMA Guidelines on Euthanasia* recommend the use of very-low or low flow rates of 100% carbon dioxide to euthanize small rodents. Although inhalation of high concentrations of carbon dioxide are generally recognized as painful in humans, whether the use of these low-flow methods of euthanasia increase potential distress for rats is unclear. This study compared physiologic and behavioral markers of animal wellbeing for rats euthanized by using 10% volume displacement per minute (VD/min), 30% VD/min, and 70% VD/min of 100% carbon dioxide. Rats were recorded during euthanasia for subsequent behavioral scoring, and blood samples were taken after euthanasia for assessment of blood glucose and serum corticosterone levels. In this study, rats euthanized with 10% or 30% VD/min of 100% carbon dioxide demonstrated increases in various behaviors, such as rearing and standing, concurrent with increases in serum corticosterone. Rats euthanized with 70% VD/min of 100% carbon dioxide did not exhibit these changes. The results suggest that a euthanasia method of 70% VD/min of 100% carbon dioxide may minimize potential pain and distress and thus be more humane for rats, as compared with very-low– and low-flow methods of carbon dioxide euthanasia.

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The 2013 *AVMA Guidelines on Euthanasia* recommend the use of 100% carbon dioxide at very-low–flow (10%) and low-flow (30%) volume displacement per minute (VD/min) rates to euthanize small rodents.[2] Because the inhalation of high concentrations of carbon dioxide is generally recognized as painful in humans,[7,9] using very-low– and low-flow methods is hypothesized to minimize pain and distress in rodents.[2,7] However, it is unclear if the use of these low flow methods of euthanasia result in increased distress for the animals, especially rats.[4]

The 2000 *AVMA Guidelines on Euthanasia* encouraged the use of 70% VD/min of 100% carbon dioxide.[1] This method was removed from the updated version because of concerns regarding the potential for pain experienced by the rodents. However, this concern may not be valid. Loss of consciousness is estimated to occur when carbon dioxide concentrations reach approximately 40%,[4] but humans do not experience pain until concentrations exceed 50% (with the percentage of people reporting pain increasing as the concentration of carbon dioxide increases).[9] At a 70% VD/min flow rate of 100% carbon dioxide, the concentration of carbon dioxide in the euthanasia chamber would be approximately 35% after 30 s of flow, meaning that consciousness is lost prior to chamber concentrations of carbon dioxide reaching painful levels. However, there is concern that significant distress could be associated with the use of very low flow (that is, 10% VD/min flow rate of 100% carbon dioxide) and low flow (30% VD/min flow rate of 100% carbon dioxide), because the concentrations of gas will exceed the threshold of detection (around 7% to 10%) early, with a delay in time to loss of consciousness (around 40%) that can last from 1 to 3 min.[4] Inhaling concentrations of 20% carbon dioxide is a proven model of anxiety in laboratory rats through the induction of a sensation of air hunger, associated with dyspnea.[14,15,16] This model suggests that exposure to 1 to 3 min of carbon dioxide prior to the loss of consciousness has a high likelihood of distress.

The current study was designed to compare measures of animal wellbeing in rats euthanized by using several volume displacement rates (VD/min) of 100% carbon dioxide and to determine the method most likely to minimize potential pain and distress for rats during euthanasia. The hypothesis was that rats euthanized with 10% and 30% VD/min of 100% carbon dioxide would display increased evidence of distress according to behavioral and physiologic markers of animal wellbeing.

Materials and Methods

Ethical statement. All procedures were reviewed and approved by the Indiana University School of Medicine IACUC prior to initiation of the project. The program is accredited by AAALAC and compliant with all applicable federal regulations.

Animals. The P-rat was used as the animal model for this study. The P-rat is an alcohol-preferring rotational outbred stock of rat that was generated at Indiana University for the investigation of alcohol addiction. These rats were behaviorally selected for a propensity to imbibe alcohol when provided.[3] This rat stock is generally more anxious and more prone to stress-induced changes in manipulation, especially when not habituated to handling, than are other lines.[19] Both male (24 rats; age, 108 to 399 d [mean, 213 d]) and female (24 rats; age, 130 to 276 d [mean, 189 d]) rats were used in this study. These animals were surplus offspring from the campus production colony, and none had been used for any other research project prior to enrollment in this euthanasia project.

