Euthanasia of rats with carbon dioxide — animal welfare aspects

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Summary
A method of inducing euthanasia by carbon dioxide (CO₂) inhalation in the home cage of an animal is described and tested for distress by behavioural as well as by hormonal measures. The animals were maintained in their home cage while CO₂ was induced at a flow of 6 l/min. The behaviour of the animals was measured continuously as were the serum concentrations of glucose, ACTH and corticosterone 30, 75 and 120 s after the CO₂ was introduced into the cage. In order to test for distress, two groups of rats were pre-treated with acepromazine (orally) and pentobarbiturate [i.p. injection] respectively, in order to reduce possible distress caused by CO₂ euthanasia, and were compared with control groups. There were no signs of distress by behavioural or by hormonal changes. All changes seen could be attributed to experimental effects and, especially as there was no difference between the pre-treated and the control groups of rats, it must be assumed that the described method of euthanasia is in concordance with animal welfare, it leads to rapid death without severe distress or pain, and it seems therefore to be 'humane'.

Keywords Euthanasia; animal welfare; CO₂

Anaesthesia and euthanasia of small laboratory rodents with CO₂ is a common and recommended method (Annis et al. 1963, Smith et al. 1972, Harkness & Wagner 1983, Smith 1986, Derr 1991, Andrews et al. 1993, Close et al. 1996, 1997). The inhalation of concentrations of CO₂ higher than 60% is known to act as an anaesthetic and leads to a rapid loss of consciousness (Green 1987). Carbonic acid, a derivate of CO₂, is known to irritate the mucous membranes, leading to discomfort for the animal (Lucke 1979). In most publications it is recommended to place the animal into a concentration of CO₂ higher than 70% (Forslid et al. 1986, Blackshaw et al. 1988, Danneman et al. 1997), although van Zutphen et al. (1993) were able to show that 100% CO₂ leads to dispnoea and distress in conscious animals. Some publications report that averse reactions of the animals can only be seen when the animal is already unconscious (Forslid et al. 1986, Erhardt et al. 1989, Andrews et al. 1993). Efforts were made to reduce these effects by adding oxygen [O₂] (Iwarsson & Rehbinder 1993, Blackmore 1993, Coenen et al. 1995), but the interpretation of these results remains controversial (Smith & Harrap 1997), therefore some authors still recommend pre-filling the euthanasia chamber with CO₂, whilst others (Hewett et al. 1993) do not. Moreover it is known that the administration of CO₂ can interfere with experimental results (Concas et al. 1993, Engel et al. 1996) and should therefore be exercised carefully to avoid confounding the experiment.

The present study was performed to investigate the distress of the animals during euthanasia with CO₂ not only by behaviour
but also by the measurement of hormones and glucose. This was done to test the hypothesis that sedation or even narcosis should reduce the stress-induced effects of CO₂ euthanasia. A sedated animal, and even more an animal in full anaesthesia, should show less or no reaction to the effects of CO₂ euthanasia. Therefore groups of animals were sedated (acepromazine) or anaesthetized (pentobarbiturate) and compared with the non-sedated or non-anaesthetized groups of animals, which were treated in exactly the same way.

Materials and methods

Animals
Male SPF rats of the inbred CDF/F-344 strain/CrlBR were acquired from Charles River Wiga GmbH (Germany) at the age of 8 weeks. This strain was chosen because of its low rate of defecation in the open-field test as well as its low locomotor activity (Harrington 1971, 1972). Males were chosen because of their lower corticosterone concentration in comparison to females (Sutano & de Kloet 1994).

Housing
Immediately after arrival at the laboratory the rats were housed singly in type III Makrolon cages (Scanbur A/S, Køge Denmark) on soft wood bedding with a light cycle of 12 h (07:00 to 19:00 h) at a temperature of 22 ± 2°C and a relative humidity of 55 ± 5%. They were fed a standard diet (Altromin 1324, Altromin International, Lage, Germany) ad libitum containing 19% protein, 4% fat and 6% fibre. The animals had access to tap water via water bottles.

Handling
To minimize stress prior to the final experiment the animals were handled over 3 weeks for about 10 min each day (Laties 1987). This way they were accustomed to being restrained for injection and later decapitation. Following each handling the animals were rewarded with a piece of chopped meat.

