

Exposure of Research Personnel to Carbon Dioxide during Euthanasia Procedures

Ashlee A Amparan,¹ Shelly M Djoufack-Momo,¹ Beverly Grunden,² and Gregory P Boivin^{3-5,*}

CO₂ is one of the most commonly used euthanasia agents for laboratory animals. Considerable research has gone into the effect of the agent on animals, but little has been done to examine potential human exposure during these procedures. In this study, we examine the CO₂ concentrations to which personnel are exposed while euthanizing rodents with CO₂. To examine the environmental levels of CO₂ generated during euthanasia, we examined several variables including flow rate, inclusion of a cage in the euthanasia chamber, inversion of the euthanasia chamber, chamber size, distance from the euthanasia chamber, and room size. Under all conditions, CO₂ concentrations in the room temporarily increased significantly to 600 to 4000 ppm. The results of this study show that, under several testing scenarios, occupational levels of CO₂ did not exceed governmentally mandated allowable exposure limits during routine rodent euthanasia procedures.

Abbreviations: STEL, short-term exposure limit; TWA, time-weighted average.

CO₂ is one of the most commonly used euthanasia agents in laboratory animal facilities. Euthanasia typically is performed by using a compressed gas cylinder to supply CO₂ to a small chamber. Considerable research has gone into the CO₂ effect on animals, but little has been done to examine the potential effects of human exposure. To protect workers exposed to CO₂, several organizations have set CO₂ exposure levels with which employers must comply. The Occupational Safety and Health Administration has set a permissible exposure limit,¹⁹ American Conference of Governmental Industrial Hygienists set a threshold limit value,² and the National Institute of Safety and Health⁷ has a recommended exposure limit. Normal atmospheric CO₂ is approximately 300 to 500 ppm. These 3 entities have all set the time-weighted average (TWA) for CO₂ as 5000 ppm and short-term exposure limit (STEL) is 30,000 ppm. TWA is an average exposure over 8 h, and STEL is the maximal exposure, typically over a 15-min interval. The National Institute of Safety and Health considers 40,000 ppm to be immediately dangerous to life.¹⁶

Rooms dedicated for euthanasia typically are small, and in some institutions, dedicated personnel are responsible for overseeing all euthanasia activities. This is especially evident during rodent colony health surveillance, in which rodent CO₂ euthanasia may involve several days. This situation potentially can place employees at risk for both exposures to STEL and TWA limits. To our knowledge, there are no reports of CO₂ over-exposure incidents in animal care facilities. However, there are multiple incidents of CO₂ mortality and morbidity in other settings, including a research laboratory.^{3,8} Events are commonly associated with CO₂ gas-line leaks in restaurants and manufacturing facilities¹ and pooling of CO₂ during fermentation processes in basements or low areas. One case report involved 25 people at an ice factory who required hospitalization when the discharge valve of a truck containing liquid CO₂ was knocked open in an

enclosed environment.¹⁰ These events warn us that exposure to harmful and potentially lethal CO₂ levels can occur.

The current study examined personnel CO₂ exposure in an animal research facility during mock mice euthanasia procedures. We hypothesized that the CO₂ room concentrations depend on gas flow rate, room size, room ventilation, chamber position, and chamber size. We also hypothesized that CO₂ room concentrations remain below 5000 ppm.

Materials and Methods

A dedicated room (30 m³; Figure 1) at Wright State University (Dayton, OH) was used for most experiments. The room had negative pressure to the corridor and 8.8 air changes hourly. The door to the room remained open during all procedures, as is standard practice at the facility. Informed consent was obtained from 2 people (AAA and SMDM), who were present in the room during all measurements, and both persons remained relatively inactive (standing or sitting in one location) while recording data, to minimize air disturbance. The CO₂ concentrations in the room were measured every 15 s by using a Veloci Calc meter (model 9555-P, TSI, Shoreview, MN). The monitor was placed on a shelf 0.48 m above and 0.62 m away from a euthanasia chamber (1.42 m above the floor). Additional measurements were obtained at a shelf 1.52 m away from the chamber at the same height. The chamber was on a counter 0.94 m above the floor. A plastic tube connected to the CO₂ tanks was inserted under the chamber lid.

