Euthanasia using gaseous agents in laboratory rodents

A M Valentin\(^1,2,*\), S R Guedes\(^3,*\), A M Pereira\(^3,*\)
and L M Antunes\(^1,2,3\)

Abstract
Several questions have been raised in recent years about the euthanasia of laboratory rodents. Euthanasia using inhaled agents is considered to be a suitable aesthetic method for use with a large number of animals simultaneously. Nevertheless, its aversive potential has been criticized in terms of animal welfare. The data available regarding the use of carbon dioxide (CO\(_2\)), inhaled anaesthetics (such as isoflurane, sevoflurane, halothane and enflurane), as well as carbon monoxide and inert gases are discussed throughout this review. Euthanasia of fetuses and neonates is also addressed. A table listing currently available information to ease access to data regarding euthanasia techniques using gaseous agents in laboratory rodents was compiled. Regarding better animal welfare, there is currently insufficient evidence to advocate banning or replacing CO\(_2\) in the euthanasia of rodents; however, there are hints that alternative gases are more humane. The exposure to a volatile anaesthetic gas before loss of consciousness has been proposed by some scientific studies to minimize distress; however, the impact of such a measure is not clear. Areas of inconsistency within the euthanasia literature have been highlighted recently and stem from insufficient knowledge, especially regarding the advantages of the administration of isoflurane or sevoflurane over CO\(_2\), or other methods, before loss of consciousness. Alternative methods to minimize distress may include the development of techniques aimed at inducing death in the home cage of animals. Scientific outcomes have to be considered before choosing the most suitable euthanasia method to obtain the best results and accomplish the 3Rs (replacement, reduction and refinement).

Keywords
euthanasia, laboratory rodents, welfare, carbon dioxide, volatile agents

The word ‘euthanasia’ is derived from the Greek words ‘eu’ meaning good and ‘thanatos’ meaning death. A ‘good death’ would be one that occurs with minimal pain and distress. In the case of animals, the word ‘euthanasia’ is often substituted by terms such as ‘humane death’ or ‘humane killing’. For simplicity, the term ‘euthanasia’ will be used throughout this review.

Laboratory rodents are euthanized for various reasons: to provide tissues for scientific purposes, at the end of an experiment, when adverse effects (pain, distress, suffering, etc.) become excessive, and when animals become unwanted stock.\(^1\)

Killing by gas is one of the techniques used for rodent euthanasia. It has advantages for both operators and animals. However, the onset of loss of consciousness (LOC) may be delayed compared with other techniques; thus, the question arises regarding the extent to

\(^1\)Laboratory Animal Science, Institute of Molecular and Cell Biology (IBMC), University of Porto, Rua do Campo Alegre, Porto, Portugal
\(^2\)Institute for Research and Innovation in Health (I3S), University of Porto, Rua Alfredo Allen, Porto, Portugal
\(^3\)Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB) and Veterinary Sciences Department, University of Trás-os-Montes and Alto Douro (UTAD), Quinta de Prados, Vila Real, Portugal

*These authors contributed equally to this review.

Corresponding author: Luís M Antunes, University of Trás-os-Montes and Alto Douro, Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB) and Veterinary Sciences Department, University of Trás-os-Montes and Alto Douro (UTAD), Quinta de Prados, 5001-861 Vila Real, Portugal. Email: lantunes@utad.pt
which the exposure to gas induces distress, or even pain. Carbon dioxide (CO₂) is widely used for the euthanasia of rodents; however, concerns that CO₂ may induce pain or distress have emerged. Ongoing discussions regarding this controversial matter suffer from a lack of updated reviews on the effects of CO₂. Inert gases and inhaled anaesthetics have been indicated as better options for inducing unconsciousness prior to the administration of CO₂, although time to death is largely delayed when using volatile gas anaesthetics. Coenen's suggestion of minimizing pain and distress rather than enhancing a fast LOC⁵ has been addressed and supported by many researchers. This review summarizes the published works in which gases have been used as euthanasia agents for rodents. A table listing currently available information to ease access to data regarding euthanasia methods using gaseous agents in laboratory rodents was compiled.

Euthanasia methods

It is interesting to note that the most suitable euthanasia techniques that use chemical agents have much in common with the best practice in anaesthesia. Good anaesthesia practice is based on the simultaneous existence of three reversible components: unconsciousness, analgesia and muscle relaxation. The best euthanasia techniques aim for the induction of rapid unconsciousness, followed by fast death, which can be effectively achieved by a physical method. Therefore, the period prior to LOC is a main concern in euthanasia, as animals may experience distress, anxiety, apprehension and pain, which may be reduced with proper handling of animals before euthanasia. The use of home cages, consistent group compositions (cage mates rather than unknown animals) and the performance of euthanasia in a room with no signs/odours of blood are positive examples of such handling conditions. Operator safety and aesthetics of the method should also be considered. There is a risk that the operator may feel an emotional burden and refuse to perform the euthanasia, not because it is inhumane for the animal, but because it is not aesthetic.

