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Wellbeing of Mice Euthanized with Carbon Dioxide in Their Home Cage as Compared with an Induction Chamber

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The AVMA Guidelines on Euthanasia state that, to decrease potential distress of animals, the home cage should be used for the euthanasia of mice. The current study evaluated this recommendation by comparing behavioral and physiologic changes in ICR and SJL mice that were euthanized by using a 30% volume per minute displacement rate of 100% CO_2 in either their home cage or an induction chamber. Blood samples were collected to assess blood glucose, serum corticosterone, and serum noradrenaline as markers of physiologic wellbeing. Behavioral assessment was performed (with emphasis on behaviors including rearing, jumping, sniffing at the gas inlet, and grooming) from the introduction of gas to the estimated time to loss of consciousness (i.e., the time period when the animal would be expected to experience pain or distress). Despite significant differences between mouse strains, no significant differences were detected in the physiologic or behavioral parameters assessed when comparing the home cage with the induction chamber. This finding suggests that— from the perspective of a mouse—either the home cage or an induction chamber can be used for induction of anesthesia with CO_2 during the euthanasia procedure.

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General recommendations for the process of euthanizing rodents used in research include the maintenance of stable groups, reduction of transport prior to the euthanasia process, and selection of euthanasia methods that minimize pain and distress.[7,16] An additional recommendation is that the wellbeing of rodents is improved when the rodent is euthanized within its home cage.[7,16] This recommendation is based on a large body of literature that demonstrates transient increases in blood pressure, heart rate, and corticosterone after a cage change.[8,17,23,24,26] Few studies have evaluated the effect of using an induction chamber or home cage during anesthesia or euthanasia with anesthetic overdose.[11,22]

This question is critical because of an apparent professional dichotomy in best practices for the improvement of animal wellbeing in euthanasia and anesthesia. Although the best practice recommendation for euthanasia is to use the home cage during induction with an inhaled anesthetic (generally CO_2 or halogenated anesthetics),[7,16] common inhalant anesthesia recommendations support the use of an induction chamber. Both CO_2 (especially when combined with O_2)[9] and halogenated anesthetics are anesthetics, which can create a surgical level of anesthesia and, if used as an overdose, euthanasia,[28] therefore the wellbeing of the animal during induction is the same in both situations. In other words, because the sequence of experiences for anesthesia and euthanasia are initially similar, there will be pain or distress associated with the induction of anesthesia for any anesthetic used (such as CO_2[10] or isoflurane[4] or any injectable combinations of anesthetic agents),[13] regardless of the intended outcome of anesthesia or euthanasia. If the home cage does improve animal wellbeing during euthanasia, then it follows that the home cage will also improve animal wellbeing during the induction of anesthesia. However, in both cases, practical reasons may underlie the use of an induction chamber for anesthesia. For example, there may be a need to anesthetize only select members of a cage cohort, or it may be beneficial to use a smaller induction chamber that can be sealed to minimize personnel exposure to the gases in use.

To determine whether induction of anesthesia in the home cage promotes animal wellbeing during the euthanasia process as compared with the use of an induction chamber, I compared behavioral and physiologic changes of individually housed ICR and SJL mice that were anesthetized prior to euthanasia using a 30% volume displacement per minute rate of 100% CO_2 in either their home cage or an induction chamber. The physiologic responses assessed included rapid markers of activation of the HPA axis (e.g., noradrenaline) and more delayed markers of activation of this axis (e.g., corticosterone and blood glucose).[4,27] The behavioral parameters assessed included the frequency in which each mouse engaged in behaviors suggestive of anxiety (for example, jumping, digging) or exploration (for example, rearing, sniffing).[4,27] I hypothesized that mice anesthetized in their home cage would demonstrate fewer indices of distress than those anesthetized in the induction chamber.

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 animal care and use program is AAALAC-accredited and is compliant with all federal regulations overseeing the use of animals in research in the United States.

Animals. The study population was composed of ICR mice (an outbred stock; 12 female [age: range, 103 to 295 d; mean, 184 d];

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12 male [age: range, 73 to 240 d; mean, 143 d]) and SJL mice (an inbred strain; 12 female [age: range, 135 to 236 d; mean, 209 d]; 12 male [age: range, 120 to 236 d; mean, 210 d]). These strains were selected because they are commonly used as research models.

