Developments in Euthanasia Protocols for Laboratory Mice (*Mus musculus)*

Discusses recent research on the aversiveness of euthanasia agents isoflurane and carbon dioxide in mice and the reliability of insensibility measures.

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Introduction

The domestic mouse (*Mus musculus*) has been used extensively in biomedical research for more than 100 years (Vandenbergh, 2000) and the utilisation of specialised mice in research is rapidly growing (Simpson *et al*., 1997). After research trials are completed, almost all animals are required to be euthanased. This may be to facilitate sample collection, or because animals are sick, injured or surplus to requirements (Leach *et al*., 2002).

In Australia, the Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes cover the use of animals for scientific purposes (NHMRC, 2008). Currently, the guidelines for the euthanasia of rats and mice recommend only one inhalant: carbon dioxide. The gaseous anaesthetic isoflurane is classed as acceptable, though there are reservations due to cost, occupational health and safety issues and the need for specialised equipment (NHMRC, 2008).

However, the use of carbon dioxide is contentious: some recent research suggests that other inhalants, such as isoflurane, may be more humane. The findings of previous studies are equivocal, with some suggesting carbon dioxide euthanasia causes pain and distress (Danneman *et al*., 1997; Conlee *et al*., 2005), while others did not observe aversion to the inhalant (Valentine *et al*., 2012; Hackbarth *et al*., 2000).

Discussion

To address this, Moody and Weary (2014) investigated the reaction of mice (n=30) to carbon dioxide and isoflurane at concentrations sufficient to achieve insensibility. The study exploited the animals' unconditioned preference for dark over light, as well as aversion to open spaces. By filling a dark compartment with the test gas and measuring when the mice left the dark compartment to move into a brightly lit compartment (not containing the gas), the authors could assess the relative levels of aversion to each stimulus.

The results of the study showed that mice were quicker to leave the dark compartment when carbon dioxide was used. Further to this, they were less likely to re-enter the dark compartment and, if they did, they spent less time in that compartment compared with the vaporised isoflurane group of mice. This evidence suggests that the mice found the carbon dioxide more aversive than the vaporised isoflurane. Some mice even persisted in the vaporised isoflurane dark compartment long enough to become recumbent. No mice became recumbent in the carbon dioxide group.

As isoflurane may be used as an anaesthetic for procedures, it was suggested that the mice might react differently when re-exposed. To test this, the mice exposed to vaporised isoflurane were re-exposed a week later. The mice spent less time initially in the compartment than the original exposure, suggesting that the isoflurane was more aversive than in the first instance. More research is needed to understand this. The authors concluded that vaporised isoflurane was less aversive that carbon dioxide administration when used in a euthanasia protocol. Re-exposure to isoflurane was also found to be more aversive and should be avoided.

Moody *et al*. (2014) then investigated further welfare concerns relating to carbon dioxide. Exposure to carbon dioxide causes dyspnoea in humans, a subjective experience of breathing discomfort of varying intensity (Manning & Schwartzstein, 1995). It is reasonable to suggest that mice may experience dyspnoea also. The interval from onset of laboured breathing until insensibility was used to indirectly assess dyspnoea in 23 female mice. Different flow rates of carbon dioxide were investigated on the basis that minimisation of the duration of the laboured breathing period would be more humane.

The authors proposed that higher flow rates of carbon dioxide would reduce the duration of laboured breathing. The onset of laboured breathing occurred at a similar time for all the flow rates tested. Increasing flow rate decreased the time between onset of laboured breathing and recumbency. However, loss of pedal reflex (a more conservative measure of insensibility) did not differ significantly with gas flow. This finding is

not well understood and further research is required. The study also found increasing carbon dioxide flow rates reduced the time to recumbency.

Both the above studies relied on an assessment of insensibility. Currently, scientifically rigorous measures of insensibility are not well established. Therefore, it is possible that animals could regain sensibility, such as regaining their righting reflex, during procedures, which would be very distressing (Moody *et al*., 2015).

A recent study evaluated three progressive measures of insensibility in order to address this concern: onset of recumbency, loss of righting reflex, and loss of pedal withdrawal reflex were each assessed (Moody *et al*., 2015). Pedal withdrawal was tested using toe pinches and preliminary trials showed no evidence of desensitisation over a short period and also that this response was consistent among the mice tested. Criteria for loss of the pedal reflex was set at three consecutive non-responses applied at 10-second intervals on alternating hind paws.

Using these three criteria for insensibility, the authors then compared the animals' responses to carbon dioxide and isoflurane. There was no significant difference in the time of onset of recumbency or loss of righting reflex in the two treatment groups. However, time until loss of pedal reflex was significantly increased in the isoflurane group. Time until last breath was also significantly increased in the isoflurane group.

The authors were concerned by the occurrence of purposeful movement observed after recumbency, as this suggested that the mice were probably sensible. Mice also continued to show indications of sensibility during righting reflex tests. Hence, the authors suggest that these are not reliable measures of insensibility and the more conservative measure of loss of pedal withdrawal reflex should be used to ensure insensibility of mice.

Conclusion

The impact of euthanasia protocols on animal welfare is very significant. The research discussed here suggests isoflurane is a less aversive stimulus than carbon dioxide when used in a euthanasia protocol. More research is required to test the ability of these agents to cause insensibility. By continuing to improve protocols to reduce the aversiveness of agents used and the period before insensibility is experienced, researchers can hope to reduce suffering in research animals.

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