The most commonly accepted techniques for the euthanasia of adult rodents are divided into chemical and physical methods. The latter have an impact on their brains and result in an immediate LOC which reduces distress for the animals. However, physical methods require animal handling and restraint, which induce distress.⁶ Cervical dislocation, cerebral concussion, decapitation and microwave irradiation using appropriate equipment are accepted methods under certain conditions.⁷ Physical methods have the disadvantage of requiring training, which increases the possibility of errors during the killing process, thus failing the achievement of a rapid LOC. Physical methods are also considered to be time-consuming and anaesthetic techniques.⁸ However if performed well, they may provide a fast and likely humane death; though their use for the euthanasia of a large number of animals is limited.

Chemical methods include inhalational or injectable agents. Barbiturates and sodium pentobarbitone are the most commonly used and accepted agents for euthanasia. Injectable anaesthetic agents may be used for euthanasia when employed at doses far higher than those used for anaesthesia, leading to overdose.⁴ The administration route (i.e. intraperitoneal, intravenous or subcutaneous) should be considered when selecting the dosage. The administration itself is a source of distress, as it involves withdrawing the animal from the home cage, followed by handling and restraint to perform the injection.⁴ Although injectable anaesthetic agents are an aesthetical method, their use in mass killing is limited and they have the disadvantage of requiring expertise, careful handling and proper restraint.

Additional information can be found in reports from several groups that have provided recommendations for euthanasia of laboratory animals in Europe⁵ and in the USA.⁶,⁷ Inhalational gases, such as halogenated anaesthetics, inert gases (argon [Ar] and nitrogen [N₂]) and CO₂, have been suggested as euthanasia agents. Their use requires placing the rodents inside a gas chamber that should then be filled with the inhalational substance. The volume and concentration of gases administered are controlled by a flowmeter and a calibrated vaporizer. Waste anaesthetic gas should be scavenged to protect the operator.⁸ The use of inhalational agents requires equipment, which may be a disadvantage; however, it enables mass killing with good animal welfare results. Furthermore, it requires minimal animal handling compared with the physical and other chemical methods described above. The importance of inhalational agents in euthanasia refinement is further discussed below.

Carbon dioxide

Carbon dioxide has been used to euthanize groups of rodents in specially designed chambers. Among the advantages argued for continuing the use of CO₂ alone is that it is a practical and effective technique associated with a good balance of costs and benefits. However, evidence from human studies has shown that the inhalation of CO₂ at different concentrations causes pain and/or distress.⁹ According to Leach, in humans, rats and cats, most nociceptors are activated at a concentration of approximately 40% of CO₂.¹⁰ Moreover, in mice, CO₂ at a concentration of 10% has been shown to evoke fear behaviour via the expression of freezing
and activation of the limbic structures, including the amygdala. Inhalation of CO₂ causes respiratory acidosis and produces a reversible anaesthetic state by decreasing the intracellular pH. The physiological effects/actions of CO₂ are revised elsewhere. To clarify CO₂ suitability for rodent euthanasia, several studies have been performed in which the use of CO₂ was addressed.

During CO₂ exposure, the time to the achievement of unconsciousness depends on the concentration, chamber volume and flow rate at which the gas is delivered. In rats, unconsciousness is achieved at CO₂ concentrations of 30%-40%14; however, no such data are available for mice. Other studies of mammals and birds have shown that LOC is achieved at higher CO₂ concentrations (>40%), whereas it should be above 70% for killing.11 Even though euthanasia using 100% CO₂ pre-filled chambers induces a rapid loss of cortical brain activity within 39s in rats15 and 30s in mice,16 it is considered unacceptable1 because of the significant pain inflicted until LOC. Placing the animals in a chamber containing room air followed by a gradual fill of CO₂ is a recommended and acceptable procedure.9 In this sense, the determination of the gas flow rate is critical for the humane use of CO₂.4,16,17 Table 1 shows the information available from euthanasia studies that have been performed using several flow rates.

It is common to assess the subjective experiences of animals in order to answer scientific questions.18 The assessment of animal welfare is usually done by studying animals' emotions, via an analysis of their behaviour and decisions when facing a certain environment. Aversion is a negative emotional response that is described by humans, for example, when experiencing dyspnoea.19,20 Several studies aimed at assessing gas aversion used approach-avoidance paradigms, which consist in providing a goal that is both appealing (presence of rewards) and unappealing (presence of gas). Thus, the animal has to make a compromise between the two stimuli, depending on the degree of aversion or motivation of each. The results of approach-avoidance tests indicate that the latency to leave the CO₂ chamber is lower compared with the time to LOC, with flow rates ranging from 3% to 27% V/min.21-23 Some studies showed that rats receiving CO₂ at a flow rate of 17% V/min exhibited signs of avoidance after one minute of exposure,24 whereas gradual displacements of 14%23 and 10% V/min25 appeared to be less aversive. In addition, it has been shown that, in rats, the administration of CO₂ at a flow rate of 17.25% V/min led to the achievement of recumbency after 106s. At that stage, the concentration of CO₂ inside the chamber was approximately 33%, which is under the pain threshold for the majority of the nociceptors located in the nasal mucosa. This suggests that the animals did not feel pain related to the procedure, although there were signs of distress, such as increases in the frequency of rearing, escape behaviour, vocalizations and time spent with the nose contacting the chamber lid.26 Makowska reported rats escaping from an environment containing CO₂ when its concentration reached 13.5%-18.2%.22 Another study performed by Niell reported similar results: rats left the gas chamber when CO₂ concentrations reached an average of 18.4%.24 These studies suggest that, even during gradual-fill procedures using low concentrations, aversion arising from mechanisms other than pain may cause distress. Animals placed inside a chamber with rising concentrations of CO₂ may find it aversive and may experience dyspnoea and 'air hunger', which is known to be very distressing in humans.27 Other adverse effects documented in regard to CO₂ exposure include a gasping/forced breathing pattern in rats29 and increase in dyspnoea scores in mice.28 In contrast to the use of low flow rates, a recent study has advocated that a flow rate of 50% V/min with a concentration of CO₂ inside the chamber below 40% reduces dyspnoea onset and insensibility and, therefore, stressful events.29

