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Evaluating methods of gas euthanasia for laboratory mice

I. Joanna Makowska *, Lori Vickers, Jane Mancell, Daniel M. Weary

Animal Welfare Program, Faculty of Land and Food Systems, University of British Columbia, MacMillan building, 2357 Main Mall, Vancouver, BC, V6T 1Z4, Canada

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ABSTRACT

Laboratory mice are most commonly killed with carbon dioxide (CO_2), a gas they may find aversive. The aim of this study was to use approach-avoidance testing to record mouse responses to CO_2 and to four alternatives: argon, carbon monoxide (CO), and the inhalant anaesthetics halothane and isoflurane. Mice were trained to enter the bottom cage of a two-cage apparatus for a reward of sweetened shredded coconut. Treatment gases were turned on at a pre-determined flow rate as soon as mice started eating, and we recorded the latency to leave the test cage and the gas concentration when mice left the test cage during the CO_2, argon and CO experiments. For the CO and inhalant anaesthetics experiments, we also conducted preliminary testing to determine the time to recumbency with the chosen flow rates and concentrations, and calculated the amount of time between when mice chose to leave the test cage and when they would have been recumbent. When exposed to CO_2, argon, and CO, mice never remained in the test cage long enough to lose consciousness. Mice left the test cage when CO_2 concentrations were 13.5–18.2%, when argon reduced oxygen concentration to 8.3–9.3%, and when CO concentration was approximately 2.5%. When exposed to inhalant anaesthetics, mice never remained until recumbency with halothane but two mice did so during exposure to isoflurane. Mice also remained in the test cage longer with isoflurane than halothane. We conclude that CO_2, argon, CO, and the two inhalant anaesthetics are all aversive to mice, so the search for a non-aversive agent should continue. However, aversion to the inhalant anaesthetic isoflurane appears to be weaker than aversion to the other agents, suggesting that this is a preferred alternative to CO_2.

1. Introduction

Mice are the most commonly used species in biomedical research, accounting for nearly half of all research animals used worldwide (Ormandy et al., 2009). Virtually all these mice are killed at the end of a study or to collect tissue samples, and many more are killed to reduce surplus breeding stock. Animal care policy in many countries stipulates that death should be painless and should minimize fear and anxiety (e.g. Canada: CCAC, 1993; United States: USDA, 1985; European Union: CEC, 1986; Australia and New Zealand: Reilly and Rose, 2001).

The most common method of killing laboratory rodents is exposure to carbon dioxide (CO_2). Recent evidence demonstrates that rats find exposure to CO_2 aversive (e.g. Leach et al., 2004; Kirkden et al., 2008; Niel et al., 2008), but little is known about mouse aversion to this gas. Leach et al. (2002a, 2004) showed that mice that are free to travel between chambers filled with various gases will spend most time in a chamber filled with air, followed by inhalant anaesthetics, argon, and finally CO_2. The authors concluded that CO_2 is both aversive and likely to cause considerable distress, pain and suffering, and recommended instead the use of inhalant anaesthetics

* Corresponding author. Tel.: +1 604 822 5715; fax: +1 604 822 4400.
E-mail address: makowska@interchange.ubc.ca (I.J. Makowska).

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initially to cause unconsciousness, followed by CO_2 or argon to produce death. However, Leach et al. (2002a, 2004) gave animals no incentive to remain in the chambers, so the strength of aversion to CO_2 and the other gases is unknown.

The aim of the present study was to use approach-avoidance testing to assess mouse aversion to CO_2, argon, carbon monoxide (CO), and the inhalant anaesthetics halothane and isoflurane.

