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Hyperbaric Carbon Dioxide: a possible refinement of euthanasia in laboratory rodents

Thesis

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Index

1 Introduction

Carbon dioxide (CO_2) is widely used to euthanize laboratory rodents but it is controversially discussed from an animal welfare's point of view. Animal welfare is the main factor to consider when choosing a suitable euthanasia method(American Veterinary Medical Association 2001, Boivin et al. 2017, Conlee et al. 2005, Flammer et al. 2019).

1.1 Aim of the study

Within the scope of refinement, the aim of this study is to evaluate the potential and the feasibility of a method to euthanize rodents by using hyperbaric carbon dioxide. Therefore published evidence was reviewed and included information on the effects of CO_2 on laboratory animals. Additionally other refinement options which may also lead to a quicker death of the rodents and the simultaneous use of other gases like nitrous oxide were reviewed.

Time to death is crucial in the euthanasia process and the hypothesis is that by using hyperbaric carbon dioxide unconsciousness and death occur quicker so that distress and pain are less or may even not occur. Also the necessary concentration of CO_2 might be lower under hyperbaric conditions which will cause less dyspnea and mucosal irritation. This study is a review on CO_2 euthanasia in laboratory rodents and explores the rationale and the possibility of using hyperbaric CO_2 as a possible refinement.

1.2 Materials and methods

For answering the research questions, a systematic literature search and an exploratory search for literature was conducted. The literature found was evaluated after the first sighting, filtered for its relevance and categorized, and used as a

starting point for a further literature search according to the "snowball system". This is a system that refers to a search procedure that searches bibliographies or references and footnotes for appropriate literature. It starts with a concrete source that is relevant to the topic, and thus encounters references that are of interest for the scientific work (Flatscher et al. 2011). Decisive sources for the present paper were Hawkins et al.(2006) paper on the Newcastle Consensus Meeting, Thomas et al.(2012) work on combining Nitrous oxide with carbon dioxide for the euthanasia of mice and general work on CO_2 euthanasia from Conlee et al.(2005) and Makowska et al.(2009). Additional information was found in reports from several groups that have provided recommendations for euthanasia of laboratory animals in Europe(EFSA 2005) and in the USA (Leary et al. 2013). Further information were retrieved by the EURL ECVAM Search Guide which has been developed to inform and support untrained database users to find high quality information on relevant alternative strategies and methods to animal experiments in an easy and effective way (Grune 2013). An extensive library of online resources is available on the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) website with information to put refinement into practice. These resources are used around the world and include information-rich websites with supporting references used in this work.

Reference books are obtained from the inventory of the University Library of the University of Veterinary Medicine Vienna. The literature search was carried out in the period from May 2018 to May 2020 in German and English.

The literature search was conducted using search engines and databases as Vetmed:seeker University Library of the University of Veterinary Medicine Vienna, PubMed, ISI Web of Science, Scopus and Google Scholar.

Studies were identified to evaluate the association between CO_2in a hyperbaric setting as well as euthanasia of laboratory animals and animal welfare. In addition, bibliographies of systematic reviews on the current euthanasia guidelines were searched.

The scientific publications and anthologies used are listed in the bibliography. The method of citation within the work follows the guidelines of the University of Veterinary Medicine Vienna.

Keywords in this research were: Carbon dioxide, hyperbaric, compressed CO_2, euthanasia, anesthesia, laboratory animals, rodents, refinement, flow rate, nitrous oxide, hyperbaric chamber, hypercapnia, supercritical CO_2, Überdruck, Kohlenstoffdioxid, and so on.

Apart from the search terms used, the bibliographies of the publications or literary work found were reviewed and publications suggested in the online search under "related articles" were taken into account.

2 Euthanasia of laboratory animals and the definition of refinement

2.1 Euthanasia

The American Veterinary Medical Association (AVMA), states in its latest report that a “good death” is one “that occurs without pain and distress”. The method should minimize any stress, pain and anxiety experienced by the animal prior to unconsciousness. Unconsciousness should occur quickly, followed by cardiac and respiratory arrest and loss of brain function (Hawkins et al. 2016). Pain and suffering can alter an animal’s behavior, physiology and immunology. The reliability and repeatability of studies might be influenced by it and lead to variation in experimental results (Prescott and Lidster 2017).

When speaking about animals, the word euthanasia is often substituted by ‘humane death’ or‘ humane killing’ (Valentim et al. 2016). The present work will stick to the term euthanasia.

In current policies, euthanasia is not seen as a harm to animals that must be minimized, but as the ultimate painkiller. It is not killing animals what requires justification. This principle is in fact crucial to the ethical justification of current practices in animal research. Euthanasia is the main strategy for managing pain for animals, especially those on chronic studies (Carbone 2011).

In Europe two important documents were issued to control the use of animals in experiments. In 1985the Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) of the Council of Europe and in 1986 the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (86/609/EEC) of the EU, based on ETS 123, but which was more stringent and updated in 2010 by the European Directive (2010/63) (Baumans 2004).The European Directive (2010/63) says “member states shall ensure that animals are killed with minimum pain, suffering and distress.”

Permitted methods to kill rodents are:

- an anesthetic overdose
- exposure to rising CO_2 concentrations
- exposure to argon, nitrogen
- cervical dislocation, concussion
- decapitation if no other method possible

Other methods are acceptable if the animal is anesthetized before (European Commission 2010). In Schedule 1 of the Animals Scientific Procedures Act 1986, the exposure to carbon dioxide in a rising concentration appropriate to rodents, rabbits and birds up to 1.5 kilogram (kg) (UK Animal Procedure Committee 1986).

In evaluating euthanasia methods Leary et al.(2013) included in the AVMA Guidelines for the Euthanasia of Animals the assessment of the time required to induce loss of consciousness, and the reliability and ability to induce loss of consciousness and death with a minimum of pain and distress. In this context, best practice in anesthesia is similar to the most suitable euthanasia techniques. A good anesthesia practice is based on unconsciousness, analgesia and muscle relaxation. Euthanasia aims for the induction of rapid unconsciousness, followed by fast death. The period prior to loss of consciousness is one of the most important parameters in euthanasia, as animals may experience distress, anxiety and pain. It is also important to consider the safety of the operators and aesthetics of the method (Valentim et al. 2016).

2.2 Refinement

The breakthrough of anesthetics and the search for biological similarities between man and animal promoted the use of animals in experimental research. The higher demand for standard animal models together with the call for animal welfare made the introduction in the 1950s of Russell and Burch's Three Rs (Replacement, Reduction and Refinement) as guiding principles for laboratory animal science (Baumans 2004). The term “the Three Rs” refers to a more humane use of animals in

research and testing. It means a replacement of animals with alternatives, a reduction of the number of animals without impeding scientific objectives, and a refinement of procedures to improve animal welfare (Prescott 2016).

In detail, refinement is defined as an optimization of animal welfare so that adverse effects can be avoided or at least minimized. Therefore, animals should be maintained under conditions that promote their health and wellbeing (Russell and Burch 1959). Refinement methods should minimize pain, suffering, distress or lasting harm in research animals and improve their welfare. Refinement is an iterative process and can be applied to all aspects of animal use (housing, husbandry, scientific procedures). Using appropriate anesthesia and analgesia to minimize pain are a good example of refinement (Baumans 2004, Lloyd et al. 2008, Prescott and Lidster 2017).

Nowadays, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), an independent scientific organization, was established by the UK government, to discover, develop and promote new ways of replacing, reducing and refining the use of animals in science. NC3Rs refinement vision is to support an effective pipeline of animal welfare research being put into practice to benefit the lifetime experience of research animals and to improve the quality of science(Prescott and Lidster 2017). Russell and Burch's Three R's receive widespread support because they directly address the serious moral dilemma of causing animals harm for scientific purposes without impairing scientific progress(Prescott 2016). In many European countries, it is mandatory by national law to grade the level of discomfort for animals in experiments. On average, 50percent (%) of the laboratory animals experience minor discomfort (a needlestick), 30%moderate and the rest severe discomfort like it is the case in e.g. toxicity tests.