The addition of nitrous oxide (N₂O) or oxygen (O₂) has been proposed to improve CO₂ exposure, by trying to reduce the onset of unconsciousness and dyspnoea, respectively. N₂O works as a carrying gas for CO₂; this second-gas effect shortens the time to LOC by 10% compared with the use of CO₂ alone.30 O₂ can be added to prevent hypoxia, thus reducing distress. At high concentrations (30%), O₂ causes hyperoxia, which reduces the ventilator and dyspnoea responses to hypcapnia.2,21,32 However, studies have shown that the addition of O₂ to CO₂ causes only a slight reduction in CO₂ aversion in the gradual-fill procedure,33 or that this procedure results in the same degree of aversion.34 Moreover, it has been shown that O₂ supplementation may provoke lung haemorrhage before LOC in mice.35

Although a consensus regarding the use of CO₂ has not been reached, recommendations can be made based on data from the studies presented above. The use of compressed CO₂ in cylinders combined with calibrated flowmeters, which allows the precise regulation of the inflow to the chamber, is recommended. The gas flow should be constant at a rate of 15% to 35% V/min, and it may be increased after LOC, to speed up death. Users should wait a minimum of 49s before increasing the flow rate of CO₂.36 The gas flow should be maintained for at least one minute after apparent clinical death.4

Volatile anaesthetics

The use of inhaled anaesthetics to induce unconsciousness has been suggested as a more humane technique
Table 1. Summary of euthanasia studies in mice and rats with several agents and flow rates.

<table>
<thead>
<tr>
<th>Gas</th>
<th>Reference</th>
<th>Specie &amp; strain</th>
<th>[Concentration]/flow rate (V/min)</th>
<th>[Estimated concentration]/time to LOC (s)</th>
<th>[Estimated concentration]/time to death (s)</th>
<th>Parameters measured</th>
<th>Conclusions</th>
<th>Comments/recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>Niel 2008⁴⁶</td>
<td>M Wistar rats</td>
<td>GF 17%</td>
<td>NA</td>
<td>NA</td>
<td>Approach–avoidance (food rewards)</td>
<td>Rats showed avoidance and escape responses to CO₂</td>
<td>Re-exposure to CO₂ does not cause habituation.</td>
</tr>
<tr>
<td></td>
<td>Niel 2008⁴⁶</td>
<td>M Wistar rats</td>
<td>GF 3%; 7%; 14%; 27%</td>
<td>NA</td>
<td>NA</td>
<td>Approach–avoidance (food rewards)</td>
<td>A flow rate of 14% V/min is optimal in terms of initial aversion; after this initial aversion (forced exposure) it induces distress with all flow rates.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moody 2014⁴⁸</td>
<td>F albino C57BL/6J-Tyr mice</td>
<td>GF 20%*</td>
<td>119.2 ± 10</td>
<td>NA</td>
<td>Dyspnoea onset; LOC; LOPWR.</td>
<td>Gradual-fill with higher flow rates reduce period from onset of dyspnoea until LOC; thus it is a refinement.</td>
<td>When using higher flow rates, a gas holding technique should be used to ensure that painful CO₂ concentrations (&gt;40%) are not reached until insensibility occurs.</td>
</tr>
<tr>
<td></td>
<td>Wong 2013⁴²</td>
<td>M Sprague Dawley rats</td>
<td>24% CO₂ [5%] Iso 100% O₂</td>
<td>NA</td>
<td>NA</td>
<td>Aversion behaviour (dark/light compartments).</td>
<td>Iso is a refinement over CO₂ exposure; though its re-exposure is as aversive as CO₂.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Valentine 2012⁷⁷</td>
<td>F CD1 mice</td>
<td>20% CO₂ 100% CO₂ [5%] Iso in 20% O₂</td>
<td>NA</td>
<td>NA</td>
<td>c-fos in the brain; ACTH; corticosterone; behaviour.</td>
<td>20% V/min CO₂ alone is the most humane method of euthanasia for mice.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moody 2014⁴₅</td>
<td>M C57BL/6J mice</td>
<td>6% CO₂ 20% Iso [5%] in 40% O₂</td>
<td>NA</td>
<td>NA</td>
<td>Aversion behaviour (dark/light compartments).</td>
<td>Isoflurane is an alternative to CO₂ exposure; however it should be avoided if recent exposure occurred.</td>
<td>It is suggested to use [5%] Iso delivered at a rate of 40% V/min to induce LOC.</td>
</tr>
<tr>
<td></td>
<td>Moody 2015⁵⁶</td>
<td>M C57BL/6J mice</td>
<td>20% CO₂*</td>
<td>15.7 ± 10.9</td>
<td>114.8 ± 5.8</td>
<td>Time to recumbency:</td>
<td>Isoflurane</td>
<td></td>
</tr>
</tbody>
</table>