2. Materials and methods

2.1. Subjects and housing

We used surplus male CD-1 mice that had been designated for euthanasia by the University of British Columbia's Animal Care Centre. Seven mice were used in the main experiments and six others were used in preliminary testing. Mice were housed in groups of four or five in an apparatus that consisted of two polycarbonate cages (Lab Products Inc., Seaford, DE, USA) connected by a sloped, opaque, ABS tube that was 4 cm in diameter and 33 cm in length, such that one cage was 10 cm higher than the other. The top cage was larger (27 cm × 20 cm × 15 cm) and contained bedding (Aspen Chip, Northeastern Products Corp., Warrensburg, NY, USA), strips of paper towel for nesting, an opaque nest box, and unlimited access to food (Lab Diet 5001, PMI Nutrition International, Richmond, USA). The bottom cage was smaller (28 cm × 17 cm × 12 cm) and contained bedding and a water bottle filled with tap water. Animals were housed under a 12-h light: 12-h dark cycle (lights on at 0800 h), with all testing done during the light phase around the same time each day. Temperature and relative humidity (mean ± standard deviation) during the study period were 23.5 ± 1.0 °C and 25 ± 6%, respectively. Mice were individually marked with brown hair dye (Garnier Belle ColorEase Crème, Garnier Canada, Montreal, QC, Canada). At the end of the study that spanned 4 months, animals were 8 months old and weighed (mean ± standard deviation) 52 ± 4 g.

2.2. Experimental apparatus

The experimental apparatus consisted of the animals' home apparatus (Fig. 1). During testing this apparatus was transferred to a fume hood in a procedures room, where the bottom cage was fitted with a Plexiglas lid that had a gas inlet in the centre, a sampling tube near the far end of the cage, and two air outlets (1.8 cm in diameter) covered with mesh at the end closest to the tube. The sampling tube, which reached down to about 1 cm above a mouse's head when it was eating, was connected to an oxygen (O_2) analyser (Series 2000 Percent Oxygen Analyzer, Alpha Omega Instruments, Cumberland, RI, USA). The nest box, nesting material, and water bottle were removed before testing.

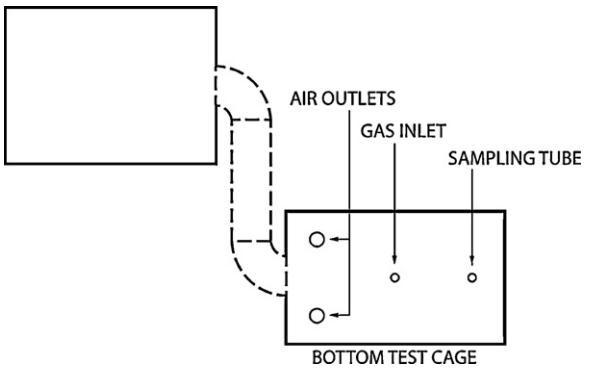

Fig. 1. Overhead view of the test apparatus, drawn to scale.

Air, O_2, CO_2, argon and CO (9% CO in air) were delivered to the bottom test cage from compressed gas cylinders (Praxair, Richmond, BC, Canada). The temperature of these gases was regulated by passing them through a copper coil in a room temperature water bath. All gas flow, except for O_2, was controlled using a variable area flow meter (Model VFB-67, Dwyer Instruments, Inc. Michigan City, IN, USA). Because the flow meter was calibrated for air, measured flow rates were adjusted for density using a correction factor of 0.812 for CO_2 and 0.849 for argon. CO flow rates were not adjusted since the gas mixture contained 91% air and CO has a density similar to that of air. O_2 flow was controlled using a flow meter (Model GL-616, Porter Instruments Company, Hatfield, PA, USA) that was attached to a table-top anaesthetic machine (ARVS, Langley, BC, Canada). Halothane (Halocarbon Laboratories, River Edge, NJ, USA) and isoflurane (Baxter Corporation, Mississauga, ON, Canada) were delivered from Fluotec 4 and Isotec 4 vaporizers (Ohmeda, Steeton, West Yorkshire, England), respectively, with O_2 as a carrier.

