

# Sedation or Inhalant Anesthesia before Euthanasia with CO<sub>2</sub> Does Not Reduce Behavioral or Physiologic Signs of Pain and Stress in Mice

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CO<sub>2</sub> administration is a common euthanasia method for research mice, yet questions remain regarding whether CO<sub>2</sub> euthanasia is associated with pain and stress. Here we assessed whether premedication with acepromazine, midazolam, or anesthetic induction with isoflurane altered behavioral and physiologic parameters that may reflect pain or stress during CO<sub>2</sub> euthanasia. Mice were assigned to 1 of 6 euthanasia groups: CO<sub>2</sub> only at a flow rate of 1.2 L/min which displaces 20% of the cage volume per minute (V/min; control group); premedication with acepromazine (5 mg/kg), midazolam (5 mg/kg), or saline followed by 20% V/min CO<sub>2</sub>; induction with 5% isoflurane followed by greater than 100% V/min CO<sub>2</sub> (>6L/min); and 100% V/min CO<sub>2</sub> only (6 L/min). Measures included ultrasonic sound recordings, behavioral analysis of video recordings, plasma ACTH and corticosterone levels immediately after euthanasia, and quantification of *c-fos* from brain tissue. Compared with 20% V/min CO<sub>2</sub> alone, premedication with acepromazine or midazolam did not significantly alter behavior but did induce significantly higher *c-fos* expression in the brain. Furthermore, the use of isoflurane induction prior to CO<sub>2</sub> euthanasia significantly increased both behavioral and neuromolecular signs of stress. The data indicate that compared with other modalities, 20% V/min CO<sub>2</sub> alone resulted in the least evidence of stress in mice and therefore was the most humane euthanasia method identified in the current study.

**Abbreviations:** CCAC, Canadian Council on Animal Care in Science; GABA,  $\gamma$ -aminobutyric acid.

CO<sub>2</sub> administration is a frequently used form of euthanasia in laboratory mice due to its ease of use, availability, low expense, and high level of personnel safety.<sup>3,7</sup> Furthermore, because this method does not require handling or manipulation of mice prior to euthanasia, it avoids stress associated with these activities. However, questions remain regarding the pain and distress that mice may experience during CO<sub>2</sub> euthanasia.<sup>7,13,23</sup>

CO<sub>2</sub> stimulates receptors in the nasal mucosa.<sup>31</sup> Pain associated with this stimulation is concentration-dependent, and studies in humans indicate that a single full breath of CO<sub>2</sub> at concentrations ranging from 50% to 100% can be painful.<sup>9</sup> Human subjects reported that exposure to 50% CO<sub>2</sub> induced tingling sensations and was associated with an unpleasant odor or taste; high CO<sub>2</sub> concentrations (100%) induced pain that was described as piercing or stabbing.<sup>9</sup> Rodents, like humans, possess these receptors in the nasal mucosa.<sup>31</sup> Rodent studies assessing the use of CO<sub>2</sub> as a euthanizing agent are unclear, and some suggest that CO<sub>2</sub> induces pain and distress, whereas others indicate there are no signs of pain or distress.<sup>7,9,13</sup>

Current euthanasia guidelines from both the American Veterinary Medical Association and the American College of Laboratory Animal Medicine recommend that CO<sub>2</sub> be administered in a gradual fill method so that the influx of CO<sub>2</sub> fills at least 20% of the euthanasia chamber volume per minute.<sup>1,3</sup> Concerns over the use of CO<sub>2</sub> have led the Canadian Council for Animal Care (CCAC) to recommend the use of an inhalant anesthetic followed by CO<sub>2</sub> for euthanasia of rodents.<sup>6</sup> In addition,

the Morris Animal Foundation does not consider the use of CO<sub>2</sub> alone to be an appropriate method of euthanasia and will not fund projects that propose to use CO<sub>2</sub> for euthanasia without premedication.<sup>29</sup> In some larger species, premedication prior to euthanasia is standard practice; however, the potential benefit of this practice has not been analyzed prospectively in mice. Therefore, we tested the hypothesis that premedication with a sedative or inhalant anesthesia alleviates pain and stress associated with CO<sub>2</sub> euthanasia in mice. Specifically, we evaluated 3 types of premedication to determine whether their use before euthanasia affected behavioral, physiologic, or neuromolecular indicators of pain and stress during CO<sub>2</sub> euthanasia. In addition, we evaluated mouse euthanasia due to CO<sub>2</sub> alone at a flow rate of 20% chamber volume displacement per minute (V/min) and at the higher rate of 100% V/min. Behavioral measures analyzed in the current study included ultrasonic sound recordings to assess any change in vocalization which might be associated with distress calls<sup>14,40</sup> and posthoc evaluation of videorecordings by a blinded observer trained in assessing pain and stress in rodents. Physiologic parameters measured in this study included plasma ACTH and corticosterone levels. We also used quantitative PCR to measure the *c-fos* transcript in the brain. The *c-fos* gene is an immediate early gene that is rapidly and transiently expressed in neurons in response to stimulation and has been used in numerous studies as an indicator of pain and stress in rodents.<sup>15,36</sup>

## Materials and Methods

**Animals.** Female CD1 mice ( $n = 10$  per group; age, 8 to 11 wk) were purchased from Charles River Laboratories (Wilmington, MA) and acclimated for at least 1 wk prior to experimental procedures. All mice were screened by the vendor and were

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deemed to be SPF for all commonly tested bacterial and viral pathogens and parasites. All animal procedures were approved by Cornell's IACUC.

**Housing.** Mice were housed in an AAALAC-accredited facility in groups of 2 to 3 in individually ventilated polycarbonate cages (29.2 cm × 16.5 cm × 12.7 cm) with autoclaved corncob bedding (7097A; Harlan Teklad, Frederick, MD). Cages were maintained on a rack system (Micro-FLO/Micro-VENT Environmental Rack System, Allentown Caging Equipment Company, Allentown, NJ). The mice were housed in a room with controlled temperature (20 to 22 °C), humidity (30% to 70%), and photoperiod (14:10-h light:dark cycle). All mice had free access to food (irradiated maintenance mouse diet 7912, Harlan Teklad) and acidified reverse-osmosis water through an automated watering system (Edstrom, Waterford, WI). All cages were provided with sterile nesting pads for enrichment.