The mice were first-generation offspring from a study evaluating the effect of cage color (clear, red, or opaque) on reproductive success. The parents (HSD:ICR [CD1] and SJL/jCrHsd) had been obtained from Envigo (Indianapolis, IN), and the mice used in this study were surplus offspring from the parent study. These mice were reared in the cage color-treatment environment until weaning at 21 d, with no other experimental manipulation performed. All animals had lived in standard mouse shoebox caging on an IVC rack system (Alt Design, Siloam Springs, AR) for a minimum of 45 d after weaning, prior to use in the present experiment, which was conducted under the assumption that the preweaning rearing environments would not confound the results of this study. Mice were individually housed in the IVC rack system with corncob bedding (Bed-O-Cobs, The Andersons, Maumee, OH) and nesting materials (Enviro-Dri, Shepherd Specialty Papers, Milford, NJ). Food (Teklad 2018SX, Envigo) was provided free choice. Reverse-osmosis–treated water was provided without restriction through a water pouch (HydroPac, Lab Products, Seaford, DE). Cages were changed at least weekly in a laminar flow workstation (Nuaire, Plymouth, MN) and autoclaved prior to reuse. Hands and implements were disinfected by using MB10 (Quip Labs, Wilmington, DE) between cages. The macroenvironment included a 12:12-h light:dark cycle (lights on, 0700), temperature of 72 ± 1 °F (22 ± 0.5 °C), and 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 the following pathogens: Sendai virus, pneumonia virus of mice, mouse coronavirus, parvoviruses (mouse parvovirus types 1 and 2 and mouse minute virus), murine rotavirus (EDIM), mouse adenovirus 1 and 2, GDVII, reovirus, lymphocytic choriomeningitis virus, *Clostridium piliforme*, *Mycoplasma pulmonis*, pinworms (*Apsicularis tetraptera*, *Syphacia* spp.), and fur mites (*Radfordia ensifer*, *Ornithonyssus bacoti*).

Experimental design. All mice were individually housed for at least 10 d prior to euthanasia to remove the potential confounding effect of social buffering on the stress responses of the mice.[1,2] Each mouse was randomly assigned to either the induction chamber or home-cage treatment group via draw. To perform the random assignment, tiles that stated home cage or induction chamber were placed in separate boxes for males and females. After a cage was selected, a tile was drawn from the box for the appropriate sex, and the indicated treatment was used.

Euthanasia process. To obtain an accurate measurement of fasting blood glucose values, mice were fasted for approximately 6 to 12 h prior to euthanasia. Feed was removed at about 0700, to coincide with the initiation of the light cycle. In all cases, the induction-chamber mouse was removed from the home cage and placed in an empty mouse cage of the same size as the home cage, but without bedding, to ensure that the volume and cage configurations were the same between the 2 treatment groups. For mice that remained in the home cage, enrichment remained, but the filter top and wire top were removed from the cage. A modified lid was placed over each cage; this lid had a port at the center for gas delivery from the top of the cage. The cage had a calculated volume of 7 L. CO_2 (100%) was delivered from a compressed air cylinder (PraxAir, Indianapolis, IN) to the cage at a volume displacement rate of 30% per minute (approximately 2 L/min) by using a flow meter. This volume displacement rate was selected to balance the minimizing of potential pain or distress while creating conditions for a sufficiently long induction phase (projected to be 45 to 60 s,)[18] for neuroendocrine and behavioral changes to occur and be measured. Mice were observed, and their behavior was scored from initiation of gas treatment until 'head bob,' defined as the point when the animal stopped moving and its head dropped down, even if movement continued after this point. This behavioral parameter allowed approximation of the loss of righting reflex (within approximately 5 to 10 s),[18] which occurs at the end of stage 1 of anesthesia and is correlated with the loss of consciousness. Spontaneous movement can continue during stage 2 of anesthesia (from loss of righting reflex to lateral recumbency); however, the animal is unconscious during that stage.[28] After a surgical plane of anesthesia (lateral recumbency, observation of regular and even respiration, confirmed by loss of response to pedal reflex) was achieved (approximately 30 s after approximated loss of consciousness),[28] the mice were removed from the cage, and terminal blood collection was performed by cardiac exsanguination, followed by cervical dislocation to confirm euthanasia. Blood glucose was measured immediately by using a glucometer, and the remainder of the blood sample was placed in a serum separator tube, centrifuged, and then stored at –80 °C until further assessment.

Behavioral assessment. All mice were digitally recorded from the time of placement in the cage until removal from the cage. A single observer performed all behavioral scoring. Blinding was not possible because it was clear which animals were in a home cage compared with an induction chamber. Mice were observed from the initiation of the CO_2 gas delivery until head bob. The number of times that the mouse reared (defined as raising both front feet in the air and balancing on the hindlimbs), jumped (defined as all 4 limbs leaving the floor at the same time), sniffed (purposeful movement toward the gas inlet), and engaged in digging behaviors during this period were recorded. The total number of rears, jumps, sniffs, or digs was divided by the number of minutes from induction to head bob to obtain the number of rears per minute, jumps per minute, sniffs per minute, and digs per minute (that is, the frequencies of these behaviors). These behaviors were extrapolated from published mouse ethograms[5,25] as being reflective of anxiety, agitation, or escape behaviors, thus suggesting distress experienced by the mice during the euthanasia process. All data were collected between 1200 and 1700 to minimize the potential for circadian patterns to confound data analysis.