Although there is no specific provision in the European Directive which demands the establishment of animal ethics committees, as such committees are operational, several EU countries are specifically dedicated to review ethical aspects of animals (Baumans 2004). The three Rs have been embraced in policies, regulations and

scientific papers contributing to the improvement of the well-being of laboratory animals(Carbone 2011, Lloyd et al. 2008)

Trevor Poole (1997) highlighted the relationship between research animal welfare and the quality of science with his mantra “good welfare equals good science”. Animal welfare is defined by how an animal is coping with the conditions in which it lives. The animal should be healthy, comfortable, well-nourished, safe, able to express innate behavior and not suffering adverse effects such as pain, fear and distress(Prescott and Lidster 2017).

Before a new refinement method is defined as such, it is important to coordinate the approach to it, validate the science behind, ensure training of new techniques and collect feedback on the refinement (Lloyd et al. 2008). Choosing the most suitable euthanasia method, the aim of the study and planned scientific outcomes have to be considered, all in the context of the Three Rs. New techniques need to be developed which minimize distress (Valentim et al. 2016). But as good animal welfare is linked to the quality of research data, the number of animals required and the reproducibility of the studies, it is important that these new techniques are developed under the consideration of animal welfare improvement and also communicated to the scientific world accordingly. Ethics, law and reputation of animal research in the public is also of major concern (Prescott and Lidster 2017). Especially research funders tend to have high expectations in terms of the use and care of animals in funded research and set up policies and regulations of grant awards (Fleetwood et al. 2015). Awareness of the existing opportunities for refinement can be variable, and in some cases there is a reluctance to question and challenge established practices and cultures (Prescott and Lidster 2017).

Obstacles to refinement are various. They are real like lack of money, personnel and equipment but also perceived as lacking of evidence-based strategies and knowledge (Lloyd et al. 2008). The struggle is to find the best for the animal.

It requires clear ethical reasoning as well as the best available knowledge of animal biology and behavior to determine which experiments may permissibly cause pain

and distress in laboratory animals(Carbone 2011).Researchers sometimes misunderstand refinement as they believe improving the quality of a scientific technique or experimental design, or reducing animal use is the key objective rather than relating to the animals' experience during the experiments and minimizing their harm (Prescott 2016). Further literature and internet research as well as the exchange with colleagues and with respective professional associations is necessary for the recognition of possibilities for each of the Three Rs. Each researcher should be able to provide examples of alternative methods and research strategies that can replace, avoid or complement the use of animals in a variety of research programs. It is important to determine and apply the most painless endpoints; determine appropriate criteria for determining when this endpoint is reached and possible conflicts between refinement and reduction (in case of reuse) to consider and avoid (Medina et al. 2012). Experiments under the consideration of optimized animal welfare are likely to be more valid, sensitive, and reliable (Prescott 2016).

3 Euthanasia with Carbon dioxide

3.1 The gas Carbon dioxide

First described by Baptist van Helmont in the 17th century, Carbon dioxide is a heavy, colorless, invisible gas and a chemical compound with two double bounds (two oxygen atoms and one carbon atom are covalently bound: O=C=O, respectively 27.3 percent carbon and 72.7 percent oxygen (O_2)) (Compressed Gas Association 1990). The molecule has a linear geometry and a molecular weight of 44 gram (g). In normal concentrations it is odorless but in high concentrations it has a burning, acid smell. It is naturally present in Earth's atmosphere and it is involved in numerous cellular biochemical reactions as all humans and animals exhale carbon dioxide when they breathe. It is a normal component of air. Air contains 78 % Nitrogen, 21 % Oxygen, 0.9 % Argon and 0.038 % Carbon Dioxide. Plants absorb CO_2 during photosynthesis to turn it into sugar that the cell can use as energy (Horn et al. 2009, Klocke 2011).

As CO_2 is fully oxidized, it is not very reactive and inflammable and at common levels also not toxic, but slightly acidic, and it is generally considered as a very useful compound. Gaseous CO_2 is used as a supplement to carbonated drinks, in greenhouses and other plant growing operations, to increase crop production whereas solid CO_2 is used to keep food and other items frozen. If CO_2 is more concentrated, 10% or more of the composition of atmospheric air, it can be toxic and cause poisoning at prolonged exposure (Bühlmann and Froesch 1989, Klocke 2011, Poulsen 1952, Toxicology Data Network 2015).

$CO_2$is only water-soluble under maintained pressure. When it dissolves in water it forms carbonic acid, which is a weak acid as its ionization in water is incomplete. If the pressure drops, the CO_2 gas will try to escape to air as CO_2 bubbles formed in the water. When put in cold conditions, like below minus 78.5°Celsius (C), it transforms into a solid, also known as dry ice. Dry ice is non-toxic, non-flammable, inert, odorless, and bacteriostatic and has an enormous cooling capacity. Under

pressure, above 5.2 bar and less than 74 bar and a temperature between -56.6 ° C and 31 ° C, CO_2becomes a liquid. At 5.2 bar and -56.6 ° C all three forms exist (solid, liquid and gaseous state), this is called the triple point. It is one of the most commonly used compressed gases for pressurized gas systems in portable pressure tools. The CO_2 cylinders available on most anesthesia machines are under pressure of approximately 150 bar and therefore the content is liquid until the valve is opened and the pressure drops and it becomes a gas again (Engelhardt et al. 2010, Forslid and Augustinsson 1988, Horn et al. 2009, Klocke 2011).

Carbon dioxide is vital for life. The body uses CO_2 to maintain proper pH levels and to synthesize fatty acids. Basically all aerobic organisms produce CO_2 when they oxidize carbohydrates, fatty acids and proteins. The human body produces approximately 1 kg of CO_2 per day per person (Engelhardt et al. 2010).Blood carries carbon dioxide in three different ways (Engelhardt et al. 2010, Scheunert and Trautmann 1987).In the lungs about 80 % of CO_2 is converted to bicarbonate ions by carbonic anhydrase, an enzyme in the red blood cells. CO_2 diffuses into red blood cells and combines with water (H_2O) to form carbonic acid which quickly dissociates into hydrogen ions and bicarbonate ions. Ten percent is dissolved in the plasma and another 10 % is bound as carbamino compounds (Edsall 1958, Weaver et al. 2009). CO_2 dissolves easily in blood and has a high diffusion coefficient (Marquardt et al. 2018). The carbon dioxide content of the blood is often given as partial pressure (PCO_2) (Engelhardt et al. 2010, Scheunert and Trautmann 1987).

Hemoglobin, the main oxygen-carrying molecule in red blood cells, carries also carbon dioxide as O_2 and CO_2 are not binding at the same site. CO_2 combines with the amino-terminal groups on the four globin chains and because of allosteric effects on the hemoglobin molecule the binding of CO_2 decreases the amount of oxygen at a certain partial pressure of O_2. So the quantity of CO_2 transported is affected by the partial pressure of oxygen (PO_2) but the transfer capacity is limited. The lower the PO_2 and hemoglobin saturation with oxygen are, the more CO_2 can be carried in the blood. This is called the Haldane Effect. It describes how O_2 concentrations determine hemoglobin’s affinity for CO_2 and this is important for the transport of CO_2

from the tissue to the lungs. Conversely, the Bohr Effect describes a rise in PCO_2or a lower pH and how CO_2 and hydrogen ions affect hemoglobin's affinity for oxygen. The offloading of oxygen from hemoglobin gives CO_2 the possibility to combine with hemoglobin and form more bicarbonate ions (Edsall 1958, Engelhardt et al. 2010, Scheunert and Trautmann 1987, Weaver et al. 2009).

3.2 The principles of CO_2 euthanasia of laboratory animals

First of all, the mechanism of CO_2 euthanasia is still not fully clear. Nevertheless, nowadays CO_2 is a common euthanasia agent because it is safe and easy to use and a large number of animals can be killed simultaneously in a short time (Conlee et al. 2005).The use of CO_2 as an euthanasia agent was researched in several studies and various recommendations exist in the current published literature. Physiological effects of CO_2 can cause a range of neurochemical, respiratory and cardiac responses (Woodbury et al. 1960). Blood pressure, heart rate, electroencephalogram (EEG) activity, blood pH and respiratory rate are typical measurements which are used to evaluate the efficacy of CO_2asthese are all parameters which are affected by CO_2 (Brosnan et al. 2007, Conlee et al. 2005). Procedural factors such as the inhaled concentration of CO_2, flow rate and the presence of oxygen are also important for the assessment of $CO_2$and attempts of refinement which will be discussed in the next chapters.

CO_2 influences the blood pH as bicarbonate ions are crucial for its regulation. The pH of the blood is regulated within a narrow range via a buffering system. It tries to keep it constant at a level between 7.35 and 7.5. During anesthesia the pH in the brain can change from 7.35 to 6.8. Usually the brain protects itself very well against such acidosis because of its blood brain barrier but carbon dioxide can pass this barrier and reach the brain. A pH shift below 7 can lead to hyperacidity, which causes severe central nervous system depression and in the worst case coma and death. The pH is closely monitored by the neurons in the medulla oblongata. The same nerve cells control respiration through several independent mechanisms, including

peripheral and central chemoreceptors for CO_2 and O_2. But the buffering system has its limits and can only manage a certain amount of CO_2 (Boron and Boulpaep 2005, Forslid and Augustinsson 1988).

The breathing rate influences the level of CO_2 in the blood. Too rapid breathing leads to hyperventilation, which can cause a respiratory alkalosis, too slow or shallow breathing causes a respiratory acidosis. At hyperventilation, the expiratory CO_2 decreases, therefore the PCO_2 decreases in the lung and the blood. New CO_2 will be produced by the combination of bicarbonate and hydrogen ions and as hydrogen ions in the blood decrease the pH will rise. With hypoventilation it is the other way around and the pH decreases because CO_2 accumulates in the blood and the result is a respiratory acidosis (Boron and Boulpaep 2005, Engelhardt et al. 2010). Due to this increased concentration of carbon dioxide, the gas exchange in the lung of the animal is impaired. A high concentration of CO_2lowers the levels of carbon dioxide leaving the circulating blood and / or alveoli and it cannot be oxygenated (Bühlmann and Froesch 1989).

In humans, if inspiratory CO_2 concentration increases up to 1 to 1.5 percent at normal atmospheric pressure, the ventilation increases, but this is barely noticed. The arterial PCO_2 is already in the upper normal range. In the case of continuous exposure, the bicarbonate content in the blood and in the interstitial fluid increases, so the pH can still be normalized. With 5% CO_2 in the respiratory gas the arterial PCO_2 rises to values around 50 mmHg and the ventilation is already 3 times the resting value. This arterial PCO_2 is sufficient to significantly increase cerebral blood flow and increase intracranial pressure. At 9 %, most human subjects are unconscious (Bühlmann and Froesch 1989). CO_2 concentrations starting from 5 % change significantly the heart rate and blood pressure in rats (Smith and Harrap 1997).Thomas et al.(2012) observed a rise in pulse and respiratory rate in CO_2anesthetized rats. Even though it is known that CO_2 has no direct effect on the breathing it seems that the hydrogen ion concentration causes this effect as there are receptors that sense a change in pH which leads to a change in the breathing.

Neural activity, basal and evoked, are depressed after inhalation of 100% CO_2 (Martoft et al. 2002). The inhalation also leads to altered neurotransmitters in the brain and causes an activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis activity (Barbaccia et al. 1996).The sympathetic nervous system and the HPA axis activation are well-accepted as stress response markers. Therefore, norepinephrine and lactate levels are commonly used to assess levels of stress. Increased lactate levels may be due to a rise in norepinephrine, as its release causes an increase in cardiac rate, lowers pH, and produces an accumulation of lactate (Meyer 2015, Minton 1994). These neurochemical responses are a reason why neuroscientist dislike CO_2 as an euthanasia agent if the need exists to collect specific postmortem material (Conlee et al. 2005). A study on acute stress and time dependent changes in rat brain neuroactive steroid concentrations and acid receptor function showed one minute after the inhalation of a CO_2 / O_2 gas mixture a significant stress response. Thus, there is a correlation of CO_2 and neuroactive steroid concentrations (Barbaccia et al. 1996).

Basically, there are four stages of anesthesia while using inhalational agents - analgesia, excitation, surgical anesthesia and the fourth overdose, which is only required when euthanizing an animal. In these stages the sensitivity to carbon dioxide shown by the central nervous system is different. Normally, with classic inhalation agents, the mechanism of action is a depression of signal transmission of the nerve cells but with carbon dioxide it is caused by nervous system acidification (Boivin et al. 2017, Forslid and Augustinsson 1988). These stages fit to the observation by Coenen et al.(1995) of four phases during CO_2 euthanasia. In phase one the animals show normal behavior and in phase 2 when CO_2 reaches the brain, abnormal activity, excitation and marked agitation. In phase 3,weakeningof hind legs and loss of body control occurs as well as disappearance of muscle tone and head sinking. In phase 4 the animal reaches the anesthetic state and finally death.

According to Ludders et al.(1999) CO_2 anesthesia does not occur before the partial pressure of CO_2 in arterial blood exceeds 200 millimeters of mercury (mm Hg). Stage three of the before mentioned anesthesia stages only occurs at PCO_2 of 200 mm Hg.

This fits to Hewett et al.(1993) observations that rats become uncoordinated at $PCO_2$of 123 mm Hg, immobile after PCO_2 exceeded 212 mm Hg and resisted a painful stimulation (toe pinch) at 332 mm Hg. Eisele et al.(1967) were one of the first who investigated the anesthetic properties of CO_2in dogs. They found out that the depth of CO_2 anesthesia is linked to a low pH of the cerebrospinal fluid. The anesthetic effect of CO_2 occurs if the partial pressure of CO_2 in arterial blood exceeds 245mmHg. The highest effect was seen at a pH of 6.8 (Eisele et al. 1967).

It seems likely that the nervous system acidification (“brain acidosis”) is the main mechanism of a CO_2 induced death as the inhalation of CO_2 causes respiratory acidosis (and a rapidly decreasing intracellular pH) (Martoft et al. 2002) and inhibits neurons which leads to a loss of consciousness, insensibility and finally death (EFSA 2005).

3.4 The problems of CO_2 euthanasia

The use of CO_2 as an agent for euthanasia received increased attention over the past years (Valentim et al. 2016).The published data on whether or not CO_2 causes distress are inconclusive and a routine use should be questioned (Conlee et al. 2005).A number of more recent studies confirmed already that CO_2 causes significant distress and pain in rodents (Makowska et al. 2009).Exposure to low concentrations of carbon dioxide causes distress and higher concentrations also cause pain (EFSA 2005).

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage. The inability to communicate verbally does not negate the possibility that an animal is experiencing pain and is in need of appropriate pain-relieving treatment. Pain is quite subjective as individuals can differ in their perceptions of intensity as in their responses (Baranowski et al. 2012, Meyer 2015). According to the ethical and legal obligations of researchers it is the main aim to avoid pain in research animals (Carbone 2011).Distress is the result of an animal’s response to stimuli which interfere with its well-being and comfort. It is created by

conditions experienced prior to loss of consciousness like transport and handling, or by conditions under which the euthanasia methods are applied (gradual filling of the CO_2 chamber, high concentration of CO_2, etc.) (Meyer 2015).It seems that rodents are sensitive enough that the sheer introduction of CO_2 into the euthanasia chamber causes distress behaviors. Distress describes both, physiological and psychological states, shown by, for example, increased locomotion, rearing, defecation and urination (Ambrose et al. 2000, Hackbarth et al. 2000). The stress associated with the induction of unconsciousness is of serious welfare concern(EFSA 2005). Unconsciousness, induced for the purpose of euthanasia, is the result of a pharmaceutical effect on the central nervous system, hypoxia or brain disruption. Loss of consciousness is defined as loss of the righting reflex (LORR). It is an old definition but still used as it is easy observable and applicable to a wide variety of species. Vocalization and non-purposeful movements observed after LORR do not necessarily indicate a conscious state(Meyer 2015).Moody et al.(2015)used as indicator of unconsciousness an absent pedal withdrawal reflex in his study. This might reduce the risk that an animal may be conscious during exposure to a noxious stimulus for an absent spinal reflex, as the pedal withdrawal, reflects a greater depth of anesthesia than that required for loss of consciousness(Chisholm and Pang 2016).

Animal welfare is determined by the animals' behavioral and physiological responses, which are good measures of emotional arousal and are widely used as welfare indicators such as cortisol / corticosterone levels or heart rate(Prescott and Lidster 2017). A quite new method to assess the pain of the animals besides monitoring behavior and clinical signs, is the grimace scale. Changes in facial expression, such as narrowing of the eyes or changes in the position of the whiskers, provide a reliable and rapid method to assess pain in rodents (Sotocinal et al. 2011). The assumption that rats exposed to CO_2 are in pain and distress was supported by the fact that rats produce ultrasonic vocalization during exposure which is only shown during negative states(Chisholm et al. 2013).

These different interpretations of behavioral signs of animals to CO_2 euthanasia make it crucial that scientists are aware of the different stages of anesthesia and how

those stages correlate to conscious and unconscious responses to noxious and distressing stimuli (Tranquilli and Grimm 2015). Conlee et al.(2005) discussed the topic of CO_2euthanasia with people working in laboratory animal medicine and care (e.g. veterinarians, vivarium directors, technicians) and found that there are conflicting CO_2 practices and recommendations within the animal research community.

As CO_2 has the potential to create pain it has been used in several pain research studies in humans and animals as a noxious stimulus (Conlee et al. 2005, Danneman et al. 1997, Thürauf et al. 1991). Krohn et al.(2003)found out in his study that rats are sensitive to CO_2 as low as 3%.Burkholder et al.(2010) identified the exposure to CO_2 as a potential source of distress accompanied with dyspnea and the sensation of breathlessness. The accumulation of CO_2 causes an acidification of the nasal mucosa(Anton et al. 1992). Rodents have non-myelinated nerve endings that sense chemicals and these nociceptors have a similar density in their nasal and ocular epithelia as in humans (Danneman et al. 1997, Thürauf et al. 1991). So, based on the comparative anatomy and physiology of humans and animals, it is reasonable to hypothesize that the pain humans experience when exposed to CO_2 is also experienced by rodents (EFSA 2005). The guidelines state that “unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals”(United States National Institutes of Health 1985).

Aversion to CO_2 is seen not only in rodents but in many species of animals (e.g. fish, poultry and pigs). All of them avoid atmospheres containing high concentrations of CO_2 or try to escape atmospheres containing low concentrations of CO_2 as soon as they start to experience breathlessness induced by elevated blood CO_2 levels (EFSA 2005). Banzett (1996) showed in his study that humans assess CO_2 at a concentration of 7 to 8% as breathable but unpleasant due to the occurrence of dyspnea. Dyspnea is described as labored breathing which is associated with a negative affective experience in rodents (Moody et al. 2014). This air hunger can be a frightening experience that motivates escape behavior (Banzett 1996), which is in

line with the observation by Kirkden et al.(2005)that rats escape from an atmosphere containing as low as 10% CO_2. Mice exposed to 10% CO_2 show fear-related freezing behavior and increased anxiety. Common escape behaviors such as jumps(Thomas et al. 2012), increased activity, rearing and vocalization, increase during a gradual fill of the chamber with CO_2 (Niel and Weary 2006).These findings indicate that distress and pain occur at CO_2 levels where the animal is still conscious (Kirkden et al. 2005, Niel and Weary 2007). The aversion to CO_2 and the resulting pain and distress are of major concern during CO_2 euthanasia (Hawkins et al. 2016) and it is important to induce the loss of consciousness in the most humane manner (Marquardt et al. 2018).

4 Attempts for refinement

4.1 CO_2 flow rate and CO_2 concentration

The concentration of a gas is content based on the volume of the mixture and cited as percentage (%). Thus it indicates how much of a substance is present in a comparison volume of the mixture (Baker 2016). It is the assumption that at any given time the gas is homogenously mixed with the residing gas inside the test cage (Marquardt et al. 2018).

Gas flow defines the number of gas-molecules streaming through a particular point during a certain time period. The flow is based on the different quantities for an amount of gas: mass, volume and standard volume. To calculate the flow rate it takes the volume of the chamber in centimeters (cm) converted to liters (L) by the factor 1000 (conversion rate into liter) and this is multiplied by the percentage of desired displacement (chamber volume per minute, CV / min) which results in the flow rate in liters per minute (Baker 2016).

e.g.
(height in cm) x (width in cm) x (length in cm) / 1000 = chamber volume (in L)
chamber volume (in L) x % displacement / min = flow rate (in L/min)

Instructions on how to fill a euthanasia chamber are often given as a concentration of e.g. 100 % CO_2 at a flow rate of 30 % of chamber volume per minute.

This shows, that the gas displacement rate is crucial for a human application of compressed gases for euthanasia and an appropriate combination of pressure-reducing regulator and flow meter for generating the desired displacement rate for the size of the chamber is absolutely necessary (Leary et al. 2013).

Based on studies from Niel et al.(2008) and Hawkins et al.(2006), it seems that the current best practice for carbon dioxide euthanasia of rodents is to first place the animals in the euthanasia chamber and then introduce a concentration of 100% CO_2 at a flow rate of 20 to 30% of chamber volume per minute. A displacement less than 20% of chamber volume per minute is too slow to induce loss of consciousness and more than 30% are likely to cause pain prior to the loss of consciousness(Ambrose et al. 2000). It is recommended, that the flow rate of CO_2 gas introduced in the chamber should be increased once the animals are unconscious and to keep track of the rising concentrations. However, CO_2 is also a general anesthetic and loss of consciousness occurs below 50% CO_2if the chamber is slowly filled at 20% CV / min(Hawkins et al. 2016).Burkholder et al.(2010) reports even a loss of consciousness in rats at concentrations of 21 % CO_2 at a flow rate of 10 % CV / min. Moody et al.(2014) found out that the onset of labored breathing was similar for all tested flow rates. They also concluded that dyspnea begins soon after exposure to even low concentrations of CO_2. Gas flow rate had a strong effect on the time until the animals became recumbent and lost their righting reflex. Niel et al.(2008) observed that CO_2 is aversive to rats over all flow rates tested. At the highest tested flow rate of 27% CV / min rats left the chamber at aCO_2 concentration of 13%. This result is in line with earlier studies showing that rats became recumbent after 106 seconds at a flow rate of 17.25% CV / min when a concentration of 33% was reached suggesting they felt no pain but distress at this level (Niel and Weary 2006).The suggestion is to slowly increase the CO_2 concentration for a gradual onset of unconsciousness, without the animal experiencing CO_2concentrations which might cause pain and dyspnea (Niel et al. 2008).

Conlee et al. (2005) provided an extensive comparison of results which clearly shows concerns regarding pain and distress at CO_2 concentrations ranging from 25% to 100%. A concentration of 7.5% CO_2 seems enough to cause pain whereas concentrations of 30% and higher cause deep anesthesia and death after prolonged exposure (Niel and Weary 2006, Smith and Harrap 1997). Concentrations of 60% CO_2 and higher cause many adverse effects as observed by Ambrose et al. (2000)

and Danneman et al.(1997). Several histopathological alterations of the lung, like edema and hemorrhage, where found in rats at all CO_2 concentrations but the severity of the lesions increased with increasing concentration (Boivin et al. 2017, Danneman et al. 1997).

For a long time it was a matter of discussion if the chamber should be pre-filled or if a start with an empty chamber followed by gradual filling is the better option. The study of Danneman et al.(1997) was indicatory for this discussion. Rats were exposed to concentrations of CO_2 from 50 to 100 % obtained after pre-filling or gradual-filling and the addition of O_2. Endpoints were time to anesthesia and time to death. At 60 % concentration the difference till anesthesia between pre-filled and gradual filled chamber was 1.5 min, at 100 % 40 seconds. Time to death took 15 minutes longer at 60 % than at 100 % concentration. The results mirror the dilemma of CO_2 as an euthanasia agent at high concentrations induce a rapid death but cause pain and distress whereas low concentrations might be inefficient and have a high incidence of adverse effects. Additionally Danneman et al.(1997)exposed humans to CO_2 who reported a concentration above 50 % as unpleasant and even painful. This is why the conclusion was made to rather use gradual filling at a low flow rate so conscious animals were never exposed to concentrations higher than 70%.The AVMA Guidelines also opt for a gradual filling (Leary et al. 2013) even though the loss of pedal reflex occurs in a pre-filled chamber six times faster than in a gradually filled (Hewett et al. 1993). However, the main concern remains animal welfare during the time till the loss of pedal reflex occurs (Conlee et al. 2005). In this time the animals should not experience pain and distress (Niel and Weary 2007) but they do regardless of induction method. The time between apnea and loss of pulsatile blood flow was approximately one minute using a 22% chamber CV / min gradual fill technique with 100% CO_2(Smith and Harrap 1997).Despite the fact that it takes longer to euthanize the animals, the gradual filling method is at the moment seen as best practice as it shows less pain and distress (Conlee et al. 2005). The results of the study of Moody et al.(2014) indicate that a gradual-fill at a CO_2 flow rate of 50% CV / min versus a 20% CV / min reduces the period till loss of pedal withdrawal reflex

by 15 seconds. But when using 50% CV / min it is necessary to make sure that a gas holding technique (e.g. manual control of the flow meter) is used to avoid painful CO_2 concentrations (>40%) until after insensibility occurs. With this refinement, they concluded, mice might only experience 30 seconds of dyspnea until onset of recumbency. The worst case in this setting might be the experience of dyspnea until loss of the pedal withdrawal reflex (which takes around another 90 seconds) with no benefit from a faster flow rate.

A high inspiratory CO_2 concentration provides a fast onset and, if intended as in the case of anesthesia, a fast recovery but there are species-specific differences (Kohler et al. 1999). Leach, M. C. et al.(2002) studied extensively the aversion of gaseous euthanasia agents including CO_2 in rodents at various concentrations. One key finding was that the aversion is similar at concentrations ranging from 25 to 50% CO_2, dependent if it were rats or mice.

Flammer et al.(2019) came to the conclusion that CO_2 exposure is never a humane method to euthanize rodents as it causes pain at high concentrations and fear or panic in low concentrations. This conclusion is supported by the findings of Johnson et al. (2011) who reasoned that as the concentration of CO_2 in the blood is highly regulated, conditions that influence the homeostasis of blood CO_2 concentrations leads to quick adaptive changes in breathing (e.g. hypoventilation) and behavioral arousal occur. If the concentrations continue to rise, panic and dramatic physiologic responses are initiated. An argument against the panic theory might be the study of Bailey et al.(2005) who examined the effects of a single inhalation of 35% CO_2 in human volunteers. The response was interpreted more as a stress stimulus rather than an anxiety-panic response. The physiological response comprised the activation of the autonomic nervous system and the hypothalamic-pituitary-adrenal axis, as reported by Barbaccia et al. (1996), but did not result in panic.

Even though the discussion on what is the best flow rate and concentration goes on more than a decade since the Newcastle Consensus Meeting on Carbon Dioxide

Euthanasia of Laboratory Animals there is still no best practice or clear guidance in place on how to administer CO_2 humanely(Hawkins et al. 2006).

4.2 Addition of Oxygen

In the name of refinement, it was considered that an addition of oxygen to CO_2might reduce pain and distress. But existing studies show conflicting results and the potential benefit of using a mixture of CO_2 and O_2 for euthanasia is controversial (Conlee et al. 2005).

Studies have shown that the addition of O_2 to CO_2 causes only a slight reduction of aversion or no reduction at all during the gradual-fill procedure (Kirkden et al. 2005, Leach, Matthew C. et al. 2002). Moreover, it has been shown that O_2 supplementation may provoke lung hemorrhage before loss of consciousness in mice (Valentim et al. 2016).

Coenen et al.(1995) conducted a study to examine if the supplementation of oxygen can minimize the signs of agitation and asphyxia which is normally shown during CO_2 euthanasia. Animals of the CO_2 100 % group suffered from asphyxia as they gasped with their mouth wide open and head tilted back. The signs of strong behavioral agitation and excitation were less pronounced when oxygen was added. Another quite extensive study showed that the addition of supplemental oxygen during CO_2 euthanasia substantially increases time to apnea. The time between onset of apnea varied between 14 minutes in the CO_2/O_2 group in contrast to 45 seconds in the CO_2 group (Chisholm and Pang 2016). Ambrose et al.(2000) compared 30% CV /min of CO_2 with and without supplementation of oxygen and no behavioral difference were seen. Increased alveolar consolidation was seen in the oxygen group and this is probably more stressful than the adverse reactions seen with CO_2 as it might induces a feeling of drowning. Kirkden et al.(2005) came to the conclusion that the combination of CO_2 and O_2 has only a modest effect on reducing aversion to the gas mixture in comparison to CO_2 alone. A mixture of 70% CO_2 and 30% O_2 is almost as aversive asCO_2 alone. Furthermore, the study reported a prolonged time to death

with CO_2/O_2 compared with CO_2 alone and therefore alternative killing methods are still urgently required (Kirkden et al. 2005).

The AVMA guidelines for evaluating killing methods do recommend to avoid the addition of O_2 to CO_2 as the reports of behaviors associated with respiratory distress are conflicting (Leary et al. 2013).

4.3 Addition of other gases like nitrous oxide

Nitrous oxide (N_2O) is, as CO_2, a cheap, nonflammable and non explosive inert gas (Rault et al. 2013).Nitrous oxide is also a low solubility anesthetic agent which is commonly referred to as laughing gas and is widely used in human anesthesia surgery and in dental medicine for its analgesic pain relieving, sedative, and anxiolytic effects(Parbrook 1968, Rault et al. 2013). Still, it is necessary that N_2O is handled with care, as animal workers are at risk as it results in health effects if exposed to it on a chronic base and it also might lead to addiction and physical abuse(Leary et al. 2013).

N_2O is the least potent inhalation anesthetic known and has not the same potency in animals compared to humans. Therefore, it is not suitable to be used alone as anesthetic agent or for an euthanasia of animals as it causes an alveolar hypoxic atmosphere (Leary et al. 2013)Actually, it is also not commonly used in rodents at all (Thomas et al. 2012).

Nitrous oxide solubility in tissue is close to that of CO_2, but CO_2 is still three times more potent (Eisele et al. 1967). The minimum alveolar concentration (MAC) in humans is 104 % and in other species it is nearly less than half of that (Leary et al. 2013). In rats there are MAC values mentioned between 136 and 235 % (Russell and Graybeal 1998, Weiskopf and Bogetz 1984).Russell and Graybeal(1998) had to use hyperbaric compression to determine the N_2O MAC in rats as it cannot be used alone at 1 atmosphere of pressure in any species without producing hypoxemia prior to

respiratory or cardiac arrest. Animals might be as stressed as with CO_2. But N_2O combined with other inhalant anesthetic lowers the time to lose consciousness by the so called “second-gas effect” (Thomas et al. 2012). So it is mostly used as a mixture of up to 70% N_2O and other inhalation anesthetics to speed the onset of anesthesia(Leary et al. 2013). If N_2O is used alone, for whatever reason, concentrations should be kept as close as possible to 100% by volume and be monitored until death. The competence of those placing animals into the chamber must be given and the chamber must fulfill the purpose and be gas tight. But owing to human health and safety concern, nitrous oxide is not suitable for euthanasia and should be avoided(EFSA 2005).

Thomas et al.(2012) considered nitrous oxide as a possible refinement when added to CO_2 or isoflurane as it can shorten the time to lose consciousness and might minimize the duration of stress and distress. N_2O, as a carrying gas for CO_2, and its rapid absorption induces a second- gas effect which causes an increase in alveolar CO_2 concentration This second-gas effect shortens the time to lose consciousness by 10 % if 20% (CV/min) CO_2 and 60% CV/min N_2O was used compared to 20% CV/min CO_2 alone. The conclusion is that the addition of N_2O improves CO_2 exposure, by trying to reduce the time till the onset of unconsciousness and dyspnea. Additionally, the study suggests to addO_2 to the gas mixture to prevent hypoxia and this might reduce distress.

5 Pro and contra of the scientific community on Carbon Dioxide euthanasia

There are two main questions to consider to develop an ideal euthanasia protocol. First, how long does it take till the animal is unconscious and second, do they experience any adverse effect before it (Hawkins et al. 2006).

117 experts summarized that a good death for animals:

• is not associated with suffering, pain, anxiety, stress, or distress

• is reliable and under control and expert supervision until death

• is immediate, without delay, and irreversible

• is safe for the staff and other animals

• avoids contamination of the environment

(Flammer et al. 2019).

In 2006, at the Newcastle Consensus Meeting on Carbon Dioxide Euthanasia of Laboratory Animals, speakers and observers concluded that “the humane killing of laboratory animals is an issue of great importance to the scientific community as a whole“(Hawkins et al. 2006).

In 2013, the American Veterinary Medical Association (AVMA), set new guidelines for euthanasia of animals and provided recommendations for the use of carbon dioxide for the humane killing of rodents. A fill rate of gradual 10% to 30% chamber volume per minute with 100% CO_2 is stated as acceptable with conditions (Leary et al. 2013).

The Canadian Council on Animal Care(2010)states in their guidelines 12 and 13 that “carbon dioxide should not be used where other methods are practical for the experiment and the species”. If carbon dioxide use is required for non-anesthetized rodents, a gradual-fill rate of less than 30% CV / min and greater than 20% CV / min should be used”. The American Veterinary Medical Association (Leary et al. 2013)

recommends in general that “Carbon dioxide is acceptable with conditions for euthanasia in those species where aversion or distress can be minimized”. So basically each country set up its own regulations and various organizations and agencies which are mentioned before provide guidelines on euthanasia. These different guidelines provide conflicting recommendations in regards to CO_2 euthanasia, especially on the optimal concentrations, rates of induction and sources of CO_2.The Office of Laboratory Animal Welfare (OLAW) published a guidance that high concentrations of CO_2 may be distressful and therefore prefilling the chamber is only recommended when the use does not show distress (Brown and Newcomer 2002).

Various scientists have measured behavioral and physiological responses as indicators of pain and distress in studies of CO_2, such as behavioral signs of asphyxia (Coenen et al. 1995), hyperventilation (Kohler et al. 1999), escape behaviors (Leach, M. C. et al. 2002) and physiological signs of nociception (Anton et al. 1992). From the data of all these studies, it can be concluded that CO_2 euthanasia should raise concerns about the associated pain and distress (Conlee et al. 2005).

Hackbarth et al.(2000), on the other hand, concluded that the euthanasia of rats and mice using carbon dioxide is a humane procedure as it is fast and, at least in their study, caused no distress.

So why using CO_2while it is so controversially discussed? So far it is the best option to euthanize big groups of rodents, it is practical and an effective technique associated with a good balance of costs and benefits (Valentim et al. 2016).

Valentim et al.(2016) came to the conclusion, that there is no hard evidence to advocate banning CO_2, although its flow rate should be low for animal welfare reasons. They also suggest to use a combination of volatile gas anesthetics and CO_2. This approach of a bi-phased euthanasia (loss of consciousness achieved by anesthetics and death by CO_2) is probably best for the animal. Also Hawkins et al.(2006) advocate that for practical reasons and on basis of research results, depending on the species and number of animals being used that the loss of

consciousness should typically be achieved with an anesthetic, before the rodents are killed by CO_2.

A move away from the use of CO_2 would face two obstacles: practicality and economics, as anesthesia-based techniques require more time, drugs and equipment (Marris 2006).

6 Hyperbaric administration – a possible refinement?

6.1 Principles of hyperbaric administration of gas

Gases underlie several physical principles and are based on the laws of physics. If hyperbaric applications are considered, several gas laws must be taken into account such as Boyle-Mariotte's Law, Dalton's Law and Henry's Law. Of particular importance for gases in overpressure is the last one. Henry's law describes the solubility of gases in liquids. Gas dissolves in liquid in proportion to the pressure on the liquid in the system of gas and liquid. So, the higher the external pressure, the more gas can be dissolved in a liquid. As blood is a liquid, according to Henry's law, gas dissolves and accumulates in body fluids in proportion to the applied pressure(Mathieu 2006, Neuman and Thom 2008).

In mammals, oxygen is mainly transported by chemical binding to the hemoglobin contained in the red blood cells. The amount of hemoglobin is therefore crucial for the oxygen transport capacity of the blood. Normal breathing air (contains 21 % oxygen) saturates red blood cells at 95 to 100%, so an increase of the blood oxygen content via the oxygen bound to hemoglobin is nearly impossible, as transport capacity is limited. Oxygen also dissolves in the blood and the amount depends on the oxygen partial pressure in the air. By increasing the concentration and so the partial pressure of O_2in the breathing air from 21 to 100 %, the amount of oxygen dissolved in the blood proportionally increases. A further increase in the oxygen partial pressure is possible by increasing the ambient pressure, which is the case in a hyperbaric chamber(Mathieu 2006, Neuman and Thom 2008).

If an animal breathes 100 % oxygen in a pressure chamber at a pressure of e.g. 2.5 bar, approximately 20 times the amount of dissolved oxygen reaches the blood. The diffusion distance for oxygen is determined through the partial pressure gradients from the capillary to the tissue. Peripheral tissue and body cells will be better

oxygenated, which is the main purpose of hyperbaric oxygen therapy (HBOT) (Bergo and Tyssebotn 1999, Mathieu 2006, Neuman and Thom 2008).

Boyle-Mariotte's law describes the pressure and volume relationship of gas. It basically states that if the pressure decreases, the volume increases and conversely. Affected are anatomically predetermined gas-filled cavities (e.g. paranasal sinuses, lungs, etc.), as well as gas bubbles in the body tissue or in the vascular system. This law explains the reduction of the volume of gas bubbles and arterial gas embolism by hyperbaric treatment as is the case with decompression sickness (Bühlmann and Froesch 1989, Mathieu 2006, Neuman and Thom 2008).

Dalton's gas law relates to the partial pressure of gases in a mixture. The total pressure of a gas mixture is composed of the partial pressures of the individual gases in the mixture. As a result under hyperbaric conditions, the partial carbon dioxide pressure increases proportionally in the inspiratory air (Mathieu 2006, Neuman and Thom 2008).

Charles law, the temperature-volume law, explains why the temperature increases when a hyperbaric chamber is pressurized and decreases when depressurized. The volume of a given amount of gas held at constant pressure is directly proportional to the temperature (Compressed Gas Association 1990).

In summary, if environmental pressure is raised, increased amounts of the gas breathed in (CO_2), are delivered to the lungs (Dalton's Law) and transferred to the blood and tissues in their soluble state (Henry's Law) (Bergo and Tyssebotn 1999, Mathieu 2006, Neuman and Thom 2008).

Hyperbaric oxygen therapy is a treatment in which a patient breathes 100% oxygen while inside a pressure vessel or treatment chamber at a pressure that is higher than sea-level atmospheric pressure. HBOT is a relatively old therapy and usage increases as more and more chambers are available. Indications are e.g. carbon monoxide poisoning, compartment syndrome and delayed wound healing as those

conditions benefit from an elevated capillary plasma oxygen transport (Edwards 2010).

Hyperbaric CO_2 is commonly used in applied and environmental microbiology and in the food industry. The effect of hyperbaric CO_2 on microorganisms has been studied more thoroughly. These studies showed that that microbial inactivation by CO_2depends on many parameters as temperature, pressure and water content of the microorganism as these increase the diffusivity of CO_2. Exposure time can be decreased by increasing temperature and the release rate, with the outcome the faster the better. Carbon dioxide dissolves in the water and forms carbonic acid which lowers the pH. This acidity leads to disturbance of the biological systems (Debs-Louka et al. 1999).

Data on the use of hyperbaric CO_2 to euthanize animals are to the best of our knowledge not existent.

6.2 Hyperbaric chamber construction

In a common euthanasia chamber rodents are placed inside it and then it will be filled with CO_2 (EFSA 2005). The molecular weight of CO_2 is higher than that of ambient air. Therefore CO_2 accumulates at the bottom of an euthanasia chamber (Kohler et al. 1999). That is why the chamber should be filled from the top and not from the bottom (Leary et al. 2013). The volume and concentration of gases administered are controlled by a pressure reducing valve, connected to the gas cylinder, a flowmeter and, if needed, the calibrated vaporizer (Kohler et al. 1999). The Canadian Council on Animal Care (2010) recommends to flush the chamber with air if euthanasia is performed on several groups of animals using the same chamber to scavenge waste gas out to protect the operator (EFSA 2005).

Carbon dioxide is delivered in compressed gas cylinders in liquid form. To avoid cold shock in animals, as the temperature can be reduced to as low as minus 78°C, CO_2 must be vaporised using specialised electrical heaters, prior to administration. Large

capacity vaporisers require a 3-phase electrical supply, which may not be readily available (EFSA 2005). If CO_2would be delivered as dry ice, or administered straight from a liquid source without a vaporiser, the temperature in a chamber will fall drastically as it sublimates or evaporates. This would be a welfare problem as inhalation of dry and cold CO_2 gas is painful, leading to freezing of tissues, cold burns and nasal bleeding (Ambrose et al. 2000, Leach et al. 2004).

In the literature only a few examples of hyperbaric chambers suitable for rodents are available. They were used for HBOT but not for euthanasia.

Djasim et al.(2012)used for their animal experiments a hyperbaric oxygen (HBO) chamber that is conform to 97/23/EC (Pressure Equipment Directive), Conformity Assessment Module G Product Group 1.The Pressure Equipment Directive (PED) (2014/68/EU) applies to the design, manufacture and conformity assessment of stationary pressure equipment with a maximum allowable pressure greater than 0,5 bar(European Parliament 2014). The chamber provides easy access, and can be run in various modes as manual, semi-automatic and full-automatic. Sensors for pressure level, oxygen level, temperature, humidity and carbon dioxide level allow full control during the animal experiments and meet the criteria regarding safety and ease of use. The HBO chamber has a diameter of 70 cm, a volume of 420 L, is 115 cm long and weighs 600 kg but is still mobile and can be transported, if necessary. The maximum working pressure is 5 bar. The animals are placed in compartmentalized cages (which allow to treat 24 mice or 12 rats at the same time) custom made out of corrosion-resisting punctured steel. High pressure proof acrylic windows and LED lights provide a clear view into the vessel. A circulating water system allows heating /cooling and a ventilator, an alarm buzzer and a data recorder can be controlled by the control box outside of the chamber. The financial costs of developing this HBO chamber were high.

Rech et al.(2008) constructed a steel carbon chamber like a tube with an internal diameter of 50.5 cm and 83.0 cm in length; weight 160 kg and internal area of 150 cubic meters ($cm^{3)}$. The internal space accommodates 2 acrylic baskets for

approximately twenty mice, ten rats/hamsters, six rabbits, four cats, a dog or a swine and its measurements are 15 cm x 28 cm x 69 cm. The chamber supports of up to 4 bar without gas escape through the sealant areas. The frontal door has an acrylic window to observe the animals.

Pereira et al.(2001) described in technical detail a chamber made totally out of acrylic material. It is only suitable for rodents and only a limited number of animals can be used in each session. Rech et al.(2008) raises concerns about this chamber type from a technical point of view. Obviously, the material presents precocious fatigue and instability of the different parts fixed by screws. The latter leads to a high risk of escaping of gas. Pereira et al.(2001) reports that the acrylic chamber had some cracks in the covers after 4 years of intermittent use at a pressure of 3 bar and after their test at 3.5 bar. This acrylic chamber might be a low cost and easy to build option to confirm the findings of this work. Animals could be monitored throughout the experiment and the chamber withstands pressure up to 3 bar. Nevertheless, it is not a suitable option for a serial production and daily use in a laboratory research setting, as of the aforementioned concerns of Rech et al.(2008).

A CO_2 euthanasia chamber for rodents capable to work under hyperbaric conditions should fulfil the following criteria:

1. It should not be operated in the room where the animals are kept.
2. The chamber must be large enough that each animal can stand in physiological posture and has enough space to turn around.
3. The realisation of a hyperbaric CO_2 environment in the chamber must be as silent as possible to avoid additional stress due to noise.
4. CO_2 from gas cylinders must be used and the chamber pressure monitored.
5. CO_2 flow rate and concentration must be measurable (so a corresponding measuring device is necessary).
6. If animals are killed one after the other in the same container, care must be taken that carbon dioxide in the chamber is removed before inserting new animals.

7. An optimal chamber filling rate for hyperbaric conditions is still unknown but it is characterized by an onset of unconsciousness with no obvious signs of pain and ideally no signs of dyspnea.
8. The chamber construction must allow visual inspection of the animals all the time

(American Veterinary Medical Association 2001, Conlee et al. 2005, EFSA 2005, Hawkins et al. 2006, Hawkins et al. 2016, Leary et al. 2013)

6.3 Hyperbaric administration of CO_2 – theoretical basis and literature review

Aversion and fear linked to CO_2 exposure of rodents make it necessary to shorten the duration of exposure to the gas without increasing either the CO_2 concentration nor the stress level of the animals (Thomas et al. 2012). “It needs an ethical discussion whether a longer procedure with less maximum stress counterbalances a shorter procedure with more maximum stress” (Marquardt et al. 2018). In this context, a theoretically expected quicker death following exposure to hyperbaric CO_2 could be a way to refinement. But the theoretical use of hyperbaric CO_2 for euthanasia of rodents raises a couple of questions.

As mentioned before, time is crucial during the euthanasia process. As with any other administration of $CO_2$the animal welfare is in danger and has consequences such as an irritation of the mucous membranes, breathlessness and lung hemorrhaging. If loss of consciousness is prolonged animals will suffer these avoidable adverse effects for longer than necessary. Pain and distress will be inevitable as they cannot escape from the chamber (Leach et al. 2004). Guidelines recommend a flow rate of 20 to 30% CV / min and 100% CO_2 which would lead in common euthanasia chambers for rodents to a time to death in about 5 to 15 minutes (Leary et al. 2013). In a hyperbaric chamber the flow rate cannot be raised as the animals need to be placed before euthanasia into the chamber and it will always need a certain time to flood the chamber with the necessary concentration and raise it to a hyperbaric level. The question is, if this induction time might be shorter and with less distress for the animals as in a common chamber. The indications to support this theory are low. Xu

et al.(1991) showed that intracellular pH levels of 5.8 in astrocytes to 6.2 in the interstitial space lead to brain acidosis from cerebral ischemia but as long as the brain is adequate oxygenated during hypercapnia it is not injured. But 50% inspired CO_2 at 2.1 bar for 15 minutes in the hyperbaric chamber led to cardiovascular collapse and sudden death. The rats were cardiovascular stable at hyperbaric conditions of PCO_2 just below 750 mm Hg but died when it rose a bit above it. So it will take around 15 minutes at a concentration of 50% $CO_2$to kill the animals at 2 bar. But 50% are still too much to call it a refinement as pain and distress are already high at this level. Exposing rats to CO_2 concentrations as low as 7% are reported to cause distress and 10% to 20% CO_2induces panic-associated behavior and physiology and loss of consciousness does not occur until at least 40% (Hickman et al. 2016). Danneman et al.(1997) also only needed 15 minutes in total to kill rats with a gradual filling of 60 % CO_2 till anesthesia and 100 % till euthanasia. So, the hyperbaric chamber was not able to accelerate the procedure but to reduce the concentration a bit.

The idea of adding oxygen to limit distress needs to be abandoned as concluded in chapter 4.2. Pure oxygen might also bring a risk to a hyperbaric chamber as there is always a possibility of ignition of the pressurized oxygen within the chamber and it might catch fire (Birnie et al. 2018).