**Experimental design.** Cages of mice were allocated randomly into 6 euthanasia groups; mice were weighed the day prior to euthanasia to ensure correct dosing. Group 1 (control) mice were euthanized by CO<sub>2</sub> only; the mice in group 2 received acepromazine (5 mg/kg IP, 0.20 mL in sterile saline) 10 min prior to euthanasia. Mice in group 3 received midazolam (5 mg/kg IP, 0.20 mL in sterile saline) 10 min prior to euthanasia. Group 4 mice received 0.20 mL saline IP 10 min prior to euthanasia (negative control group for handling and intraperitoneal injection). Mice in these groups were all euthanized with CO<sub>2</sub> at a flow rate of 20% chamber air displacement per minute (20% V/min; 1.2 L/min). Mice in the group 5 were anesthetized with 5% isoflurane at a flow rate of 1.2 L/min O<sub>2</sub> until all mice in the cage were unconscious and then immediately euthanized with CO<sub>2</sub> at a flow rate exceeding 100% V/min (more than 6 L/min; consistent with the CCAC recommendation<sup>6</sup>). Mice in group 6 were euthanized by using CO<sub>2</sub> at flow rate of 100% V/min (6 L/min). Flowmeter settings were calculated by using the following formula: (% air displacement per min) × (volume of cage in L; without filter top).

The cages were moved one at a time from the housing room to a procedure room across the hall. The mice were maintained in their social groups of 2 or 3 mice per cage. Mice that received intraperitoneal injections were injected in the procedure room and left undisturbed for 10 min prior to CO<sub>2</sub> exposure. CO<sub>2</sub> was administered at a set flow rate from a compressed tank through a tube that was inserted into the home cage of the mice through the lEXIT port (Figure 1).

**Behavioral measures.** Digital videorecordings of each cage were collected for approximately 2 min immediately after intraperitoneal injection, if applicable (induction), 2 min immediately prior to starting either isoflurane or CO<sub>2</sub> (preeuthanasia), and for the duration of isoflurane and CO<sub>2</sub> exposure (euthanasia). Videorecordings were collected by using a digital videocamera (Sony, San Jose, CA) directed at the long axis of the cage. Each recording was assigned a random number and blindly scored for increased respiratory effort (dyspnea); abnormal activity such as flipping and spinning or an abnormal alteration in activity level (a marker of agitation); and presence of any behaviors indicative of pain (such as pawing at the face) on a scale of 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe) by a laboratory animal veterinarian trained in assessing rodent behavior but who was unfamiliar with the study design and group identity. Times to unconsciousness and death were recorded for each mouse, starting from the onset of gas exposure (isoflurane or CO<sub>2</sub>). Behavioral scores were assessed only until unconsciousness was reached (regardless of whether unconsciousness was due to anesthesia or CO<sub>2</sub>). Unconsciousness was defined as

cessation of voluntary movement, and death was defined as complete cessation of breathing.

**Ultrasonic sound recordings.** Sound recordings in the range of 0 to 120 kHz were collected for 2 min prior to (preeuthanasia) and during (euthanasia) gas administration by using an ultrasonic microphone (USG 116-200, UltraSoundGate Kit, Avisoft, Berlin, Germany) to capture vocal emissions made by the mice.<sup>14,40</sup> Sounds were recorded until mice lost consciousness (regardless of whether unconsciousness was due to anesthesia or CO<sub>2</sub>). The microphone was directed into the cage through a hole in a platform that was placed on top of the cage after removing the bonnet and wire rack (Figure 1). An averaged power spectrogram of each cage recording was created by using software provided by the manufacturer (SASLab Pro, version 4.3, Avisoft). Sonograms from 2 cages of each group were averaged and plotted on a graph comparing preeuthanasia and euthanasia values.

**Physiologic measures.** Immediately after euthanasia, mice were exsanguinated by cardiocentesis, and blood was collected into EDTA tubes. Euthanasia of all groups was coordinated to occur at the same time (0900 to 1100) in a random order to ensure no fluctuations in circadian rhythm and that order of euthanasia was not a factor in the parameters analyzed. Plasma was isolated and stored at -20 °C until assayed for ACTH and corticosterone levels. A chemiluminescent ELISA (Calbiotech, Spring Valley, CA) was used to measure plasma ACTH levels, and a competitive ELISA (VWR, Radnor, PA) was used to measure plasma corticosterone levels.

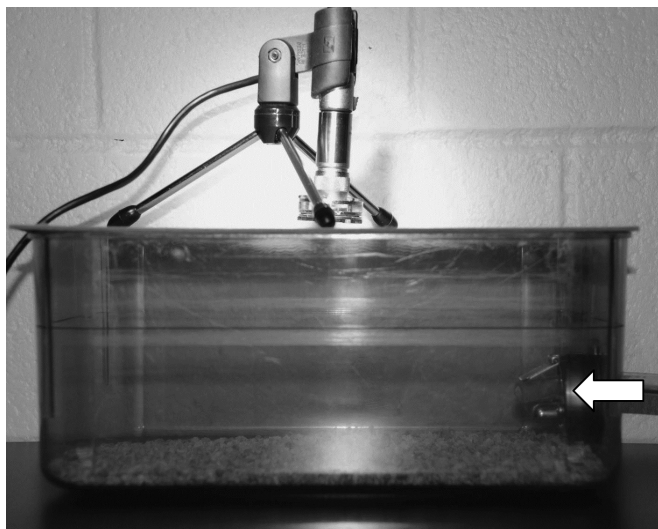
**Quantification of *c-fos* mRNA in brain.** A straight razor blade was used to cut a 2-mm coronal section of brain tissue at the level of the hypothalamus, which was processed for *c-fos* mRNA quantification. This section was chosen because differences due to distress in mice typically occur in this region.<sup>27</sup> Total RNA was isolated from the samples by using the EZNA Tissue RNA kit (Omega Bio-Tek, Norcross, GA), and 1 µg of RNA was converted to cDNA (Quanta Biosciences, Gaithersburg, MD). Quantitative PCR was performed for *c-fos* and *GAPDH* mRNA expression (SYBR/Lo Rox, Quanta Biosciences) and run on an ABI Fast 7500 machine. Expression of *c-fos* was normalized to that of *GAPDH* and analyzed by using the  $\Delta\Delta C_t$  method as described previously.<sup>28</sup>

**Statistics.** All statistical analyses were conducted by using GraphPad Prism (version 5.04, GraphPad Software, San Diego, CA). All parametric data sets, including time measurements, ACTH, corticosterone, and *c-fos* mRNA expression were analyzed by using one-way ANOVA and Tukey posttest. Behavior data were analyzed by using a nonparametric Kruskal-Wallis test followed by Dunn posttest. Differences were considered significant at a *P* value of less than 0.05.

