SYSTEMATIC REVIEW PROTOCOL FOR ANIMAL INTERVENTION STUDIES

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Item #	Section/Subsection/Item	Description	Check for approval
	A. General		
1.	Title of the review	The use of carbon dioxide as a method for euthanasia of laboratory mice and rats– a systematic review	
2.	Authors (names, affiliations, contributions)	PV Turner (PVT) –University of Guelph, Guelph, ON, Canada – study design, search design, search, study selection, RoB assessment, data extraction, data analysis, MS preparation, MS editing J Sargeant – University of Guelph, Guelph, ON, CANADA – study design, process oversight, analytical support, MS editing D Hickman (DH), Indiana University School of Medicine, Indianapolis, IN, USA - study selection, data extraction, data analysis, RoB assessment, MS editing TM Kurosawa (TMK) - Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan - data extraction, data analysis, RoB assessment, MS editing B Mercer – University of Guelph, Guelph, ON, Canada – study design, search design, search, MS editing M Ritskes - Radboud University, The Netherlands - study design, process oversight, MS editing J van Luijk (JVL), SYRCLE, Radboud University, The Netherlands – search design, search, study selection, process oversight, MS editing	
3.	Other contributors (names, affiliations, contributions)		
4.	Contact person + e-mail address	Dr. Patricia V. Turner (pvturner@uoguelph.ca)	
5.	Funding sources/sponsors	Ontario Ministry of Agriculture Food and Rural Affairs (CO2 euthanasia), International Association of Colleges of Laboratory Animal Medicine (IACLAM), Stichting Reinier Post (Radboudumc, Nijmegen, the Netherlands)	
6.	Conflicts of interest	The authors report no conflicts of interest	
7.	Date and location of protocol registration	April 2017	
8.	Registration number (if applicable)	NA	
9.	Stage of review at time of registration	Systematic searches completed. An updated search will be performed by the library to be as complete as possible.	
	B. Objectives		
	Background		
10.	What is already known about this disease/model/intervention? Why is it important to do this review?	Humane endpoints are required for all laboratory research projects. When endpoints are identified requiring	

		euthanasia, the euthanasia method selected must be rapid and should minimize the potential pain and distress experienced by an animal prior to the loss of consciousness. Carbon dioxide has been conditionally approved for laboratory rodent euthanasia because it is an inexpensive, simple, and relatively safe method to use that results in rapid death of rats and mice when used appropriately. The technique is used around the world for laboratory rodent euthanasia. Given that carbon dioxide gas is an inhaled gas many comparisons are made between the experience that rodents have during induction to unconsciousness with CO2 inhalation and the experience of rodents during the process of anesthetic induction via inhalant anesthetic agents, such as isoflurane in oxygen. There is conflicting information regarding the impact of the induction experience of rodents for euthanasia (vs anesthesia) on animal well-being and on operator/observers conducting or viewing the technique, leading to questions regarding whether CO_2 inhalation continues to be a suitable method for rodent euthanasia. In addition to a need for safe and effective laboratory rodent euthanasia methods, it is occasionally necessary to humanely kill larger numbers of rats and mice. Appropriate techniques for depopulation of large numbers of mice/rats have not been well explored. The outcome of this review is expected to inform the international research community about the options and acceptability of different inhalant euthanasia procedures available for laboratory mice/rats.	
	Research question		
11.	Specify the disease/health problem of interest	Euthanasia of laboratory mice and rats, healthy and diseased animals	
12.	Specify the population/species studied	Laboratory rats and mice	
13.	Specify the intervention/exposure	Carbon dioxide gas euthanasia	
14.	Specify the control population	No CO2, control mixture or exposure to oxygen or medical air only or other control types. Studies with no control group (observational studies) will also be eligible for inclusion.	
15.	Specify the outcome measures	Any quantifiable outcomes related to gas aversion in mice/rats during induction (e.g. behavioural and physiological parameters related to discomfort, distress, pain and suffering)	
16.	State your research question (based on items 11-15)	What are the quantifiable effects of CO_2 gas exposure on mice/rats during euthanasia (as it relates to pain/ aversion/ distress)?	

