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Carbon Dioxide for Euthanasia: Concerns Regarding Pain and Distress, with Special Reference to Mice and Rats

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Carbon dioxide for euthanasia: concerns regarding pain and distress, with special reference to mice and rats

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Summary

Carbon dioxide (CO_2) is the most commonly used agent for euthanasia of laboratory rodents, used on an estimated tens of millions of laboratory rodents per year worldwide, yet there is a growing body of evidence indicating that exposure to CO_2 causes more than momentary pain and distress in these and other animals. We reviewed the available literature on the use of CO_2 for euthanasia (as well as anaesthesia) and also informally canvassed laboratory animal personnel for their opinions regarding this topic. Our review addresses key issues such as CO_2 flow rate and final concentration, presence of oxygen, and prefilled chambers (the animal is added to the chamber once a predetermined concentration and flow rate have been reached) versus gradual induction (the animal is put into an empty chamber and the gas agent(s) is gradually introduced at a fixed rate). Internationally, animal research standards specify that any procedure that would cause pain or distress in humans should be assumed to do so in non-human animals as well (Public Health Service 1986, US Department of Agriculture 1997, Organization for Economic Cooperation and Development 2000). European Union guidelines, however, specify a certain threshold of pain or distress, such as 'skilled insertion of a hypodermic needle', as the starting point at which regulation of the use of animals in experimental or other scientific procedures begins (Biotechnology Regulatory Atlas n.d.). There is clear evidence in the human literature that CO_2 exposure is painful and distressful, while the non-human literature is equivocal. However, the fact that a number of studies do conclude that CO_2 causes pain and distress in animals indicates a need for careful reconsideration of its use. Finally, this review offers recommendations for alternatives to the use of CO_2 as a euthanasia agent.

Keywords Carbon dioxide; euthanasia; pain; distress; anaesthesia; welfare; rodents

Carbon dioxide (CO_2) is commonly used for euthanasia and anaesthesia of laboratory rodents, largely because of its ease of use, relative safety, and low cost, as well as its capacity to euthanize large numbers of animals in a short time span (Ambrose *et al.* 2000). In large institutions and those with significant rodent-breeding programmes, large numbers of rodents are often euthanized in a short time (Kline *et al.* 1963) and an appropriate gas agent is often the best way to approach such a challenge. However, CO_2 is not physiologically inert and the published evidence on whether or not CO_2 administration causes pain or distress in animals raises questions about its routine use. This paper reviews this published evidence and includes information on the effects of CO_2 in both humans and non-humans. Methodological details are included when possible in order to provide a clear

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picture of how studies were conducted. Alternatives to the use of CO_2 as a sole agent for euthanasia are also suggested.

Animal welfare should be the main factor taken into consideration when choosing an appropriate method of euthanasia (American Veterinary Medical Association [AVMA] 2001). The least stressful procedure should be utilized whenever possible – the term 'euthanasia' being derived from a Greek term meaning 'good death.'

The current standards for euthanasia in the US are set out in the *2000 Report of the AVMA Panel on Euthanasia*. This report, written by the AVMA (2001), indicates that a 'good death' is one 'that occurs without pain and distress' and 'the technique should minimize any stress and anxiety experienced by the animal prior to unconsciousness'. Similar statements are indicated in various international guidelines and recommendations regarding euthanasia (e.g. Canadian Council on Animal Care [CCAC] 1993, Close *et al*. 1996, 1997, ANZCCART 2001).

Existing guidelines offer no clear, quantitative guidance on what techniques might be considered 'without pain and distress' nor what would qualify as 'rapid unconsciousness' – seconds, tens of seconds, minutes? Nonetheless, the literature on decapitation gives some sense of what may not be acceptable in regards to length of time. The 1986 and 1993 AVMA reports on euthanasia cautioned against the use of decapitation because a single study of rats found that the decapitated head continued to produce electroencephalogram (EEG) traces for an average of 13.6 s after decapitation (5.6–29.5 s) (see references in AVMA 2001). Therefore, due to lack of information, such times specified in guidelines can start to guide us. The published literature indicates that CO_2, at least under some circumstances, causes pain and distress and does so for longer than 10 s (there are conflicting reports regarding duration, but 10 s is the minimal amount of time reported in the literature reviewed). Despite the evidence in the published literature that CO_2 causes distress, the authors have received anecdotal reports from laboratory animal veterinarians that CO_2 appears humane when euthanasia is done properly by well-trained personnel.

Existing policies relevant to this issue

The Organization for Economic Co-operation and Development (OECD) guidance document on humane endpoints follows the principle that if something is known to cause pain, distress and suffering in humans, it should be assumed to cause the same in animals (OECD 2000). This principle is also included in animal research regulations and guidelines in the United States, including those promulgated by the United States Department of Agriculture (1997) and the Public Health Service (1993), as well as those in other countries (e.g. CCAC 1993). As mentioned, European Union guidelines follow this principle but specify a certain threshold of pain or distress for regulating animal use, for example using 'skilled insertion of a hypodermic needle' as guidance (Biotechnology Regulatory Atlas n.d.).

Physiological effects/actions of CO_2

CO_2 causes a range of neurochemical, respiratory and vascular responses in both humans and non-humans (Woodbury & Karler 1960). Low to moderate concentrations of CO_2 (ranging from 5% to 35%) cause changes in respiration rate (Thomas & Spyer 2000), heart rate and blood pressure (Dripps & Comroe 1947, Kety & Schmidt 1947, Smith & Harrap 1997), as well as HPA axis activity (Barbaccia *et al*. 1996). High concentrations of CO_2 initially cause similar responses and may induce hyperventilation before respiratory and cardiac depression and subsequent failure (Martoft *et al*. 2003). The accumulation of CO_2 also causes acidification of nasal mucosa (Anton *et al*. 1992, AVMA 2001). There is evidence that non-myelinated nerve endings that sense chemicals (Thurauf *et al*. 1991) and CO_2-sensitive olfactory receptors are present in the nasal mucosa of mammals, including rats (Coates 2001) and humans (Alvaro *et al*. 1993). The existence of these nerve endings

and olfactory receptors indicate the ability to sense any pain that may be associated with CO_2.

Relevant human data

There is evidence from human studies that inhalation of CO_2 at various concentrations can cause pain and/or distress. For example, Danneman *et al*. (1997) asked 20 adult humans to take a full breath of CO_2 at different concentrations ranging from 50% to 100% and to score each concentration according to the level of discomfort. Results indicated that higher concentrations of CO_2 were perceived to be increasingly noxious. Danneman's subjects used the following terms in reference to *every* concentration of CO_2 tested: burning, tingling or prickling, and unpleasant (taste or odour); these terms were used more frequently at higher concentrations. Many described 100% CO_2 as piercing, stabbing, painful or causing the eyes to burn or water, and 18 out of 20 subjects indicated that they were unable to take a full breath of this concentration.

Dripps and Comroe (1947) found that 7–10% CO_2 in oxygen caused increases in pulse rate, respiratory rate, and blood pressure in male humans. Symptoms upon inhalation of CO_2 included headache, dizziness and dyspnoea. Additional descriptions by the subjects included irritation of the nose, palpitation, faintness, 'generally uncomfortable,' muscle tremor and substernal pain. McArdle (1959) examined the effects of 30% CO_2:70% O_2 on the heart rate of humans and found no serious disturbance to cardiac function. However, CO_2 caused hyperventilation, severe acidosis, a significant rise in arterial pressure, and was associated with an overall substantial degree of stress.