Preliminary testing. Previous studies^{6,11,12,18} and a preliminary test of our system were used to determine the length of time for the CO₂ gas to be turned on and reach a peak level. Several studies have shown that a CO₂ displacement rate of 20%/min produces unconsciousness in rodents in about 106 s.^{6,11,12,18} The time to onset of unconsciousness increased to 156 s when the slower displacement rate of 10% CO₂/min was used.⁵ The length of time to unconsciousness and death was examined to model real-time mouse euthanasia. The preliminary data were obtained from IACUC-approved protocols. Gas exposure times for euthanasia were determined according to these preliminary results (data not shown) and were consistent with previous reports.^{5,6,11,12,18}

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Departments of ¹Community Health, ²Mathematics and Statistics, ³Pathology, and ⁴Orthopaedic Surgery, Wright State University, Dayton, Ohio; ⁵Veterans Affairs Medical Center, Cincinnati, Ohio.

*Corresponding author. Email: gregory.boivin@wright.edu

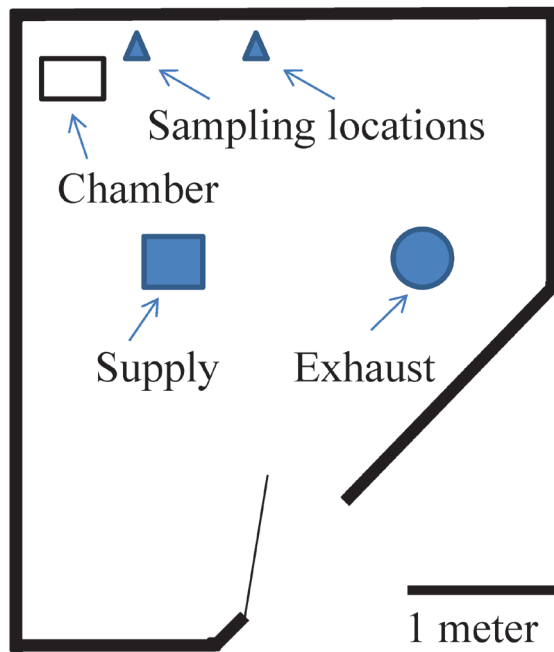


Figure 1. Schematic of the test room at Wright State University.

Testing procedure. All trials were conducted without animals in the chamber. Tests were performed in a 22-L euthanasia chamber volume (44 cm × 23.5 cm × 21 cm) with different variables. The chamber had a removable lid that was removed (except during the lid-on experiment) after the CO₂ tank was turned off. CO₂ escaped the chamber through a 1-cm hole in the lid and emptied directly into the room. Prior to the initiation of a new trial, the chamber CO₂ concentration was 500 ppm or lower, and room CO₂ levels were 600 ppm or lower. Five replicates of each experimental condition were performed.

Two flow rates were analyzed, to model the most common euthanasia techniques. In the first paradigm, the CO₂ gas cylinder was turned to a flow rate of 3 L/min (about a 15% fill rate of 100% CO₂) for 7 min, thus replicating a continuous slow-fill rate. The second method (3 to 10 L/min) consisted of a flow rate of 3 L/min of 100% CO₂ for 2 min; the rate then increased to 10 L/min (about a 50% fill rate) for 3 min to replicate a slow fill rate until unconsciousness of the mouse, followed by a fast fill rate. In both cases, the lid was removed 15 s after turning the gas off. The procedure was performed with the chamber empty or containing a 5.8-L cage (Allentown Caging, Allentown, NJ), to model direct placement of a mouse into the chamber compared with placement of a shoebox-style cage containing a mouse. When present, the cage was removed directly after lid removal.

Because CO₂ is heavier than air, we considered that inverting the chamber might affect room CO₂ levels. This additional condition was tested by repeating the previous experimental conditions. For the trials in which the chamber was turned over, the inversion was performed right after the lid was removed. The chamber then was placed so that 17.8 cm (40% of its length) extended over the counter edge in all trials involving the upside-down orientation.