2.3. Preliminary testing

Little data exists on times to recumbency (i.e. loss of the righting reflex) or concentrations causing recumbency during exposure to CO. For this reason, we recorded the time to recumbency and the CO concentration at recumbency for mice exposed to CO at the flow rate chosen for this experiment. Average time to recumbency (±standard deviation) was 39 ± 5 s. The average CO concentration in the cage at recumbency was 5.1 ± 0.4%.

Slightly higher concentrations are required for halothane than isoflurane to achieve similar times to recumbency. To facilitate comparisons between the two anaesthetics, we used additional mice to measure the time until recumbency with each of two concentrations of halothane (3% and 4.5%) and isoflurane (2% and 3%) to ensure that the concentrations were well matched in terms of time to cause recumbency. Four mice were tested with each treatment. A period of at least 20 h was allowed between exposures. Times to recumbency with these concentrations averaged (±standard deviation) 93 ± 9 s and 68 ± 5 s for halothane and 100 ± 4 s and 64 ± 8 s for isoflurane. The overall average across the two anaesthetics was 96 s and 66 s.

2.4. Training

Mice were trained to cross the tube connecting their two cages at the sound of gentle tapping on the cage side, using a reward of one piece of sweetened shredded coconut

(Safeway Ltd., Calgary, AB, Canada). Mice were also trained to enter the bottom cage of their home apparatus for a reward of 1/4 teaspoon of sweetened shredded coconut while air or O_2 was delivered to this test cage.

2.5. Testing procedure

Once the home apparatus was in place in the fume hood, mice were transferred to a holding cage that contained familiar bedding. The experimental mouse was picked-up and placed in the experimental apparatus, where it was given 60 s for exploration. After 60 s the mouse was called to the top cage (if necessary), and the tube between the two cages was disconnected for 60 s. During this time, the experimenter placed 1/4 teaspoon coconut in the bottom cage. When the tube was reconnected, the mouse was able to access the reward while the experimenter stood out of sight. As soon as the mouse started eating, the experimental or control gas was turned on at a pre-determined flow rate. Trials ended when mice returned to the top cage. Mice were not allowed to re-enter the test cage after they returned to the top cage.

2.6. Experimental design

In the CO_2 experiment, seven mice were tested with flow rates of 18%, 35%, 53% and 70% of the test cage volume per min. The same set of flow rates was used in the air control trials. We ran three replicates, so each mouse was tested three times under each condition. Responses were averaged to provide one value per mouse for each of the eight treatments.

In the argon experiment, the same seven mice were tested with argon and air at flow rates of 66%, 92%, 119% and 160% of the test cage volume per min. Each mouse was tested twice under each condition; these replicate values were then averaged to provide one value per mouse for each of the eight treatments.

In the CO experiment, the same seven mice were tested with a flow rate of 88% of the test cage volume per min for both the CO and the air control trials. Because the CO tank contained 9% CO in air, the actual flow rate of CO was equivalent to 8%.

In the inhalant anaesthetics experiment, six mice were tested with two concentrations of halothane (3% and 4.5%) and two concentrations of isoflurane (2% and 3%). These were the same mice as those used in the other experiments, minus one that failed to eat in the control trials. Inhalant anaesthetics were delivered with O_2 as a carrier at a flow rate of 70% of the test cage volume per minute. The same O_2 flow rate was used during control trials. We ran two replicates; these replicate values were averaged to provide one value per mouse for each of the five treatments.

Testing was done twice a day, with each mouse undergoing one control and one experimental trial each day. Experimental trials were interspersed with control trials to avoid extinction. Roughly half the mice were tested with control trials in the morning and experimental trials in the afternoon, and the other half were tested in the reverse order. Morning and afternoon trials were always 5–6 h apart. In the CO_2, argon, and inhalant anaesthetics experiments, treatment order for experimental trials was balanced across mice and days using a Latin square design. For the CO_2 and argon experiments, another Latin square design was used to balance treatment order for control trials. We were concerned that mice would be reluctant to return to the test cage after testing with CO, so during this experiment all control trials were performed in the morning and all experimental trials were done in the afternoon of the same day.