## Results

**Time measurements.** Mice euthanized with 100% V/min CO<sub>2</sub> had the most rapid loss of consciousness (39.6 ± 1.9 s), which was significantly (*P* < 0.05) faster than that of the 20% V/min CO<sub>2</sub> (137.2 ± 10.5 s), saline (112.9 ± 12.7 s), and isoflurane (122.9 ± 16.0 s) groups (Figure 2 A). Mice that received 100% V/min CO<sub>2</sub> also reached death (79.9 ± 4.2 s) significantly (*P* < 0.05) faster than did all other groups (range, 222.3 ± 22.5 s to 385.2 ± 46.7 s; Figure 2 B).

Premedication of mice with acepromazine or midazolam significantly (*P* < 0.05) decreased the time to unconsciousness (74.5 ± 8.3 s and 71.6 ± 6.6 s, respectively) compared with 20% V/min CO<sub>2</sub> alone (137.2 ± 10.5 s; Figure 2 A). However, premedication with midazolam significantly (*P* < 0.05) lengthened the



**Figure 1.** The euthanasia set-up. Mice remained in the home cage, CO<sub>2</sub> was administered through the lixit port (arrow), and an ultrasonic microphone was placed over the cage.

time to death ( $385.2 \pm 46.7$  s) compared with 20% V/min CO<sub>2</sub> alone ( $234.6 \pm 18.4$  s; Figure 2 B). Induction with isoflurane did not reduce time to unconsciousness or death but instead produced the unwanted side effect of 5 of the 10 mice recovering consciousness while the cage was being treated with greater than 100% V/min CO<sub>2</sub>.

**Behavior analysis.** Blinded evaluations of behavior were conducted for the induction, pre euthanasia, and euthanasia periods. Groups of mice were compared according to both treatment and time point (induction, pre euthanasia, euthanasia). Behavior scores at induction were compared among the acepromazine, midazolam, and saline groups. Induction with midazolam resulted in significantly ( $P < 0.05$ ) higher agitation scores compared with induction with acepromazine ( $1.4 \pm 0.3$  and  $0.4 \pm 0.2$ , respectively) but did not differ significantly from the saline control ( $0.9 \pm 0.1$ ; Figure 3 A). Midazolam induction resulted in significantly ( $P < 0.05$ ) higher agitation scores compared with pre euthanasia and euthanasia time points ( $0.3 \pm 0.2$  and  $0 \pm 0$ , respectively). Two mice received a pain score of 1 (mild): one midazolam-treated mouse during induction and one acepromazine-treated mouse during pre euthanasia, both due to pawing at the face (data not shown).

During euthanasia, the isoflurane group had agitation scores ( $3.0 \pm 0$ ) that were significantly ( $P < 0.05$ ) higher than those of the 20% V/min CO<sub>2</sub> ( $0.8 \pm 0.1$ ), acepromazine ( $0.4 \pm 0.2$ ), midazolam ( $0 \pm 0$ ), and saline ( $0.7 \pm 0.2$ ) groups (Figure 3 A). Because the observer stopped scoring at unconsciousness, this effect is entirely due to isoflurane and not to CO<sub>2</sub> administration after isoflurane. Euthanasia caused a significant ( $P < 0.05$ ) increase in dyspnea in all groups of mice compared with their respective pre euthanasia scores, but dyspnea scores did not differ significantly between any of the groups (Figure 3 B).

**Ultrasonic sound recordings.** Ultrasonic sound recordings were taken to capture any altered vocalizations made during euthanasia. All pre euthanasia and euthanasia sound spectrograms displayed sound peaks at 26.5 kHz that were not present in background noise control recordings for which no mice were present (black line, Figure 4 A); therefore, this peak is presumed to be mouse vocalization (arrows, Figure 4). Mice euthanized with 20% V/min CO<sub>2</sub> or premedicated with acepromazine had euthanasia spectrograms that were identical to their respective pre euthanasia spectrograms (Figure 4

A and B). Mice premedicated with midazolam had an overall lower-amplitude spectrogram during euthanasia than during pre euthanasia for all data points greater than 7 kHz, except at the 26.5-kHz vocalization peak (Figure 4 C). This finding is consistent with the marked decreased activity noted in this group but demonstrates that vocalization was unaltered. The saline-premedicated group had a higher amplitude spectrogram during euthanasia compared with pre euthanasia for all data points greater than 18 kHz, consistent with increased activity and increased vocalization (Figure 4 D). Mice induced with isoflurane had a large increase in amplitude during euthanasia, which differed from the pre euthanasia values for all data points greater than 8 kHz, including the 26.5-kHz vocalization peak (Figure 4 E), consistent with the noted agitation in this group and increased vocalization. Mice euthanized with 100% V/min CO<sub>2</sub> had some fluctuations of higher amplitude during euthanasia (9 to 11 kHz, 13 to 18.6 kHz, 23 to 27 kHz, 28 to 32 kHz, and 33 to 37 kHz), some of which were similar to fluctuations seen in background noise control recordings of 100% V/min CO<sub>2</sub> in which no mice were present (Figure 4 F); however, there was an increase at the 26.5-kHz vocalization peak similar to that noted in isoflurane-treated mice.

