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SCIENTIFIC OPINION

Scientific Opinion on the assessment of studies on the use of carbon dioxide for stunning rabbits[1]

EFSA Panel on Animal Health and Welfare (AHAW)[2,3]

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ABSTRACT

The EFSA Panel on Animal Health and Welfare (AHAW Panel) was asked by the European Commission to deliver a scientific opinion on two studies concerned with the use of carbon dioxide for stunning rabbits. The European Commission had received from the Spanish authorities a report of a study entitled 'Carbon dioxide stunning of rabbits' and another study entitled 'Stunning of rabbits with carbon dioxide', provided as a complement to the first study. The latter was undertaken in a commercial abattoir where a commercial carbon dioxide stunner was installed. The results of electrocardiography and animal-based measures (nasal discomfort and vocalisation) produced in the experimental slaughterhouse study clearly indicated that the rabbits were subjected to pain and suffering prior to the loss of consciousness. The data presented do not describe stable, controlled or repeatable experimental conditions. The statistical tests used to analyse the data were not appropriate. The study in the commercial abattoir was not based on sound scientific conclusions resulting from the experimental slaughterhouse study. For all of these reasons, the submitted studies did not meet the minimum criteria for eligibility in the EFSA guidance on the assessment criteria for studies evaluating the effectiveness of stunning interventions. Therefore, they were not further assessed.

KEY WORDS

rabbits, stunning, carbon dioxide, animal welfare, slaughter

[1] On request from the European Commission, Question No EFSA-Q-2014-00186, adopted on 4 February 2015.

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[3] Acknowledgement: The Panel wishes to thank the members of the Working Group on the assessment of studies on the use of carbon dioxide for stunning rabbits: Howard Browman, Massimiliano Petracci and Mohan Raj, for the preparatory work on this scientific opinion, and EFSA staff member: Renata Leuschner, for the support provided to this scientific opinion.

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Animal Health and Welfare (AHAW Panel) was asked to deliver a scientific opinion concerning use of carbon dioxide for stunning rabbits.

The European Commission received from the Spanish authorities a report of a study entitled 'Carbon dioxide stunning of rabbits' and another study entitled 'Stunning of rabbits with carbon dioxide'. The latter study was undertaken in a commercial abattoir in Spain where a commercial carbon dioxide stunner was installed. These studies were sent to EFSA for a scientific evaluation.

The Commission provided EFSA with four terms of reference (ToRs). It was requested that the assessment focus on the stunning of rabbits (ToR 1). For ToR 2, a review of the study was carried out to conclude if it provides sufficient scientific details to evaluate the stunning procedure applied and its welfare outcome. Each of the three working group experts independently considered whether the eligibility criteria set out in the guidance for carbon dioxide stunning at high concentrations (EFSA AHAW Panel, 2013a) were met by the study. If the eligibility criteria were met, the assessment would proceed to ToRs 3 and 4, which are outlined in the section 'Background provided by the European Commission' in this opinion.

The assessment revealed sufficient evidence in the experimental slaughterhouse study that the animals were subjected to pain and suffering prior to the loss of consciousness, as indicated by the results of the animal-based measures (nasal discomfort and vocalisation).

The temperature and humidity of the gas in the experimental slaughterhouse study varied between the treatments and were not controlled. It is difficult to estimate the effect of this as a confounding factor when determining the welfare of the animal during the induction of unconsciousness, as inhalation of cold and dry carbon dioxide gas would lead to drying of the nasal mucous membrane, making inhalation painful (EFSA, 2004).

The statistical analysis is not appropriate for several reasons. The experimental unit used was the individual animal. Rabbits observed together in one cage are not statistically independent and, therefore, they cannot be considered as true replicates (as a result, the sample sizes and degrees of freedom were not calculated correctly); several of the variables are measured repeatedly over time, yet the statistics applied were not those normally applied to repeated measure data; no measures of variability were presented.

The methods used in the experiments were not supported by proper citations to studies and protocols used in previously published articles. In addition, the few previous studies concerning electrical stunning in rabbits were not considered in order to make a comparison with the data obtained in the present study.

The study in the commercial abattoir was not based on sound scientific conclusions resulting from the experimental study. The study parameters selected in the commercial slaughterhouse study were different from those evaluated in the experimental study.

The submitted studies failed to pass the assessment criteria for eligibility in the EFSA guidance document and were therefore not further assessed (EFSA AHAW Panel, 2013a). As the submitted study did not meet the eligibility criteria, a full assessment of the animal welfare implications of the proposed stunning procedure was not carried out.

The use of live animals in experimental procedures should be minimised as far as possible: specifically, the 3R principles (replacement, reduction and refinement) shall be considered in accordance with Directive 2010/63/EU).

TABLE OF CONTENTS

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Article 4 (2) of Council Regulation (EC) No 1099/2009[4] on the protection of animals at the time of killing allows the Commission to amend stunning parameters laid down in Annex I to this Regulation to take into account scientific and technical progress on the basis of an EFSA opinion. Any such amendments shall ensure a level of animal welfare at least equivalent to that ensured by the existing methods. At present, the use of carbon dioxide is not allowed for stunning rabbits.

Article 2 (f) of Regulation (EC) No 1099/2009 defines 'stunning' as 'any intentionally induced process which causes loss of consciousness and sensibility without pain including any process resulting in instantaneous death'. Furthermore, Article 4 states that 'The loss of consciousness and sensibility should be maintained until the death of the animal'.