The ability of CO_2 to induce pain in humans is underscored by the use of CO_2 as a pain stimulus in humans. For example, Anton *et al*. (1992) examined pain thresholds induced by CO_2 in the nasal mucosa in humans. A linear relationship was found between CO_2 concentration and pain sensation. Average individual tolerance thresholds ranged from 40% to 55% CO_2.

Animal studies regarding CO_2 as a euthanasia agent: general information

CO_2 euthanasia occurs via administration of the inhalant gas in a sealed container, with the purpose of inducing unconsciousness and death (Britt 1986). One source of CO_2 is 'dry ice,' but a pressurized cylinder of CO_2 is now viewed by a number of international animal research oversight authorities as the only acceptable source (United Kingdom 1997). CO_2 may be administered in the home cage or in a specialized compartment and may be used to kill individuals or small groups of animals.

Research regarding CO_2 use in different species has examined several parameters such as blood pressure (Smith & Harrap 1997), heart rate (Coenen *et al*. 1995, Smith & Harrap 1997, Leach *et al*. 2002a,b), behaviour (e.g. Smith & Harrap 1997, Leach *et al*. 2002a,b), times to anaesthesia and death (Blackshaw *et al*. 1988, Danneman *et al*. 1997, Kohler *et al*. 1999), EEG activity (Thurauf *et al*. 1991), histology (Britt 1986, Danneman *et al*. 1997) and blood pH (Hewett *et al*. 1993).

Procedural factors such as the CO_2 concentration, flow rate and the presence of oxygen have also been included in the assessment of CO_2. There are wide variations in the methods used, measurements taken and the resulting recommendations throughout the published literature; these factors limit the comparability of results, although some trends can be discerned. Discussions of CO_2 euthanasia with various people working in laboratory animal medicine and care (e.g. veterinarians, vivarium directors, technicians) reveal that there are conflicting CO_2 practices and recommendations within the animal research community. As one example, some institutions require that the euthanasia chamber be prefilled with CO_2, while others prohibit the use of prefilled chambers because they appear to cause animal distress.

Similar discrepancies in practice have also been noted in regards to concentration, flow rate and presence of oxygen.

Pain and acute stress studies utilizing CO_2

CO_2 has been used as a pain and/or stress stimulus in animals. In some cases, the concentrations used correlate with concentrations used for CO_2 euthanasia. Thurauf *et al*. (1991) exposed rats to various concentrations of CO_2 (0–90%) and measured evoked potentials in EEG recordings in order to determine the origin of negative mucosal potential (NMP – negative potential recorded from the respiratory mucosa following painful stimulation with CO_2). Local anaesthetics eliminated NMPs and EEG cortical responses, signifying that the pain response had ceased. When no local anaesthetic was administered, the result was *increased* NMPs, indicating an increased nociceptive response.

Barbaccia *et al*. (1996) used CO_2 to elicit a stress response in rats in order to examine the effects of acute stress on brain steroid concentrations and $GABA_A$ receptor function. A combination of 35% CO_2 and 65% O_2 inhaled from gas cylinders for one minute caused a sufficient stress response for the study. It was concluded that exposure to CO_2 is correlated with an increase in various brain neuroactive steroid concentrations.

In sum, it is noteworthy that CO_2 is used to induce pain and stress in animals, and the concentrations used in these studies are the same or similar to those used for anaesthesia and euthanasia.

Effects of CO_2 administration

CO_2 effects have been examined at various concentrations. A comparison of results illustrates concerns regarding pain and distress at concentrations ranging from 25% to 100%. The results of a number of studies are discussed here and specifics are provided in Tables 1 and 2 for mice and rats.

Danneman *et al*. (1997) euthanized rats with various concentrations of CO_2 (50–100%) combined with oxygen via either prefilled or gradual induction (GI) chambers and found a number of adverse effects. Measures included time to recumbency, time to anaesthesia (shallow breathing or lack of response to toe pinch) and any clinical adverse effects. Adverse reactions, prior to death, included seizures, convulsive chewing, nasal haemorrhage, sero-sanguinous nasal discharge and excessive salivation. The frequency and severity of the adverse reactions were inversely related to CO_2 concentration. It appears that these reactions occurred prior to or at onset of anaesthesia, but the publication does not make a clear distinction. Whether adverse reactions occur prior to or after unconsciousness is an important consideration and should be made clear in publications. Based on Danneman's results, it appears that lower concentrations (at least when considering 50% CO_2 and higher) cause increased incidence of adverse effects.

Ambrose *et al*. (2000) found that a concentration of 60% CO_2, supplied from a gas cylinder, caused many adverse effects in mice, whereas 30% was not as problematic. Simply introducing CO_2 into the chamber caused behaviours such as increased locomotion, rearing, defaecation and urination. These behaviours are considered by Ambrose and others to be indicative of distress, but further investigation is needed for verification. Overall, the authors concluded that 60% CO_2 'caused an undue amount of stress' (Ambrose *et al*. 2000).

Concentration, induction method and presence of oxygen with CO_2 were examined in rats by Coenen *et al*. (1995). Gas cylinders were used and gas was humidified in all conditions. The authors reported four phases in each case of CO_2 use: normal behaviour (phase I); continuous abnormal activity, excitation and agitation at a higher rate than normal (phase II); sagging of hindlegs and loss of body control (phase III); disappearance of muscle tone and head sinking (phase IV). Phase II was seen more frequently when 100% CO_2 was used. The same adverse signs were also observed at lower concentrations (without O_2), but less frequently than in 100% CO_2. Animals experienced asphyxia (as evidenced by gasping with mouth open

Table 1 Specifics of mice studies that examined carbon dioxide exposure

Reference	Prefilled (PF) or gradual induction (GI)	Gas mixture/final concentration	Estimated time to unconsciousness (s)	Estimated time to death (s)	Behaviour before unconsciousness	Physiology/ neurological measures	Comments
Ambrose *et al.* (2000)	GI	CO_2:O_2/50%:50% within 110 s	93.4	250.5	↑Locomotion, rearing, defaecation and urination	Postmortem: ↑alveolar haemorrhagic consolidation relative to CO_2 alone	
	GI	CO_2:O_2/50%:50% within 30–40 s	65.0	177.8	↑Locomotion, rearing, defaecation and urination (significantly higher than the two other groups)	Postmortem: ↑alveolar haemorrhagic consolidation relative to CO_2 alone	60%:20% produces faster unconsciousness; Authors: 'distress' behaviour may be due to rapidity of ↑in CO_2 concentration
Ambrose *et al.* (2000)	GI	CO_2 alone/50% within 110 s	93.4	235.6	↑Locomotion, rearing, defaecation and urination	Postmortem: ↑alveolar haemorrhagic consolidation relative to CO_2 + O_2	
Britt (1986)	GI	CO_2:O_2/ 70.8%:5.5%	55.0	242.0	Forced breathing Hyperventilation Urination, defaecation	Some lung haemorrhage	
Iwarsson & Rehbinder (1993)	GI	CO_2:O_2/80%:20%	13.0	130.0	Mild hyperpnoea and shivering, mild stress response	Lungs: oedema and haemorrhage	
Blackshaw *et al.* (1988)	PF	CO_2 alone/97%	12.8	48.0	None recorded (only movement was measured and only for 10 s)	NS	Used dry ice – may be difficult to maintain consistent concentration
Britt (1986)	PF	CO_2 alone/100%	14.0	38.0	Forced breathing, hyperventilation; shaking; urination, defaecation	High concentration= more lung haemorrhaging before death	

(continued)

Table 1 (continued)