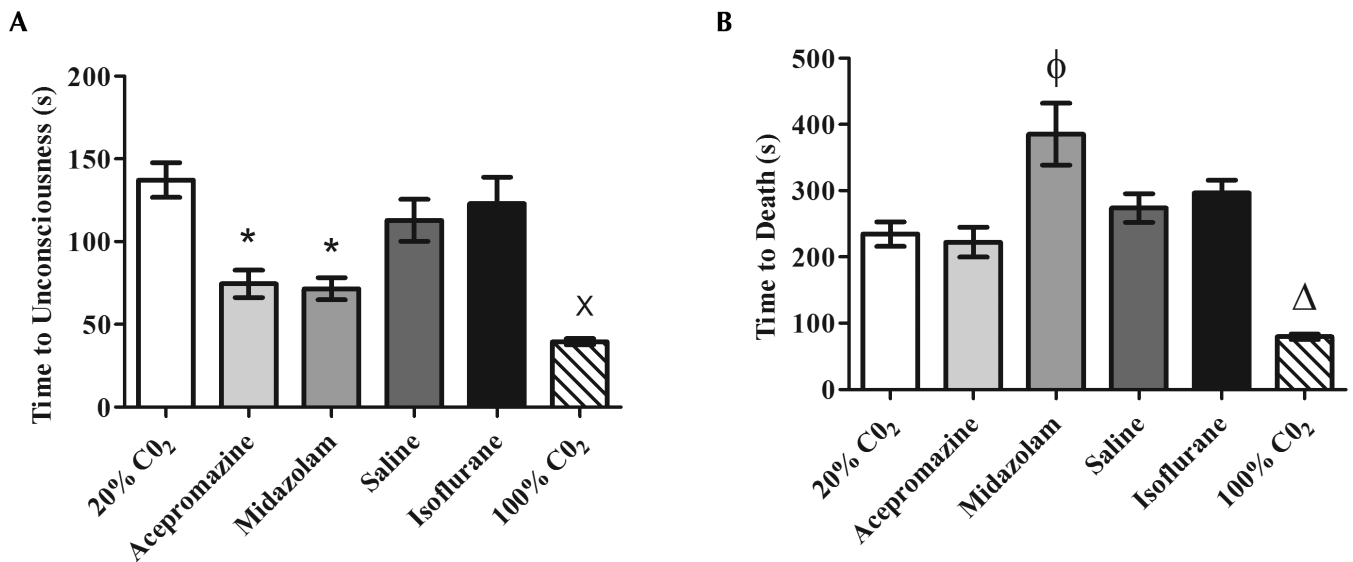
**Physiologic measures.** Plasma ACTH and corticosterone concentrations are used often to quantitatively assess stress. No significant differences in ACTH concentration were noted between any treatment groups (Figure 5 A). In contrast, midazolam-treated mice had significantly ( $P < 0.05$ ) higher corticosterone concentrations ( $147.9 \pm 37.0$  ng/mL) than did all other groups (range,  $26.7 \pm 9.0$  ng/mL to  $65.8 \pm 3.5$  ng/mL; Figure 5 B).

**Measurement of c-fos expression.** All premedication groups and the isoflurane anesthetized group displayed significantly ( $P < 0.05$ ; range, 3- to 7-fold) higher c-fos expression as compared with the 20% V/min CO<sub>2</sub> control group (Figure 6). There was no significant difference in c-fos expression between the 2 CO<sub>2</sub> flow rates.

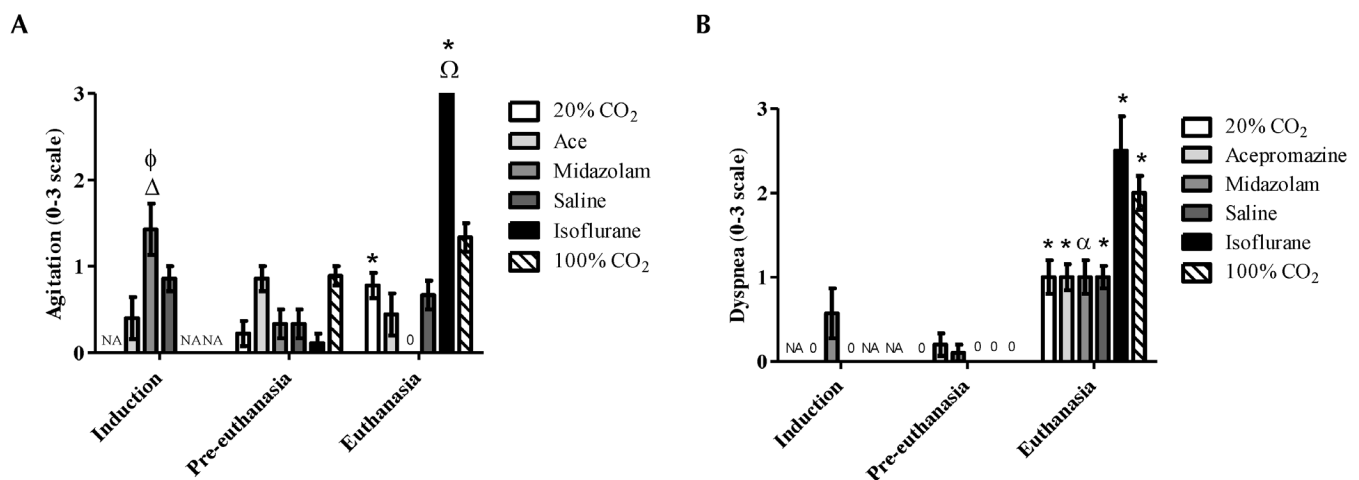
## Discussion

The hypothesis of the current study was that premedication with acepromazine, midazolam, or anesthetic induction with isoflurane prior to CO<sub>2</sub> euthanasia would alleviate pain and stress compared with CO<sub>2</sub> administration alone in mice. The data described agree with a similar study that assessed the use of sedation or anesthesia prior to CO<sub>2</sub> euthanasia of rats.<sup>13</sup> Specifically, premedication or anesthetic induction of mice prior to CO<sub>2</sub> euthanasia did not provide any advantage over the use of CO<sub>2</sub> alone, in that premedication or anesthesia did not decrease behavioral, physiologic, or neuromolecular markers of pain and stress. Furthermore, all measured parameters were minimal in mice euthanized with CO<sub>2</sub> at a flow rate of 20% chamber air displacement per minute. In addition, to our knowledge, the current study is the first that provides direct evidence that, within the flow rates examined, isoflurane induction results in higher stress measurements than does euthanasia with CO<sub>2</sub> alone.