2.7. Data collection

Mice were video recorded with a Panasonic CCTV camera (Model WV-BP330, Laguna, Philippines) and scored for the latency to leave the test cage (from the time gas was turned on until the tip of the tail disappeared in the tube). If mice did not leave the test cage within 240 s, the trial was ended and a latency of 240 s was recorded. For the inhalant anaesthetics and the CO trials, we also calculated the difference between the time at which mice left the test cage and the time at which recumbency was expected to occur (i.e. 40 s for CO, and 66 s and 96 s for the two concentrations of inhalant anaesthetics) to give an indication of how long conscious mice would be exposed to aversive concentrations if forced to remain in the chamber. Finally, we recorded the O_2 concentration at the time mice left the test cage in the CO_2, argon and CO trials. Changes in measured O_2 concentration were used to calculate concentrations of the treatment gases.

2.8. Statistical analysis

For the CO_2 and the argon experiments, dependent variables were analysed using mixed models (SAS v9.1) that included mouse (6 d.f.) as a random effect, and tested for linear and quadratic effects of flow rate (1 d.f. for each) against an error term with 19 d.f. For the inhalant anaesthetics experiment, dependent variables were analysed using a mixed model that included mouse (5 d.f.) as a random effect, and tested for the effects of anaesthetic, treatment, and anaesthetic by treatment interaction (1 d.f. each) against an error term with 15 d.f. Descriptive statistics are provided for the CO experiment since only one flow rate was tested.

3. Results

When exposed to CO_2, mice never remained in the test cage long enough to lose consciousness. Latency to leave the test cage and CO_2 concentration when mice left the test cage are presented in Table 1. In this experiment, mice left the test cage more quickly (linear: $F_{1,19} = 32.68$, $P < 0.0001$; quadratic: $F_{1,19} = 9.2$, $P = 0.0068$) with increasing CO_2 flow rates. Regardless of flow rate, mice always left the test cage when CO_2 reached a similar threshold concentration (linear: $F_{1,19} = 2.05$, $P = 0.17$; quadratic: $F_{1,19} = 1.96$, $P = 0.18$). During exposure to air (control), all mice remained in the test cage until the end of the trial, regardless of flow rate.

Table 1
Response (±S.E.M.) of mice ($n = 7$) during exposure to CO_2 and air at four flow rates.

Flow rate (%/min)	Latency to leave (s)	CO_2 concentration when left (%)
18	32 ± 3	18.2 ± 2.0
35	17 ± 3	13.5 ± 2.0
53	18 ± 3	15.3 ± 2.0
70	15 ± 3	14.6 ± 2.0

Table 2
Response (±S.E.M.) of mice ($n = 7$) during exposure to argon and air at four flow rates.

Flow rate (%/min)	Latency to leave (s)	O_2 concentration when left (%)
66	82 ± 5	9.3 ± 0.5
92	59 ± 5	8.5 ± 0.5
119	48 ± 5	8.3 ± 0.5
160	37 ± 5	8.3 ± 0.5

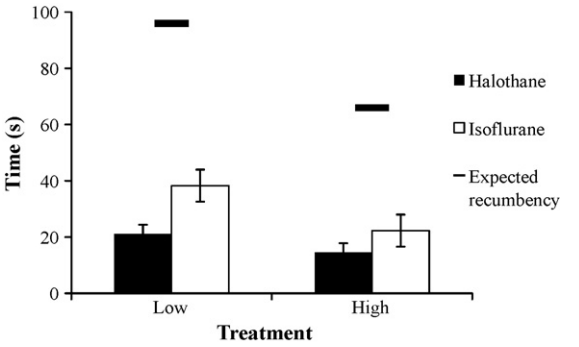

Fig. 2. Least square mean (±S.E.M.) latency to leave the test cage during exposure to low and high concentrations of halothane (3% and 4.5%) and isoflurane (2% and 3%) ($n = 6$ mice). The times at which mice were expected to become recumbent with each of the two treatments are indicated by the horizontal bars.