Reference	Prefilled (PF) or gradual induction (GI)	Gas mixture/final concentration	Estimated time to unconsciousness (s)	Estimated time to death (s)	Behaviour before unconsciousness	Physiology/ neurological measures	Comments
Iwarrson & Rehbinder (1993)	PF	CO_2 alone/100%	8.0	35.0	Moderate hyperpnoea, body stiffness and excitement before loss of righting reflex, mild stress response	Lungs: mild to moderate lung emphysema	
Leach *et al.* (2002a)	PF	CO_2 alone/28% or 36% or 53%	NA	NA	Shorter withdrawal and dwelling times compared to halothane, isoflurane and enflurane (therefore more aversive)	NS	This study did not involve euthanasia
Leach *et al.* (2002b)	PF	CO_2/28% or 36% or 53%	NA	NA	Shortest withdrawal and dwelling times in comparison to other mouse groups (therefore most aversive); all concentrations equally aversive	NS	This study did not involve euthanasia
	PF	CO_2:argon/ 20%:13% or 20%:7.9% or 20%:4.5%	NA	NA	Shorter withdrawal and dwelling times in comparison to argon alone; all concentrations equally aversive	NS	This study did not involve euthanasia
	PF	CO_2:argon/ 30%:14% or 30%:10.1% or 30%:5.3%	NA	NA	Shorter withdrawal and dwelling times in comparison to argon alone; all concentrations equally aversive	NS	This study did not involve euthanasia

This table includes the results of studies of mice only, for groups that were exposed to CO_2. Therefore, if a study compared CO_2 with other agents, information of other agents was not included in order to keep the table as short as possible. Time to unconsciousness was determined conservatively as the onset of postural collapse and loss of muscle tone (Woodbury *et al.* 1958, Coenen *et al.* 1995); the present authors calculated these times when they were not specified directly by the authors of the original papers. NS = not specified

Table 2 Specifics of rat studies that examined CO_2 exposure

Reference	Prefilled (PF) or gradual induction (GI)	Gas mixture/final concentration	Estimated time to unconsciousness (s)	Estimated time to death (s)	Behaviour before unconsciousness	Physiology/neurological measures	Comments
Britt (1986)	GI	CO_2:O_2/61.5%: 7.88%	70.0	395.0	Forced breathing Hyperventilation Urination, defaecation	Some lung haemorrhage	
Coenen *et al.* (1995)	GI	CO_2 alone/NS	65.0	530.0	Forced breathing; unspecified signs of agitation and excitation	EEG stages: normal; lowered amplitude; slow waves and large spikes Heart rate: normal; rapid decrease; cessation	Slow wave EEG commenced at onset of postural collapse
	GI	CO_2 alone/NS	40	395	Forced breathing; author reported signs of agitation and excitation (not described)	Same as above	Slow wave EEG commenced before postural collapse
	GI	CO_2:O_2/80%:20%	50.0	618.0	No signs of asphyxia or forced breathing	Same as above	Slow wave EEG commenced after postural collapse
Danneman *et al.* (1997)	GI	CO_2 alone/60%	NS	27.3	NS	Lungs: marked perivascular haemorrhage; perivascular oedema; intra-alveolar haemorrhage Haemorrhage from nose; excessive salivation; nasal discharge	
	GI	CO_2 alone/60% until anaesthetized then 100%	NS	15.1	NS	Haemorrhage from nose; excessive salivation; serosanguinous nasal discharge	

(continued)

Table 2 (continued)

Reference	Prefilled (PF) or gradual induction (GI)	Gas mixture/final concentration	Estimated time to unconsciousness (s)	Estimated time to death (s)	Behaviour before unconsciousness	Physiology/neurological measures	Comments
Danneman *et al.* (1997)	GI	CO_2 alone/70%	NS	16.4	NS	Lungs: marked perivascular haemorrhage; perivascular oedema and intra-alveolar haemorrhage; Serosanguinous nasal discharge; nasal haemorrhage	
	GI	CO_2 alone/70% until anaesthetized then 100%	NS	16.1	NS	Serosanguinous nasal discharge; nasal haemorrhage	
	GI	CO_2 alone/80%	NS	13.5	NS	Lungs: marked perivascular oedema; perivascular and intra-alveolar haemorrhage Serosanguinous nasal discharge; nasal haemorrhage	
	GI	CO_2 alone/80% until anaesthetized then 100%	NS	15.4	NS	Serosanguinous nasal discharge	
	GI	CO_2 alone/100%	NS	6.0	NS	Lungs: perivascular oedema and intra-alveolar haemorrhage	
Hackbarth *et al.* (2000)	GI	CO_2 alone/55%	45–50 s (loss of movement) 85 s (muscle relaxation)	NS	Tachypnoea followed by stationary phase and subsequent relaxation of muscles	No changes in ACTH and corticosterone levels	Convulsions occurred between loss of movement and muscle relaxation
	GI	CO_2 + acepromazine/55% CO_2	Same as above	NS	Same as above	Higher ACTH and corticosterone levels than CO_2 alone group	Same as above
	GI	CO_2 + pentobarbitone/55% CO_2	Same as above	NS	Same as above	Same as above	Same as above

Hewett *et al.* (1993)	GI	CO_2 alone/100%	103–109.0 (loss of righting) 139–144.4 (loss of pedal reflex)	374.0	NS	CO_2 leads to ↓in pH, PaO_2, and % Sat. of Hb; ↑in $PaCO_2$	
	GI	CO_2:O_2:N_2/ 75%:20%:5%	97.9 (loss of righting) 140.8 (loss of pedal reflex)	NS	NS	NS	Same as above
Hornett & Haynes (1984)	GI	CO_2 alone/NS	~160 for each group	NS	Apprehension; laboured breathing	NS	
Iwarrson & Rehbinder (1993)	GI	CO_2:O_2 80%:20%	27.0	196.0	Mild uneasiness and laboured breathing, mild stress response	Lungs: marked hyperaemia, oedema, and haemorrhages	
Smith & Harrap (1997)	GI	CO_2 alone/75%	97.2	541.2	Immediate gasping or laboured breathing	Rapid drop in blood pressure after 4 min followed by elevation to normal levels for 2 min prior to collapse	
Blackshaw *et al.* (1988)	PF	CO_2 alone/97%	7.8	135.0	None recorded (only movement was measured and only for 10 s)	NS	Used dry ice – may be difficult to maintain consistent concentration
	PF	CO_2 alone/97%	11.8	78.0	Same as above	NS	Same as above
Britt (1986)	PF	CO_2 alone/100%	18.0	171.0	Hyperventilation Shaking Urination, defaecation	Some lung haemorrhage	
Coenen *et al.* (1995)	PF	CO_2 alone/100%	30	408	Signs of asphyxia; author reported signs of agitation and excitation (not described)	EEG stages: normal; lowered amplitude; slow waves and large spikes Heart rate: normal; rapid decrease; cessation	Slow wave EEG commenced before postural collapse

(continued)

Table 2 (continued)