		Sub-questions: How do these effects compare to other euthanasia methods (i.e., isoflurane, argon, and halothane)? How do the effects compare between different ages of mice/rats (ie, neonates vs mice >1 week of age)? What is the impact of different gas delivery technique on the outcome (e.g., slow vs fast fill vs pre-charged chambers, humidification, temperature)? What is the effect of mixing unfamiliar or a large volume of mice/rats during euthanasia?	
	C. Methods		
	Search and study identification		
17.	Identify literature databases to search (*e.g.* Pubmed, Embase, Web of science)	X MEDLINE via PubMed X Web of Science ☐SCOPUS ☐EMBASE X Other, namely: Cabdirect, Agricola, Agricola (USDA National Agricultural Library) ☐Specific journal(s), namely:	
18.	Define electronic search strategies (*e.g.* use the step by step search guide[15] and animal search filters[20, 21])	When available, please add a supplementary file containing your search strategy: Main search components: - Mice/rats - CO2 euthanasia A scoping search has been conducted, the results of this scoping search will be used to determine the final search strategy (search terms and databases) - Evaluation of evidence found from scoping search (selection based on the relevance for review question) - Where was relevant evidence found (identification of relevant databases/ sources) - Which relevant terms have been used (terms will be added to optimalize search string) Search strategies will be adapted to the specific databases as mentioned under item 17 and other identified relevant sources.	
19.	Identify other sources for study identification	X Reference lists of included studies ☐Books X Reference lists of relevant reviews ☐Conference proceedings, namely: ☐Contacting authors/ organisations, namely: ☐Other, namely:	
20.	Define search strategy for these other sources	Grey literature sources will be determined based on the evaluation of the scoping search. reviewers.	
	Study selection		

21.	Define screening phases (*e.g.* pre-screening based on title/abstract, full text screening, both)	Phase 1: Pre-screened on title to remove obvious irrelevant references to the review topic Phase 2: Screening on title and abstract content Phase 3: Inclusion or exclusion based on full-text	
22.	Specify (a) the number of reviewers per screening phase and (b) how discrepancies will be resolved	Phase 1: one reviewer (PVT) assesses all references for relevance to the review topic. Excluded references are checked by TMK or DH or JVL. Phase 2: each reference is assessed by two independent reviewers (PVT and DH or TMK) using EROS. Disagreements are resolved through discussion. Phase 3: each reference is assessed full-text by two independent reviewers (PVT and DK or TMK) using EROS. Disagreements are resolved through discussion by consulting a 3rd reviewer.	
	Define all inclusion and exclusion criteria based on:		
23.	Type of study (design)	Inclusion criteria: Controlled and observational studies. Controlled studies using control groups such as no exposure to carbon dioxide or oxygen/air only compared with cardon dioxide euthanasia groups Exclusion criteria: None	
24.	Type of animals/population (*e.g.* age, gender, disease model)	Inclusion criteria: Laboratory mice/rats of any age or sex Exclusion criteria: Other species	
25.	Type of intervention (*e.g.* dosage, timing, frequency)	Inclusion criteria: Exposure to carbon dioxide for euthanasia in a laboratory/experimental setting Exclusion criteria: Exposure to carbon dioxide for pest control	
26.	Outcome measures	Inclusion criteria: Any outcomes related to discomfort, distress, pain or suffering (e.g. behavioural and physiologic and pathologic parameters) Exclusion criteria: None (Any other parameters not related to discomfort, distress, pain or suffering)	
27.	Language restrictions	Inclusion criteria: all languages. In case of non-English studies a suitable translator will be approached (preferably within the task force). Exclusion criteria: None	
28.	Publication date restrictions	Inclusion criteria: All years of publication Exclusion criteria: None	
29.	Other	Inclusion criteria: NA Exclusion criteria: Not a primary studies with primary data (e.g. Reviews)	
30.	Sort and prioritize your exclusion criteria per selection phase	Tiab selection phase: 1. Article without original data (e.g. review, editorial) 2. Not an *in vivo* animal study 3. Not looking at carbon dioxide/inhalant exposure in mice/rats 4. Not looking at euthanasia Full text selection phase: 1. Article without original data (e.g. review, editorial)	