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 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 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 (BA E-5200; LDN Immunoassays and Services, Nordhorn, Germany). Serum samples were diluted 1:20 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). Concentrations were calculated by using the 4-parameter logistic curve assay on MyAssays.com.

Statistical analysis. Each animal represented the unit of analysis in this study. For the statistical analysis, a general linearized

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Table 1. Data from both strains of mice

Description	ICR	SJL	*P*
Time to head bob (s)	50 ± 1	42 ± 1	<0.0001
Blood glucose (mg/dL)	111 ± 8	203 ± 8	<0.0001
Serum corticosterone (ng/mL)	46 ± 8	59 ± 8	0.2438
Noradernaline (pg/mL)	584 ± 107	1,456 ± 107	<0.0001
No. of jumps (per minute)	0.6 ± 0.2	0.4 ± 0.2	0.4790
No. of rears (per minute)	5.3 ± 0.6	1.6 ± 0.6	0.0002
No. of digs (per minute)	0	0	not applicable
No. of sniffs (per minute)	1.8 ± 0.3	0.1 ± 0.3	<0.0001
No. of grooming events (per minute)	0.0 ± 0.2	0.4 ± 0.2	0.0636

All data are presented as mean ± SEM and compared between strains. Significance was set at $P < 0.05$.

model was used to determine whether there were interactions between stock or strain, sex, and treatment. After this analysis, the raw data were assessed for normal distribution, followed by a comparison of the means between treatments (home cage compared with induction chamber by using 2-way ANOVA). Only differences with a probability less than 0.05 were considered to be significant. All statistical analyses were conducted by using JMP 8.0 (SAS Institute, Cary, NC). A sample size of approximately 12 animals per treatment group was estimated for time to loss of consciousness (α, 0.05; 80% power; mean difference, 20 s; 1 SD 17 s) prior to IACUC review of the study.

Results

No animals or samples were excluded from the analysis. In the general linearized model analysis, all data were compared by identifying significant differences between treatments (home cage compared with euthanasia chambers) and strain or stock and by identifying interactions between strain or stock, sex, and treatment (Table 1). Differences between strain or stock were identified in the time to head bob ($P < 0.0001$), blood glucose ($P < 0.0001$), noradrenaline concentration ($P < 0.0001$), frequency of rears ($P = 0.0002$), and frequency of sniffs ($P < 0.0001$). The frequency of jumps differed ($P = 0.0425$) between treatments. An interaction of strain or stock with treatment was identified in the frequency of grooming events ($P = 0.0496$). No significant differences or interactions were identified in corticosterone concentration ($P = 0.2773$) or the frequency of digging events ($P = 0.4296$). Given these results, the data set for each strain or stock was analyzed separately.

ICR mice. All data from ICR mice are presented in Table 2. There were no significant differences between home cage compared with the induction chamber in any of the parameters. Males were more likely to sniff at the gas inlet as compared with females, but there was no interaction of sex and treatment in these behaviors ($P = 0.1377$).

SJL mice. All data from SJL mice are presented in Table 3. There were no significant differences between home cage compared with the induction chamber in any of the parameters. Males were more likely to rear as compared with females, and females took a significantly longer time to reach the head bob, but there was no interaction of sex and treatment in these behaviors ($P = 0.0676$ and $P = 0.9294$, respectively).

Discussion

Best practices for the euthanasia of mice suggest that the home cage should be used instead of an induction chamber.[7,16] The results of this study did not show an inherent advantage to animal wellbeing by following this recommendation in individually housed mice. No significant differences were detected between the induction chamber compared with the home cage in the physiologic and behavioral indices measured for either stock or strain. The assessment of corticosterone and blood glucose could be influenced by the experimental design of this study. Mice were individually housed for at least 10 d prior to the initiation of the study and fasted during the light cycle, which may have altered these parameters. But, in performing welfare assessments of animals during euthanasia, these parameters may not be sensitive or rapid enough to reflect the acute stress experience of the animal.[4,27] In contrast, catecholamines are much more sensitive to acute stressors associated with activation of the HPA axis and are less sensitive to other external forces, such as hunger or social isolation.[4,27] No significant differences were detected in the noradrenaline and the proportional expression of behaviors by these mice, suggesting that the home cage and an induction chamber are equivalent when used to induce anesthesia prior to euthanasia with CO_2.