Following a previous request, the EFSA adopted an opinion on the use of carbon dioxide for stunning rabbits (EFSA AHAW Panel, 2013b) as well as a document on the guidance on the assessment criteria for studies evaluating the effectiveness of stunning interventions regarding animal protection at the time of killing (EFSA AHAW Panel, 2013a).

The Spanish authorities have sent the Commission new data that they would like to be examined (see attachment). In order to reply to this request, the Commission would like to request the EFSA to review the scientific knowledge on the stunning of rabbits of these studies.

TERMS OF REFERENCE (TOR) AS PROVIDED BY EUROPEAN COMMISSION

The Commission therefore considers it opportune to request EFSA to prepare a scientific opinion on the use of a carbon dioxide for stunning rabbits.

ToR 1: The scope of this request is limited to the stunning of rabbits.

ToR 2: Review if the study provides sufficient scientific details as to evaluate the stunning procedure applied and its welfare outcome;

ToR 3: In the case of a favourable reply, carry out a full assessment of the animal welfare implications of the proposed stunning procedure, taking into account other relevant references. In its assessment, EFSA should give its view on the following issues:

The extent to which the use of carbon dioxide is, in principle, an acceptable alternative for the stunning of rabbits compared to the welfare advantages/disadvantages related to other stunning methods used for rabbits under commercial conditions;

The extent to which the findings of the study are consistent with other sources of information;

Requirements possibly attached to the use of carbon dioxide for stunning rabbits, (minimum or maximum gas concentration, duration of exposure, stun-to-stick interval, quality of the gas, temperature of the gas, type of recording and maintenance etc.);

The extent to which the findings of the study can be valid in the context of other rabbit slaughterhouses in the EU;

ToR 4: Assess whether this method can be considered as equivalent to the ones listed in Annex I to Regulation (EC) No 1099/2009.

[4] Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. OJ L 303, 18.11.2009, p. 1–30.

ASSESSMENT

1. Introduction

Inhalation of carbon dioxide (CO_2) induces respiratory and metabolic acidosis, leading to neuronal inhibition via a reduction in the pH of the cerebrospinal fluid. The use of high CO_2 concentrations to stun animals is described in detail in previous EFSA opinions (EFSA 2004, 2005, 2006; AHAW Panel 2013a, b). A lawful application of new stunning methods in the European Union must ensure a level of welfare at least equivalent to that ensured by the methods already provided in Council Regulation (EC) 1099/2009. The term 'acceptable alternative' is defined as an alternative stunning intervention that is at least as good as those listed in Council Regulation (EC) 1099/2009. In particular, for interventions that do not induce immediate unconsciousness, the alternative procedure should ensure (1) that there is absence of pain, distress and suffering until the onset of unconsciousness; and (2) that the animal remains unconscious and insensible until death (EFSA, 2004; EFSA AHAW Panel, 2013a).

The EFSA guidance (EFSA AHAW Panel, 2013a) defines the eligibility criteria for studies on alternative stunning methods that are based on the legal framework provided in Council Regulation (EC) No 1099/2009 and its Annex I. For consistency with the legislation, the eligibility criteria defined in this opinion specify only the minimum requirements. The criteria concerning the outcome of the intervention are based on the legal definition of stunning and consequently focus on the onset and duration of unconsciousness and insensibility, as well as the absence of pain, distress and suffering in the event that the onset of unconsciousness and insensibility is not immediate.

EFSA herein presents its opinion on the findings of the studies received this year for a scientific evaluation entitled 'On the use of carbon dioxide for stunning rabbits' in a manner consistent with the terms of reference provided by the European Commission. EFSA assessed only the stunning procedure itself and did not take into account any pre-stunning phases. A full assessment of the welfare implications, which would need to take into account both the pre-stunning and the stunning phases of the slaughter process, is beyond the scope of this mandate.

2. Documentation assessed by EFSA

The European Commission received a report (Dalmau, Pedernera, Pallisera et al.[5]) from the Spanish authorities entitled 'Carbon dioxide stunning of rabbits' and another report (Villagrá and Gómez[6]) entitled 'Stunning of rabbits with carbon dioxide', provided to complement the laboratory study with a report on trials carried out in a commercial abattoir in Spain. These studies were sent to EFSA for a scientific evaluation.

The report of the study entitled 'Carbon dioxide stunning of rabbits' (Dalmau, Pedernera, Pallisera et al.) outlines its objectives as (1) to study the assessment of pain, distress and/or suffering by means of aversion reaction during exposure to 70%, 80%, 90 % and 98 % CO_2 in atmospheric air; (2) to study the onset and duration of unconsciousness at 70%, 80%, 90 % and 98 % CO_2 in atmospheric air; and (3) to ascertain the concentrations and exposure times to produce effective stunning with a stun-to-stick interval of 70 seconds and 120 seconds. The study is divided into three phases entitled 'pain, suffering and/or distress' (phase 1 (as defined by the authors of the document)), 'onset and duration of unconsciousness' (phase 2) and 'stun with sticking' (phase 3). Phase 1 was carried out in two trials. The three phases are reflected in the materials and methods and in the results sections of the manuscript.