*After LOPWR, a flow rate of 60% V/min was delivered to speed up death.
<table>
<thead>
<tr>
<th>Gas</th>
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<th>Species &amp; strain</th>
<th>[Concentration]/flow rate (V/min)</th>
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<th>Parameters measured</th>
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</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>Makowska</td>
<td>M CD-1 mice</td>
<td>[5%] Iso in 17% O₂*</td>
<td>10–70% CO₂</td>
<td>NA</td>
<td>NA</td>
<td>Approach-avoidance [food rewards].</td>
<td>is a humane alternative to CO₂ exposure. This table as LOC measure. It is recommended to wait a minimum of 77s after the appearance of recumbency before switching to a high flow rate of CO₂.</td>
</tr>
<tr>
<td>Isoc</td>
<td>2089³⁶</td>
<td></td>
<td>GF 66–160% Ar</td>
<td></td>
<td>93 ± 9</td>
<td>Re-exposure: 68 ± 5</td>
<td></td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Halo</td>
<td></td>
<td></td>
<td>GF CO (8%) in air</td>
<td>[5.1 ± 0.4%]/39 ± 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ar</td>
<td></td>
<td></td>
<td>GF [3%], [5%,]</td>
<td></td>
<td>68 ± 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td></td>
<td></td>
<td>Ga in 70% O₂</td>
<td></td>
<td>64 ± 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>Niel 2006²⁹</td>
<td>M Sprague Dawley rats</td>
<td>GF 17.25% CO₂</td>
<td>[33%/106 ± 12</td>
<td>[80%]/443 ± 14</td>
<td>Behaviour (activity, rearing, nose to lid, escape behaviours, vocalization).</td>
<td>GF CO₂ euthanasia causes distress in rats, and the concentrations involved suggest that this distress is due to dyspnoea rather than pain. Nose to lid slightly</td>
<td></td>
</tr>
<tr>
<td>Ar</td>
<td></td>
<td></td>
<td>GF 17.25% Ar</td>
<td>&gt; 105</td>
<td>NA</td>
<td></td>
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</table>

(continued)
<table>
<thead>
<tr>
<th>Gas</th>
<th>Reference</th>
<th>Specie &amp; strain</th>
<th>[Concentration]/flow rate [L/min]</th>
<th>[Estimated concentration]/time to LOC [s]</th>
<th>[Estimated concentration]/time to death [s]</th>
<th>Parameters measured</th>
<th>Conclusions</th>
<th>Comments/recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Niel 2007&lt;sup&gt;27&lt;/sup&gt;</td>
<td>M Wistar rats</td>
<td>PF 5% CO&lt;sub&gt;2&lt;/sub&gt;, PF [10%] CO&lt;sub&gt;2&lt;/sub&gt;, PF [15%] CO&lt;sub&gt;2&lt;/sub&gt;, PF [20%] CO&lt;sub&gt;2&lt;/sub&gt;, SF CO&lt;sub&gt;2&lt;/sub&gt; 17%, PF [90%]</td>
<td>NA</td>
<td>NA</td>
<td>Approach–avoidance (food rewards).</td>
<td>Rats tolerated extended exposure to 5% and 10% CO&lt;sub&gt;2&lt;/sub&gt;, but this was not sufficient to cause unconsciousness.</td>
<td></td>
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</tr>
<tr>
<td>Burkholder 2010&lt;sup&gt;20&lt;/sup&gt;</td>
<td>M Sprague Dawley rats</td>
<td>SF 10% CO&lt;sub&gt;2&lt;/sub&gt;, SF 50% Ar [21% ± 2%] 156 ± 12</td>
<td>[100%] 138 ± 41</td>
<td>NA</td>
<td>Physiological parameters (temperature, heart rate, and activity); behaviour; Pathologic examinations (lungs, nares, brain, adrenals).</td>
<td>Ar and CO&lt;sub&gt;2&lt;/sub&gt; induce stress; however CO&lt;sub&gt;2&lt;/sub&gt; is preferable to Ar.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;/N&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Thomas 2012&lt;sup&gt;21&lt;/sup&gt;</td>
<td>M, F C57BL/6J mice</td>
<td>SF 20% CO&lt;sub&gt;2&lt;/sub&gt;, SF 20% CO&lt;sub&gt;2&lt;/sub&gt;/40% N&lt;sub&gt;2&lt;/sub&gt;O, SF 20% CO&lt;sub&gt;2&lt;/sub&gt;/60% N&lt;sub&gt;2&lt;/sub&gt;O [26%] CO&lt;sub&gt;2&lt;/sub&gt; 108.7 ± 6.4</td>
<td>[20%] CO&lt;sub&gt;2&lt;/sub&gt; 96.7 ± 7.9</td>
<td>[27%] CO&lt;sub&gt;2&lt;/sub&gt; 112.46 ± 6.9</td>
<td>Time to LOC; blood analysis (pH, arterial partial pressure of oxygen, lactate); behaviour (rearing, jumping).</td>
<td>The addition of N&lt;sub&gt;2&lt;/sub&gt;O is a refinement since it shortens the time to LOC by 10% without triggering any obvious increase in behavioural signs of aversion or distress.</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>Makowska, 2009&lt;sup&gt;27&lt;/sup&gt;</td>
<td>M Wistar rats</td>
<td>GF 3%* [5% ± 0.6%]/104 ± 24</td>
<td>GF 6%* [5.5% ± 0.5%]/64 ± 5</td>
<td>GF 7%** [5.1% ± 0.6%]/53 ± 7</td>
<td>Approach–avoidance (food rewards).</td>
<td>All animals exposed to CO exhibited convulsions after they were recumbent, but it is not clear if they were unconscious when this occurred.</td>
<td></td>
</tr>
</tbody>
</table>