Numerous studies, primarily using rats, have attempted to determine whether CO<sub>2</sub> is a humane and acceptable method of euthanasia; however, there has been great variation in methodology. The major questions that remain over the use of CO<sub>2</sub> for euthanasia stem from the ability of CO<sub>2</sub> to induce pain in human<sup>2,9,18,21</sup> and rodent studies.<sup>22,31,38</sup> Unfortunately, the manner in which those studies were conducted differs greatly from the way that rodents typically are euthanized with CO<sub>2</sub>, in that specific concentrations of CO<sub>2</sub> were applied directly on mucosal surfaces.<sup>2,9,18,21,22,31,38</sup> In a human study, the mean



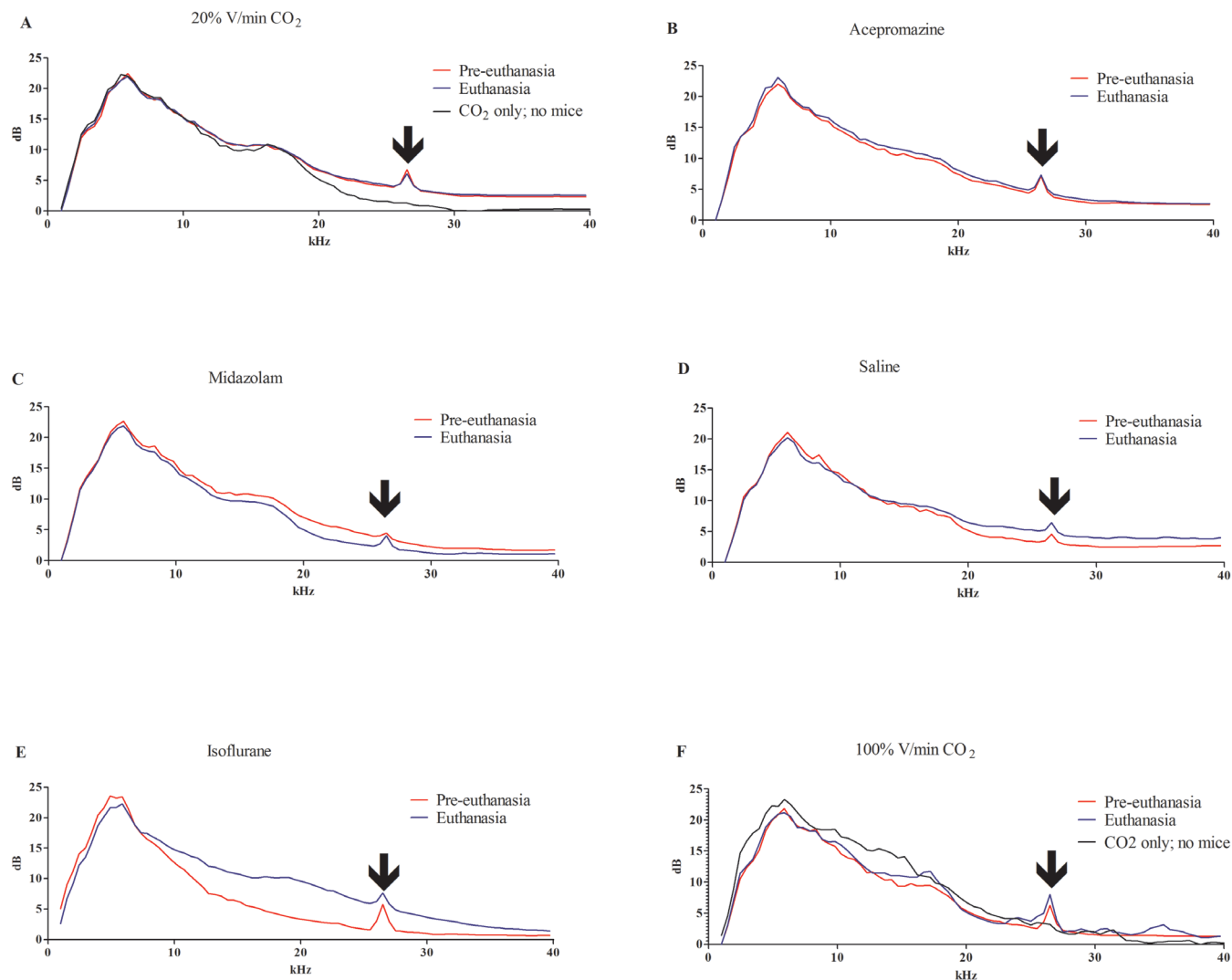
**Figure 2.** Times (mean  $\pm$  SEM) to (A) unconsciousness and (B) death were analyzed for each euthanasia group. Mice given either acepromazine or midazolam or euthanized with 100% V/min CO<sub>2</sub> experienced the most rapid time to unconsciousness, but only mice euthanized with 100% V/min CO<sub>2</sub> experienced a significantly more rapid death compared with other groups. Despite a shorter time to unconsciousness, mice euthanized with midazolam experienced a significantly increased time to death compared with that of mice that received 20% V/min CO<sub>2</sub>. (A) \*, Significant ( $P < 0.05$ ) decrease in time compared with values for 20% V/min CO<sub>2</sub> and isoflurane groups; <sup>X</sup>, significant ( $P < 0.05$ ) decrease in time compared with 20% V/min CO<sub>2</sub>, saline, and isoflurane groups; (B) <sup>Φ</sup>, significant ( $P < 0.05$ ) increase in time compared with values for 20% V/min CO<sub>2</sub>, acepromazine, and saline groups; <sup>Δ</sup>, significant ( $P < 0.05$ ) decrease in time compared with values for all other groups.



