RESEARCH PAPER

Is xenon a suitable euthanasia agent for mice?

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Abstract

Objective To compare behavioural and electrophysiological variables of mice undergoing gas euthanasia with either xenon (Xe) or carbon dioxide (CO_2).

Study design Single animals chronically instrumented for electroencephalography (EEG) recording were randomized to undergo euthanasia with either CO_2 or Xe ($n = 6$ animals per group).

Animals Twelve adult (>6 weeks old) male C57Bl6/n mice.

Methods Mice were surgically instrumented with EEG and electromyogram electrodes. Following a 7-day recovery period, animals were placed individually in a sealed chamber and a 5-minute baseline recorded in 21% O_2. Gas [100% Xe ($n = 6$) or 100% CO_2 ($n = 6$)] was then added to the chamber at 30% chamber volume minute^{-1} (2.8 L minute^{-1}) until cessation of breathing. EEG, behaviour (jumping and freezing) and locomotion speed were recorded throughout.

Results Mice undergoing single gas euthanasia with Xe did not show jumping or freezing behaviours and had reduced locomotion speed compared to baseline, in contrast to CO_2, which resulted in increases in these variables. EEG recordings revealed sedative effects from Xe but heightened arousal from CO_2.

Conclusions Our data suggest that Xe may be less aversive than CO_2 when using a 30% chamber volume minute^{-1} fill rate and could improve the welfare of mice undergoing gas euthanasia.

Keywords 3Rs, animal welfare, carbon dioxide, euthanasia, inert gases, mice.

Introduction

Carbon dioxide (CO_2) is the most commonly used method of gas euthanasia for laboratory animals and is frequently used for the slaughter of pigs and poultry (Hawkins et al. 2016). However, its use has been associated with fear, pain and aversion (Mongeluzi et al. 2003; Ziemann et al. 2009; Moody & Weary 2014; Hawkins et al. 2016; Boivin et al. 2017). These behavioural elements are considered to result from the formation of carbonic acid in the mucous membranes of the nose and mouth which some humans report as painful (Danneman et al. 1997) and which can be sensed in the amygdala of mice resulting in a fear response (Ziemann et al. 2009). There is currently a concerted effort to identify and implement alternative euthanasia agents and methods to CO_2-only euthanasia in rodents (Hawkins et al. 2016).

One avenue to explore is the use of inert gases (Gent et al. 2018) which are colourless, odourless and do not readily form biologically reactive compounds in animals whilst in the gaseous phase. Therefore, exposure to them should not result in the same welfare problems as CO_2 (Hawkins et al. 2016). Xenon (Xe) is an inert gas with unique anaesthetic properties at atmospheric conditions (Rylova & Maze 2019). It is considered to possess many of the qualities required to be an 'ideal' anaesthetic agent including cardiac stability, neuroprotection and profound analgesia (Franks 2008; Rylova & Maze 2019). The undesirable characteristics associated with clinical use as an anaesthetic include a high incidence of postoperative nausea and vomiting (Rylova & Maze 2019), which is not a factor for consideration as a euthanasia agent. We previously demonstrated that unlike other inert gases, Xe is not associated with epileptiform activity in mice at hypoxic conditions, suggesting that it might offer a refinement to inert gas euthanasia (Gent et al. 2018). Based on these qualities, it is a strong candidate to offer improved animal welfare conditions as a euthanasia agent and the need to assess its suitability as such has been identified (Hawkins et al. 2016). Therefore, we sought to compare behavioural and electrophysiological variables of adult male C57Bl6/n mice undergoing euthanasia with either CO_2 or Xe.

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Materials and methods

Animals

All work received ethical approval from the Canton of Zürich veterinary office (license number 58/14). We used adult male (8–12 weeks old, 25–30 g) C57Bl6/n mice (Charles Rivers Laboratories, Germany). Animals were kept in exhaust individual ventilated cages (EIVC) cages with wood chip bedding material (Safe Select; Safe Lab, France) on a 08:00–20:00 light–dark cycle and given access to standard laboratory rodent food (Granovit 3436; Granovit AG, Switzerland) and autoclaved water *ad libitum*. The housing room was kept at 22 ± 1 °C and 55 ± 5% relative humidity. Animals were housed with littermates until instrumentation and then individually following instrumentation until experimentation. Animals were placed into clean cages every 7 days as part of routine husbandry. Cages were not changed less than 2 days before experimentation. All animals were considered healthy based on health reports from the supplying unit and absence of behaviours indicative of poor health, for example hunched back, piloerection, inappetence and lethargy. All animals were naïve to any drug treatment before the start of the study. To reduce the number of animals used, the data reported here were collected during a previous experiment (Gent et al. 2018).