Husbandry. All rats were pair-housed in standard rat shoebox caging on an IVC system rack (Lab Products, Seaford, DE).

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The cages were bedded with pine chip bedding (Sani-Chip, PJ Murphy Forest Products, Montville, NJ) and nesting material (for enrichment), and each cage was changed weekly. Food (Teklad 2018SX, Envigo, Indianapolis, IN) was provided without restriction. Reverse-osmosis–treated water was provided without restriction through an automatic watering system. Cages were changed at least weekly in a laminar flow workstation (Nuaire, Plymouth, MN) and were autoclaved prior to reuse. Hands and implements were disinfected with MB10 (Quip Labs, Wilmington, DE) between cages. The macroenvironment was on a 12:12-h light:dark cycle (lights on, 0700), and the temperature was maintained at 72 ± 1 °F (22.2 ± 0.5 °C), with humidity maintained between 30% and 70%. 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*).

Experimental design. The euthanasia chamber had the same dimensions as a standard rat shoebox cage (12.1 × 12.1 × 7.4 in.). All rats were removed from their home cage and placed in the nonbedded euthanasia chamber to facilitate recording of rat behavior during the euthanasia process. Each rat was euthanized individually, and all euthanasia was performed between 1200 and 1600 to control for circadian variation. Three 100% carbon dioxide displacement rates were used for this study: 10%, 30%, and 70% VD/min. The 10% (that is, very low flow) and 30% (that is, low flow) VD/min of 100% carbon dioxide are currently approved with considerations by the AVMA;[2] 70% VD/min of 100% carbon dioxide was previously conditionally approved by the AVMA.[1]

To randomly assign animals to treatment groups, slips of paper with each of the 4 treatment groups were placed in a container. Groups of 12 same-sex rats were brought to the euthanasia room. Prior to euthanasia of each rat, a paper was drawn to determine which treatment that animal would experience. This process was repeated for each group of 12 male and 12 female rats. The researcher (DH) performed all euthanasia procedures and was not blinded to treatment group.

Euthanasia process. The rats were fasted for approximately 12 to 18 h prior to euthanasia to allow for collection of fasting blood glucose values. Rats were brought to the euthanasia room in groups of 12 and were allowed to acclimate for 10 min prior to euthanasia of the first rat. After placement in the euthanasia chamber, each rat was allowed 30 to 60 s for acclimation before the initiation of 100% carbon dioxide gas infusion at the designated rate. A flow meter was used to ensure controlled delivery. The gas was introduced at the center of the top of the euthanasia chamber through a single lid modified to fit the chamber. On one side of the chamber, a red line was drawn at 5 in. from the bottom and a blue line was drawn at 7 in. from the bottom. The camera and chamber were maintained at the same distance and angle for all euthanasias. Each rat was digitally recorded from the side to measure vertical behaviors, such as rearing. Each recording was collected from placement of the rat in the cage, with verbal notation of initiation of gas delivery, until the rat was removed from the chamber.

After the rat achieved 'nose down' (estimated as the approximate beginning of stage II of anesthesia)[24] and respirations had transitioned to a slow, regular pattern (consistent with stage III of anesthesia),[24] the rat was removed from the euthanasia chamber for terminal cardiac blood collection and secondary euthanasia (bilateral pneumothorax). Blood glucose was recorded immediately as described later. The balance of the blood sample was placed in a serum separator tube, centrifuged, and stored in a –80 °F freezer until further processing. Between animals, the euthanasia chamber was cleaned with MB10 (Quip Labs) and inverted to facilitate the dispersion of carbon dioxide prior to reuse.[10]