**Figure 3.** A blinded observer examined videotapes taken at induction, preeuthanasia, and euthanasia and scored levels of (A) agitation (increased and altered activity) and (B) dyspnea (increased respiratory effort) on a scale of 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe). For both agitation and dyspnea, mice anesthetized with isoflurane displayed the highest mean scores at euthanasia. Regardless of treatment, all groups displayed significant ( $P < 0.05$ ) dyspnea at the time of euthanasia compared with either preeuthanasia or induction levels. NA, no applicable data. (A) <sup>Δ</sup>, Significant ( $P < 0.05$ ) increase in agitation compared with values at preeuthanasia and euthanasia time points; <sup>Φ</sup>, significant ( $P < 0.05$ ) increase in agitation compared with that of with mice induced with acepromazine; \*, significant ( $P < 0.05$ ) increase in agitation compared with that of respective preeuthanasia score; <sup>Ω</sup>, significant ( $P < 0.05$ ) increase in agitation at euthanasia compared with that of mice treated with 20% V/min CO<sub>2</sub>, acepromazine, midazolam, or saline. (B) \*, Significant ( $P < 0.05$ ) increase in dyspnea compared with that of respective preeuthanasia and induction scores (if applicable); <sup>α</sup>, indicates a significant increase in dyspnea compared with that for the respective preeuthanasia score.

concentration of CO<sub>2</sub> that was reported to induce pain was 47.1%;<sup>2</sup> similarly in a study involving rats, nociceptors in the nasal mucosa responded to concentrations of 37% to 50% CO<sub>2</sub>, although no assessment of pain was made.<sup>31</sup> Human subjects reported that increasing concentrations of CO<sub>2</sub> were progressively more noxious, with 50% V/min CO<sub>2</sub> being considered "highly unpleasant" bordering on "uncomfortable" and 100% V/min CO<sub>2</sub> being rated as "painful."<sup>9</sup> In contrast to the conduct

of those cited studies, our mice experienced a chamber filled with 100% CO<sub>2</sub> mixed with ambient air at a specified flow rate (20% chamber volume air displacement per minute). Mice generally lost consciousness approximately 2 min after initiation of CO<sub>2</sub>, indicating that they lost consciousness prior to experiencing concentrations (approximately 50%) that are reported to be painful in humans. Specifically, concentrations of CO<sub>2</sub> in the cage after 2 min of exposure would be approximately 40%.



**Figure 4.** Spectrograms of ultrasonic sound recordings for each euthanasia group were graphed. Preeuthanasia recordings were generated for 2 min prior to administration of gas. Euthanasia recordings were initiated at the beginning of gas administration until all mice in the cage were unconscious. Each figure represents the averaged values from 2 cages of mice from each group. All groups displayed a peak at 26.5 kHz (arrow), during both recordings, which was absent when mice were not in the cage. This peak represents the only peak readily attributable to vocalization. Mice (A) euthanized with 20% V/min CO<sub>2</sub> or (B) administered acepromazine had identical baseline preeuthanasia and euthanasia recordings. In contrast, (C) mice treated with midazolam had lower dB recordings in the 10- to 25-kHz range during euthanasia, consistent with heavy sedation and decreased movement, but not a decrease at the 26.5-kHz vocalization peak. Mice given (D) saline, (E) isoflurane, or (F) 100% V/min CO<sub>2</sub> had higher amplitude recordings at the vocalization peak, consistent with increased vocalization at euthanasia. (E) Isoflurane euthanasia also produced a large degree of background noise (10- to 26.5-kHz range), consistent with increased movement and agitation noted in this group.

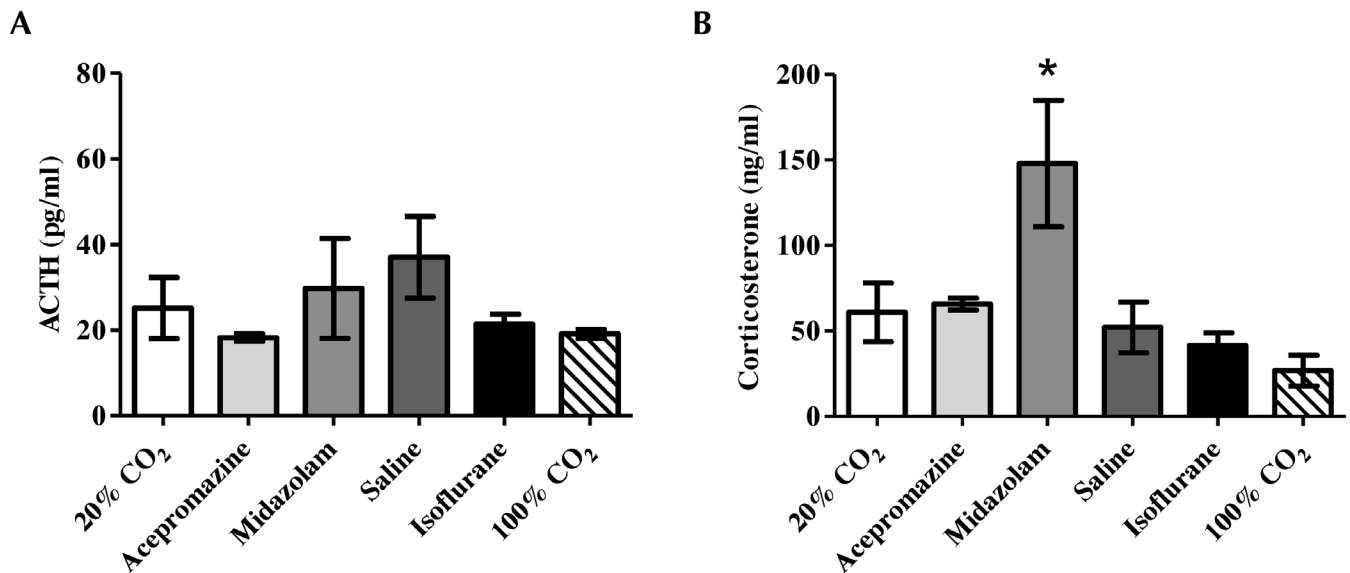
Therefore, gradually filling the cage with CO<sub>2</sub> likely achieves unconsciousness before reaching the exposure level that is reported to be painful in humans.