The report entitled 'Stunning of rabbits with carbon dioxide. Commercial study' (Villagrá and Gómez) states that it is intended to be complementary to the above-mentioned report (Dalmau, Pedernera, Pallisera et al.). It states that the main objective is to assess the suitability of 80%, 90 % and 98 % CO_2 to stun rabbits in commercial conditions and to assess its efficiency in producing a state of

[5] Report is listed under item 1 in the Section 'Documentation provided to EFSA'.
[6] Report is listed under item 2 in the Section 'Documentation provided to EFSA'.

unconsciousness until the death of the animals by means of the assessment of physiological reflexes already used in the laboratory study. The commercial study investigates 'stunning with sticking' and 'stunning without sticking'.

3. Assessment approach

The assessment of the submitted studies was carried out in a manner analogous to the approach and specific criteria outlined in the guidance document (EFSA AHAW Panel, 2013a). The assessment was first conducted independently by each working group (WG) member. The individual assessments were then discussed to reach a consensus on issues (if any) over which the experts had expressed different opinions. A detailed evaluation of the reported parameters in the submitted study is presented in Appendix A. The basis for the conclusions drawn by the AHAW Panel is summarised below. Only those aspects that were not considered adequate are commented upon in the text of this opinion.

The assessment focuses on the first term of reference (ToR 1), limiting the scope of this request to the stunning of rabbits. A review of the study was carried out to conclude if it provides sufficient scientific details to evaluate the stunning procedure applied and its welfare outcome (ToR 2). If the eligibility criteria were met, the assessment would proceed to ToRs 3 and 4. However, as the submitted study did not meet the eligibility criteria, neither a full assessment of the animal welfare implications of the proposed stunning procedure (ToR 3) nor an assessment of whether or not this method can be considered equivalent to the ones listed in Annex I to Regulation (EC) No 1099/2009 (ToR 4) was carried out.

4. Assessment of eligibility criteria

The information provided in the submitted studies was assessed following the procedure detailed in the EFSA guidance (EFSA AHAW Panel, 2013a).

4.1. Intervention and outcome of the study carried out in an experimental slaughterhouse

The submitted study involved three objectives:

Objective 1. Assess pain, distress and/or suffering during the induction of unconsciousness by means of recording aversive reactions during exposure to air (sham/control), or 70 %, 80 %, 90 % or 98 % by volume of CO_2 in atmospheric air.

Objective 2. Determine the time to onset and duration of unconsciousness induced with 70 %, 80 %, 90 % or 98 % CO_2 in atmospheric air.

Objective 3. Ascertain the concentrations and exposure times required to produce effective stunning and prevent recovery of consciousness following stunning and severance of jugular veins and carotid arteries.

4.1.1. Objective 1 of the experimental slaughterhouse study

Two trials were carried out. In trial 1, unrestrained animals were exposed in pairs to the gas. Aversive reactions occurring until the loss of posture, which is considered to be a behavioural indicator of unconsciousness, were studied in animals by recording (1) spontaneous activity (similar to open field test); (2) vocalisation; and (3) nasal discomfort. In trial 2, animals were exposed to the gas in pairs, but one animal implanted with electrocardiographic (ECG) recording electrodes was restrained. Heart rate was recorded in the restrained animal for 10 minutes prior to, and 2 minutes during, exposure to the gas, and aversive reactions were studied in the unrestrained animal.

4.1.1.1. Intervention of objective 1

A detailed evaluation of the reported parameters in the submitted study carried out in the experimental slaughterhouse is presented in Table 1 (Appendix A). It is reported that, in trial 1, the animals were housed in pairs in a crate, with both animals free to move. In contrast, in trial 2, one of the two

experimental animals was restrained, but whether or not this restriction of the movement of one animal influenced the behavioural outcome recorded in the other animal was not reported. Additional information concerning trails 1 and 2 are summarised in Tables 2 and 3. The reported temperature range of the gas, of 13 to 15 °C, is not reflected in the data presented in Figures 9 to 13 of the report, which show higher temperatures of up to 22 °C. There is no description of how the gas composition of the atmosphere was measured. The relative humidity reported is inconsistent, varying between 40 and 70 % in the various treatments. Inhalation of dry and cold CO_2 leads to drying of the nasal mucous membrane, making inhalation painful (EFSA, 2004). The breath holding caused by such an atmosphere could also lead to delayed onset of unconsciousness. Therefore, the conditions described do not represent stable, controlled or repeatable experimental conditions in which to reliably assess the effects of the treatments.

4.1.1.2. Outcome of objective 1

Information provided by the submitted study in relation to the onset of unconsciousness and insensibility is summarised in Table 4 in Appendix A.

The WG experts noted that it is difficult to associate the activity of the animals with the magnitude of aversion. However, reduced activity was found in CO_2 compared with air, which could be interpreted as an indication of aversion-induced tonic immobility. Nasal discomfort did not occur during exposure to air, but about 60 % of the animals showed nasal discomfort during exposure to CO_2 of all the gas treatments and vocalisation increased with increasing CO_2 concentrations. These results suggest that the animals are subjected to pain and distress prior to the onset of unconsciousness.

Data presented in Figure 16 of the submitted study suggest that, based on loss of posture as a behavioural indicator of onset of unconsciousness, the duration of suffering is very similar (about 13 seconds) in 80 %, 90 % and 98 % CO_2 and was longer in 70 % CO_2 (about 20 seconds).

The reporting and interpretation of the heart rate results are inconsistent, making it difficult to compare the results between treatments and experiments. In addition, the authors imply that human contact, physical handling and restraint were confounding factors.