When exposed to argon, mice never remained in the test cage long enough to lose consciousness. Latency to leave the test cage and O_2 concentration when mice left the test cage are presented in Table 2. In this experiment, there was a linear effect of flow rate on the latency to leave the test cage (linear: $F_{1,19} = 51.33$, $P < 0.0001$; quadratic: $F_{1,19} = 3.85$, $P = 0.065$), with mice leaving more quickly with increasing flow rates. Regardless of flow rate, mice always left the test cage when O_2 was depleted to a similar threshold concentration (linear: $F_{1,19} = 2.13$, $P = 0.16$; quadratic: $F_{1,19} = 1.08$, $P = 0.31$). During exposure to air (control), all mice remained in the test cage until the end of the trial, regardless of flow rate.

When exposed to CO, mice never remained in the test cage long enough to lose consciousness. Mice left the test cage after (mean ± standard deviation) 23 ± 7 s, when CO concentration averaged 2.5 ± 0.6%. The average amount of time between when mice left the test cage and expected recumbency was approximately 16 s. During exposure to air (control), all mice remained in the test cage until the end of the trial.

When exposed to inhalant anaesthetics, mice never remained in the test cage long enough to lose consciousness when tested with halothane, but two mice became recumbent in the test cage during one of their trials with isoflurane. One mouse became recumbent when exposed to 2% isoflurane and the other when exposed to 3% isoflurane. Mice remained in the test cage longer (Fig. 2; $F_{1,15} = 4.76$, $P < 0.05$), and remained closer to the time of expected recumbency ($F_{1,15} = 4.76$, $P < 0.05$), when exposed to isoflurane versus halothane. Mice also remained closer to the time of expected recumbency when exposed to higher concentrations of the anaesthetic gases ($F_{1,15} = 10.71$, $P = 0.0051$). During O_2 (control) trials in this experiment, all mice remained in the test cage until the end of the trial.

4. Discussion

In this study, mouse motivation to avoid gas exposure was measured against motivation to access a highly palatable sweet food reward. Evidence suggests that mice fed *ad libitum* are at least moderately motivated to consume sweet foods (Bachmanov et al., 2001; Sclafani, 2006), so if they choose to abandon this sweet food to escape gas exposure, we may infer that exposure to the gas is at least moderately aversive. The results of the current study indicate that CO_2, argon, CO and halothane are sufficiently aversive that mice forego access to a preferred food reward. Exposure to isoflurane is also aversive to most mice, but some tolerated the agent until recumbency, indicating that motivation to avoid isoflurane is sometimes weaker than motivation to consume a preferred food reward. Aversion was not due to sound or air currents associated with gas entry, since exposure to the same set of flow rates of air or O_2 had no effect on the variable measured.

When tested with CO_2, mice left the test cage when CO_2 concentrations were, on average, between 13.5% and 18.2%, well below the concentrations required to render the animals unconscious (>30%). In similar studies on rats, animals left the test cage when CO_2 concentrations were 13.0–18.4% (Niel and Weary, 2007; Niel et al., 2008), suggesting that rats and mice have similar aversion thresholds to CO_2. In humans, dyspnea begins at concentrations of approximately 8% CO_2 and becomes severe at approximately 15% (Hill and Flack, 1908; Liotti et al., 2001). Dyspnea is a likely cause of aversion to CO_2 in rats (e.g. Hawkins et al., 2006; Niel and Weary, 2007). Based on the aversion thresholds in this study, we suggest that dyspnea is also a cause of aversion to CO_2 in mice. Exposure to CO_2 also causes pain due to the formation of carbonic acid on the nasal mucosa, but this occurs at higher CO_2 concentrations (<40%; Peppel and Anton, 1993) than those avoided by mice in this study. Pain in the nasal mucosa is therefore not a likely cause of aversion in this study.