In CO<sub>2</sub> euthanasia studies, despite observing the same behaviors, some observers describe the animals as experiencing no pain or stress, whereas others conclude that the animals experienced considerable pain or stress.<sup>7,9</sup> To avoid basing recommendations on subjective data alone, authors also have evaluated objective measures, including heart rate and blood pressure monitoring, plasma ACTH, corticosterone, and glucose level quantifications, and ultrasonic vocalizations; the resulting data supported the notion that CO<sub>2</sub> is a humane method of euthanasia in rodents.<sup>5,13,35</sup>

We included acepromazine in the current study because it is a frequently used premedication and tranquilizer in veterinary medicine. There are species-specific differences in the reported effectiveness of acepromazine prior to euthanasia. Specifically,

acepromazine reduces agitation in dogs<sup>8</sup> but provides no beneficial effect in rats.<sup>13</sup> In the present study, the use of acepromazine reduced time to unconsciousness, but it also increased *c-fos* expression.

We evaluated midazolam because it is a fast-acting anxiolytic with strong sedative properties. Like acepromazine, midazolam significantly reduced the time to unconsciousness but significantly prolonged the time to death compared with all other euthanasia groups except isoflurane. This prolonged time to death after unconsciousness may be explained by the mechanism of action of midazolam, which increases the efficiency of  $\gamma$ -aminobutyric acid (GABA), an inhibitory neurotransmitter in the brain. GABA is neuroprotective against ischemia during hypoxia,<sup>33,41</sup> and the protective effects of GABAergic drugs, such as midazolam, against neural hypoxic damage may have prolonged the time to death in these mice. Induction with midazolam was associated with significantly more agitation



**Figure 5.** Plasma levels (mean  $\pm$  SEM) of (A) ACTH and (B) corticosterone for each euthanasia group were evaluated. The mice that received midazolam displayed a significant (\*,  $P < 0.05$ ) increase in corticosterone but not ACTH; this result was suggestive of a hypoxic ACTH-independent activation of adrenal steroidogenesis. This finding was consistent with the increased time to death following unconsciousness in this group.

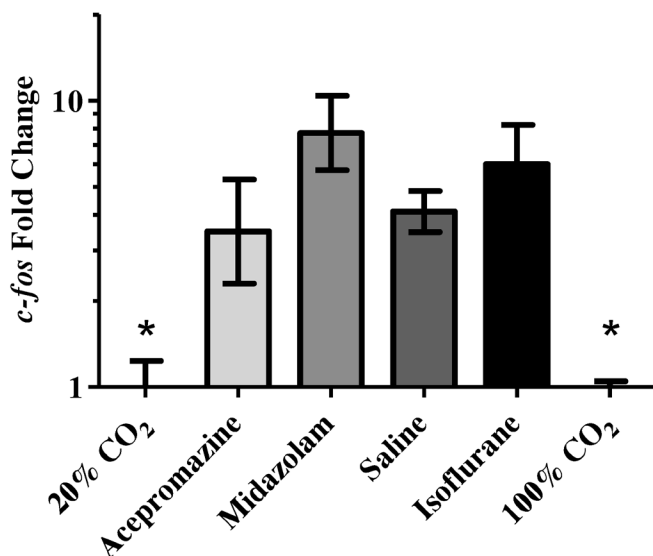
than was induction with acepromazine. This greater agitation at induction is likely due to paradoxical excitement, which occasionally occurs with sedative doses of benzodiazepines, such as midazolam. This benzodiazepine-induced excitation has been reported in numerous species including humans and may include restlessness, agitation, violent behavior, and self-mutilation.<sup>12,19,26</sup> The mechanism of action for this reaction is unknown. Mice pretreated with midazolam also had similar ACTH levels to those of the other treatment groups; however, their corticosterone levels were significantly higher. This finding suggests a hypoxic ACTH-independent activation of adrenal steroidogenesis.<sup>32</sup> The elevated corticosterone in the midazolam-treated group was consistent with the extended time period of hypoxia that the group experienced during the prolonged time to death, although direct activation by midazolam cannot be completely discounted. The *c-fos* levels in the midazolam treated group were the highest of all the groups, 7.7-fold higher than the control. Therefore, midazolam does not appear to provide any benefit as a premedication for euthanasia. These data are similar to studies in rats, in which sedation with oral acepromazine or anesthesia with pentobarbital intraperitoneally did not appreciably alter behavioral or biochemical parameters of pain in rats euthanized with CO<sub>2</sub>.<sup>13</sup>

Like midazolam and acepromazine treatment, saline-treated mice showed greater *c-fos* expression. Handling and intraperitoneal injection may be more potent stimuli in this respect than is CO<sub>2</sub> euthanasia itself. These findings imply fundamental differences between mice and standard companion animals in that standard pre-euthanasia regimes used in companion species appear deleterious in animals for which human contact and handling is itself a stressful event.

We included anesthetic induction with isoflurane prior to CO<sub>2</sub> euthanasia in the current study in acknowledgement of current recommendations in the CCAC guidelines regarding the euthanasia of animals used in science. According to the CCAC, where practical, animals should be anesthetized, preferably with an inhalant anesthetic, prior to the use of CO<sub>2</sub>.<sup>6</sup> The 2 studies that were cited in support of this recommendation used approach-avoidance tests to assess rodent aversion to gas stimuli.<sup>23,25</sup> However, one study assessed rat and mouse

aversion to prefilled chambers of CO<sub>2</sub>, CO<sub>2</sub>-argon mixture, or argon without the presence of a food reward in the test chamber,<sup>23</sup> whereas the other study compared rat aversion to gradual-fill halothane or isoflurane with a food reward in the test chamber.<sup>25</sup> In the CO<sub>2</sub>-argon study, the authors concluded that induction with CO<sub>2</sub> either alone or in combination with argon is likely to cause considerable distress in rodents before they lose consciousness.<sup>23</sup> This finding was based on significantly shorter initial withdrawal times and total dwelling times in the test chamber that was prefilled with CO<sub>2</sub> at concentrations that ranged from 25.5% to 50.8% compared with the control of room air. The halothane-isoflurane study concluded that both inhalant anesthetics were aversive to rats.<sup>25</sup> However, because some of the rats remained in the test cage long enough to become ataxic, the authors concluded that the rats likely were sedated at the time they chose to leave the test cage; therefore, continued forced exposure from the onset of aversion to unconsciousness may be more humane than forced exposure to CO<sub>2</sub>.<sup>25</sup> The 2 studies were not comparable in their design (prefill compared with gradual fill; no food reward compared with food reward), and neither study directly compared aversion of CO<sub>2</sub> with isoflurane. In addition, neither of these studies<sup>23,25</sup> involved euthanizing the rodents nor assessed any direct or indirect indicators of pain or stress.