To assess the effects of leaving the lid on after euthanasia, an experiment was performed in which the 3-L/min CO₂ flow rate was used for 7 min and the lid was kept on for 20 min after the gas was turned off.

To evaluate the effect of chamber size on the room CO₂, the 4 experimental conditions described were repeated with a 7.6-L chamber (32 cm × 19 cm × 12.5 cm; Anesthesia Chamber,

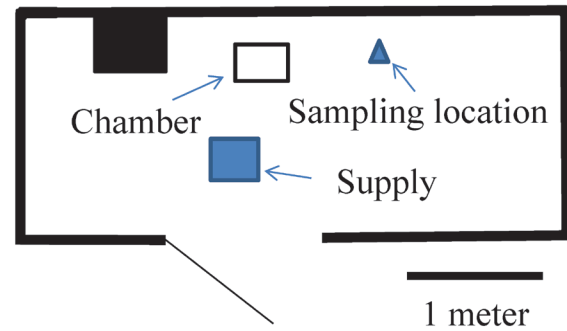


Figure 2. Schematic of the test room at Cincinnati Veterans Affairs Medical Center.

Braintree Scientific, Braintree, MA). With this smaller chamber, both 1.3-L/min and 1.3-to-10-L/min flow rates were analyzed at distances of 0.62 m and 1.52 m. The lower flow rate was used to maintain consistency in the percentage fill rate between the 7.6- and 22-L chambers.

To evaluate the effect of room size, measurements were repeated by using the 22-L chamber at the Cincinnati Veterans Affairs Medical Center (Cincinnati, OH), where the room size was only 13.5 m³, with 13.1 air changes hourly and positive pressure to the hallway (Figure 2). The door to the procedure room remained open during testing, consistent with practice at this facility. We measured CO₂ concentrations at a distance of 0.53 m from and 0.31 m above the chamber (0.94 m above the floor) with a flow rate of 3 L/min, without a cage in the chamber, and the chamber right side up.

Statistical analysis. In total, 22 combinations of the described variables were tested. Each combination was replicated 5 times. The averages of the 5 replicates are reported for the 22 groups. All statistical analyses were completed by using SAS software (version 9.3, SAS Institute, Cary, NC). Prior to interpretation of results, data were checked for normality and homogeneity of variance. Data that were in violation were transformed before additional testing. The only transformation required was one that squared the outcome values in the 3-way ANOVA for the 0.62 m distance. In one case for which transformations were not helpful (comparing the 2 distances for the 3-to-10-L/min rate with cage included and chamber right side up, for which variances were not equal), a Kruskal–Wallis test was used instead. The outcome variable for all analyses was peak CO₂, and the means were compared by using 3-way ANOVA under different conditions involving cage presence, chamber position, and flow rate. *t* tests were used to compare the peak CO₂ at 2 different distances from the source of CO₂, at 2 locations, and with 2 chamber sizes. Significance was set at an α value of 0.05, and, where appropriate, the location of significant effects was determined by using a Tukey post hoc test or a Holm–Bonferroni correction. Data are presented as group means \pm 1 SD.

Results

Peak CO₂ levels were measured for 22 trials involving 6 variables (Table 1). The CO₂ levels increased steadily in the room at both distances measured when the CO₂ tank was turned on (Figure 3). The room CO₂ levels reached peak values shortly after the gas was turned off and the lid was removed from the chamber. The decline in room CO₂ back to baseline levels took less than 10 min. In the 5 trials examining the chamber with the lid on, CO₂ levels peaked at 728 ppm on average and were slightly above ambient CO₂ levels for 13 of the 27 monitored minutes.