Argon is often suggested as an alternative to CO_2 because it is odourless, affordable, and safe to use. Moreover, this inert gas causes little to no aversion in terrestrial mammals (pigs: Raj and Gregory, 1995) and poultry (hens: Webster and Fletcher, 2004; turkeys: Raj,

1996; broilers: Gerritzen et al., 2000). Argon acts by displacing air, leading to hypoxia and death. In this study, mice avoided argon-induced hypoxia when O_2 was depleted to 8.3–9.3%. In a similar study (Makowska et al., 2008), rats were found to avoid argon-induced hypoxia when O_2 reached 6.6%, suggesting that mice may be more sensitive than rats to hypoxia. Both species are burrowers and may have developed the ability to detect hypoxia, but which sensations they find aversive remains unclear. This study was not designed to compare aversion between the various euthanasia agents as gases were not matched in terms of the time they took to induce recumbency. Leach et al. (2002a, 2004) found that mice spent more time in chambers containing argon than ones containing CO_2, but the scope for suffering if forced to remain in the euthanasia chamber beyond the point of aversion is unknown.

CO also causes hypoxia, but the mechanism of action is different than that of argon. CO impairs O_2 delivery to the tissues by preferentially binding to iron in haemoglobin, and then by diminishing the ability of the other binding sites to off-load O_2. During preliminary testing, it was determined that mice became recumbent when CO concentration in the cage averaged approximately 5.1%; this value is comparable to the concentration at which rats became recumbent in a similar study (Makowska and Weary, 2009a). Contrary to rats, however, no mouse was willing to tolerate exposure to CO until recumbency. Which sensations mice find aversive when exposed to CO is also unclear.

Inhalant anaesthetics cause rapid chemical depression of the nervous system and lead to a loss of sensation in the body (Kohn et al., 1997). Halothane and isoflurane are two of the most commonly used volatile liquid anaesthetics for animals (Flecknell, 1996). In this study, two mice tolerated isoflurane until recumbency (in both cases during just one of four trials), indicating that aversion to isoflurane is weaker than aversion to the other agents tested. Moreover, anaesthetics induce a state of conscious sedation even before consciousness is lost (Heinke and Schwarzbauer, 2001; Trevor and White, 2006). This suggests that exposure to anaesthetics beyond the point of aversion may be less unpleasant than exposure to non-sedative agents beyond this point, because the animals are becoming progressively sedated.

On average, mice spent more time in the test cage when exposed to isoflurane than to halothane. Preference for isoflurane over halothane was also found in rats (Makowska and Weary, 2009). Leach et al. (2002b, 2004) reported the opposite finding perhaps because they used pre-filled cages while we used the gradual-fill method. Isoflurane has a pungent odour (Yentis et al., 1996; Gallacher and Hutton, 2002) that may be more unpleasant when encountered at high concentrations. In addition, unlike Leach et al. (2002b, 2004), we provided animals with an incentive to remain in the test cage, and this incentive may have enticed them to tolerate the anaesthetics beyond the point that caused avoidance in the Leach et al.'s (2002b, 2004) studies.

5. Conclusion

Our results indicate that the most common method of euthanasia for laboratory mice – exposure to CO_2 – is at least moderately aversive to mice. Mice show aversion to CO_2 concentrations as low as 13.5–18.2%, but concentrations of greater than 30% are needed to render them unconscious. Mice also showed aversion to argon, CO, and the two inhalant anaesthetics, so the search for a non-aversive agent should continue. Aversion to the inhalant anaesthetic isoflurane appears to be weaker than aversion to the other agents, so we recommend the use of this agent to render mice unconscious before euthanasia with any method.

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