Each flow rate was compared to air delivered at a flow rate of: *63%: **78%.
### Table 1. Continued

<table>
<thead>
<tr>
<th>Gas</th>
<th>Reference</th>
<th>Specie &amp; strain</th>
<th>[Concentration] /flow rate [V/min]</th>
<th>[Estimated concentration] / time to LOC [s]</th>
<th>[Estimated concentration] / time to death [s]</th>
<th>Parameters measured</th>
<th>Conclusions</th>
<th>Comments/ recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar</td>
<td>Makwoska 2008</td>
<td>M Wistar rats</td>
<td>GF 40–120%*</td>
<td>NA</td>
<td>NA</td>
<td>Approach–avoidance (food rewards).</td>
<td>Ar is not a suitable alternative to CO₂ for the euthanasia of rats.</td>
<td>Sound or air currents associated with gas entry were not the cause of aversion.</td>
</tr>
<tr>
<td>Halo Iso</td>
<td>Each flow rate was compared to air delivered at a flow rate of: *63%; **120–239%.</td>
<td></td>
<td>Halo* [2%]</td>
<td>158 ± 55</td>
<td>NA</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Makwoska 2009</td>
<td>M Wistar rats</td>
<td></td>
<td>[2.5%]</td>
<td>138 ± 7</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>[3.25%]</td>
<td>114 ± 3</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>[5%]</td>
<td>88 ± 16</td>
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<tr>
<td></td>
<td>Iso* [1.25%]</td>
<td></td>
<td>153 ± 14</td>
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<tr>
<td></td>
<td>[2%]</td>
<td></td>
<td>135 ± 14</td>
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<tr>
<td></td>
<td>[2.5%]</td>
<td></td>
<td>111 ± 8</td>
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<tr>
<td></td>
<td>[3.75%]</td>
<td></td>
<td>79 ± 18</td>
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</tr>
<tr>
<td>Iso Sevo</td>
<td>Bertolus 2015</td>
<td>M Sprague Dawley rats</td>
<td>[*89 IQR = 81–91]**65.5 [IQR = 78–88]</td>
<td>*80 IQR = 78–80**79 [IQR = 69–82]</td>
<td>NA</td>
<td>*Aversion–avoidance; **Approach–avoidance.</td>
<td>GF Sevo and Iso are similarly aversive: they are a 'humane' alternative to CO₂ exposure, if no recent exposure to those anaesthetics occurred.</td>
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</tbody>
</table>

for euthanasia compared with CO₂, as these anaesthetics may be less aversive and do not inflict pain. Nonetheless, there is no consensus regarding whether the level of distress during induction is lower with these anaesthetics than it is with CO₂. In addition, to protect personnel from exposure to these anaesthetic gases, the use of scavenge systems for the elimination of waste gases is mandatory, which have health and safety implications. Air or O₂ should be provided during induction when using volatile anaesthetic agents to avoid hypoxia. As agents have to reach a certain alveolar concentration before they become effective, this method takes some time, during which animals may suffer distress. Animals may struggle and become anxious during the induction of anaesthesia because drugs may irritate and can cause excitement between the beginning of the procedure and LOC. The expression of distress caused by anaesthetic properties (odour, hypoxia and hypercarbia) may be hard to differentiate from the expected excitatory phase of anaesthesia induction, when animals exhibit an increase in their activity and speed of movements. Excitation is also observed when injectable anaesthesia is administered, but not jumping, indicating a behaviour that is more in line with the distress induced by volatile agents.

Isoflurane is an anaesthetic that is commonly used in most laboratories, is less soluble than halothane and generally induces anaesthesia more rapidly. However, it has a slightly pungent odour and animals often hold their breath, thus delaying the onset of LOC and increasing levels of distress. As halothane has a lower minimum alveolar concentration (MAC) and higher potency compared with isoflurane, a greater quantity of isoflurane may be needed to kill an animal. Although isoflurane is acceptable as a euthanasia agent, halothane is less irritating and its odour is not as intense, thus causing less disturbance in the respiratory airways, at least when used with humans. A great disadvantage of halothane is the difficulty of obtaining it in the market currently.

Leach and colleagues have shown that the level of aversion is proportional to the increase in isoflurane and halothane concentrations, and have recommended the use of a medium concentration of halothane for rats and halothane or enfurane for mice. A study performed by Makowska and Weary using approach-avoidance testing has shown that most rats reach a state of conscious sedation, before choosing to leave a cage that is gradually filled with isoflurane or halothane; without, however, finding any differences between the two anaesthetics in this respect. Another study performed by the same group compared several inhaled agents and showed that mice took more time to leave an environment containing isoflurane than they did one containing halothane. Moreover, two mice remained in the isoflurane chamber until recumbency, suggesting that isoflurane may be an alternative to CO₂. The different outcomes reported by the Leach and Makowska studies may be explained by the Leach group using pre-filled chambers which induce an unpleasant contact with high concentrations of isoflurane, known for its more pungent odour than halothane. Moreover, Leach did not use rewards, compared with the studies by Makowska, which may have affected the latency of the animals to leave the gas chamber measured in these studies. Thus, in the presence of food rewards, two animals achieved LOC in the isoflurane chamber, because the potential aversion induced by isoflurane was lower than the motivation to eat the reward.