Experimental outcomes. ***Nose down.*** Rats were observed and their behavior was scored from induction until nose down, defined as the point where the head dropped and the nose of the rat touched the bottom of the cage, even if movement continued after this point. This behavioral parameter allowed approximation of the loss of righting reflex (plus or minus approximately 5 to 10 s),[20] which occurs at the end of stage I of anesthesia and is correlated with the loss of consciousness.[24] Spontaneous movement can continue during stage II of anesthesia (from loss of righting reflex to lateral recumbency), but the animal is not conscious during that stage.[24]

Blood glucose. Blood glucose in samples of whole blood were measured by using a glucometer (FreeStyle Lite, Abbott, Abbott Park, IL). Blood glucose increases as a component of the HPA response to stressors and can be an indicator of potential distress when evaluated in tandem with behavioral data.[4]

Serum corticosterone. Serum corticosterone was measured by using an ELISA kit (rat/mouse corticosterone kit, catalog no. 07DE-9922, MP Biomedicals, Santa Ana, CA). Serum samples were undiluted. The plates were read at 450 nm on a plate reader by using SoftMax Pro 7.0 (Molecular Devices, Sunnyvale, CA). Concentrations were calculated by using the 4-parameter logistic curve assay on MyAssays.com. In addition, corticosterone increases as a component of the HPA response to stressors and can be an indicator of potential distress when evaluated in tandem with behavioral data.[4]

Behavioral assessment. All rats were videorecorded from the side, as described earlier. The time from initiation of gas infusion to the presumptive loss of consciousness (defined as the point when the rat's head dropped and its nose touched the bottom of the cage; that is, nose down) was calculated for each rat. The recordings were then scored for the number of rears and the amounts of time during which the rat's head was at or above 5 in. and 7 in. Rearing can be an indirect measure of anxiety in a novel environment (that is, induction chamber and gas exposure).[6,23] Because carbon dioxide is heavier than room air, the time spent in the bipedal stance, effectively avoiding the bottom of the chamber, was hypothesized to be indicative of the distress experienced by the rats.[4] The proportions of these measurements (number of rears and time spent at or above 5 in. and 7 in.) were calculated by dividing each assessments by the total time from initiation of gas infusion to nose down. The amount of time per rear was calculated as the time (in seconds) at or above 5 in. divided by the number of rears. These recordings were scored manually by using continuous behavior tracking methods. The researcher (DH) was blinded in regard to treatment group during behavioral scoring.

Statistical analysis. Using the data for serum corticosterone (200 ± 100 pg/mL) from previous studies[12,13] in a power analysis with an α-value of 5% and power of 0.8 indicated that a sample size of 6 was sufficient to identify statistically significant differences between treatment groups, including possible sex-associated differences (www.powerandsamplesize.com/calculators/). Statistical analysis was performed by using JMP (SAS, Cary, NC). Prior to analysis, all parameters evaluated were assessed for normal distribution by using the Shapiro–Wilk test. Double-sided ANOVA was used for overall model analysis to

Table 1. Results from data collection during euthanasia of rats by using 100% carbon dioxide at various volume displacement rates (% VD/min)