The statistical analysis is not appropriate for several reasons: rabbits observed together in one cage are not statistically independent and, therefore, cannot be analysed as if they are true replicates. As a result, the sample sizes and degrees of freedom were not calculated correctly; several of the variables were measured repeatedly over time, yet the statistics applied were not those normally applied to repeated measure data; no measures of variability are presented.

4.1.2. Objective 2 of the experimental slaughterhouse study

4.1.2.1. Intervention objective 2

A detailed evaluation of the reported parameters in the submitted study carried out in the experimental slaughterhouse is presented in Table 5 (Appendix A). The time to onset of unconsciousness was determined using several EEG criteria:

(a) appearance of low-frequency (up to 4 Hz), high-amplitude activity with two criteria: (i) the dominant low frequency should account for 50 % of the EEG power and (ii) this dominant low frequency should occur at least in the first three of six one-second epochs;

(b) appearance of a significant change in the EEG power, as determined from the time taken to decrease the total EEG power content below the baseline levels (pre-exposure power content) at least in 6 of the 15 one-second epochs;

(c) appearance of profoundly suppressed or quiescent EEGs, as determined from the time to onset of less than 10 % of the pre-exposure EEG total power content at least in nine of 15 one-second epochs;

(d) appearance of a continuous reduction of the EEG total power content to less than 10 % of the pre-stun EEG power content, as determined from the occurrence of less than 10 % of the pre-exposure EEG total power content in more than 80 % of 50 one-second epochs.

It was noted that none of these criteria are validated. The interpretation of EEG data, and conclusions drawn based on these criteria, are not supported by the literature.

4.1.2.2. Outcome of objective 2

Information provided by the submitted study in relation to the onset of unconsciousness and insensibility is summarised in Table 6 in Appendix A.

The WG experts noted a number of inconsistencies; for example, the number of surviving animals does not seem to add up. An assessment was not possible owing to incomplete reporting of data (Table 7, Appendix A).

It was noted that the estimated time to onset of unconsciousness varied according to the EEG criterion. For example, the average time to onset of unconsciousness during exposure to 70 % CO_2 was estimated to be 27.5 ± 14.67 seconds according to criterion (a), 31.6 ± 30.52 seconds according to criterion (b), 270 seconds according to criterion (c) and 360 seconds according to criterion (d) (Figures 31–33 in the report).

One of the reasons for this discrepancy could be the high background noise and movement artefacts in the EEG signal. It was nevertheless noted that none of the EEG criteria are validated and the interpretations of EEG data are not supported by relevant scientific literature. In addition, according to data presented in Figure 34 in the report, the time to onset of 10 % or less of the pre-stun total EEG power increases with the higher concentrations of CO_2 of 80 % and 98 % CO_2. An explanation for these reported results, which are contradictory to what would be expected, is not provided. Possibly because of this, the authors concluded that the time to loss of posture, which is an animal-based measure, is the most valid indicator of unconsciousness, making the EEG data redundant.

Regarding time to onset of unconsciousness based on animal-based measures in Section 4.4 of the report, entitled 'Phase 2. Onset and duration of unconsciousness', the duration of exposure for the different CO_2 concentrations is not mentioned and original data are not reported.

For the duration of unconsciousness based on animal-based measures, as reported in the submitted study, the data presented in Figure 26 suggest that animals that survived the treatment were breathing rhythmically and showed a positive corneal reflex. If these animal-based measures were considered to be signs of consciousness, then the interpretation of these data should be that these animals were not stunned. It is not clear why some animals died while others were judged to be conscious after the treatments.

4.1.3. Objective 3 of the experimental slaughterhouse study

4.1.3.1. Intervention of objective 3

The intervention of phase 3 of the laboratory study is summarised in Table 8 and additional information is provided in Table 9 (Appendix A).

4.1.3.2. Outcome of objective 3

Information provided for phase 3 is summarised in Table 10 (Appendix A).

The WG experts noted that in the commercial study in the abattoir (Villagrá and Gómez) (slaughterhouse study) an exposure time of 200 seconds was used, which was not tested in the experimental slaughterhouse study (Dalmau, Pedernera, Pallisera et al.).

4.1.4. Conclusions on the experimental slaughterhouse study

4.1.4.1. Onset of unconsciousness and insensibility

The time to onset of unconsciousness reported in the study varied highly depending upon the EEG criterion used, and the authors did not rely on the EEG data to draw conclusions on the outcome.

4.1.4.2. Absence of pain, distress and suffering until loss of consciousness and sensibility

The WG experts noted that evidence of suffering prior to loss of consciousness was presented in the study. The results showed that none of the animals exposed to air showed vocalisation or nasal discomfort but a very large percentage of animals showed these behavioural manifestations of aversion during exposure to CO_2 and the incidence did not vary between treatments.

In addition, it is known that inhalation of cold and dry CO_2 is painful (EFSA, 2004). It is therefore likely that the lack of control of temperature and humidity in the gas chamber contributed to the stress during induction of unconsciousness.

4.1.4.3. Duration of unconsciousness and insensibility

The number of animals surviving each treatment is not always clearly reported in the text. It is reported that 12 animals survived after exposure to 70 % CO_2, but the number of animals surviving in each exposure time is not reported. For this reason, it is difficult, if not impossible, to ascertain the number of animals surviving each treatment combination (CO_2 concentrations and exposure times) and, therefore, to assess the consequences for animal welfare.