Peak CO₂ levels in the smaller euthanasia room (13.5 m³) with 3-L/min flow rate, chamber right side up, and measurements

Table 1. Peak CO₂ room concentrations (mean ± 1 SD, *n* = 5) during mock euthanasia procedures

Room size (m ³)	Distance (m)	Flow rate (L/min)	Cage present?	Chamber size (L)	Chamber position	CO ₂ concentration (ppm)
30	0.62	3	No	22	Up	1114 ± 68
30	0.62	3–10	No	22	Up	1435 ± 61
30	0.62	3	Yes	22	Up	1059 ± 101
30	0.62	3–10	Yes	22	Up	1331 ± 32
30	0.62	3	No	22	Up	728 ± 38 ^a
30	0.62	3	No	22	Down	986 ± 42
30	0.62	3–10	No	22	Down	1461 ± 44
30	0.62	3	Yes	22	Down	1085 ± 59
30	0.62	3–10	Yes	22	Down	1486 ± 94
30	1.52	3	No	22	Up	2047 ± 306
30	1.52	3–10	No	22	Up	1956 ± 224
30	1.52	3	Yes	22	Up	1663 ± 229
30	1.52	3–10	Yes	22	Up	2429 ± 522
30	1.52	3	No	22	Down	1605 ± 228
30	1.52	3–10	No	22	Down	2277 ± 233
30	1.52	3	Yes	22	Down	1910 ± 157
30	1.52	3–10	Yes	22	Down	2440 ± 223
30	0.62	1.3	No	7.6	Up	657 ± 22
30	0.62	1.3–10	No	7.6	Up	1144 ± 133
30	1.52	1.3	No	7.6	Up	716 ± 56
30	1.52	1.3–10	No	7.6	Up	2638 ± 251
13.5	0.53	3	No	22	Up	3613 ± 226

^aLid left on the chamber.

taken 0.53 m from the chamber averaged 3613 ± 226 ppm (Table 1). The peak value for similar conditions in the larger room (measurements taken at 0.62 m) was 1114 ± 68 ppm (*P* < 0.0001).

The highest mean peak CO₂ concentration (2440 ppm) for the large chamber in the 30-m³ room occurred with the chamber turned upside down, a 3-to-10-L/min flow rate, a cage in the chamber, and measurement of the CO₂ concentration at 1.52 m (Table 1). There was a significant (*P* = 0.0022) increase due to distance with all conditions at 1.52 m compared with 0.62 m. Increasing the flow rate and turning over the chamber (Figure 3) both resulted in significant (*P* < 0.0001 and *P* = 0.0436, respectively) increases in peak CO₂ at 0.62 m. Having a cage in the chamber and removing it when the lid was taken off had no significant impact on CO₂ levels compared with trials with no cage in the chamber (*P* = 0.4790). With the lid on, the chamber was tested at the 0.62-m distance only. There was a significant decrease in peak CO₂ when the lid was left on (*P* < 0.0001). The highest mean peak with the small chamber in the 30-m³ room was 2638 ppm at 1.52 m with a 1.3-to-10-L/min flow rate. There were only mild increases in the room CO₂ levels when the 1.3-L/min flow rate was used with the small chamber.

At 1.52 m, the 3-way interaction among flow rate, chamber, and cage was significant (*P* = 0.0092), but the significant differences were due only to flow rate. Increasing flow rate with a cage in the chamber positioned right side up increased peak CO₂ compared with those at the lower flow rate (*P* = 0.0038). A similar increase was seen without a cage and the chamber turned upside down (*P* = 0.0152). The main effect for flow rate was significant (*P* < 0.0001).

The effect of chamber size on room CO₂ levels was evaluated in the 30-m² room by using both flow rates, chamber right side up, and no cage (Table 1). There was a significant increase in peak CO₂ in the room when comparing the large chamber with the small chamber at low (3 L/min and 1.3 L/min, respectively) flow (*P* < 0.0001) and low-to-high (3 to 10 L/min and 1.3 to 10

L/min, respectively) flow (*P* < 0.0022) at 0.62 m and at low flow at 1.52 m (*P* = 0.0005). However, at 1.52 m with low-to-high flow, there was an increase in peak CO₂ with the small chamber compared with the large chamber (*P* = 0.0019).