There are, in fact, contradictory reports regarding isoflurane, probably because of different approaches and interpretations. Valentine et al. reported that the use of isoflurane induction prior to CO₂ euthanasia increased c-fos expression considerably in the brain; c-fos has been described as a neural marker of pain and is related with distress. Agitation scores were also higher in the presence of isoflurane compared with a recommended flow rate of CO₂ (20 V/min); without, however, alterations in plasma ACTH and corticosterone levels. Different outcomes were reported by a study that compared the effect of isoflurane and a CO₂/O₂ mixture on corticosterone in rats during serial blood collections. It was indicated that, after one hour, a significantly lower corticosterone concentration was achieved when isoflurane anaesthesia was used compared with CO₂. Lower concentrations of corticosterone suggest that animals experienced less distress prior to LOC. Despite these different results, several authors agree that isoflurane represents a refinement over exposure to CO₂ alone for euthanasia. However, this only applies if no previous exposure to the anaesthetic has occurred, as re-exposure to isoflurane and sevoflurane induced an aversion behaviour in rats, based on a decrease in the number of animals that stayed, or took longer to leave, the gas compartment.

Sevoflurane is less soluble than halothane and does not have an objectionable odour, but it is less potent than isoflurane or halothane and has a lower vapour pressure. Recent studies performed by our group suggested that mice have a low degree of aversion to sevoflurane, as they spent more time in the sevoflurane chamber, where food rewards were presented, than they did in the chamber that was filled with environmental air; which did not occur in the cases using CO₂ or isoflurane. In fact, mice spent less time in the isoflurane chamber with food rewards than they did in the chamber with air, which indicated an aversion to this gas. By contrast, a recent study suggested that rats find sevoflurane and isoflurane to be similarly aversive, probably because this study had a higher flow rate.
than ours.\textsuperscript{45} Studies performed using different gas concentrations and flow rates may be required to understand the smaller differences between these two halogenated gases. Despite the information presented above, volatile anaesthetic gases induce some degree of aversion in rodents. It would be of great interest to obtain more information about the advantages of using newer inhaled anaesthetics, such as sevoflurane, desflurane or enflurane, to complete and consolidate the information presented previously by Leach and colleagues.\textsuperscript{39}

**Inert gases**

N\textsubscript{2} and Ar are inert colourless and odourless gases that have no inflammable or explosive properties. For euthanasia, a container is usually pre-filled with a minimum of 98% (volume) of N\textsubscript{2} or Ar, to induce death by hypoxaemia. As N\textsubscript{2} is lighter than air, specialized equipment is needed for its administration; Ar is denser than air and is easily contained. Studies performed by Leach et al. have found rodents showing less aversion to Ar compared with CO\textsubscript{2}, which may have been attributable to its odourless, tasteless and inert properties. However, the animals could enter and leave the chamber at will, so LOC was never achieved.\textsuperscript{34,39}

Previous approach–avoidance tests with food rewards have shown that rats are able to detect decreases in O\textsubscript{2} concentration almost immediately after the onset of Ar delivery, and that they stop eating when hypoxia becomes sufficiently aversive.\textsuperscript{46} In another study, rats refused to enter a chamber containing Ar, and none of them ate food rewards, which highlighted the aversive properties of Ar.\textsuperscript{24} It is possible that cognitive impairment, dizziness and visual changes associated with low O\textsubscript{2} are the causes of the aversion of rats toward Ar.\textsuperscript{46} The physiological effects of hypoxia become aversive at approximately 7.7% of O\textsubscript{2}; however, this O\textsubscript{2} concentration is too high to cause unconsciousness or death.\textsuperscript{46} Thus, according to these findings, an effective O\textsubscript{2} concentration for Ar euthanasia would always be aversive. In fact, studies have shown that Ar induces back arching with an open mouth in rats, i.e. abnormal gasping.\textsuperscript{25} Rats exposed to Ar and N\textsubscript{2} exhibited muscle spasms and were hyperreflexic to touch and handling when they appeared to be unconscious. Prolonged tachycardia following short-term exposure is also associated with Ar.\textsuperscript{47} Similarly, N\textsubscript{2} at approximately 100% is not very effective, as it is slow to produce unconsciousness and death and induces hyperreflexia during short-term exposure.\textsuperscript{47}

There is still no consensus in the guidelines and legislation regarding the use of Ar or N\textsubscript{2}. According to the American Veterinary Medical Association guidelines on euthanasia, these techniques are acceptable conditionally, and so should only be used if O\textsubscript{2} concentrations <2% can be achieved rapidly and animals are heavily sedated or anesthetized.\textsuperscript{4} Conversely, this method is accepted in the European Directive.\textsuperscript{48} Either way, the welfare implications of using inert gases regarding asphyxiation have to be considered, as these can cause alveolar haemorrhage and the displacement of O\textsubscript{2}, thus inducing hypoxaemia before LOC.