4.2. Intervention and outcome of the commercial abattoir study

It was noted that the commercial slaughterhouse study does not represent an evaluation of findings derived from the experimental slaughterhouse study and that the parameters (gas concentrations and exposure times) were different. A direct relationship between the two studies is missing, and hence the outcome of the experimental study is not validated by the submitted study carried out under commercial slaughterhouse conditions. Parameters used in the slaughterhouse, such as stocking density of animals and exposure times to the gas, were not evaluated in the experimental study.

4.2.1. Intervention of the commercial abattoir study

The commercial abattoir was equipped with a commercial CO_2 stunner and kills about 25 000 rabbits per day at the throughput rate of 3 120 rabbits per hour.

The intervention carried out in the study under commercial conditions is summarised in Table 11 (Appendix A). In the slaughterhouse an experimental trial was carried out that involved stunning but not sticking, which seems out of place in this setting because the stunning parameters should have been investigated in the experimental slaughterhouse study. Such operations seem not relevant in slaughterhouses. In the slaughterhouse context only stunning with sticking seems to be relevant and appropriate.

4.2.2. Outcome of the commercial abattoir study

Information provided regarding the onset and duration of unconsciousness and insensibility and in relation to animal-based measures associated with pain, distress and suffering during the induction of unconsciousness (Section 3.2.1 of EFSA guidance (EFSA AHAW Panel, 2013a)) is presented in Tables 12 and 13 (Appendix A).

4.2.3. Conclusions on the commercial abattoir study

An assessment of the study under commercial abattoir conditions was not possible. Scientific data derived from the laboratory study were inconsistent and were consequently not a sound basis for the trials in the commercial abattoir. Study parameters selected in the commercial slaughterhouse study

were different from those evaluated in the experimental study. In the slaughterhouse stunning experiments were performed without sticking, which is not the normal practice.

5. Reporting and methodological quality

5.1. Assessment of the reporting and methodological quality of the submitted studies based on the selected parameters

The assessed studies did not pass the eligibility assessment and, therefore, reporting and methodological quality was not assessed (EFSA AHAW Panel, 2013a).

6. The extent to which the findings are consistent with other sources of information

Methods used in the experiments were not supported by proper citations to studies and protocols used in previously published articles. In addition, previous studies concerning both gas (Llonch et al., 2012) and electrical stunning (Anil et al., 1998, 2000; María et al., 2001; Rota Nodari et al., 2009) and its effects on animal welfare in rabbits were neither considered nor compared with the data obtained in the present report. In particular, Llonch et al. (2012) reported that rabbits are more averse to exposure to CO_2 than to gas mixtures containing predominantly nitrogen, suggesting that exposure of rabbits to high concentrations of CO_2 may not be the best option on animal welfare grounds.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Although the descriptive information provided in the submitted study fulfil the EFSA guidance requirement for reporting the intervention, the animal welfare outcomes raise serious concerns.

There is sufficient evidence from the experimental slaughterhouse study that the animals were subjected to pain and suffering prior to the loss of consciousness, as indicated by the results of animal-based measures (nasal discomfort and vocalisation).

The temperature and humidity of the gas in the experimental slaughterhouse study varied between the treatments and were not controlled. It is difficult to estimate the effect of this as a confounding factor when determining the welfare of the animal during the induction of unconsciousness, as the inhalation of cold and dry CO_2 gas is painful.

The statistical analysis is not appropriate for several reasons:

- Rabbits observed together in one cage are not statistically independent and, therefore, they cannot be considered as true replicates (as a result, the sample sizes and degrees of freedom were not calculated correctly).
- Several of the variables were measured repeatedly over time, yet the statistics applied were not those normally applied to repeated measures data.
- No measures of variability are presented.

The estimated times to onset of unconsciousness varied highly depending upon the EEG criterion used, and the authors did not rely on the EEG data to formulate conclusions. Instead, they used the time to loss of posture, which is an animal-based measure, as the most valid indicator of unconsciousness, making the EEG data redundant.

As animals were subjected to human contact, physical handling and restraint, which are confounding factors, ECG data failed to yield useful results.

The methods used in the experiments were not supported by proper citations to studies and protocols used in previously published articles. As it has been reported that rabbits are more averse to exposure

to CO_2 than to gas mixtures containing predominantly nitrogen, this suggests that exposure of rabbits to high concentrations of CO_2 may not be the best option on animal welfare grounds. In addition, the few previous studies concerning electrical stunning in rabbits were not considered in order to make a comparison with the data obtained in the present study.

The study in the commercial abattoir was not based on sound scientific conclusions resulting from the experimental study. The study parameters selected in the commercial slaughterhouse study were different from those evaluated in the experimental study.

The submitted studies failed to pass the assessment criteria for eligibility in the EFSA guidance document and, therefore, were not further assessed.

RECOMMENDATIONS

When a particular parameter aimed at achieving effective stunning and slaughter without causing avoidable pain and suffering, the procedure should be terminated on ethical and animal welfare grounds.

The use of live animals in experimental procedures should be minimised as far as possible: specifically, the 3R principles (replacement, reduction and refinement) shall be considered in accordance with Directive 2010/63/EU[7].

DOCUMENTATION PROVIDED TO EFSA

1. Dalmau A, Pedernera C, Pallisera J, Muñoz I, Carreras R, Casal N, Mainau E and Rodríguez P. Report of the study entitled carbon dioxide stunning of rabbits. IRTA, Spain.