Bergo and Tyssebotn(1999)tested the hypothesis that HBOT would be more efficient in deliveringO_2 to poorly perfused tissues if the vasoconstriction induced by elevated O_2 could be abolished adding CO_2 to the breathing gas. In the experiment only 1 % CO_2 was added but it resulted in an increased PCO_2 and decreased pH and even though the rats could be kept in O_2 atmosphere at 3 bar for hours, they became restless after 45 minutes after CO_2 was added. So, hyperbaric CO_2might result quickly in a brain acidosis but may cause severe problems of distress and pain.

Another point to consider is that hyperbaric conditions intensify the adverse effects of the components of the breathing mixes. Doboszyński et al.(2017) reported from a study with rats swimming stress test in a hyperbaric chamber at a pressure of 2.4 bar

the greatest increase in PCO_2 in the brain when air was mixed with argon. The result is associated with disorders in the respiratory mechanics resulting from the high density of this mixture. Argon and oxygen at 4 bar was 6.61 times denser than air and it seems that in a long-term it would lead to respiratory failure. The rats showed a decrease of physical capacity regardless of the breathing mix used. The conclusion was that hyperbaric conditions involve the risk of CO_2retention, therefore respiratory acidosis, and leads to organism exhaustion. These aspects would support the theory of a quick euthanasia under hyperbaric conditions.

In the case of exposure to hyperbaric pressure, also the following points should be taken into account: the pressure equalization with the gas-filled organs such as lungs, middle ear and paranasal sinuses and the difficulty in breathing due to increased gas density (Bühlmann and Froesch 1989).

Insufficient pressure compensation during changes in the atmosphere pressure can cause barotrauma and so damage the middle ear or lungs. A barotrauma of the middle ear with tympanic membrane rupture is painful and quite common in a hyperbaric chamber if the study participant does not clear its ears (Bühlmann and Froesch 1989). Dogs and cats undergoing a hyperbaric oxygen therapy show sometimes minor adverse effects which cause minimal patient distress as intermittent head shaking, ear flicks and vocalization. Nevertheless these clinical signs can be suggestive of barotrauma (Birnie et al. 2018).

Experts try to find a solution besides CO_2. It is doubtful that a refined CO_2 delivery method would render the method humane. However, better handling and delivery methods could be helpful. Distress may be reduced by conducting procedures in the home cage and minimize handling distress. In a meeting of the Swiss Federal Food Safety and Veterinary Office, which took place in 2018 to find a suitable replacement for the usage of CO_2 as euthanasia agent, they came again to the conclusion that a replacement is urgently required, and the next step would be to draft a research strategy to find a suitable option. The idea to use hyperbaric CO_2 did not come up in the meeting as they try to avoid CO_2 at all (Flammer et al. 2019). This is the same

conclusion as already in 2005 where “new methods of humane killing of animals using gas mixtures other than those containing CO_2 need urgently to be developed” (EFSA 2005).

7 Conclusion

Areas of inconsistency within the euthanasia literature have been highlighted and are related to insufficient knowledge regarding the best methods of euthanasia. By reviewing the published studies on the issue of animal welfare and the method of CO_2 euthanasia it becomes clear that there is no standardized implementation instruction to date and animal welfare cannot be guaranteed.

Conflicting data in the literature, the discussion around prefilled versus gradually filled chambers, presence or absence of oxygen, the lack of agreement on time to unconsciousness and the fact that CO_2 produces significant indicators of pain and distress, still call for a reassessment of the current application of CO_2 as a euthanasia agent.

In case that all mentioned possible advantages are confirmed and other disadvantages like discomfort following inadequate pressure compensation are declared as negligible, a hyperbaric method can be an option to qualify as refinement. The biggest issue in search for refinement might be time. What is an acceptable time period to experience pain? The referred studies with CO_2 report various times and there is no agreement on what is the ideal timeframe.

As none of the reviewed studies used hyperbaric CO_2 to kill animals and time to death with a hyperbaric application is unknown, the assumption that it will be quicker cannot be made. From the present protocol a flow rate of 20 to 30 % CV / min has to be taken over, as the animals cannot be placed in a prefilled chamber with hyperbaric atmosphere.

Unfortunately, the data from the literature search are not sufficient and too inconclusive to develop a mathematical model to predict the best procedure to euthanize laboratory animals with hyperbaric CO_2. But the data provide information in which setting it might work and which parameters need to be considered. Such data

are e.g. flow rate, chamber volume per minute and what a hyperbaric euthanasia chamber should look like.

However, technical aspects of the chamber construction, the numbers of animals that can be euthanized at once and the costs of chamber construction and maintenance will always be a big obstacle. As the use of CO_2 per se remains a highly controversial issue and difficult to bring in line with animal welfare, the chances that a hyperbaric euthanasia chamber will attract attention and find financial resources to perform pilot and proof of principle studies are not high.

8 Abstract

The aim of the study on “Hyperbaric Carbon Dioxide: a possible refinement of euthanasia in laboratory rodents” was to find a humane method for killing rodents in a laboratory setting. Carbon dioxide is the most used gas to euthanize laboratory rodents but it also includes major animal welfare problems.

The study is based on a literature search and clarifies the terms euthanasia and refinement at the beginning. The gas carbon dioxide is explained with its physical and physiological properties and how euthanasia with carbon dioxide works. The next chapters are dedicated to the problems that arise with CO_2 and how these are counteracted in the sense of refinement, such as by adding Oxygen or other gases such as Nitrous Oxide. Very important in the CO_2euthanasia process are flow rate and concentration. These parameters are controversially discussed among experts and so individual opinions of these specialist groups are examined and analyzed.

The study continues with an investigation of hyperbaric administration of CO_2.The physical and physiological peculiarities of hyperbaric gases are highlighted and studies on examples of hyperbaric chambers for laboratory animals are presented.

The final part highlights whether hyperbaric administration is a refinement or not. The conclusion is that $CO_2$is a controversial topic and nearly impossible to bring in line with animal welfare. Therefore it will probably not be possible to find financial and scientific support for carrying out an experiment to confirm the findings in practice.

9 Zusammenfassung

Ziel der Studie zum Thema „Hyperbares Kohlendioxid: eine mögliche Verfeinerung der Euthanasie bei Labornagetieren“ war es, eine humane Methode zur Tötung von Nagetieren im Labor zu finden. Kohlendioxid ist das am häufigsten verwendete Gas zur Euthanasie von Labornagetieren, aber es entstehen dabei auch große tierschutzrelevante Probleme.

Die Studie basiert auf einer Literatursuche und klärt zu Beginn die Begriffe Euthanasie und Verfeinerung. Danach wird das Gas Kohlenstoffdioxid mit seinen physikalischen und physiologischen Eigenschaften erklärt und wie eine Euthanasie mit Kohlenstoffdioxid erfolgt. Weitere Kapitel widmen sich den Problemen die dabei entstehen und wie diesen bereits im Sinne einer Verfeinerung entgegen gewirkt wird, wie zB durch die Hinzugabe von Sauerstoff oder anderen Gasen wie Lachgas. Ein wichtiger Punkt bei der Euthanasie mit CO_2 ist die Durchflussrate und Konzentration. Diese Parameter werden in den Fachkreisen sehr unterschiedlich und kontrovers behandelt. Die einzelnen Meinungen dieser Fachgruppen werden beleuchtet und analysiert.

Die Arbeit geht dann in die Untersuchung einer hyperbaren Administration von CO_2 über. Es werden die physikalischen und physiologischen Besonderheiten von Gasen in Überdruck beleuchtet und Studien zu möglichen Überdruckkammern für Labortiere vorgestellt.

Den Abschluss bildet dann die Abwägung, ob hyperbares Kohlenstoffdioxid tatsächlich eine Verfeinerung darstellt, mit dem Ergebnis, dass die Verwendung von CO_2 ein äußerst kontroverses Thema ist und nur schwer mit dem Tierschutz in Einklang gebracht werden kann und es daher kaum möglich sein wird finanzielle und wissenschaftliche Unterstützung für die Durchführung eines solchen Experiments in der Praxis zu finden.

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