Reference	Prefilled (PF) or gradual induction (GI)	Gas mixture/final concentration	Estimated time to unconsciousness (s)	Estimated time to death (s)	Behaviour before unconsciousness	Physiology/neurological measures	Comments
Danneman *et al.* (1997)	PF	CO_2 alone/60%	NS	19.0	NS	Lungs: marked perivascular and alveolar haemorrhage; perivascular oedema Haemorrhage from nose; excessive salivation; serosanguinous nasal discharge	
	PF	CO_2 alone/60% until anaesthetized then 100%	NS	19.0	NS	Haemorrhage from nose; excessive salivation; serosanguinous nasal discharge	
	PF	CO_2 alone/70%	NS	15.3	NS	Serosanguinous nasal discharge; haemorrhage from nose	
	PF	CO_2 alone/70% until anaesthetized then 100%	NS	8.8	NS	Serosanguinous nasal discharge; haemorrhage from nose	
	PF	CO_2 alone/80%	NS	11.1	NS	Lungs: marked perivascular haemorrhage; perivascular oedema and intra-alveolar haemorrhage, Serosanguinous nasal discharge	
	PF	CO_2 alone/80%/until anaesthetized then 100%	NS	8.7	NS	Serosanguinous nasal discharge	
	PF	CO_2 alone/100%	NS	4.6	NS	Lungs: perivascular oedema; perivascular and intra-alveolar haemorrhage	

Hewett *et al.* (1993)	PF	CO_2 alone/100%	19.0 (loss of righting) 27.8 (loss of pedal reflex)	109.2	NS	CO_2 leads to ↑$PaCO_2$; ↓pH and percent saturation of Hb	Reported no indication of distress, but behaviours other than locomotion not measured
Iwarsson & Rehbinder (1993)	PF	CO_2 alone/100%	13.0	116.0	Moderate uneasiness, tachypnoea, urination, defaecation, mild–moderate stress response	Lungs: emphysema, oedema and extravasation of blood	
	PF	CO_2 alone/100%	13.0	116.0	Moderate uneasiness, tachypnoea, urination, defaecation, mild-moderate stress response	Lungs: emphysema, oedema and extravasation of blood	
Leach *et al.* (2002a)	PF	CO_2 alone/25.5% or 34.9% or 50.8%	NA	NA	Shorter withdrawal and dwelling times in comparison with halothane, isoflurane and enflurane (therefore more aversive); significant increase in washing	NS	This study did not involve euthanasia
	PF	CO_2 alone/25.5% or 34.9% or 50.8%	NA	NA	Shortest withdrawal and dwelling times in comparison with other rat groups (therefore most aversive): medium and high concentrations aversive		This study did not involve euthanasia

(continued)

Table 2 (continued)

Reference	Prefilled (PF) or gradual induction (GI)	Gas mixture/final concentration	Estimated time to unconsciousness (s)	Estimated time to death (s)	Behaviour before unconsciousness	Physiology/neurological measures	Comments
Leach *et al.* (2002a)	PF	CO_2:argon/ 25.5%:5% or 31.7%:5% or 59.2%:5%	NA	NA	Shorter withdrawal and dwelling times in comparison with argon alone; medium and high concentrations most aversive	NS	This study did not involve euthanasia
	PF	CO_2:argon/ 19.1%:7% or 32.1%:7% or 54.2%:7%	NA	NA	Same as above	NS	This study did not involve euthanasia
Smith & Harrap (1997)	PF	CO_2 alone/75%	26.4	311.4	Immediate circling and urination; gasping or laboured breathing;	Rapid ↓in pulse rate and blood pressure followed by slow drop in blood pressure	Visual signs of death precede vascular collapse by 1 min, therefore must maintain gas flow for at least 1 min after signs of death

This table includes the results of studies of rats only, for groups that were exposed to CO_2. Therefore, if a study compared CO_2 with other agents, information of other agents was not included in order to keep the table as short as possible. Time to unconsciousness was determined conservatively as the onset of postural collapse and loss of muscle tone (Woodbury *et al.* 1958, Coenen *et al.* 1995); the present authors calculated these times when they were not specified directly by the authors of the original papers. NS = not specified

and head turned up and backward) in some conditions and those in the 100% CO_2 group showed most evidence of this behaviour. These results support the notion that higher concentrations of CO_2 lead to increased adverse reactions; this is in contrast to the conclusion by Danneman *et al*. (1997) that lower concentrations of CO_2 lead to increased incidence of adverse reactions.

Leach *et al*. (2002a,b) studied aversion to various gaseous euthanasia agents, including CO_2, in rats and mice at low, medium and high concentrations. Measures of avoidance of, or preference for, different environments are well-established tools in the study of farm animal welfare. The animal's choice of environment is considered to reflect its priorities to access or avoid specific stimuli. In one study (Leach *et al*. 2002a), the agents were CO_2 (25%, 35%, 50%) and argon (93%, 95%, 98%), as well as two combinations of CO_2 and argon. A second study (Leach *et al*. 2002b) compared halothane, isoflurane, enflurane and CO_2 at low, medium and high concentrations. Aversion measures included time taken to initially withdraw from the test chamber, time spent in test chamber and frequency of entries and exits to and from the test chamber. Additional behavioural measurements indicative of aversion included washing, rearing, sniffing and excreting.

Overall, CO_2, alone or in combination with argon, caused the highest degree of aversion in both rats and mice in both studies, with higher concentrations leading to significantly shorter withdrawal times (i.e. 0.6 s at highest concentration). In regards to the additional behavioural measurements examined, increase in frequency of 'washing' was the only clear trend, possibly the result of nasal irritation. The mice and rats differed in regards to response threshold, with mice having a lower threshold to CO_2. Leach *et al*. concluded that CO_2, either alone or in combination with argon, cannot be used humanely at any concentration and is therefore unacceptable as a euthanasia agent for laboratory rodents, particularly when more humane methods exist.

The findings of the studies discussed above demonstrate that CO_2 causes adverse effects that lead to stress, and perhaps distress, at various concentrations ranging from 25% to 100%.

A number of studies have examined the histological effects of CO_2 and some results raise further concerns regarding pain and/or distress. Some physiological evidence of the effects of CO_2 suggests that increasing physical trauma occurs when the extent of exposure to the inhalant gas increases. Terminal CO_2 inhalation has been shown to lead to alveolar extravasation (bleeding into the tissue) and pulmonary oedema (Danneman *et al*. 1997), haemorrhaging, lung oedema and emphysema (Iwarsson & Rehbinder 1993), and degeneration of myocardial tissue and other organs (Iwarsson & Rehbinder 1993). Britt (1986) reported increased pulmonary haemorrhaging in higher concentrations of CO_2. However Danneman *et al*. (1997) found that the severity of pulmonary oedema and haemorrhage were inversely correlated with the concentration of inhaled CO_2; this may be the result of increased time of exposure. Fawell *et al*. (1972) found oedema of perivascular connective tissue in the lungs of all rats subjected to CO_2 euthanasia (concentration and flow rate were not indicated). An increased incidence of extravasation was considered to be related to the trauma of asphyxiation.

Only one recent study (Ambrose *et al*. 2000) has assessed the extent of oedema and alveolar consolidation (inflammatory induration of lung tissue) from samples taken immediately at the point of unconsciousness instead of following death. This study compared the use of 30% CO_2 with 30% CO_2 plus 20% O_2 in two strains of mice and it demonstrated greater alveolar consolidation in the $CO_2 + O_2$ condition. This consolidation may have occurred just prior to loss of consciousness and led the authors to propose that the animal may not only demonstrate hyperventilation with exposure to $CO_2 + O_2$, but may also experience a state similar to drowning while the animal is still conscious (Ambrose *et al*.

2000). Danneman *et al*. (1997) also noted damage to the lungs and/or respiratory tract via observations of serious serosanguinous nasal discharge during exposure to 50% CO_2, but it is unclear whether this occurred when animals were still conscious. This evidence does raise the question of whether, without visible behavioural effects, the animal may experience a highly distressing state while still conscious, as oedema and haemorrhaging develop.