		2. Not an *in vivo* animal study 3. Not looking at carbon dioxide/inhalant exposure in mice/rats 4. Not a euthanasia study 5. Outcomes not relevant for direct assessment of behavioural or physiologic impact of euthanasia on mice/rats 6. Article not retrievable	
	Study characteristics to be extracted (for assessment of external validity, reporting quality)		
31.	Study ID (*e.g.* authors, year)	Author, title, year of publication	
32.	Study design characteristics (*e.g.* experimental groups, number of animals)	Number of experimental groups or control groups, number of animals per group, housing and husbandry history	
33.	Animal model characteristics (*e.g.* species, gender, disease induction)	Species, strain, sex, weight, age, genetic condition, health status, health history, diseased models.	
34.	Intervention characteristics (*e.g.* intervention, timing, duration)	Flow rate of gas, duration of exposure, type of gas, concentration/ratio, temperature, humidity, additives, additional gasses (mixtures)	
35.	Outcome measures	Time/frequency of outcome assessment, type of outcome measures observed (only OM with quantifiable measures will be included)	
36.	Other (*e.g.* drop-outs)	'reversed ' dropouts (e.g. survivors and how/why)	
	Assessment risk of bias (internal validity) or study quality		
37.	Specify (a) the number of reviewers assessing the risk of bias/study quality in each study and (b) how discrepancies will be resolved	At least 2 reviewers will assess risk of bias and study quality. Discrepancies will be dealt with through consensus decisions by consulting 3rd reviewer.	
38.	Define criteria to assess (a) the internal validity of included studies (*e.g.* selection, performance, detection and attrition bias) and/or (b) other study quality measures (*e.g.* reporting quality, power)	☐By use of SYRCLE's Risk of Bias tool[4] **X**By use of SYRCLE's Risk of Bias tool, adapted as follows: additional scoring of reporting of any randomisation, reporting of any blinding, reporting of a power calculation and any conflict of interest (e.g. CO2 cage manufacturers) ☐By use of CAMARADES' study quality checklist, e.g [22] ☐By use of CAMARADES' study quality checklist, adapted as follows: ☐Other criteria, namely:	
	Collection of outcome data		
39.	For each outcome measure, define the type of data to be extracted (*e.g.* continuous/dichotomous, unit of measurement)	Any outcome will be documented, we expect and prioritize the following outcome measures: - Behavioural 1 vocalization 2 urination 3 defecation 4 anxiety or distress or avoidance behaviour 5 convulsions or seizures 6 time to insensibility 7 time to death	

		8 aversion - Physiologic 1 heart rate 2 brain activity 3 corticosterone levels 4 lung pathology (surrogate measure).	
40.	Methods for data extraction/retrieval (*e.g.* first extraction from graphs using a digital screen ruler, then contacting authors)	Numerical data will be extracted from text or tables. In case of missing data, we will contact authors in an attempt to retrieve additional information. If there is no response within 3 weeks (including a reminder), the study will be excluded from the analysis	
41.	Specify (a) the number of reviewers extracting data and (b) how discrepancies will be resolved	At least two reviewers will independently extract data. Discrepancies will be dealt with through consensus Discussion. If no consensus is reached, a 3rd reviewer will be consulted.	
	Data analysis/synthesis		
42.	Specify (per outcome measure) how you are planning to combine/compare the data (*e.g.* descriptive summary, meta-analysis)	A descriptive summary of all included studies and their outcome measures. A meta-analysis will be conducted if there are sufficient studies (3 or >) using the same or similar outcome measures	
43.	Specify (per outcome measure) how it will be decided whether a meta-analysis will be performed	If greater or equal to 3 studies are conducted using similar outcome measures a meta-analysis will be performed.	
	If a meta-analysis seems feasible/sensible, specify (for each outcome measure):		
44.	The effect measure to be used (*e.g.* mean difference, standardized mean difference, risk ratio, odds ratio)	To be determined	
45.	The statistical model of analysis (*e.g.* random or fixed effects model)	Significant heterogeneity is expected between studies, thus, we will use a random effects model.	
46.	The statistical methods to assess heterogeneity (*e.g.* I^2, Q)	(residual) I2 and adjusted R2	
47.	Which study characteristics will be examined as potential source of heterogeneity (subgroup analysis)	-age of animal -species - strain - sex? -method of chamber fill - gas concentration/ratio? - use of home cage y/n - Individual versus group euthanasia - group euthanasia: mixing unfamiliar animals or not	
48.	Any sensitivity analyses you propose to perform	To be determined	
49.	Other details meta-analysis (*e.g.* correction for multiple testing, correction for multiple use of control group)	If applicable, we will perform a Bonferroni correction for testing multiple subgroups. If one or more subgroup analyses cannot be performed due to insufficient data, the p-value will be adjusted accordingly. Also correction for multiple use of control groups will be performed by	

		dividing the number of animals in the control group by the number of comparisons performed with this control group	
50.	The method for assessment of publication bias	Produce funnel plots and visual analysis of these plots for outcome measures containing 20+ studies. We are aware that funnel plots of SMD are susceptible to distortion and will omit the assessment of publication bias if this is suspected for our dataset. In addition, we aim to perform Egger's test for small study effects for outcome measures containing 20+ studies	
Final approval by (names, affiliations):			Date: