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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

humans and animals via a direct activation of the excitatory ion-channel transient receptor potential (TRP)-A1.[2]

d. In mice, corticosterone concentration increases significantly after anesthesia with isoflurane, sevoflurane, ether, and CO_2, with no significant difference between the anesthetic groups, and mice respond similarly in open field testing with all agents, indicating that all likely induce similar stress responses.[4]

e. Finally, in recognition of the stress associated with chamber induction using isoflurane, the Association of Shelter Veterinarian's Spay-Neuter Task Force discourages the use of isoflurane mask or chamber induction of anesthesia citing the "…severe sympathomimetic effects and bronchial irritation…" associated with isoflurane induction.[3]

Makowska and colleagues also seem to misinterpret our dyspnea score. This score was based on increased respiratory effort prior to loss of consciousness and was determined by observing whole body breathing, not on increased respiratory rate associated with activity, as suggested by Makowska and colleagues.

3) Makowska and colleagues suggest that the increased ultrasonic peak frequency noted in the isoflurane-treated group was an artifact of increased physical activity rather than an actual vocalization. As described in the results section, we do account for activity level. An overall increase in amplitude throughout the spectrogram compared to the preeuthanasia baseline was consistent with increased activity observed in this group; likewise, a decrease in overall amplitude in the midazolam treated group was consistent with decreased activity.[7] The 26.5-kHz peak was not present in the background noise control recordings in which no mice were present, and was therefore assumed to be a vocalization. Because vocalizations in mice have not been defined as in rats, we stated in the discussion that the increased vocalization could potentially be indicative of stress.[7] We agree that further research into mouse vocalization and its potential association with painful or distressful procedures is needed. Ultimately, we made no definitive conclusions from this finding except that CO_2 spectrograms did not change from preeuthanasia levels; this was consistent with our blinded behavioral observation, which also indicated minimal changes.

4. Makowska and colleagues criticize our interpretation of *c-fos* mRNA expression levels, citing a paper that found differences in immunohistochemical positive foci in the hypothalamus of mice exposed to CO_2. This criticism seems misguided. One must be cautious when comparing immunohistochemistry to quantitative PCR (QPCR) as the first method merely indicates positive compared with negative cells without necessarily being capable of determining the expression of a given cell compared with another. In contrast, QPCR determines expression level of a given tissue or cell, often in relation to another cell or condition. In short, the 2 measures are completely different and provide different information (protein compared with mRNA, cellular presence compared with relative mRNA produced). Makowski and colleagues make this error in interpreting the data to mean that *c-fos* was not present in the CO_2-treated group. Because the data were normalized to that of mice exposed to the 20% flow rate CO_2, the brains of CO_2-treated mice did indeed show *c-fos* expression; however, those mice had 5 to 7 times less expression than did other groups. With those caveats in mind, we will specifically address several issues.

a) Makowski and colleagues imply that we may have missed differences because we evaluated the hypothalamus and a thin slice of surrounding cortex. This could be true; however, despite any potential lack of sensitivity, we nonetheless detected a nearly 7-fold induction of *c-fos* in isoflurane treated mice.

b) Makowski and colleagues also imply that because the immunohistochemical studies had longer exposure times, we did not wait long enough for *c-fos* induction. Two compelling reasons negate this argument. First, transcription of *c-fos* is rapid whereas protein translation is a more gradual process (15 to 30 min). Second, because the time to death was not statistically different for isoflurane and 20% CO_2, we see no mechanism by which we would have been able to detect an increased signal in isoflurane-treated but not CO_2-treated mice. Indeed Makowski and colleagues contradict their own argument on this point by claiming that the *c-fos* increase was due to increased activity during the excitatory phase, which occurred just moments before unconsciousness and only 1 min or so before death. Again, the timing and loss of signal argument must apply to all experimental groups not merely the CO_2 group in order to be a compelling and convincing argument.

As discussed in our article, no single methodology can be used to determine definitively whether mice are experiencing pain or distress. Therefore, we based our conclusions on a panel of methodologies (blinded behavioral analysis by a board certified laboratory animal veterinarian, physiologic and neuromolecular signs of pain/distress, and spectrographic analysis). All of these methods indicated that augmenting CO_2 euthanasia with premedication or isoflurane did not provide any perceptible benefit.

We appreciate the feedback given by Makowska and colleagues and hope that this discussion will encourage other investigators to rigorously analyze the best practices concerning euthanasia methods in rodents. Our manuscript was not meant to be the definitive analysis on this but rather is meant as a starting point. We hope that others will conduct similar studies. Only after these methods are analyzed in an appropriate fashion during actual euthanasia of target species in a controlled setting can we come to reasonable conclusions. Further, we would argue that aversion or lack thereof should not be the benchmark for a humane euthanasia method. Rather, we need to analyze distress (not stress), pain, and importantly the potential for failure (as noted for isoflurane in our study). Aversion can be elicited by far too many nonstressful or momentarily stressful (that is, not distressful) and nonpainful procedures to serve as an appropriate bar of success.

Sincerely,

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Total IgE as a Serodiagnostic Marker to Aid Murine Fur Mite Detection

Dear Editor,

This is a letter in reference to the article "Total IgE as a Serodiagnostic Marker to Aid Murine Fur Mite Detection" by Roble and colleagues.[8]

I am a Professor of Veterinary Anatomy in a higher education institution (graduated in 1955 and been an anatomist ever since) and one of the 5 members and the sole representative of the 2 Americas in the editorial board of the 5th edition of the Nomina Anatomica Veterinaria (NAV),[6] the international nomenclature of veterinary anatomy. I am also the author of 414 publications and familiar with hundreds of publications in the mammalian anatomy (domestic and wild animals, and laboratory animals alike), as well as the author of one poster on the Anatomy of the Mouse,[2] one poster on the Anatomy of the Rat,[3] and most recently of the Comparative Anatomy of the Mouse and the Rat – A Color Atlas and Text.[1] I am also the Editor of the 3rd edition of the Illustrated Veterinary Anatomical Nomenclature[4] based on the 5th edition of the NAV.[6]

Regarding the correct naming of the submandibular vein or the submandibular bleeding method in mice, in this letter I am presenting the details of the roots of the external jugular V. (V. jugularisexterna), which are targets for the bleeding method.

Three references are taken into consideration: 1) Popesko and colleagues,[7] 2) Cook,[5] and 3) Takamasa and colleagues.[9]

Popesko[7] illustrated the external jugular V. with its 2 roots, the linguofacial V. and the maxillary V., all correct anatomical terms (the illustration does not show where the lingual V. originated from the linguofacial and separates it from the facial V.). However, during the above mentioned blood collection method, either the linguofacial or the facial V. is subject to puncture, and not the lingual V.

Cook[5] illustrated the facial blood vessels (Figure 84), the superficial vessels of the head (Figure 85), and the dorsolateral dissection of the head (Figure 86). In Figure 84, the Facial V. is wrongly labeled as "Anterior facial V" and the Transverse facial V. is wrongly labeled as "Superficial temporal V." In Figure 85, the Superficial temporal V. is wrongly labeled as "Posterior facial V," the Transverse facial V. is wrongly labeled as "Superficial temporal V," and the Facial V. is wrongly labeled as "Anterior facial V." In Figure 86, the Facial V. is wrongly labeled as "Anterior facial V," the Superficial temporal V. is wrongly labeled as "Internal maxillary V," and the Transverse facial V. is wrongly labeled as "Superficial temporal V."

The Takamasa team's[9] illustrations of the veins of interest in the head are labeled correctly in some cases but not in others. The facial V. is correctly labeled on pages 9, 11, 12B, 18, 19, 23A, 57, 63, 97, 99, 101, and 105. On page 17B, the transverse facial V. is wrongly labeled as "facial V." On page 109, the facial V. and the maxillary V. are correctly labeled. On page 113, the facial V. is wrongly labeled as "transverse facial V," whereas the maxillary V. is correctly labeled. On page 116, the maxillary V. is wrongly labeled.

In conclusion, the bleeding method should be called either "linguofacial" or "facial", depending on the site of puncture. The site close to the connection to the external jugular V. is the linguofacial V., whereas the site far from the external jugular V. rostrally is the facial V. These are the internationally correct names that everyone should use.

I have to add that other JAALAS articles have used this misnomer and the misnomer is widely used in the field, so that I am not pointing a finger specifically (or only) at the authors of the above-cited paper.

Respectfully submitted,
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Response to Dr Constantinescu's Letter to the Editor:

In response to the letter from Dr Constantinescu regarding our recent article entitled "Total IgE as a Serodiagnostic Marker to Aid in Murine Fur Mite Detection,"[3] we have the following remarks. We thank Dr Constantinescu for the comments and in-depth references that he provided and agree that scientific accuracy would have been improved with the use of these anatomical terms. Based on the information provided by Dr.