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Letters to the Editor

Sedation or Inhalant Anesthesia before Euthanasia with CO_2 Does Not Reduce Behavioral or Physiologic Signs of Pain and Stress in Mice

Dear Editor,

Valentine and colleagues[13] tested the effects of anesthetic induction with isoflurane on behavioral and physiologic signs of pain and stress in mice euthanized with CO_2, and concluded that induction with isoflurane prior to euthanasia with CO_2 is worse for the animals' welfare than euthanasia with CO_2 alone. This conclusion seems to contradict a growing body of literature[9,11,14] that shows that exposure to CO_2 is strongly aversive to rodents, likely due to feelings of dyspnea[11] (that is, "air hunger") or anxiety[14] as reported in humans, and that induction with isoflurane is a less aversive alternative. For example, a key study[9] (not cited by Valentine and colleagues) showed that mice would sometimes choose to stay in a chamber filling with isoflurane until they were recumbent rather than abandoning a sweet food reward, but always abandoned the reward when the chamber was filling with CO_2.These results correspond with those from an earlier preference study[7] showing that mice tolerate longer periods of exposure to isoflurane than to CO_2.

The conclusion of Valentine and colleagues[13] rests on 4 results. We argue below that each of these is based on problematic methods or interpretation.

1) 5 of the 10 mice anesthetized with isoflurane recovered consciousness while the cage was being filled with CO_2. The criterion the authors used to determine when mice were unconscious was "cessation of voluntary movement," but this is not an appropriate proxy for unconsciousness. Isoflurane has sedative properties, and after an initial excitatory phase, mice appear 'sleepy' and settle down. At this stage mice are not unconscious and will withdraw in response to touch. Unconsciousness occurs only after the animal becomes recumbent (loss of muscular tone)[5] and breathing is deep and slow. A simple method to ensure the mice have lost consciousness is to check for the absence of the righting reflex when the mice are rolled onto their backs by tilting the chamber; this measure shows a strong correlation with measures of loss of consciousness in humans.[2] At UBC, where standard practice is to anesthetize with isoflurane and switch to CO_2 only after mice are recumbent, we have never had a report of a mouse recovering consciousness during the procedure.

2) Mice had highest agitation and dyspnea scores with isoflurane. The 'agitation' score used was likely not appropriate for comparing distress between isoflurane and CO_2.[10] Isoflurane induces an excitatory phase,[8] but there is no evidence that this behavior is reflective of aversion or distress. In contrast, mice often respond to CO_2 by gasping at the bottom of the cage, a response associated with low levels of activity. The use of the term 'dyspnea' is another source of confusion; in the veterinary literature this term is generally defined as "labored breathing" while the medical literature defines this as a feeling of "air hunger." Feelings of air hunger (often extreme and distressing) are reported by humans exposed to CO_2[1] but not isoflurane. Valentine and colleagues defined dyspnea as "increased respiratory effort," and likely measured increased breathing rates associated with activity during the excitation stage of isoflurane induction rather than air hunger.

3) Mice exposed to isoflurane produced calls with a peak frequency of 26.5 kHz, potentially indicative of stress. The paper cited in support of this claim[4] discusses only vocalizations in rats in response to pain. However, in contrast to rats, there is no evidence that vocalizations in mice are indicative of negative or positive affect.[12] The observed peak may be an artifact of increased activity rather than actual vocalizations.

4) *c-fos* expression was highest in the sedative and isoflurane groups. Valentine and colleagues failed to detect an increase in *c-fos* after exposure to CO_2 alone, but a previous study[6] found that a brief exposure to CO_2 caused a specific, localized expression of *c-fos* in brain areas involved in panic and defensive reactions in rodents, including the hypothalamic-pituitary axis. Valentine and colleagues evaluated global levels of *c-fos* mRNA in a 2-mm brain slice whereas the previous study[6] examined local expression of *c-fos* using immunostaining; Valentine and colleagues' global measure may have 'averaged out' localized increases in expression of *c-fos* in specific brain areas that would have been detected by a more selective assay.

Moreover, Valentine and colleagues examined *c-fos* expression only 4 min after the onset of CO_2 whereas the previous study examined *c-fos* levels 90 min after exposure when *c-fos* expression in response to a stressor is likely to be maximal.[6] Previous work has shown that *c-fos* levels are not significantly elevated 5 min after induction and maintenance of anesthesia with isoflurane but are elevated at 30 min.[3] Consequently, the elevated *c-fos* levels obtained by Valentine and colleagues likely reflect preeuthanasia handling in the case of mice receiving premedication (explaining why the sedative and the saline control groups also had elevated *c-fos* levels) or increased locomotor activity in the case of animals receiving isoflurane.