Surgical electroencephalogram implantation

Animals were placed in an anaesthesia induction box containing room air and anaesthetized using isoflurane (3% volume; Isoflo; Abbott, Switzerland) in oxygen. They were then positioned in a stereotaxic frame (51625; Stoelting Europe, Ireland), as previously reported (Gent et al. 2018). Buprenorphine (100 μg kg^{-1}; Temgesic; Schering Plough, Switzerland), meloxicam (5 mg kg^{-1}; Metacam; Boehringer-Ingelheim, Switzerland) and 0.9% saline (10 mL kg^{-1}; B. Braun, Germany) were then co-administered subcutaneously. Anaesthesia was maintained by isoflurane in oxygen delivered by face mask, with the concentration determined by the minimum amount required to prevent toe pinch reflex or reaction to surgical manipulation. The hair was then shaved from the scalp and the skin aseptically prepared, first with iodine solution (Betadine; Mundipharma, Switzerland) and 4 minutes later with 70% ethanol (Ethanol 70; B. Braun, Germany). Following a longitudinal skin incision in the scalp, holes were drilled in the skull and three small jewellery screws (00 × 1/8″; J.I. Morris, MA, USA), soldered to 0.5 mm stainless steel, PTFE wire (W3 632; W3 Wire International, NV, USA) were inserted above the dura (not penetrating brain tissue) as electroencephalogram (EEG) electrodes. With respect to the cranial bregma suture, the ground electrode was placed +4.0 mm anterior and +1.0 mm lateral and the two recording electrodes −2.0 mm posterior and ±2.0 mm lateral. The recorded signal was a differential voltage between the two posteriorly placed electrodes. The bare ends of two 0.5 mm stainless steel, PTFE insulated wires (W3 632; W3 Wire International) were implanted in the rhomboideus muscles of the neck as electromyogram (EMG) electrodes. All electrodes were then soldered to a pin connector (M52-040023V2545; Harwin, UK) and the implant sealed using methyl-methacrylate cement (Paladur; Kulzer, Germany).

Animals were placed in a clean cage with fresh bedding, food and water and allowed to recover under a warming lamp (InfraPhil; Philips, Germany). The cage was returned to the housing room when the animals were able to walk. Following instrumentation, animals were housed individually from the time of instrumentation until experimentation, in adjacent transparent cages with holes to allow diffusion of sound and smell. This was done to reduce stress associated with single housing.

Animals were allowed 2 days to recover and were then habituated to wearing a miniature EEG recording device (Neurologger 2A; Evolocus, USA; Anisimov et al. 2014) for 15 minutes each day for 7 days whilst in the home cage. Experimentation was performed on the ninth day after surgery, during the light period (08:00–20:00).

Experimentation

Animals were randomized to be administered either CO_2 or Xe ($n = 6$ animals per group) by an automated randomization function in Microsoft Excel (RAND() function). Briefly, the cage numbers of the animals were written in column A and a series of randomized numbers generated in column B. Column B was then sorted in to descending order using the ZA↓ function thus randomising the corresponding column A. The first six animals in column A were administered CO_2 and the second six were administered Xe treatments. Experiments were performed in order of the cage number with the treatments in randomized order.

Animals were connected to the Neurologger 2A recording device (Anisimov et al. 2014) and then returned to the home cage for 30 minutes. Individual animals were then transferred to a sealed chamber (length: 25 cm, width: 25 cm, height: 15 cm; volume: 9.375 L) and a baseline in 21% oxygen and 79% N_2 recorded for 5 minutes with a gas flow rate of 30% chamber volume $minute^{-1}$. Either CO_2 or Xe was then introduced to the chamber at a concentration of 100% of total gas inflow, using an automated gas mixer (GSM-3; CWE Inc, USA), calibrated to both gases, with a gas flow rate of 30% chamber volume $minute^{-1}$ (2.8 L $minute^{-1}$). The experiment was terminated 3 minutes after cessation of breathing.

Data acquisition

We simultaneously recorded EEG and chamber oxygen concentration (Rapidox 3100; Cambridge Sensotec, UK) at the

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level of the mouse's head (3 cm from the floor) using a calibrated oxygen analyser and recorded digitally with a sampling frequency of 1 Hz on a personal computer. EEG and EMG data were sampled at 200 Hz with a low cut-off (3 dB) filter of 0.5 Hz. At the end of experimentation, data were downloaded from the Neurologger and analysed in Spike2 software (Cambridge Electronic Design, UK). Videos were acquired using a webcam (C250; Logitech, Switzerland).