A possible interpretation of these data is that the introduction of gas during the euthanasia process is stressful for the animal. Conducting the procedure in the home cage does not decrease the potential stress.[4,12,27] This result was expected because every method of euthanasia involves some possibility of pain or distress, which is why guidance documents recommend that operators take reasonable steps to minimize pain and distress.[4,7,16] Although the emphasis on pain or distress during euthanasia is usually focused on inhalant anesthetics, such as CO_2 and isoflurane,[4,10,27] injectable anesthetics are not free from the potential for pain or distress. Injectable anesthetics require restraint, which can be distressing, and injection, which can cause momentary pain, and these compounds may or may not cause pain to the animal.[13] Evaluation of other compounds, agents, or practices, such as anesthetic induction in stable groups, may provide significant differences that can guide recommendations to improve wellbeing of mice during the euthanasia process.

Evaluating questions about wellbeing can help to mitigate compassion fatigue in personnel charged with the euthanasia of these animals.[14,19,20] If best practices recommend that the home cage can be used, an internal conflict for the human is inherently created if a single mouse must be removed from a cage because it is the only affected animal that is requiring euthanasia. In that circumstance, personnel working with mice are being required to act contrary to so-called best practices for the greater good of the other animals in the cage. Likewise, a strong argument can be made regarding the negative welfare considerations that would be inherent in anesthetizing an entire cage of animals to

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Table 2. Data from ICR mice

Description	Induction chamber	Home cage	*P*
Time to head bob (s)	49 ± 2	52 ± 2	0.4474
Blood glucose (mg/dL)	114 ± 7	108 ± 7	0.5251
Serum corticosterone (ng/mL)	37 ± 13	54 ± 12	0.3641
Noradernaline (pg/mL)	722 ± 138	456 ± 138	0.1715
No. of jumps (per minute)	1.0 ± 0.3	0.1 ± 0.3	0.0550
No. of rears (per minute)	5.2 ± 1.1	5.4 ± 1.1	0.9248
No. of digs (per minute)	0.0 ± 0.1	0.1 ± 0.1	0.3282
No. of sniffs (per minute)	2.2 ± 0.5	1.5 ± 0.5	0.3448[a]
No. of grooming events (per minute)	0	0	not applicable

All data are presented as mean ± SEM and compared between the treatments of home cage and induction chamber. Significance was set at $P < 0.05$.
[a]Sex-associated difference noted ($P = 0.0113$), with males more likely to engage in this behavior (2.7 ± 0.43 sniffs/min) than were females (1.0 ± 0.4 sniffs/min).

Table 3. Data from SJL mice

Description	Induction chamber	Home cage	*P*
Time to head bob (s)	40 ± 1	42 ± 1	0.0583[a]
Blood glucose (mg/dL)	203 ± 14	203 ± 14	0.9702
Serum corticosterone (ng/mL)	49 ± 10	69 ± 10	0.1620
Noradernaline (pg/mL)	1,423 ± 164	1,489 ± 164	0.7809
No. of jumps (per minute)	0.5 ± 0.2	0.2 ± 0.2	0.4370
No. of rears (per minute)	1.3 ± 0.7	1.9 ± 0.7	0.5350[b]
No. of digs (per minute)	0	0	not applicable
No. of sniffs (per minute)	0.0	0.1 ± 0.1	0.3282
No. of grooming events (per minute)	0.9 ± 0.3	0.0 ± 0.3	0.0553

All data are presented as mean ± SEM and are compared between the treatments of home cage and induction chamber. Significance was set at $P < 0.05$.
[a]Sex-associated difference noted ($P = 0.0115$), with females taking significantly longer (43 ± 1 s) than males (40 ± 1 s)
[b]Sex-associated difference noted ($P = 0.0074$), with males more likely to engage in this behavior (2.7 ± 0.5 rears/min) than females (0.5 ± 0.5 rears/ min)

perform serial surgeries. All mice after the first animal would experience prolonged anesthesia, which can have significant effects on welfare and health. [3,6,15,21,29] Although the use of an induction chamber smaller than the cage may benefit personnel safety, one would need to determine whether the smaller, novel cage increases distress for the rodent. In that situation, a preferable strategy may be to develop appropriate scavenging practices to promote the wellbeing of the animal yet preserve protection of the personnel. The current study suggests that the use of the home cage provides no significant benefit over the use of the induction chamber with regard to decreasing the potential pain or distress experienced by the mouse. This finding allows the personnel working with these animals to select the method that provides the best clinical course or experimental manipulation for all animals within a cage, without internal conflicts regarding the implementation of best practices.

In conclusion, this study found no significant improvement in the wellbeing of mice when the home cage is used instead of an induction chamber during the induction of anesthesia for a procedure or euthanasia using CO_2.

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