Prefilled (PF) chamber versus gradual induction (GI)/times to anaesthesia and death

The manner by which animals are exposed to CO_2, PF chamber versus GI, has been examined fairly extensively. The results of these studies do not clearly indicate which method is preferred in regards to animal welfare, but these studies do demonstrate that both methods raise concerns in regards to animal welfare. Smith and Harrap (1997) compared GI (from 0% to 80% CO_2: 3% O_2) versus PF (75% CO_2: 3% O_2) methods in rats. PF caused an immediate fall in blood pressure while GI caused increased blood pressure for the first 4 min and then a rapid decline. Additionally, time to unconsciousness was 30 s in PF subjects and 99 s in GI subjects; time to death was 5.4 min in PF subjects and 9 min in GI subjects. Although head-raising, urination, defaecation and gasping or laboured breathing were reported (behaviours were predetermined by the authors to be indicators of stress, pain, distress, anxiety and fear), the authors reported that CO_2 caused few overt signs of distress in either group.

Hewett *et al*. (1993) examined the differences between PF and GI methods in rats at a final concentration of 100%. Measurements included blood gas quantitation, and times to ataxia, immobility, loss of righting ability, loss of pedal reflex and anaesthesia (two consecutive negative pedal reflexes). The shortest amount of time to loss of pedal reflex was 28 s, which occurred in the subjects exposed to the PF chamber. Loss of pedal reflex in the GI study occurred at 140 s, or six times longer than the PF method. Overall, the authors reported that the animals' responses were not indicative of distress by either method. However, the study did not clearly indicate that any specific behaviours were looked for and therefore it is impossible to determine whether the animals were observed for anything other than ataxia and loss of pedal reflex.

Britt (1986) presented information on the humaneness of CO_2 use based on a comparison of PF versus GI methods using rats and mice. Evaluations were based on types of behaviour, time spent in each activity, number of times activity was changed per unit of time, changes in breathing patterns with time and position in cabinet at loss of consciousness. Britt noted that time to collapse was shorter with PF, but PF caused more signs of distress. Abnormal behaviours (referred to by the author as signs of distress) included: shaking (frequent), moving in reverse, tail thrashing (uncommon) and increase in frequency of urination and defaecation. Urination and defaecation may result from the physiological effects of CO_2 on the autonomic nervous system and conclusions regarding their use as indicators of stress should be cautious; however, they did arise in association with increased micturation, which may indicate disturbance or distress (Britt 1986). Behavioural responses varied between species and individuals. The author concluded that neither method is stress-free; however, he favoured GI.

Again, there is evidence from these studies that CO_2 is associated with pain and distress, regardless of induction method. Overall, two studies concluded that neither method is problematic in regards to pain or distress (Hewett *et al*. 1993, Smith & Harrap 1997), while one study (Britt 1986) indicated that prefilling leads to increased adverse reactions. One could conclude, therefore, that GI is preferred due to absence of adverse reactions, despite the fact that it may take longer to euthanize the animals.

Presence of supplemental oxygen

The use of oxygen supplementation in order to minimize pain and distress during CO_2

use has also been a point of debate. Existing studies, again, provide conflicting results, depending on the effects examined.

Iwarsson and Rehbinder (1993) examined CO_2:O_2 mixtures for euthanasia of rats, mice and guineapigs. Animals were exposed to CO_2 from a gas cylinder at either 100% CO_2 or 80% CO_2: 20% O_2 via GI. The presence of oxygen doubled the amount of time to unconsciousness in rats and mice but, according to the authors, appeared to decrease distress. Stress response was determined by examining various behaviours, such as breathing, urination and defaecation. Postmortem examinations of lung tissue revealed that both methods generated adverse physiological reactions. It is not clear, however, if these changes in lung tissue occurred before or after unconsciousness.

As previously mentioned, Coenen *et al*. (1995) reported that there were four phases in the response to CO_2 inhalation in rats. The phase of continuous abnormal activity (excitation and agitation) was completely absent in the presence of oxygen. While 100% CO_2 produced the shortest time to death, the authors concluded that the presence of added oxygen produced a longer time to death but with a reduction of adverse effects. Therefore, adding oxygen was considered to be the preferred method. However, the difference in concentrations of CO_2, and not the use of supplemental oxygen, could be the cause of these results.

The results from Ambrose *et al*. (2000), however, appear to conflict with those of Coenen *et al*. (1995). Ambrose *et al*. compared 30% chamber volume per minute of CO_2 with and without supplemental oxygen. There were no behavioural differences observed between the two conditions and presence of oxygen was found to increase alveolar consolidation. The authors concluded that '[a]s haemorrhage is likely to be stressful to the mice by inducing a feeling of 'drowning', any alveolar consolidation above basal level indicates potential poor welfare' and that addition of 20% chamber volume per minute of O_2 is not recommended as a refinement to CO_2 euthanasia. The reason for the conflict with Coenen *et al*. (1995) results is unclear, but neither author described the behaviours that were assessed in order to determine aversion; differences in behavioural data collection and analysis could explain differences between results of this as well as other studies.

Time to and measurements of unconsciousness

It is important to determine the point at which unconsciousness occurs in order to clearly identify whether adverse effects occur prior to or following unconsciousness. Experienced veterinary clinicians have indicated that anaesthesia sets in so quickly when CO_2 is used that there is not sufficient time for the animals to experience significant pain and distress. However, a review of the literature finds that times to unconsciousness vary greatly when using recommended methods. As discussed, many authors use different terminology, such as: anaesthesia, collapse, recumbency, unconsciousness and immobility. The following are observations of rats exposed to the AVMA-recommended condition of prefilling the chamber with 70% CO_2: Danneman *et al*. (1997) recorded that anaesthesia (onset of slow, shallow breathing and loss of response to toe pinch with haemostats) occurred at an average of 4.01 min; Mischler *et al*. (1994) recorded anaesthesia ('appeared comatose, exhibited muscle flaccidity and were unresponsive to deep pressure applied to the tail and/or hindpaw') within a maximum of 10 s; and Smith and Harrap (1997) reported 'loss of recumbency' at an average of 26.6 s. What could account for this variability? Some possibilities are variations in gas concentrations, sample sizes, methods and equipment used, condition of the animals, and how unconsciousness was actually measured.

Although many papers have described the onset of ataxia as symptomatic of the onset of the effects of CO_2 (Hewett *et al*. 1993, Coenen *et al*. 1995, Danneman *et al*. 1997), it is not clear whether ataxia itself is distressing. It is possible that the inability to behave adaptively or complete purposeful locomotion might be unpleasant. Further

research is needed to assess whether an ataxic state is aversive.

Overall, as the inhaled CO_2 concentration increases, both time to unconsciousness (Britt 1986, Danneman *et al*. 1997, Ambrose *et al*. 2000, Leach *et al*. 2002a,b) and time to death (Britt 1986, Ambrose *et al*. 2000) decrease, while the addition of oxygen to the inhalant gases increases time to death (Coenen *et al*. 1995). Additional details are provided in Figure 1 and Tables 1 and 2. It would appear that if time to unconsciousness were the only criteria influencing distress, higher concentrations of CO_2 would be beneficial. However, before such a decision is made, it is important to determine what the intensity of such distress might be, while also taking into consideration reaction of humans to high concentrations of CO_2.

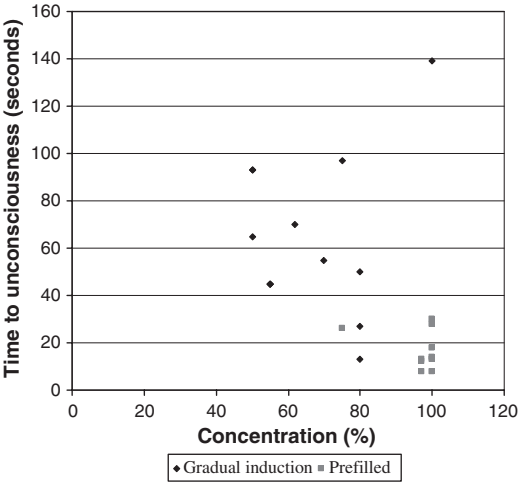

Figure 1 Effects of concentration on times to unconsciousness: gradual induction and prefilled chamber

Summary

The literature demonstrates that CO_2 is painful and/or distressful in humans at concentrations ranging from 7% to 100%, and that there is sufficient evidence that CO_2 likely causes pain and distress in animals. Table 3 summarizes the incidence of adverse reactions in the literature reviewed here. Adverse reactions to CO_2 in species other than those discussed in this review, including cats (Simonsen *et al*. 1981), dogs (Eisele *et al*. 1967) and guineapigs (Iwarsson & Rehbinder 1993), can be found in the scientific literature. In summary, the human and animal literature

Table 3 Comparison of publications reviewed for this paper: those that reported adverse effects and those that reported no adverse effects in response to carbon dioxide

Author (year)	No adverse effects reported	Adverse effects reported
Ambrose *et al*. (2000)		√
Barbaccia *et al*. (1996)		√
Blackshaw *et al*. (1988)	√	
Britt (1986)		√
Coenen *et al*. (1995)		√
Danneman *et al*. (1997)		√
Fawell *et al*. (1972)		√
Hackbarth *et al*. (2000)	√	
Hewett *et al*. (1993)	√	
Hornett & Haynes (1984)	√	
Hoenderken (1983)	√	
Iwarsson & Rehbinder (1993)		√
Jongman *et al*. (2000)	√	
Leach *et al*. (2002a)		√
Leach *et al*. (2002b)		√
Raj & Gregory (1994)		√
Raj & Gregory (1995)		√
Raj & Gregory (1996)		√
Robb *et al*. (2000)		√
Smith & Harrap (1997)		√
Thurauf *et al*. (1991)		√

signals that the use of CO_2 may be problematic.

Rodent studies that compare the use of CO_2 alone with other euthanasia methods

A fair amount of the published data on CO_2 stems from studies that compared CO_2 with other euthanasia methods. CO_2 concentration, induction method and presence of oxygen remain potentially confounding factors. The studies discussed here conclude that CO_2 is a preferable euthanasia agent for rodents in comparison to ether, chloroform, nitrogen, and that the use of anaesthesia with pentobarbital or sedation prior to induction with CO_2 has no particular benefits.

Blackshaw *et al*. (1988) compared CO_2, ether and chloroform in rats, mice and chickens. Dry ice was used to generate 97% CO_2 in a prefilled chamber. Time to collapse and death were shortest with CO_2 for all three species in comparison with ether and chloroform. Adverse behavioural signs to CO_2 were not observed, except for wing flapping in chickens. Arguing that the shortest time to death is preferable when choosing a euthanasia agent (while also taking behaviour prior to unconsciousness into consideration), the authors concluded that CO_2 was the preferred agent for rats and mice, but not chickens. One potential problem with this study is that the behaviours observed focused on the animal's movement and did not include other behaviours that may be indicative of stress (e.g. urination and defaecation) that were observed in other CO_2 studies.

Hornett and Haynes (1984) compared CO_2 and nitrogen in rats and concluded that CO_2 was the preferred euthanasia method. CO_2 from a gas cylinder was gradually induced and behaviours measured included perceived signs of fear (behaviours were not specified) and oxygen deprivation, as well as laboured breathing, loss of co-ordination and balance, collapse (correlated with unconsciousness) and respiratory arrest. Overall, the rats had an extreme response to nitrogen including panic, attempts to escape and convulsions, while CO_2 caused increased 'nasal movement' and a stage of laboured breathing. The authors concluded that CO_2 at 19.5% chamber volume per minute 'achieved a quiet delivery into unconsciousness'.

Finally, Hackbarth *et al*. (2000) examined whether sedation (acepromazine in chopped meat) or anaesthesia (injection of sodium pentobarbitone) prior to the use of CO_2 would decrease the stress-induced effects of CO_2 euthanasia. Rats were subjected to one of four conditions followed by CO_2: meat with acepromazine, meat without acepromazine, injection with pentobarbitone or injection with saline. Behavioural observations were recorded and adrenocorticotrophic hormone (ACTH), glucose and corticosterone measurements were taken at various times after CO_2 induction began. No group exhibited behavioural signs of pain or distress. Glucose and corticosterone levels did not differ between the groups. ACTH was higher in the subjects given injections in comparison with those given oral treatments (assumed to be a result of handling stress). Given the relative absence of effects of sedation and anaesthesia, the authors concluded that '…euthanasia with CO_2 [without prior sedation or anesthetization] is in concordance with animal welfare as it is rapid and does not cause distress in the animal and therefore can be recommended as ''humane'' '.

These studies do not show evidence of distress as some of the previously discussed studies have. Again, this may be a result of the behaviours examined and differences between studies in interpreting behaviour and other measures. Differences in behaviour are important to recognize because each individual, as well as each species, has a particular coping strategy and pain threshold (Leach *et al*. 2002b).

Literature on stunning for slaughter

The use of CO_2 for stunning prior to slaughter has been examined in a number of studies with different species. Overall, many studies found that CO_2 is aversive, as demonstrated by avoidance by the animals.

Raj and Gregory (1995, 1996) compared the use of argon (90%) and CO_2 (30% or 90%) for stunning in pigs and found high concentrations of CO_2 to be highly aversive. Aversion was assessed from the pigs' reluctance to enter or remain in the gaseous atmospheres for a reward of apples. When 90% CO_2 was in the box, the pigs immediately withdrew their heads, repeatedly attempted to feed, but withdrew their heads when they began to hyperventilate. The following day, three out of six pigs hesitated to enter the box. It was concluded that high concentrations (90%) of CO_2 were aversive to the majority of pigs (88%) and that they would not attempt to get the apples, even after 24 h of fasting. By contrast, pigs did not demonstrate aversion to the presence of argon and the majority did not demonstrate aversion to 30% CO_2 in air (Raj & Gregory 1995). Since the design of the study allowed for escape and did not force the animals to be exposed to the gas, distress was not examined. However, the study does demonstrate that when given a choice, pigs will avoid high concentrations of CO_2. Comparable results were found in a similar experiment on turkeys (Raj & Gregory 1994).

A second study (Raj & Gregory 1996) examined the severity of respiratory distress when pigs were placed in a well of various concentrations of argon with or without oxygen and/or CO_2 in air. Respiratory distress was scored and it was found that argon with 2% oxygen induced minimal respiratory distress, while the combination of 30% CO_2 and argon with residual oxygen induced moderate distress, and all concentrations of CO_2 in air 'induced severe respiratory distress in the pigs' (Raj & Gregory 1996).

Hoenderken (1983) compared CO_2 and electrical stunning methods in pigs and also found CO_2 to be aversive. Behaviours (specific behaviours were not indicated) and EEG were recorded while CO_2 was used; specific concentrations and induction methods were not clearly indicated in the publication. It was found that pigs show signs of excitation after 12 s of exposure to CO_2 and this excitation lasts 26 s on average (isoelectric EEG was recorded at 56 s). The author concluded that there is a long period of excitement during CO_2 exposure. Jongman *et al*. (2000), however, found shock with an electric prod to be more aversive than 90% CO_2 based on the pigs' reluctance and time to enter the treatment area following previous exposure to the stimuli. Behaviour other than time to enter treatment area was not measured in this study.

Finally, four slaughter methods (exsanguination without stunning, CO_2 stunning followed by gill cutting, percussive stunning and spiking) were compared in salmon; behaviour and visual evoked response to a flash of light (lack of response = good indicator of brain failure) were examined (Robb *et al*. 2000). CO_2 caused the fish to shake their heads and tails vigorously for 2 min, which resumed again after gill cutting. Loss of visual evoked responses also took much longer with exsanguination and CO_2. Overall, the authors recommended the use of stunning or spiking over exsanguination or CO_2.

These studies, overall, demonstrate that CO_2 stunning for slaughter causes adverse effects and is avoided when the animals are provided with the opportunity to do so. In some instances, as described by Jongman *et al*. (2000), the animals will choose CO_2 over other methods; however, this does not indicate that CO_2 is the preferred of all available methods.

Pain and distress: considerations

Pain is considered to be the experience of an unpleasant sensory and emotional state that arises from central perception of nociception mechanisms in response to actual or potential tissue damage (International Association for the Study of Pain 1979). Distress is primarily considered to encompass both physiological and psychological states, and may be observed through physiological and behavioural responses which indicate that the animal is experiencing stimuli as noxious and from which the animal is motivated to avoid or withdraw (Dawkins 1990, Rolls 1999).

There are a range of measures of physiological function and behaviour that

may be used to determine whether an animal is in a state of distress. These include heart rate, blood pressure, circulating free cortisol, measures of behavioural aversion or avoidance and behavioural correlates of pain or distress (Appleby & Hughes 1997, Mason *et al*. 2001). A range of authors have measured a variety of behavioural and physiological responses as indicators of pain and distress in studies of CO_2, such as behavioural signs of asphyxia (Coenen *et al*. 1995), hyperventilation (e.g. Britt 1986, Kohler *et al*. 1999), escape behaviours (Britt 1986, Leach *et al*. 2002a,b), tail thrashing (Britt 1986), disturbance behaviours such as washing (Britt 1986) and physiological signs of nociception (Anton *et al*. 1992). The interpretation of such behavioural measures can be difficult if the association between the behaviour and an underlying state has not been validated, or where behaviours are poorly described, or where quantitative measures are not used and the reliability of observational recordings confirmed (see Martin and Bateson (1993) for a discussion). Further complications arise as some authors may not have been consistent in measuring particular behaviours, or may have drawn different conclusions regarding similar behavioural phenomena (e.g. Britt 1986, Blackshaw *et al*. 1988, Hewett *et al*. 1993, Danneman *et al*. 1997).

As seen in Tables 1 and 2, the current evidence regarding CO_2 inhalation and pain and distress is based on studies using a diverse range of exposure methods, gas concentrations and mixtures, as well as species and genetic strains. The types of variables measured are inconsistent, and often physiological and behavioural data have not been collected from the same experiment. For behavioural data to be meaningful, rigorous ethological techniques are required, including organized sampling methods, quantifiable measurement and good quality objective description, allowing for future interpretation and replication (Martin & Bateson 1993). Behaviours interpreted as indicating underlying physiological changes should be validated where possible. The increasing sophistication of techniques from animal welfare science, such as measures of aversion, are valuable tools for understanding the animal's priorities and choices in light of exposure to environmental stimuli. The use of control animals (where CO_2 is not present) would also be useful to indicate the extent to which the behaviours and physiological responses observed in previous studies arise from the novelty, unfamiliarity or mixing with unfamiliar conspecifics. Despite some of the problems with the data in the above studies, it can be concluded that the information available is more than sufficient to raise concerns about the pain and distress associated with CO_2 euthanasia.

Existing guidelines for euthanasia

Various organizations and agencies such as the AVMA (2001), CCAC (1993), the European Commission (1996, 1997), the Australia and New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART 2001), the UK Home Office (1997b) and the Universities Federation for Animal Welfare (UFAW 1986) provide guidelines on euthanasia. These guidelines provide conflicting recommendations in regards to CO_2 euthanasia, specifically the optimal concentrations, rates of induction and sources of CO_2.

In July 2002, the Office of Laboratory Animal Welfare (OLAW) of the US National Institutes of Health published a guidance notice that high concentrations of CO_2 may be distressful to some species and therefore prefilling the chamber is only recommended when such use has not been shown to cause distress (OLAW 2002). This recommendation conflicts with the AVMA guidelines that suggest using a prefilled chamber, illustrating the continuing confusion regarding CO_2 use.

Discussion of CO_2 use

The evidence that CO_2 causes pain and distress in both humans and animals can be summarized as follows.

(1) CO_2 has been used in animals as a painful stimulus (Thurauf *et al*. 1991),

at a concentration that is typically used for euthanasia, and in studies to elicit an acute stress response (Barbaccia *et al*. 1996); this supports the claim that CO_2 causes pain and distress in rodents.

(2) Although high concentrations of CO_2 have been found to cause more pain and distress in comparison with low concentrations, pain and distress have also been reported at low concentrations, such as 20% CO_2 in pigs (Raj & Gregory 1996) and 25% in rats and mice (Leach *et al*. 2002a,b). Reported findings that there is increased noxiousness with increased concentrations of CO_2 are of concern because some literature and most available euthanasia guidelines recommend the use of higher concentrations of CO_2 due to its shorter time to recumbency, anaesthesia and euthanasia.

(3) According to published findings and anecdotal reports, GI and prefilled euthanasia chambers both lead to concerns about pain and distress.

(4) The histological effects of CO_2 on lungs, heart and other organs, including oedema and haemorrhage, raise concerns about animal distress.

Policies of the OECD and individual countries indicate that if a procedure causes pain and distress in humans, it must be considered to cause pain and distress in animals (US Public Health Service Policy also indicates 'in the absence of evidence to the contrary'). The evidence that CO_2 elicits a painful response in humans (Dripps & Comroe 1947, McArdle 1959, Anton *et al*. 1992, Hummel *et al*. 1994, Danneman *et al*. 1997) is very strong. Therefore, these policies require that CO_2 use should be considered painful and distressful in animals.

Alternatives to use of CO_2

Discontinuing use of CO_2 alone as a euthanasia agent is unlikely to happen soon because it is so widely used, but there are some reasonable alternatives.

Pre-anaesthetic followed by CO_2

The use of a pre-anaesthetic to induce unconsciousness prior to induction of CO_2 for euthanasia should be considered. Halothane was found to be the least aversive by Leach *et al*. (2002b) when distress associated with induction of halothane, isoflurane, enflurane and CO_2 were compared. Halothane, isoflurane and enflurane were found to be more aversive than air, but were significantly less aversive than CO_2.

Use of pre-anaesthetic agents with CO_2 should be acceptable for the euthanasia of large numbers of animals not involved in research protocols or for the euthanasia of small numbers of animals where the effect of the anaesthetic gas is not a problem. Where the research protocol calls for tissues unaffected by anaesthetics, decapitation may be the best method for small numbers of rats or mice. However, this method is still questioned because of the debate over the meaning of the EEG trace following decapitation and also because of the high chance for error due to inadequate operator training or guillotine maintenance.

Inhalational gas agents are particularly advantageous because they require minimal handling of the animals and larger numbers of animals can be euthanized simultaneously. As Hackbarth *et al*. (2000) report, an injectable pre-anaesthetic can cause a handling-induced stress response.

One concern about the use of volatile anaesthetics is exposure of personnel to the gas. However, there are portable scavenging units that would allow for safe use of both a pre-anaesthetic and CO_2. The European Commission recommendations for euthanasia indicate that halothane, enflurane and isoflurane 'are all acceptable agents with appropriate gas scavenging apparatus'.

Argon

Argon, an odourless, inert gas that is non-flammable and non-explosive (AVMA 2001), is another option for euthanasia that might be an improvement over the use of CO_2. Leach *et al*. (2002b) found argon to be much less aversive to rats and mice in comparison to CO_2, although all examined concen-

trations of argon (ranging from 25% to 53%) caused a degree of aversion in comparison to air. Raj and Gregory (1995) found no aversion to argon (90%) in pigs. The AVMA (2001) considers argon to be only conditionally acceptable, but this recommendation did not cite the scientific literature. The existing literature, however, does generally demonstrate that argon is preferable to CO_2 in regards to animal welfare. The Farm Animal Welfare Council (2003), for example, indicates that argon is less aversive in pigs in comparison to CO_2. The council also expresses support for additional research in order to determine how to best use argon for euthanasia purposes.

Lawson *et al*. (2003) compared the use of CO_2, nitrogen and argon for euthanasia and found that CO_2 induced 'apparent unconsciousness' within 40 s and death within approximately 3 min, and argon had similar effects (unconsciousness at 55 s and death at 4 min). However, CO_2 caused a rapid and significant increase in blood pressure (measured by radio-telemetry), while argon and nitrogen caused muscle rigidity and spasms. In this case, the authors concluded that CO_2 is preferable for euthanasia due to the rapidity of effects and lack of muscle rigidity and spasms. Finally, the authors did not rule out that CO_2 could have caused a stress effect.

Overall, argon is as easy to use as CO_2, is non-flammable, and sinks to the bottom of the chamber since it is heavier than air, therefore reducing danger to those conducting the procedure. One drawback, however, is that argon is more expensive than CO_2; however, utilizing a method that is less painful and distressful to the animals should take priority over cost. Moreover, the costs of both argon and CO_2 are trivial compared with the total financial investment in an animal when all real costs are taken into account (e.g. technician and investigator time, costs of the animal and the animal's care, etc.).

Decapitation

Decapitation is a possible alternative to the use of CO_2 for the euthanasia of (relatively) small numbers of animals, but this remains somewhat controversial. Mikeska and Klemm (1975) found that decapitation causes an EEG trace for an average duration of 13.6 s; this study led to much of the controversy surrounding decapitation and previous recommendations by the AVMA (1986, 1993) that decapitation should not be done without justification.

Various authors have since challenged Mikeska and Klemm's (1975) findings. For example, Allred and Berntson (1986) presented a counter-argument, citing references that demonstrated an activated EEG pattern after hypoxia or damage to the lower brain stem as evidence that the Mikeska and Klemm data did not necessarily provide evidence of consciousness and distress. Vanderwolf *et al*. (1988) conducted studies that examined the source of low voltage fast activity (LVFA) and found that such EEG traces are not associated with noxious stimuli or consciousness. Additionally, Derr (1991) reported that it would take 2.7 s for a decapitated rat's head to become anoxic (indicating a presumed loss of consciousness).

Finally, Holson (1992) found that, by 1986, all eight papers reporting on the EEG of decapitated rodent heads agree that decapitation triggers an immediate, slow direct current EEG trace of 2–4 s duration followed by an LVFA trace that is usually gone in 10–13 s, a pattern that is not associated with consciousness.

One reason that decapitation is sometimes chosen over other methods is that some agents, such as anaesthetics, can cause changes in tissue and alter brain metabolism parameters (see, e.g. Miller *et al*. 1988) and the aim of the study may be to collect 'undisturbed' tissue. In this case, however, it is important to consider that the stress of handling during the decapitation procedure may also modulate metabolite levels (e.g. Faupel *et al*. 1972). There may, of course, be occasions where the stress of handling is unlikely to change the parameters a scientist wishes to measure, but these should be readily justifiable.

In sum, decapitation produces an EEG trace that can last for 10–13 s, but the weight

of the evidence indicates that consciousness following decapitation is unlikely to persist for more than 3–6 s. It seems clear that decapitation produces a much quicker loss of consciousness than the recommended CO_2 protocols. Therefore, from an animal welfare perspective, decapitation properly performed may be preferable to CO_2. However, decapitation is not an easy procedure and technicians who use a guillotine must be properly trained and the equipment must be properly maintained.

Recommendations and final discussion

While CO_2 has long been used as a method of euthanasia, questions have arisen that this practice may not fulfill the criteria for an easy and gentle death, as required by both ethical concerns and policy. The primary concerns are that exposure to CO_2 gas may be painful; CO_2 may cause onset of asphyxia while the animal is still conscious; physiological effects of CO_2 on nasal mucosae and on the autonomic nervous system may be distressing; the cognitive/perceptual/behavioural effects of CO_2, such as ataxia, may be disturbing to the animal; and the process of CO_2 inhalation may be highly aversive. Some or all of these results may cause pain and distress.

Information regarding concerns with the use of CO_2 for euthanasia, described in this review, has led organizations such as The Humane Society of the United States to recommend changes, such as the use of an inhalant pre-anaesthetic in conjunction with CO_2 for euthanasia of a large number of surplus rodents or a few to moderate number of rodents being used for research. Decapitation is strongly discouraged when large numbers of animals are being euthanized because of the chances for operator error. For euthanasia of relatively few rodents being used for research that does not permit contamination of tissues, The Humane Society of the United States recommends decapitation by well-trained personnel if there is scientific justification, and when CO_2 in conjunction with a pre-anaesthetic has well-founded scientific concerns. The use of CO_2 alone is strongly discouraged in all instances.

Personnel play a big role in the issues discussed in this paper. Proper training of personnel in all techniques is essential for improved animal welfare. Centralized facilities in which the veterinary care staff performs such techniques are recommended; it is easier to ensure adequate training in centralized facilities and the expertise is typically higher among those who are performing euthanasia.

We recognize that many clinical veterinarians and laboratory animal technicians appear to be comfortable with CO_2 alone as a euthanasia agent. However, we would argue that the conflicting data in the literature and the arguments around prefilled chambers versus GI, the presence or absence of oxygen, the lack of agreement on time to unconsciousness and the fact that CO_2 produces significant physiological changes and behavioural indicators of pain and distress, all demand a careful reassessment of the use of the gas by itself as a euthanasia agent. Careful studies of distress and objective assessments of such distress using behavioural and physiological measures are clearly possible, as evidenced by the progress made in assessing farm animal welfare (see, for example, Appleby & Hughes 1997).

The limitations in the currently available data outlined above (such as small sample sizes, examination of only certain measures while excluding others, and so on) suggest the need for a structured, multi-factorial study utilizing a range of dependent physiological and behavioural measures, evaluating a representative range of modes of CO_2 and CO_2–gas mixture exposures. This type of study would reveal the effects of CO_2 exposure while controlling for methodological and procedural differences in practice between institutions. Most importantly, with effective coordination, this type of study could be completed without incurring further potential animal distress, by gathering data from existing practices, with welfare measures taken as add-on to procedures that would be performed on existing animals. This type of applied animal

welfare research has already led to significant increases in the understanding of animal distress within the farm animal arena (e.g. King 2003).

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