**Carbon monoxide**

CO binds irreversibly to haemoglobin to form carboxyhaemoglobin and blocks the uptake of O\textsubscript{2} by erythrocytes, finally leading to unconsciousness and fatal hypoxaemia.\textsuperscript{4} Rodents should be placed inside a container pre-filled with at least 6% CO (volume). CO at a concentration above 10% is highly explosive and toxic to operators; hence, it must only be used with appropriate gas scavenging in place. Commercially compressed CO is preferable to CO generated by other means because it is not contaminated with other gases and because it minimizes the problems associated with adjusting the concentration, cooling of the gas and equipment maintenance. In addition, personnel must be instructed thoroughly regarding the use of CO, to understand its hazards and limitations.\textsuperscript{4}

Concerning aversion in rats, a study showed that intermediate and high flows of CO provoked recumbency in two animals in a situation in which they could have escaped to another cage. However, these rats exhibited convulsions and it was not clear if they were completely unconscious when this occurred. Other animals showed behavioural changes, such as agitation, which suggests an aversion to CO exposure.\textsuperscript{49} Therefore, there is no clear evidence that CO can be used as a refinement in euthanasia. Furthermore, it can be dangerous for the operator.

**Euthanasia of fetuses and newborn animals**

There are not much new data regarding recommendations for the euthanasia of fetuses and newborn animals. Nevertheless, two factors must be taken into account when choosing a method of euthanasia for the fetus or newborn animal: they are resistant to hypoxia and they metabolize drugs slowly.

The specificity of euthanasia recommendations for fetuses is based on their neuronal development. A fetus of up to 15 days is believed to have minimal pain perception because of a non-functional cerebral cortex and subcortical brain structure.\textsuperscript{4,50} Thus, killing the mother is sufficient to cause rapid death of the fetus, as it is non-viable at this stage of development.\textsuperscript{51} Rats
and mice over 15 days after conception perceive pain; therefore, humane methods of euthanasia should be chosen in these cases. Skillful injections of chemical anaesthetics, decapitations with surgical scissors, or cervical dislocations are acceptable. Inhaled anaesthetics or CO₂ can be used; however, they require a long time of exposure, with the risk of distress. When fetuses are not used for further experiments, euthanizing the mother should ensure cerebral anoxia and minimal uterine disruption, for example by using CO₂ euthanasia followed by cervical dislocation.

In the case of neonatal rodents, recent evidence has confirmed that there is a huge difference in the time until death in the presence of CO₂ compared with adult rodents because of the resistance of neonates to hypoxia. In rats, the time to death decreased steadily with increasing age, with 100% of the rats being euthanized after 5 min of CO₂ exposure at 10 days of age. The time required for 100% of mortality decreased by 3 min for each day of age between days 0 and 10. Methods used for the euthanasia of neonatal animals in the presence of CO₂ must, therefore, be substantially modified from those employed for adults. Euthanasia techniques that are acceptable in neonates are the injection of chemical anaesthetics (e.g. pentobarbital), cervical dislocation or decapitation. In these rodents, decapitation can be performed using sharp knives or scissors. The bilateral pneumothorax method may be used as a secondary method to ensure death in anaesthetized newborns. Immersion in liquid N₂ is used only if preceded by anaesthesia, but it is considered acceptable if fetuses or neonates do not have fur and weigh less than 4 g. The guidelines for/acceptance of this technique may vary between countries: e.g. in Switzerland, rapid freezing is allowed without anaesthesia in fetuses and newborn animals below 10 g of body weight. The most suitable euthanasia method for rodents may also vary depending on the strain.

**Equipment**

The most common euthanasia methods involve using anaesthesia equipment, which include anaesthetic chambers, vaporizers, scavenging systems and, obviously, gases. Although the anaesthesia equipment available in laboratories may be practical for euthanizing a few animals, it may not be adequate for mass killing on a daily basis. The choice of equipment for the euthanasia of rodents should take into account both animal welfare and personnel safety, which is achieved by minimizing human occupational exposure to the agents. Although recommendations vary between countries, the concentrations of halothane, enflurane and isoflurane to which humans are exposed should be less than 2 ppm, and less than 25 ppm for N₂O. Hence, it is of the utmost importance to perform the procedure using adequate equipment with a well-designed waste-gas scavenging system to collect, remove and dispose of the gases.

From a welfare point of view, equipment should be able to reproduce a correct adjustment of the flows, according to the recommendations for each gas, and be as silent as possible, as noise and the stream of inflowing gas may induce distress. It is known that placement in a gas chamber causes physiological and behavioural changes in rats, which represent evidence of distress. Therefore, this issue raises an interest regarding developing solutions that avoid the need to handle or move the animals. In response to this concern, new products have been engineered that permit euthanasia of animals in their home cages, by developing mobile or fixed euthanasia stations and automated devices connected to lids that are adapted to the commonly used Makrolon cages. This equipment runs different cycles, using CO₂ as a single euthanasia agent or in combination with previous administration of isoflurane.

In the future, systems with automatic recognition of LOC may be available for use during the induction phase of euthanasia. After LOC is observed, euthanasia may be concluded using a potentially more averse gas or a rapid increase in the concentration of the first gas used to kill the animal quickly.