2. Villagrá A and Gómez EA. Report of the study entitled stunning of rabbits with carbon dioxide. Commercial study. IVIA, Spain.

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[7] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. OJ L 276, 20.10.2010, p. 33–79.

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APPENDIX

Appendix A. Assessment of eligibility criteria

Table 1: Phase 1 'To study pain, suffering and/or distress to four carbon dioxide concentrations'. Parameters to be provided when applying a stunning intervention based on high CO_2 concentrations or CO_2 in two/multiple phases, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad hoc expert working group (corresponding to Table 5 (EFSA AHAW Panel, 2013a))

Parameter	Component	Description presented in study (Dalmau, Pedernera, Pallisera et al.) (all specifications should be in internationally recognised units) Trial 1	Trial 2	Is the information required by the EFSA guidance present? (yes, no or not applicable)
CO_2 concentration	Initial CO_2 concentration [(a)]			Yes
	Targeted CO_2 concentration(s) [(a)]	70, 80, 90 or 98 %	70, 80, 90 or 98 %	Yes
	Final CO_2 concentration [(a)]			Yes
	CO_2 concentration gradient	Table 4, p. 22	Table 4, p. 22	Yes
	Animal stocking density and type	0.9 to 1.2 kg of rabbit/m^2, p. 7 In pairs in crates, p. 10 In crates, two animals lowered into pit, facing each other, freedom to move	0.9 to 1.2 kg of rabbit/m^2, p. 7 In pairs in crates, but one rabbit was in a restrainer for recording heart rate, pp. 10–11	Yes
	Monitoring	Carbon dioxide concentration	Carbon dioxide concentration	Yes
Duration of intervention [(b)]	Time to reach targeted CO_2 concentration [(a)]	Nine seconds	Nine seconds	Yes
	Total duration of targeted CO_2 exposure [(a)]	45 seconds kept in pit	45 seconds kept in pit	Yes
Maximum stun-to-stick/-kill interval(s) [(a),(c)]				Not applicable for this phase 1
Frequency of calibration of the equipment		The analyser is calibrated by an external company twice a year following the calibration procedures of IRTA, p. 8		Yes

Parameter	Component	Description presented in study (Dalmau, Pedernera, Pallisera et al.) (all specifications should be in internationally recognised units) Trial 1	Trial 2	Is the information required by the EFSA guidance present? (yes, no or not applicable)
Quality of the gas	CO_2 source	Supplied by Carburos Metálicos (Barcelona, Spain) in packs with liquid CO_2 that was heated before being delivered in the pit that contained only atmospheric air before delivery of CO_2		Yes
	Gas composition of the atmosphere	CO_2 concentration is reported, p. 8 'continuous gas flow analyser' (Check Mate II, PBI Dansensor, Ringsted, Denmark)		Yes
	Humidity and temperature	It was measured		Yes
Temperature of the gas		13 to 15 °C, p. 8		Yes

(a): Provide information on mean or median and range and standard deviation or interquartile range of detailed parameter.
(b): Referring to the legal parameter 'duration of exposure.'
(c): In the case of simple stunning.

Table 2: Additional information provided for phase 1, trial 1, of the submitted experimental slaughterhouse study (Dalmau, Pedernera, Pallisera et al.)

Information provided	70 % CO_2	80 % CO_2	90 % CO_2	98 % CO_2
Exposure time (seconds)	45	45	45	45
Number of animals	20	20	20	20
ABMs of aversion	Activity, vocalisation, nasal discomfort None of the animals exposed to air vocalised or showed nasal discomfort. Vocalisation occurred in a large number of animals during exposure to CO_2 and it is stated that it did not vary between treatments. However, data presented in Figure 15 of the submitted study show that the percentage of animals vocalising increased significantly as the concentration of CO_2 was increased from 70 to 90 %	Activity, vocalisation, nasal discomfort	Activity, vocalisation, nasal discomfort	Activity, vocalisation, nasal discomfort

ABM, animal-based measure.

Table 3: Additional information provided for phase 1, trial 2, of the submitted experimental slaughterhouse study (Dalmau, Pedernera, Pallisera et al.)

Information provided	70 % CO_2	80 % CO_2	90 % CO_2	98 % CO_2
Exposure time (seconds)	45	45	45	45
Number of animals	16	16	16	16
ABMs of aversion	Activity, vocalisation, nasal discomfort, heart rate Data presented in Figure 18 suggest that none of the animals exposed to air showed vocalisation or nasal discomfort, but a very large percentage of animals showed these behavioural manifestations of aversion during exposure to CO_2 and the incidence did not vary between treatments	Activity, vocalisation, nasal discomfort, heart rate	Activity, vocalisation, nasal discomfort, heart rate	Activity, vocalisation, nasal discomfort, heart rate

ABM, animal-based measure.

Table 4: Phase 1 'To study pain, suffering and/or distress to four carbon dioxide concentrations': experimental slaughterhouse study. Information provided by the submitted study in relation to the onset of unconsciousness and insensibility (Section 3.2.1 of EFSA guidance (EFSA AHAW Panel, 2013a))

Parameter	Information provided in the study (Dalmau, Pedernera, Pallisera et al.)		Is the information required by the EFSA guidance present? (yes, no or not applicable)
	Trial 1	Trial 2	
Start and end of EEG measurement	No	No	Not relevant
EEG measurement	No	No	Not relevant
EEG recording analysis	No	No	Not relevant
EEG results	No	No	Not relevant
ABM to detect onset of unconsciousness	The following variables were measured: activity, vocalisation, nasal discomfort, loss of posture	In the restrained animals in trial 2, only vocalisations and nasal discomfort (in this case assessed only by means of head shaking) were considered	Yes

ABM, animal-based measure.