In summary, we suggest that the conclusion from Valentine and colleagues[13] should be treated with caution. Our reading of the literature suggests that CO_2 is aversive to rodents, and current evidence indicates that isoflurane is less aversive than CO_2.

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Response to Makowska and colleagues' Letter to the Editor:

Dear Editor,

We are writing in response to the letter from Makowska and colleagues regarding our article entitled "Sedation or Inhalant Anesthesia before Euthanasia with CO_2 Does Not Reduce Behavioral or Physiologic Signs of Pain and Stress in Mice."[7]

Makowska and colleagues assert that our study conclusions contradict a growing body of literature indicating that isoflurane is a more humane alternative to CO_2 euthanasia in mice. Their primary argument in favor of this assertion is that CO_2 causes aversion in rodents. We agree entirely with the numerous articles that demonstrate that CO_2 may be both aversive and painful in a variety of species. However, as we describe in our manuscript, none of these studies were conducted in a fashion consistent with the gradual fill method of CO_2 euthanasia. In fact, quite the opposite, the articles indicting CO_2 use either prefilled chambers or exposure to defined concentrations of CO_2. Both of these conditions ignore the possibility, which we describe in our manuscript, that mice become sedated and lose consciousness prior to experiencing a high concentration of CO_2. Further, these studies do not allow for physiological adaptation to gradual alterations in atmospheric CO_2 levels.

In addition, the literature referred to by Makowska and colleagues rely primarily on approach-avoidance testing. To conclude that induction with isoflurane is a more humane alternative to euthanasia with CO_2 based on approach-avoidance testing alone, one must assume that any avoidance behavior mice exhibit is due to either pain or distress. As we point out in our article, mice exhibit aversion to a variety of nonpainful and nondistressful stimuli. Further, even if an avoidance behavior does indicate avoidance of stress, one must then assume that the stressful stimulus was significant enough to be considered distressful. Both of these are significant assumptions that have not been validated.

Finally, none of the papers cited by the authors actually test euthanasia under prescribed conditions. As a group, we question any recommendations for euthanasia that are not based on actual validation when used in the intended and prescribed fashion.

Makowska and colleagues specifically raise 4 concerns with our data that we address point by point below:

1) Makowska and colleagues criticized our definition of unconsciousness as the cessation of voluntary movement, suggesting that mice regained consciousness during CO_2 exposure because they were only sedated rather than unconscious when switched to CO_2. Perhaps we should have been more explicit in our definition of unconsciousness: the mice were recumbent, all voluntary movement had ceased, and breathing had slowed and become more regular than it was during the induction phase of anesthesia. In short, the mice were unconscious, not sedated, at the time of CO_2 administration. Supporting this, once the mice were switched from isoflurane to CO_2, they showed a long delay (> 1 min in all cases) before awakening from isoflurane. Had they been only sedated at the beginning of CO_2 exposure, this delay would not have occurred.

We cannot reasonably comment on the unpublished anecdotal claims of Makowska and colleagues of validation of isoflurane as an adjunctive method to CO_2 euthanasia. However, a probable reason for recovery in our study is that we euthanized the mice in their home IVC cages. When the isoflurane is switched to CO_2, the denser CO_2 would displace the isoflurane out the top of the cage. Once isoflurane is removed, recovery from anesthesia is rapid. Because the mice are anesthetized, their breathing rate is slow and they would not inhale CO_2 as rapidly as would conscious mice. Furthermore, as described in our article discussion, the hypothermic effect of general anesthesia can be neuroprotective during hypoxia, therefore increasing the duration of CO_2 exposure required to achieve death.[7] Again, we hesitate to comment on unpublished anecdotal evidence, but perhaps Makowska and colleagues used containers with sealed lids (solid plastic or metal) and not home IVC cages, thus mitigating rapid loss of isoflurane.

2) Makowska and colleagues argue that the "agitation" noted during isoflurane exposure was due to the excitatory phase of isoflurane induction and further state that no evidence is available to indicate that this behavior reflects aversion or distress. However, the data from human and animal studies of isoflurane and this excitation indicate quite the contrary, as follows.

a. In human subjects, exposure to increasing concentrations of isoflurane results in tachycardia, hypertension, and norepinephrine release.[5,6,8] Increased heart rate, blood pressure, and catecholamine release are the hallmarks of a stress response. Furthermore, tachycardia and hypertension are significantly blunted by premedication with clonidine or nasal administration of lidocaine, indicating that this stress response is due to isoflurane induced irritation of the airways rather than compensatory changes due to anesthesia.[5]

b. In humans exposed for 15 s to 4 different volatile anesthetics, isoflurane induced the greatest amount of subject-described irritation, the greatest increase in cough response and the greatest increase in respiratory rate.[1]

c. Isoflurane activates peripheral nociceptors and actually produces hyperalgesia and irritation in the airways of both