Another important issue is the provision of cost-effective euthanasia using equipment that is adjusted to the number of animals that need to be euthanized. This minimizes the waste of anaesthetics and, therefore, costs.

**Conclusions**

Areas of inconsistency within the euthanasia literature have recently been highlighted and are related to insufficient knowledge regarding the best methods of euthanasia for various species and strains at different life stages. For practical reasons, and also often for research considerations, depending on the species and number of animals being used, LOC is typically achieved with an anaesthetic, and then rodents are killed by switching the agent to CO₂ or by using an injectable agent or physical method.

A great deal of research remains to be done on the euthanasia of laboratory animals, and on the euthanasia of rodents in particular. The information currently available is based mainly on rat studies, and additional studies using mice are needed to avoid extrapolation of information between species. Furthermore, there is a lack of consensus between individual opinions regarding the best euthanasia techniques, which may reflect the wide range of experience of users of these techniques, as well as the high variability surrounding subjective concepts (e.g. distress, pain and level of expertise). Many improvements to current methods should be made, including the use of
home-cage euthanasia and the implementation of gas chambers with fill rates or gas mixtures that are tailored to minimize distress. Alternative gaseous agents need further evaluation. However, there is a theory that rats, and probably other animals, avoid anything that produces a state change. Hence, even if euthanasia agents are not aversive per se, the novel state of conscious sedation may induce fear. In agreement with this contention, different gases may never be perfect and may always have an associated disadvantage. The administration of isoflurane or sevoflurane prior to CO₂ has been suggested as a more humane death, but there is still no consensus regarding this issue and information on the advantages of sevoflurane is scarce. However, a move away from the use of CO₂ faces two obstacles: practicality and economics, as anaesthesia-based techniques require more time, drugs and equipment. Inert gases do not seem to be less aversive than CO₂. However, the combination of different gases that potentiate LOC in a short time, such as N₂O and CO₂, has been suggested as an euthanasia refinement, and other combinations as CO₂ and volatile gases would be suitable for future studies.

In conclusion, there is no evidence to advocate banning CO₂, although its flow rate should be low. However, evidence suggests a potential refinement of the method using volatile gas anaesthetics. The use of these agents increases euthanasia costs. Therefore, the use of a bi-phased euthanasia, in which LOC achieved via these anaesthetics is the first objective, and death achieved via CO₂ the second, has been shown to be advantageous in terms of animal welfare, practicability and cost. Furthermore, different anaesthetics, such as sevoflurane and desflurane, need to be further evaluated. In addition to the choice of gaseous agent, euthanasia refinement may also be achieved via the development of techniques aimed at inducing death in the home cage, to minimize handling and distress.

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Mouse reproductive fitness is maintained up to an ambient temperature of 28°C when housed in individually-ventilated cages

J Helppi¹, D Schreier¹, R Naumann¹ and O Zierau²

Abstract
Production of genetically-modified mice is strongly dependent on environmental conditions. Mice are commonly housed at 22°C, which is significantly lower than their thermoneutral zone. But, when given a choice, mice often seem to prefer higher ambient temperatures. In the current study we investigated the effect of higher ambient temperature on the production of transgenic mice, with emphasis on embryo and sperm yield and quality. Mice (C57BL/6JolaHsd) were housed under four different ambient temperatures (22, 25, 28 and 30°C). Female mice were superovulated, and mated with males. As indicators for reproductive fitness, the success of the mating was observed, including embryo yield and quality, as well as sperm count, motility and progressively. Female mice were found to produce high amounts of high quality embryos from 22 to 28°C. Sperm count dropped continuously from 22 to 30°C, but sperm motility and progressivity remained high from 22 to 28°C. We conclude that mice can be housed at significantly higher temperatures than is commonly recommended without compromising embryo production and quality, or sperm quality. These results could lead to fundamental changes in how mouse facilities are built and operated – especially in warmer climates whereby energy consumption and therefore costs could be significantly reduced.

Keywords
environmental tolerance, temperature, sexual behaviour, GM models, embryo

The mouse has become one of the major animal models in biomedical research, not only because its genome has been fully sequenced, but also because its genome can be precisely engineered.¹² Producing genetically-engineered mouse strains¹³ require a reliable and high yield of fertilized embryos. The embryos for microinjections must be collected from donor mice that have to be euthanized in the process.⁴ To generate one new transgenic strain one generally has to use many tens, and sometimes more than 100, mice as embryo donors and as recipients. With an increasing need to constantly produce more transgenic strains it has become essential to look into the production and breeding efficiency with the aim of producing more strains with fewer mice. This is both a practical and an ethical issue.

One way to improve breeding efficiency could be by ensuring that welfare is optimal. Good welfare is an essential requirement when housing and breeding animals for experimental purposes, and it is actively enforced by regulations and laws.⁵⁶ Better welfare could be achieved by ensuring that animals are content with their immediate environment.⁷⁹ Consequently, it can therefore be assumed that animals would also breed better when content with their environment.¹⁰¹¹ Mice, for example, are typically housed in a relatively narrow temperature range varying from the German standard

¹Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
²Institute of Zoology, Technische Universität Dresden, Dresden, Germany

Corresponding author:
Jussi Helppi, Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstr. 108, 01307 Dresden, Germany.
Email: helppi@mpi-cbg.de