Behavioural analysis – locomotion speed, jumping and freezing

We used video tracking software (for software development see: de Chaumont et al. 2012) to quantify locomotion as distance travelled. In the software, the number of pixels cm^{-1} were calculated using the known lengths of the sides of the chamber. The software then automatically detected the centre of the mouse, based on contrast to the background. The distance moved by each animal was then calculated at an interval of 1 second to give a unit speed which was normalized by dividing the speed during each second of the gas exposure by the average speed of the last 30 seconds of the baseline. Videos were scored *post hoc* for jumps (defined as vertical rapid jumps where all four paws simultaneously left the ground) and freezing episodes (defined as a transient period of minimum 2 seconds of complete inactivity except that which is necessary for respiration whilst the animal is standing; Mongeluzi et al. 2003) by one of the experimenters. The number of jumps and freezing episodes per animal were determined during the baseline and gas exposure periods.

EEG analysis

EEG data were opened in Spike2 software. From the original waveform, two new waveforms were created using band-pass filters; one in the delta band (1–4.5 Hz) and another in the theta band (5–9 Hz). The band passed waveforms were multiplied by themselves to create a signal of power (μV^2) and the theta:delta ratio was calculated by dividing the theta power signal by the delta power signal, such that a value >1 indicates a predominant theta oscillation and a value <1 indicates a predominant delta oscillation. The theta:delta ratio is considered a measure of vigilance (Vanderwolf 1969; Brankack et al. 2010) and was calculated in 1 second bins and normalized to the last 30 seconds of baseline. Time to loss of motion (LOM) was measured, which correlates with the onset of unconsciousness, considered to occur 2–3 seconds afterwards (Franks 2008; Hwang et al. 2010) and cessation of neocortical activity as determined by isoelectric activity in the EEG (Gent et al. 2018). LOM was determined from the calculated locomotion speed given by the video tracking software and from scoring the videos to determine that animals were not making any purposeful movements. The LOM was considered to have occurred from the time point of cessation of purposeful movement, where locomotion speed was 0 and the animal did not move again for the remainder of the experiment.

Experimenters were masked to the assigned treatment of the animal during data analysis.

Statistical analysis

Statistical analysis was performed in Graphpad Prism 6.01 and is reported as mean ± standard error mean for continuous data and median (range) for discrete data. Data were first analysed for normality of distribution using the Shapiro–Wilk test and we considered p values greater than 0.05 to indicate normal distribution. Data were analysed with Student's t-test for parametric data and Kolmogorov–Smirnov test for non-parametric data. We considered p values less than 0.05 as significant.

Exclusion criteria

Animals were excluded from experimentation if they showed signs of pain assessed by grimace scale score greater than 1 [ranging from 0 (no pain) to 10 (maximum possible pain expression); Langford et al. 2010] or if body weight had not returned to pre-surgery values by 3 days after surgical implantation. Furthermore, animals only began acclimatization to wearing the EEG recording device, if they had resumed feeding, drinking and nesting behaviours. Segments of electrophysiological data were excluded from analysis if they contained movement artefacts, defined as single deflections of more than 400 μV lasting more than 200 ms during movement of the animal, or any segments with a saturated signal, where the recorded amplitude range was ±500 μV.

Results

No mice were excluded from experimentation or analysis; therefore, data are presented for $n = 6$ mice per group. We have included the raw values for each animal in Supplementary Table S1.

Behavioural results – locomotion speed, jumping and freezing

We found that mice significantly increased locomotion during the first 60 seconds of exposure to CO_2 when compared to baseline (+15.6 ± 3.5%; $p = 0.007$; $t = 4.45$; $df = 5$; one sample t-test) but significantly decreased locomotion when exposed to Xe (−25.6 ± 3.5%; $p = 0.002$; $t = 6.27$; $df = 5$; one sample t-test; Fig. 1a). Oxygen concentrations were not significantly different between groups during this time (data not shown). We further found that all mice jumped when exposed to CO_2 [2 (1–4) jumps $minute^{-1}$; $p = 0.002$; Kolmogorov–Smirnov test; Fig. 1b]. No animals jumped during baseline or during exposure to Xe.