	10% VD/min	30% VD/min	70% VD/min
Time (s) to nose down			
Total	177.08 ± 7.91	71.33 ± 7.37[a]	37.90 ± 9.02[a]
Male	331.33 ± 45.44	73.88 ± 39.36	40.71 ± 42.07
Female	152.25 ± 39.36 (P = 0.0970)	68.43 ± 42.07 (P = 0.3321)	31.33 ± 64.27 (P = 0.0374)
Blood glucose (mg/dL)			
Total	114.73 ± 7.22	88.31 ± 7.00[a]	81.56 ± 7.00[a]
Male	131.75 ± 11.95	104.5 ± 6.48	81.38 ± 6.46
Female	96.75 ± 11.95 (P = 0.0572)	72.13 ± 6.48 (P = 0.0033)	81.75 ± 6.46 (P = 0.9678)
Serum corticosterone (pg/mL)			
Total	362.36 ± 81.97	310.05 ± 79.19	96.32 ± 79.20[a]
Male	395.17 ± 130.44	236.21 ± 166.21	79.90 ± 43.06
Female	452.42 ± 122.01 (P = 0.7536	394.44 /- 177.69 (P = 0.5268)	110.68 ± 40.28 (P = 0.6104)
Number of rears per minute			
Total	6.97 ± 0.99	10.35 ± 0.92[a]	7.26 ± 1.13
Male	7.62 ± 0.93	9.21 ± 1.48	7.83 ± 1.51
Female	6.12 ± 0.80 (P = 0.2445)	11.66 ± 1.58 (P = 0.2798)	5.91 ± 2.31 (P = 0.5062)
Proportion of rearing time spent at or above 5 in.			
Total	0.39 ± 0.04	0.35 ± 0.04	0.21 ± 0.05
Male	0.40 ± 0.09	0.36 ± 0.05	0.25 ± 0.04
Female	0.33 ± 0.08 (P = 0.5592)	0.34 ± 0.06 (P = 0.8735)	0.11 ± 0.07 (P = 0.0960)
Proportion of rearing time spent at or above 7 in.			
Total	0.23 ± 0.03	0.18 ± 0.03	0.11 ± 0.04
Male	0.24 ± 0.07	0.18 ± 0.04	0.14 ± 0.04
Female	0.19 ± 0.06 (P = 0.6037)	0.17 ± 0.04 (P = 0.7487)	0.04 ± 0.05 (P = 0.1537)
Time (s) at or above 5 in. per rear			
Total	3.56 ± 0.39	2.13 ± 0.36	1.82 ± 0.44[a]
Male	3.79 ± 0.43	2.45 ± 0.34	2.13 ± 0.37
Female	3.41 ± 0.63 (P = 0.7666)	1.76 ± 0.67 (P = 0.0473)	1.08 ± 1.03 (P = 0.1342)

All data are presented as mean ± SE. 'Total' indicates pooled data from male and female rats; 'male' and 'female' indicate data separated by sex within each treatment. Significance was set at $P < 0.05$; P values in the table refer to the differences between sexes within a treatment group.
[a]Values differ significantly ($P < 0.05$) between treatments

assess for effects of volume displacement rate or sex. Significance was set a P value of less than 0.05.

Results

Time to nose down differed significantly between treatment groups ($F_{2,36}$ = 9.3350, P = 0.0005), with a significant sex-associated difference (that is, time shorter for female than male rats) in the 70% VD/min 100% carbon dioxide group. Blood glucose levels differed significantly between treatment groups ($F_{2,45}$ = 6.2253, P = 0.0041), with a significant sex-associated difference (that is, higher in male than female rats) in the 30% VD/min 100% carbon dioxide group. Serum corticosterone levels differed significantly between treatment groups ($F_{2,42}$ = 3.7438, P = 0.0319) with no significant sex-associated differences. Number of rears per minute differed among treatment groups ($F_{2,36}$ = 4.2256, P = 0.0225), but neither the proportion of rearing time spent at or above 5 in. ($F_{2,36}$ = 2.9871, P = 0.0631) nor at or above 7 in. ($F_{2,36}$ = 1.9250, P = 0.1606) differed among treatment groups. The total time at or above 5 in. differed between treatment groups ($F_{2,36}$ = 5.4609, P = 0.0086), with less time for 30% and 70% VD/min 100% carbon dioxide compared with 10% (Table 1).

Discussion

As expected, mean time to nose down differed significantly among the 3 volume displacement rates of 100% carbon dioxide. Specifically, rats euthanized with 10% VD/min of 100% carbon dioxide took the longest time to reach nose down. This outcome has been proposed to be desirable because it has been thought to minimize pain and distress.[7,11] However, the data from the current study suggest that these rats experienced significant distress. Evaluation of their physiologic changes shows that the rats in the 10% VD/min treatment group had higher mean blood glucose and serum corticosterone levels than the other groups. In addition, rats in the 10% VD/min treatment group engaged in significantly more rears per minute and spent a high percentage of time standing, with 39% of their time spent at or above 5 in. and 23% of their time at or above 7 in. Furthermore, the mean time for each rearing event was 3.6 s, significantly longer than

the other treatment groups, suggesting the 10% VD/min rats were not simply exploring but were trying to avoid the carbon dioxide or escape the cage, consistent with published studies of aversion.[17,18] These results were not unexpected, given that the exposure of rats to 20% carbon dioxide induces air hunger and dyspnea with associated anxiety.[14,15,16]