Experimental design
In order to measure the influence of the euthanasia with CO₂ the animals were sedated with acepromazine (Ventrazzil R-drops/13.6 mg/ml [Sanofi Ceva GmbH, Düsseldorf, Germany]) per os in chopped meat (7 mg/animal), or with pentobarbital (Nembutal R/60 mg/ml [Wirtschaftsgenossenschaft Deutscher Tierärzte, Garbsen, Germany]) by i.p. injection (60 mg/kg). For each sedated group a control group received chopped meat without acepromazine or with an i.p. injection of saline respectively. These two drugs and their dosages were chosen because they are easy to administer and are commonly used with laboratory animals for reducing stress or for general anaesthesia. The animals were decapitated at 30, 75 and 120 s after the induction of CO₂, leading to 12 groups. There were four animals in each group, totalling 48 animals (Table 1). As there are no seasonal differences in the corticosterone levels in rats (Wong et al. 1983) there was no need for seasonal timing, but since there is a circadian rhythm all experiments were started at exactly 14:00 h. The animals were fed the chopped meat or injected to apply the acepromazine or pentobarbiturate respectively. Only the animals which were injected were handled for injection, weighed and injected. The regular lid of the cage was replaced by a safety glass cover with a hole in the middle (diameter 1 cm). At 14:45 h euthanasia was induced in these cages. Carbon dioxide was led into the cage by a tube at a rate of 6 l/min (Fig 1). With this flow the concentration of CO₂ in the cage was 18.2% after 30 s, 39.5% after 75 s and 55.5% after 120 s. These concentrations were calculated by the inflow of CO₂ and the volume of the Makrolon Type III cage.

| Drug treatment with acepromazine and pentobarbital with controls |
|-----------------------|---|---|---|
| Drug treatment          | 30 s | 75 s | 120 s |
| Acepromazine (per os)   | n = 4 | n = 4 | n = 4 |
| None (per os)           | n = 4 | n = 4 | n = 4 |
| Pentobarbitral (i.p. injection) | n = 4 | n = 4 | n = 4 |
| None (i.p. injection)   | n = 4 | n = 4 | n = 4 |
Assessment of behaviour

In line with the proposal of Gärtner and Militzer (1993) a catalogue of behavioural patterns was made to record the changes in behaviour during the induction of euthanasia. The eyes, the respiration, the overall appearance, defecation, urination, abnormal activity, the bearing, the locomotion and vocalization were recorded.

Assessment of stress

In the situation of stress due to the release of adrenalin the level of glucose is raised to enable the animal for fight and flight. In addition, the release of ACTH will cause glucocorticoid levels to rise (Scharmann 1988) in rats, especially the corticosterone level (Bamberg 1987). Therefore blood glucose, ACTH and corticosterone were measured using commercial available radioimmunoassays (Diagnostic Product Cooperation, Bad Nauheim, Germany) in plasma samples to assess stress at 30, 75 and 120 s following the induction of euthanasia. These time points seemed to be most suitable due to the distinct behavioural differences seen in rats during euthanasia.

Blood sampling

Since the sampling of blood should not interfere with the measurements the only possible method for this was rapid decapitation (Derr 1991, Holson 1992). This method has been proven not to influence the measurement of ACTH (Gartner & Messow 1976). The blood was collected in a cooled glass vial coated with EDTA. The blood was centrifuged at 3000 rpm at 4°C for 12 min. The plasma was separated and stored frozen at −20°C.

Blood measurements

Glucose was determined in a 32 μl sample of whole blood in a Reflotron according to Stähler 1989. ACTH and corticosterone were determined in a specialized laboratory at the University of Heidelberg by radioimmunoassays, as described above.

Statistics

The results were evaluated by a balanced three factorial analysis of variance with the factors ‘drug’ (acepromazine/pentobarbiturate), ‘treatment’ (per os/injection) and blood sampling time after induction of CO₂ (30/75/120 s). Due to skewness of some data they had to be transformed logarithmically prior to the analysis of variance. Significant mean differences were tested according to Scheffé (1953).

Results

Behaviour

The behavioural pattern observed was characterized by three distinct phases as already described by Smith and Harrap (1997). At the beginning of the CO₂ anaesthesia the animals showed tachypnoea and a higher attention span indicating more interest and curiosity. There was no vocalization, urination or defecation in any of the animals. There were no signs for pain, no sneezing and

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Fig 2 Onset of certain behaviours in seconds after inducing euthanasia with CO₂ (6 l/min). The box plot was chosen to show the mean (n = 14) and range of these behaviours.