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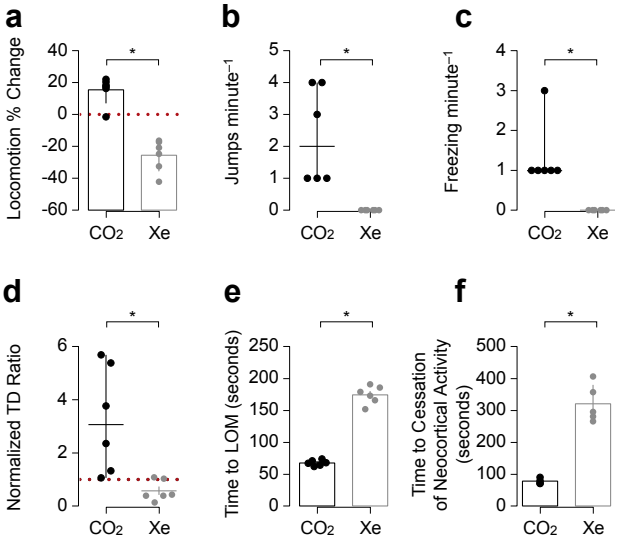

Figure 1 Quantification of behavioural and electrophysiological variables of mice exposed to carbon dioxide (CO_2) or xenon (Xe) for euthanasia. (a) Average percentage change ± standard error of measurement (SEM) in locomotion (distance travelled) for the first 60 seconds of exposure to CO_2 (black) and Xe (grey) compared to baseline (dotted red line; $^*p < 0.0001$; two-sided t-test). (b) Number of jumps per minute during exposure to CO_2 (black) and Xe (grey). Data shown as median (range) ($^*p = 0.002$; Kolmogorov–Smirnov test). Note that no jumps were observed during the baseline. (c) Number of freezing episodes per minute during exposure to CO_2 (black) and Xe (grey). Data shown as median and range ($^*p = 0.002$; Kolmogorov–Smirnov test). Note that no freezing episodes were observed during the baseline. (d) Average percentage change ± SEM in the theta:delta ratio for the first 60 seconds of exposure to CO_2 (black) and Xe (grey) compared to baseline (dotted red line; $^*p = 0.0091$; two-sided *t*-test). (e) Average time to loss of motion (LOM) ± SEM for animals exposed to CO_2 (black) and Xe (grey; $^*p < 0.009$; two-sided t-test). (f) Average time to cessation of neocortical activity ± SEM for animals exposed to CO_2 (black) and Xe (grey; $^*p < 0.009$; two-sided *t*-test). SEM, standard error of the mean; TD, theta:delta. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Given that CO_2 exposure causes a fear response in rodents (Boivin et al. 2017) and humans (Danneman et al. 1997), we tested whether there was a difference in freezing episodes (defined as a period of no muscular activity except for respiration, whilst the animal was standing), an established assay for fear response in mice (Mongeluzi et al. 2003; Ziemann et al. 2009). We found that CO_2 exposure caused a significant increase in freezing compared to baseline (CO_2: 1 (1–2) per minute; Baseline: 0 (0–0); $p = 0.002$; Kolmogorov–Smirnov test; Fig. 1c). No freezing was observed in the Xe group.

EEG and LOM

To test the sedative and hypnotic effects of CO_2 and Xe at the level of the brain, we measured the theta:delta ratio of the EEG, commonly used as proxy for behavioural arousal (Franks 2008; Brankack et al. 2010). We found that compared to baseline, CO_2 caused an increase in the theta:delta ratio (3.3 ± 0.8; $p = 0.04$; $t = 2.77$; $df = 5$; one-sample t-test) and therefore neocortical arousal, whereas Xe caused a decrease in the ratio (0.6 ± 0.4; $p = 0.04$; $t = 2.68$; $df = 5$; one-sample *t*-test; Fig. 1d) consistent with neocortical depression.

CO_2 resulted in LOM after approximately 60 seconds (67.7 ± 1.8 seconds; Fig 1e) quickly followed by cessation of neocortical activity (78.2 ± 3.4 seconds; Fig. 1f). Xe resulted in LOM after nearly 3 minutes (174.5 ± 5.8 seconds; Fig. 1e) with a heavily prolonged time until loss of neocortical activity (321.0 ± 58.8 seconds; Fig. 1f). Cessation of breathing occurred after 345 ± 25 seconds for Xe and 80 ± 2 seconds for CO_2.

Discussion

In this study we found that mice undergoing single gas euthanasia with Xe showed reduced locomotion speed and did not jump or exhibit freezing behaviour. We further found that Xe exposure resulted in a decreased theta:delta ratio of the EEG prior to LOM and that time to LOM and cessation of neocortical activity was prolonged when compared to CO_2.

These findings show that as a single gas euthanasia agent, Xe has several advantages over CO_2, namely it does not appear to cause behavioural excitation or a fear response. For example, in contrast to CO_2, Xe produces decreased locomotion and no freezing episodes, which are considered a fear response (Mongeluzi et al. 2003; Ziemann et al. 2009), or jumping, which may be considered an aversive behaviour (Thomas et al. 2012). Together these results suggest that Xe offers improved welfare conditions over CO_2 as a euthanasia agent.