Table 5: Phase 2 'Efficacy in producing an effective stunning': parameters to be provided when applying a stunning intervention based on high CO_2 concentrations or CO_2 in two/multiple phases, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad hoc expert working group (corresponding to Table 5 (EFSA AHAW Panel, 2013a))

Parameter	Component	Description presented in study (Dalmau, Pedernera, Pallisera et al.) (all specifications should be in internationally recognised units)	Is the information required by the EFSA guidance present? (yes, no or not applicable)
CO_2 concentration	Initial CO_2 concentration [(a)]		Yes
	Targeted CO_2 concentration(s) [(a)]	70, 80, 90 and 98 %	Yes
	Final CO_2 concentration [(a)]		Yes
	CO_2 concentration gradient	As reported in Table 1	Yes
	Animal stocking density and type	As reported in Table 1	Yes
	Monitoring	As reported in Table 1	Yes
Duration of intervention [(b)]	Time to reach targeted CO_2 concentration [(a)]	As reported in Table 1	Yes
	Total duration of targeted CO_2 exposure [(a)]	Table 2, p. 15	Yes
Maximum stun-to-stick/-kill interval(s) [(a),(c)]			Not applicable for this phase
Frequency of calibration of the equipment		As reported in Table 1	Yes
Quality of the gas	CO_2 source	As reported in Table 1	Yes
	Gas composition of the atmosphere	As reported in Table 1	Yes
	Humidity and temperature	It was measured	Yes
Temperature of the gas		As reported in Table 1	Yes

(a): Provide information on mean or median and range and standard deviation or interquartile range of the detailed parameter.
(b): Referring to the legal parameter 'duration of exposure'.
(c): In the case of simple stunning.

Table 6: Phase 2 'Efficacy in producing an effective stunning': experimental slaughterhouse study. Information provided by the submitted study in relation to the onset and duration of unconsciousness and insensibility (Section 3.2.1 of EFSA guidance (EFSA AHAW Panel, 2013a))

Parameter	Information provided (Dalmau, Pedernera, Pallisera et al.)	Is the information required by the EFSA guidance present? (yes, no or not applicable)
Start and end of EEG measurement	Measurement was started 5 minutes before descending into the pit, during exposure to gas treatment and continued up to 5 minutes after the end of the exposure or until animals showed signs of death, as glassy eyes	Yes
EEG measurement	The methodology is described on p. 16	Yes
EEG recording analysis	The parameters used to assess the EEG, were: (1) Time of appearance of slow waves (high amplitude and low frequency) (2) Moment of appearance of a significant change in the EEG power (3) Appearance of profoundly suppressed or quiescent EEGs (4) Appearance of a continuous reduction in the EEG total power (5) Recovery of EEG	Yes
EEG results	A description of the results is provided on p. 42 onwards. EEG results are shown together for phases 2 (without sticking) and 3 (with sticking)	Yes
ABM to detect onset of unconsciousness	At the beginning and end of EEG measurement duration, physiological reflexes, such as rhythmic breathing (in a continuous way) and corneal reflex (every 10 seconds), were also assessed	Yes

ABM, animal-based measure.

Table 7: Additional information provided for phase 2 of the experimental slaughterhouse study (Dalmau, Pedernera, Pallisera et al.)

Information provided	**70 % CO_2**			**80 % CO_2**		**90 % CO_2**			**98 % CO_2**
Exposure time (seconds)	80	270	360	90	170	50	80	90	60
Number of animals	10	30	40	40	40	40	8	32	40
Surviving animals (%)	Not in the text but data in Figure 26 suggest that all animals survived	Not in the text but data in Figure 26 suggest that 50 % of animals survived	35 (n = 14)	Not reported in the text but data in Figure 26 suggest that that about 80 % of animals survived	32 (n = 12.8)	Not reported in the text but data in Figure 26 suggest that more than 80 % of animals survived	Nothing reported	25 (n = 8)	37 (n = 14.8)
Time (seconds) to onset of unconsciousness based on EEG	Time to onset of low frequency (Figure 31) = 27.5 ± 14.67 Time to onset of a significant change in EEG power = 31.6 ± 30.52 Time to onset of EEG different to basal values reported in Figure 31 but difficult to interpret and different from the criterion set out in the methods Time to onset of 10 % or less of the pre-stun total EEG power reported in Figure 33 is different from the criterion set out in the methods			24.8 ± 14.94 30.7 ± 21.92		22.2 ± 16.27 31.2 ± 17.66		12.7 ± 8.19 33.9 ± 23.84	
	Time (seconds) to onset of 10 % or less of the pre-stun total EEG power reported in Figure 34 is about 200 seconds			120		170		300	
Duration in seconds of unconsciousness based on EEG	Reported that 12 animals survived after exposure to 70 % CO_2, but the number of animals surviving in each exposure time was not reported			Reported that 15 animals survived after exposure to 80 % CO_2, but the number of animals surviving in each exposure time was not reported		Reported that 17 animals survived after exposure to 90 % CO_2, but the number of animals surviving in each exposure time was not reported		Reported that 5 animals survived after exposure to 98 % CO_2	
Time in seconds to onset of unconsciousness based on ABMs	NR	NR	NR	NR	NR	NR	NR	NR	NR

ABM, animal-based measure; NR, not reported.