Discussion

In this study, we found that the peak CO₂ concentrations, under all conditions, remained well below the OSHA threshold limit values: 5000 ppm for TWA and 30,000 ppm for STEL. TWA is an 8-h exposure and to reach this exposure level, the CO₂ concentration would have to average more than 5000 ppm throughout the entire work day. In the current study, peak exposure levels averaged near 2000 ppm and reached 3690 ppm in one situation. The CO₂ peaks were transitory, and even if personnel were exposed to the increased CO₂ levels several times during a single day, they would still not approach TWA limits.

Exposures to increased CO₂ levels can lead to a variety of symptoms (Figure 4). Although there are no limitations below the 5000 ppm for indoor CO₂, the American Society of Heating, Refrigerating, and Air Conditioning Engineers suggests that indoor CO₂ can be used as an indicator of odor levels, that 1000 to 1200 ppm CO₂ is an indication of inadequate ventilation, and that odors often are associated with this CO₂ level.¹⁷ Despite CO₂ levels that remain below accepted exposure limits, there is concern that long-term exposure to CO₂ in excess of 300 to 500 ppm may have a negative effect on people. This concern was raised in regard to classrooms with high student density, in which CO₂ levels frequently were elevated above 1000 ppm and occasionally reached 3000 ppm.⁹ Classroom air quality had a significant effect on error rates in students taking a d2 test⁴ when CO₂ increased from 1045 ppm to 2115 ppm, although no change in concentration performance was shown.²¹ A study examining cognitive ability in young adults showed that increasing room CO₂ levels from 600 to 2500 ppm led to a significant decline in

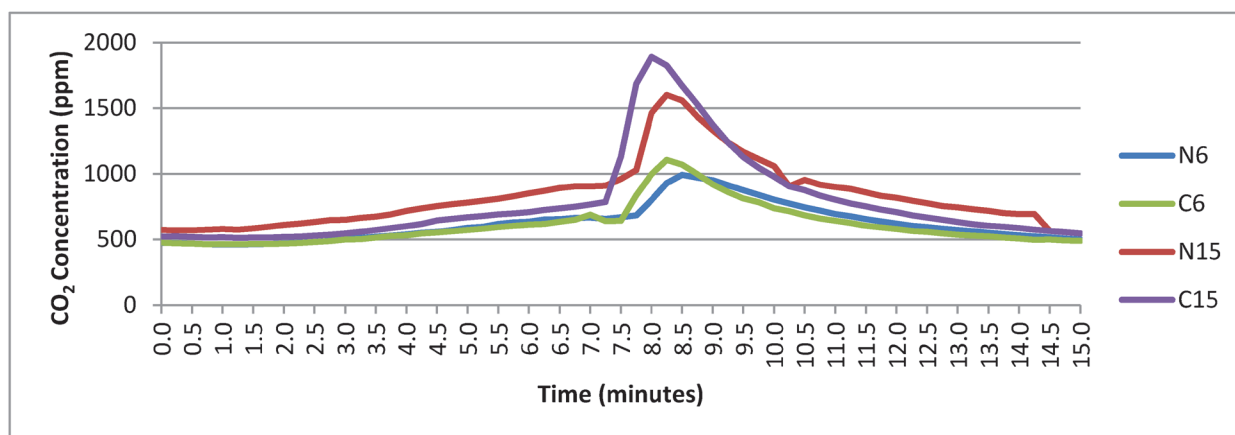


Figure 3. Average room CO₂ concentrations with a 3-L/min flow rate in a 22-L chamber turned upside down. The CO₂ was turned off at 7 min and the lid removed 15 s later. N, no cage; C, cage in chamber; 6, 0.62 m; 15, 1.52 m.

CO ₂ concentration	Symptoms	Reference
30,000 ppm	Increased breathing	8
50,000 ppm	Decreased vascular resistance	8
≥50,000 ppm in alveolar air	Hypercapnia, systemic acidosis	15
60,000 ppm for 6 to 8 min	Cardiac rhythm changes	8
75,000 ppm for 15 min	Dyspnea, headache, vision changes, vertigo, sweating, numbness, irritability, disorientation	14
100,000 ppm for seconds to minutes	Unconsciousness, death	15

Figure 4. Symptoms associated with increasing CO₂ concentrations.