If 10% VD/min of 100% carbon dioxide causes rats to experience distress, perhaps the low flow rate of 30% VD/min of 100% carbon dioxide is preferable.[4,20] However, the current study suggests that this flow rate is also associated with significant distress for rats. Although the time to nose down was shorter for 30% than 10% VD/min of 100% carbon dioxide, serum corticosterone levels did not differ significantly between these 2 treatment groups. Compared with those in the 10% VD/min animals, the lower blood glucose in the 30% VD/min group (with levels lower for female than male rats) might simply reflect insufficient time for levels to increase.[4] In addition, the rats in the 30% VD/min group exhibited a significant increase in the number of rears per minute, although female rats spent less time engaged in each rear, as compared with the 10% VD/min. Proportionally, the mean time spent at or above 5 or 7 in. did not differ between the 10% and 30% VD/min treatment groups. Overall, these findings suggest that the rats in the 30% VD/min group experienced similar levels of distress as those in the 10% VD/min group.

The rats in the 70% VD/min of 100% carbon dioxide treatment group had the shortest mean time to nose down and the lowest mean levels of blood glucose and serum corticosterone, but these values likely reflect the brief induction time and thus insufficient time for these levels to increase significantly in the peripheral circulation.[4,8,22] Assessment of a more rapid response biomarker, such as ACTH or noradrenaline,[4,12] may have been helpful for assessing the wellbeing of these rats, but those data were not collected during this study. However, note that these rats spent significantly less time at or above 5 in. and 7 in. and significantly less time per rear. Because these mean proportions are weighted against the time to nose down, these findings suggest that the rats in the 70% VD/min group did not feel the drive to engage in the rearing and standing (that is, proportions of time spent at or above 5 and 7 in.) behaviors seen in the 10% and 30% VD/min carbon dioxide treatment groups.

If pain were to be experienced during carbon dioxide anesthesia induction, it has been suggested that it would occur in the 70% VD/min of 100% carbon dioxide treatment group.[7] However, with a 70% VD/min flow rate of 100% carbon dioxide, the concentration of carbon dioxide in the euthanasia chamber would be approximately 35% after 30 s of flow. Consciousness is lost at carbon dioxide concentrations of approximately 40% (which is consistent with the mean time to nose down recorded in this study). In studies in humans evaluating pain associated with inhalation of carbon dioxide, concentrations of 50% or greater are reported to be painful (with the percentage of people reporting pain increasing as the concentration of carbon dioxide increases above 50%).[9] Because the rats were unconscious once that concentration was achieved, it is unlikely that these animals consciously experienced pain.

It is worth noting that a few rats (at least 2 in this study) repeatedly approached and sniffed the 100% carbon dioxide entering the cage without shaking their heads or rubbing their faces. However, in the human study of pain associated with inhalation of carbon dioxide, 13 of the 40 subjects rated 80% CO_2 as uncomfortable (not painful) and 2 of the 40 subjects rated 100% carbon dioxide as uncomfortable (not painful).[9] Clearly, interindividual differences regarding tolerance of exposure to high concentrations of carbon dioxide exist.

The current study supports other work that suggests that the 10% VD/min (very low flow rate) of 100% carbon dioxide is distressing to rats.[5,14,21] The data further suggest that distress is experienced by rats euthanized with 30% VD/min of 100% carbon dioxide, because many of the measures of animal wellbeing did not differ between these 2 groups. In contrast, 70% VD/min of 100% carbon dioxide appears to meet the expectations of minimizing pain (consciousness is lost prior to concentrations associated with the experience of pain) and distress (fewer behavioral changes in these rats as compared with those in the lower flow treatment groups).[2] Other volume displacement rates of carbon dioxide, such as 50%, should be evaluated in the future.

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