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no tears. The second phase was characterized by the absence of movement, a so-called 'stationary phase'. In this phase the rats sat quietly and showed reduced attention to their environment. They still showed tachypnoea, but there was no sign of fear. In the third and final phase the rats showed a total relaxation of their muscles and seemed to be unconscious. Reflexes could not be checked without interfering with the experimental design. A few animals showed convulsions before entering this phase. The average starting time of the three phases is shown in Fig 2.

**Glucose**

The blood glucose values are given in Fig 3. There is a time dependency in those animals which were fed chopped meat, whether with or without acepromazine, while there is none in those treated by i.p. injection. This time shift leads to higher glucose levels in the orally-treated group in comparison to the injected group. There is no difference between the sedated/anaesthetized and conscious animals.

**ACTH**

The plasma levels of ACTH in the different group of rats are shown in Fig 4. There is no difference in the plasma level of ACTH between the sedated and the non-sedated or between the anaesthetized and non-anaesthetized rats. The only statistical difference exists between sedation and narcosis especially at 75 s after the induction of CO₂, thus the animals treated by injection prior to the experiment show higher ACTH levels than those animals which were treated per os. During euthanasia the level of ACTH rises with time no matter whether the animals are sedated or anaesthetized or conscious. For the analysis of variance these data have to be transformed logarithmically due to skewness.

**Corticosterone**

The plasma levels of corticosterone are given in Fig 5. Animals which were injected always show statistically higher values than those treated per os. There is no time effect on the level of corticosterone and no statistical difference between the sedated and the non-sedated animals.
Discussion

The aim of this study was to investigate if euthanasia with CO₂ can be recommended with respect to animal welfare. A method for euthanasia should, according to some authors, be inexpensive and aesthetic (Breazile & Kitchell 1969, Cardin 1977, Smith 1986, Blackshaw et al. 1988). But the main point for the recommendation of a method of euthanasia should be that the animal has not suffered pain, distress or even discomfort, along with an assurance that the method is not allowed to interfere with the experimental results. So the best method would be not to touch the animal at all. That is why the inhalation of CO₂ seems to be a suitable method, especially when the animal does not need to be removed from its home cage. To measure the influence of euthanasia on the animals, the effects in conscious, sedated or anaesthetized animals were compared. The hypothesis to be tested was that a sedated/anaesthetized animal would show less reaction to euthanasia with CO₂ than conscious animals, thus if there is no difference it must be assumed that the effects of euthanasia are small and negligible and that this method can be recommended in respect to animal welfare.

The behaviour of the rats during the induction of CO₂ euthanasia revealed no changes indicating behavioural stress. In some previous studies, when the animals were not treated (fed or injected) at all, they even continued to sleep during euthanasia. A flow of 6 l/min was high enough to quickly reach an effective CO₂ concentration within the cage without disturbing the animal with flow-induced noises. The speed of the induction of euthanasia can be seen from the onset of the three stages. The serum level of glucose showed only a slight change over time during the euthanasia as described by Tabat et al. (1998). These results are in contrast to those published by Bhathena (1992). Only in those rats, which were fed chopped meat 45 min before the start of euthanasia there was a higher rise with time, no matter whether the meat contained acepromazine or not, indicating just the reabsorption of energy. The serum concentration of ACTH rises significantly during euthanasia, while the difference between the sedated/anaesthetized group and their conscious control group was not significant at any time. Injected animals reacted faster than the orally-treated rats, thus it must be assumed that there is some effect due to the prior injection. The corticosterone level seems not to be a good measure of short time effects of stress, as it is known to react more slowly (Sutano & de Kloet 1994). There is a clear increase of serum corticosterone concentration in response to the injection. This response was reduced when the animals were anaesthetized with the injection, while the orally-treated animals showed no significant change with time nor any difference between the sedated and conscious group. This influence of anaesthesia on plasma corticosterone levels has also been described for other species such as horses (Taylor 1989). Nevertheless these results indicate that any direct handling will be more stressful for the animals and should therefore be avoided.

Summarizing these results under the assumption of the hypothesis to be tested, it can be concluded that there are no signs of distress during the induction of inhalation euthanasia with the method described when the animals remain in their home cage. Behavioural as well as hormonal changes do not indicate distress in these animals undergoing CO₂ euthanasia. Especially, as there were no differences between the sedated and the non-sedated rats or between the anaesthetized and the conscious rats, it can be assumed that the described euthanasia with CO₂ 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’.

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