7 of 9 decision-making performance parameters.²⁰ At 600 ppm, subjects performed at average levels. However, at 2500 ppm CO₂, subjects performed at dysfunctional levels for initiative and basic strategy and at marginal to dysfunctional levels for information usage and breadth approach. In another study, proofreading was significantly worse when subjects were exposed to 5000 ppm, with a marginally significant reduction at 3000 ppm compared with 600 ppm CO₂.¹³ These experiments demonstrate that the CO₂ levels in rooms with a euthanasia chamber could lead to cognitive impairment, even though CO₂ levels are below the TWA of 5000 ppm and STEL of 30,000 ppm.

We noted significant differences in room peak CO₂ concentrations with alterations of flow rate, lid removal, chamber position, chamber size, room size, and distance from the chamber. Not surprisingly, there was no significant difference associated with inclusion of a cage in the chamber. Many of the alterations in room CO₂ levels were expected. For example, increasing the flow rate of CO₂ increased room CO₂ levels, as anticipated. Similarly, gas release led to increased CO₂ in the small room compared with the large room. The number of room-air changes were higher in the small room but likely were insufficient to reduce the acute elevations in room CO₂ levels. A slight increase in room CO₂ was observed when the chamber was inverted. In this orientation, the chamber was 40% off of the table and released more CO₂ into the room, given that CO₂ is heavier than air and therefore more easily escapes an inverted chamber, to contribute to overall room CO₂ levels.

Surprisingly, CO₂ peak levels were higher at 1.52 m than at 0.62 m from the euthanasia chamber. Room ventilation and air circulation patterns likely played a role in this finding, although this hypothesis was not investigated further. Another surprising result was the increased level of CO₂ at 1.52 m for the 1.3-to-10-L/min flow rate in the small chamber compared with the 3-to-10-L/min flow rate in the large chamber. The CO₂ peak in the room occurred earlier when the small chamber was used instead of the large chamber. These results may be due to

differences in how the lid was removed (slid off for the small chamber compared with lifted off for the large chamber).

There were a few limitations in our study. First, the room door was left open. Closing the door may reduce air changes, and higher levels of CO₂ may occur. In addition, there were 2 people in the room who contributed to the total CO₂ of the room. However, we believe this situation had little effect on overall results, given that room CO₂ concentrations returned to baseline levels prior to subsequent tests in all trials. A third deficiency was the absence of animals in the chamber. We do not believe that the CO₂ produced by animals in the euthanasia chamber would significantly contribute to room levels. Chamber leakage was a factor, because placement of the interior chamber meter for a companion study prevented the lid from closing completely. This feature likely had little effect because there are already holes in the chamber to allow gas escape, and peak CO₂ levels occurred after the lid was removed. In addition, we did not measure the CO₂ concentrations at floor level. Therefore, whether subsequent trials were affected by an overall accumulation of CO₂ is unknown. However, because CO₂ did not exceed 5000 ppm, this accumulation likely had minimal effect on the study. Finally, differences in ventilation patterns and air replacement rates between the 2 rooms could have influenced the observed CO₂ levels, so that direct comparisons are limited. We did not evaluate optimal placement of the CO₂ meter in the room to detect the highest CO₂ levels possible but rather selected distances at which personnel commonly stand relative to the chamber. We considered these distances to most accurately reflect the human exposure potential.

Despite these minor limitations, we believe our data are a fair representation of the CO₂ exposure of personnel involved with euthanasia procedures using compressed gas cylinders. Note that the results of this study are specific to the room sizes, frequency of euthanasia procedures, and variables tested; results in other facilities may be different. It is important that personnel who deal with potentially lethal agents, such as CO₂, are monitored

closely to ensure their safety. According to our findings, the current practice of CO₂ euthanasia of rodents by using a chamber is safe in regard to regulatory standards. However, we caution that published reports suggest that even the moderately increased CO₂ levels present in our experiments could alter human cognitive ability. Additional research in this area may clarify risks involved with chronic exposure to moderately increased CO₂ levels.

Acknowledgments

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