The hypnotic effects of Xe are likely to make it useful as a euthanasia agent; however, CO_2 also possesses anaesthetic-like qualities. Unlike CO_2, Xe was not associated with a period of excitation of brain activity as measured by EEG. During slow-wave sleep and light general anaesthesia induced by most agents (a notable exception being halothane), the brain produces slower rhythmic waves in the delta range (1–4.5 Hz; Franks 2008). Conversely, during consciousness, faster theta rhythms (5–9 Hz) predominate the EEG. During exploratory behaviours and freezing behaviour, the frequency and power of theta oscillations further increase in the EEG of rodents (Kramis et al. 1975; Sainsbury et al. 1987). The power ratio between the two bands is considered to be an accurate measure of vigilance state and behavioural excitation (Vanderwolf 1969; Brankack et al. 2010). We found increased theta:delta for mice exposed to CO_2 and decreased for Xe. These results are consistent with the increased locomotion and freezing behaviours shown during CO_2 exposure as well as the sedative properties of Xe.

One factor limiting the use of Xe is the cost (ca. 27.00 euro L^{-1} at the time of writing); however, several steps can be taken

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to reduce this. Firstly, because Xe does not produce metabolites when exhaled, the gas can be recaptured for use by a closed-loop recycling system (Rylova & Maze 2019). Secondly, Xe is traditionally extracted from air by fractional distillation using specialized industrial equipment leading to high costs. However, development of selectively porous polycarbonate membranes has allowed the extraction of Xe from air using reverse osmosis and low pressures (ca. 300 kPa; Kwon et al. 2018). Such systems offer the possibility of recovering Xe already in the chamber to be reused for subsequent euthanasia sessions. Given the high concentration of Xe in the chamber at the end of euthanasia (~98%), extraction and recovery of Xe would be significantly cheaper than purchasing Xe obtained from distilled air. Thus, an economically viable Xe euthanasia unit for animal research facilities is possible.

The prolonged time until cessation of neocortical activity of Xe compared to CO_2 makes it less practical as a stand-alone gas agent, especially in high-throughput systems. However, it might be useful as a method of inducing unconsciousness to be followed by a second method of killing such as cervical dislocation, overdose of general anaesthetics or low atmosphere pressure killing, although this remains to be experimentally verified. In this context, it would be well suited to supersede volatile anaesthetics, which produce a variable amount of aversion and are irritants (Moody & Weary 2014; Marquardt et al. 2018) as well as pollutants. Importantly, it would be necessary to determine when loss of consciousness occurs during Xe exposure which cannot be done from our current results. The neuroprotective and cardiac stabilising effects of Xe (Franks 2008; Rylova & Maze 2019) may contribute to the prolonged brain activity, whereas the rapid onset of acidosis caused by CO_2 (Thomas et al. 2012) is likely to hasten dysfunction of neuronal and cardiac tissue.

Recent studies have suggested that 30% volume minute^{-1} may not be an optimal flow rate for CO_2 euthanasia of mice (Moody et al. 2014; Boivin et al. 2016; Powell et al. 2016; Detotto et al. 2019). One study measured similar variables as this investigation and revealed that CO_2 at 80% volume minute^{-1} could be considered less aversive than when used at 30% volume minute^{-1}; however, it was still more aversive than use of nitrogen (Detotto et al. 2019). Therefore, further comparison of Xe and CO_2 at faster displacement rates may be warranted. As a potent analgesic (Rylova & Maze 2019), Xe may have further advantages for euthanasia of mice which are injured or otherwise unhealthy, especially as CO_2 is considered likely to cause pain in rodents as in humans (Danneman et al. 1997; Hawkins et al. 2016; Boivin et al. 2017). However, this remains to be studied. Although experimenters were masked to the treatment whilst analysing the data, the large differences between groups may have allowed experimenters to guess the treatment. Note, however, that locomotion analysis was entirely automated.

Conclusion

The use of Xe reduces behavioural and electrophysiological excitation in mice undergoing gas euthanasia compared to CO_2 and therefore is a strong candidate to offer a refinement to animal welfare. The commercial production of a gas chamber formulated to recapture and reuse Xe gas is required to make it financially viable for wide-scale use in laboratories, zoos and industry.

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Authors' contributions

TCG, SI, MW and RB-W: conception and design of the study. ALV: provided Neurologgers and technical assistance with the experimental setup. TCG: conducted all experiments. TCG, ALV and CD: data analysis. All authors wrote and edited the manuscript.

Conflict of interest statement

Authors declare no conflict of interest.

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Supporting Information

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