The present study tested the hypothesis that isoflurane anesthesia prior to CO<sub>2</sub> euthanasia would decrease the level of pain or stress experienced with CO<sub>2</sub> alone. However, isoflurane induction required longer CO<sub>2</sub> exposure at greater than 100% V/min than did the use of 100% V/min CO<sub>2</sub> alone. This prolonged time to death may be due to the hypothermic effect of general anesthesia, which can be neuroprotective during hypoxia.<sup>11,33</sup> In addition to prolonged euthanasia times, isoflurane generated the highest scores for both dyspnea and agitation, which were significantly higher than pre-gas exposure scores, and produced significantly higher agitation scores than did 20% V/min CO<sub>2</sub> during euthanasia. In addition, the ultrasonic spectrogram indicated increased vocalization at the 26.5-kHz peak, potentially indicative of stress.<sup>14</sup> Furthermore, *c-fos* expression was 6-fold higher in the isoflurane-treated group compared with the 20% V/min CO<sub>2</sub> group. An additional negative effect of isoflurane



**Figure 6.** Relative mean expression (mean  $\pm$  SEM) of *c-fos* in the brains of mice compared with the 20% V/min CO<sub>2</sub> control group calculated by using the  $\Delta\Delta$ ct method. Both the 20%- and 100%-CO<sub>2</sub>-treated groups expressed significantly (\*,  $P < 0.05$ ) less *c-fos* than did all other groups.

is that some mice awoke after isoflurane induction and during the euthanasia phase. This finding highlights a weakness in the CCAC recommendation, in that, due to the rapid recovery associated with isoflurane, mice may recover once the CO<sub>2</sub> displaces the isoflurane from the chamber. Indeed, with newer gas anesthetics (for example, sevoflurane), this problem may become even greater. Previous studies that recommended the use of isoflurane prior to CO<sub>2</sub> euthanasia<sup>23,25</sup> do not account for the rapid recovery from isoflurane that occurs while the chamber is filling with CO<sub>2</sub>. Furthermore, avoidance tests do not assess the possibility of pain or stress associated with anesthetic induction. The use of an air-tight induction chamber might retard recovery, but this modification would require handling of the mice and removal from the home cage. Regardless, based on behavioral and neuromolecular markers, isoflurane does not provide any benefit. In short, in the current study, prior anesthesia with isoflurane was the worst option as an adjunct to CO<sub>2</sub> euthanasia.

A group of mice euthanized with CO<sub>2</sub> at a flow rate of 100% air displacement per minute was included in this study because the American Veterinary Medical Association currently recommends concentrations of 20% V/min CO<sub>2</sub> or greater. Times to unconsciousness and death were significantly faster for 100% V/min than the 20% V/min CO<sub>2</sub> control. However, ACTH, corticosterone, and *c-fos* levels were comparable between the 100% and 20% V/min CO<sub>2</sub> groups. Furthermore, because mice were still conscious in the cage during the timeframe when the CO<sub>2</sub> concentration exceeded 50%, they might have experienced mucosal pain from CO<sub>2</sub> at this flow rate. These data support evaluation of higher CO<sub>2</sub> flow rates in future studies to determine the recommended upper flow rate limit for euthanasia.

Data that call into question the humane nature of rodent CO<sub>2</sub> euthanasia generally fall into 2 categories: those that rely on human studies indicating that CO<sub>2</sub> is painful at high concentrations and those that rely on approach-avoidance tests. With regard to the former, our data indicate that with 20% V/min flow rates, mice reach unconsciousness prior to the build-up of potentially painful concentrations of CO<sub>2</sub>. With regard

to the latter, we suggest that approach-avoidance tests are not ideal tests to determine a humane euthanasia method. Specifically, aversion does not necessarily indicate pain or distress. Several examples support this idea. Specifically, a brief puff of air induces aversion in mice,<sup>39</sup> and urine collected from male mice infected with *Polyplax serrata* induces aversion in female mice.<sup>20</sup> In these examples, nonconditioned avoidance-aversion responses are not necessarily indicators of pain or distress, but rather evolutionary survival cues (puff of air = predator, infected mouse urine = poor mate). Indeed, nonconditioned CO<sub>2</sub> avoidance is conserved evolutionarily (presumably to avoid hypoxic death) and can be demonstrated in organisms as diverse as nematodes (*Caenorhabditis elegans*), *Drosophila*, fish, and mice, which can actually sense CO<sub>2</sub> concentrations at near atmospheric levels (that is, far below those which would ever be reported as painful or unpleasant in humans).<sup>4,16,17,24,37</sup> Further, approach-avoidance tests fail to adequately distinguish between aversion and distress, which is the inability to appropriately respond (either behaviorally or physiologically) to a stressor.<sup>30,34</sup> That is, just because an animal encounters an external stressor (for example, puff of air, mouse urine, CO<sub>2</sub>) and chooses to avoid it does not mean that distress is induced. Our data indicate that behavioral, physiologic, and neuromolecular signs of pain or distress do not occur with CO<sub>2</sub> euthanasia at the 20% V/min flow rate tested. The avoidance studies provide evidence that indeed CO<sub>2</sub> is likely aversive, but all currently available euthanasia agents probably fall into this category.

In summary, the present study demonstrates that premedication with acepromazine or midazolam did not improve CO<sub>2</sub> euthanasia. Furthermore, the use of isoflurane induction prior to CO<sub>2</sub> euthanasia significantly increased *c-fos* expression and adverse behavioral signs. A limitation of the current study is that none of the methodologies used can indicate definitively whether the mice encountered pain or stress-distress. However, taken together our results indicate that, compared with the other readily accepted treatments (isoflurane, premedication) analyzed in this study, 20% V/min CO<sub>2</sub> alone is the most humane method of euthanasia for mice.

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