Table 8: Phase 3 'Stun to sticking': parameters to be provided when applying a stunning intervention based on high CO_2 concentrations or CO_2 in two/multiple phases, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad hoc expert working group (corresponding to Table 5 (EFSA AHAW Panel, 2013a))

Parameter	Component	Description presented in study (Dalmau, Pedernera, Pallisera et al.) (all specifications should be in internationally recognised units)	Is the information required by the EFSA guidance present? (yes, no or not applicable)
CO_2 concentration	Initial CO_2 concentration [(a)]		Yes
	Targeted CO_2 concentration(s) [(a)]	80, 90 or 98 %	Yes
	Final CO_2 concentration [(a)]		Yes
	CO_2 concentration gradient	As reported in Table 1	Yes
	Animal stocking density and type	As reported in Table 1	Yes
	Monitoring	As reported in Table 1	Yes
Duration of intervention[(b)]	Time to reach targeted CO_2 concentration [(a)]	9 seconds	Yes
	Total duration of targeted CO_2 exposure [(a)]	Exposure times for each gas treatment were selected according to the results of phase 2: 200 seconds in 80 % CO_2, 130 and 150 seconds in 90 % CO_2, and 110 seconds for 98 % CO_2 when the stun-to-stick interval was 120 and 110 seconds of exposure with bleeding at 70 seconds in 90 % CO_2	Yes
Maximum stun-to-stick-/kill interval(s) [(a),(c)]		70 to 120 seconds	Yes
Frequency of calibration of the equipment		As reported in Table 1	Yes
Quality of the gas	CO_2 source	As reported in Table 1	Yes
	Gas composition of the atmosphere	As reported in Table 1	Yes
	Humidity and temperature	Were measured	Yes
Temperature of the gas		As reported in Table 1	Yes

(a): Provide information on mean or median and range and standard deviation or interquartile range of the detailed parameter.
(b): Referring to the legal parameter 'duration of exposure.'
(c): In the case of simple stunning.

Table 9: Additional information provided for phase 3 of the submitted experimental slaughterhouse study (Dalmau, Pedernera, Pallisera et al.)

Information provided	**80 % CO_2**	**90 % CO_2**			**98 % CO_2**
Exposure time (seconds)	200	130	150	110	110
Stun-to-stick interval (seconds)	120	120	120	70	120
Number of animals	30	30	30	22	30
Incidence of recovery of consciousness based on ABMs	All the animals died before sticking	Two animals survived and rhythmic breathing occurred in these animals at 140 and 60 seconds after the end of exposure	All the animals died before sticking	Two animals survived and rhythmic breathing occurred in these animals at 90 and 60 seconds after the end of exposure	All the animals died before sticking

ABM, animal-based measure.

Table 10: Phase 3: Experimental slaughterhouse study. Information provided by the submitted study in relation to the onset of unconsciousness and insensibility (Section 3.2.1 of EFSA guidance (EFSA AHAW Panel, 2013a))

Parameter	Information provided (Dalmau, Pedernera, Pallisera et al.)	Is the information required by the EFSA guidance present? (yes, no or not applicable)
Start and end of EEG measurement	Before exposure to gas until death (p. 19)	Yes
EEG measurement	Outlined previously on p. 15, but not in Section 3	Yes
EEG recording analysis	Outlined previously (p. 15), but not in Section 3	Yes
EEG results	A description is given in Section 4.6 starting on p. 42	Yes
ABM to detect onset of unconsciousness	Absence of rhythmic breathing, vocalisations and righting reflex and every 10 seconds by means of absence of corneal reflex	Yes

ABM, animal-based measure.

Table 11: Commercial study in the abattoir: parameters to be provided when applying a stunning intervention based on high CO_2 concentrations or CO_2 in two/multiple phases, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad hoc expert working group (corresponding to Table 5 (EFSA AHAW Panel, 2013b))

Parameter	Component	Description presented in study (Villagrá and Gómez) (all specifications should be in internationally recognised units)	Is the information required by the EFSA guidance present? (yes, no or not applicable)
CO_2 concentration	Initial CO_2 concentration [(a)]	Air	Yes
	Targeted CO_2 concentration(s) [(a)]	80 or 90 %	Yes
	Final CO_2 concentration [(a)]	80 and 90 %	Yes
	CO_2 concentration gradient		No
	Animal stocking density and type	29 animals per cage 7 cages were in the tunnel at the same time Stocking density of 38.36 animals/m^2 (p. 3, second paragraph)	Yes
	Monitoring	Gas measurer (ADEV srl EC2001 Analyser (name and location of the manufacturer not provided))	Yes
Duration of intervention [(b)]	Time to reach targeted CO_2 concentration [(a)]	3 seconds + 3 seconds = 6 seconds	Yes
	Total duration of targeted CO_2 exposure [(a)]	80 % for 200 seconds 90 % for 150 seconds (anticipated); however, 200 seconds done	Yes, the intervention did not result in irreversible stunning of all animals
Maximum stun-to-stick/-kill interval(s) [(a),(c)]		26.4 ± 2.88 for the first and 95.2 ± 13.03 seconds The time between the first hung rabbit and its bleeding was 31.8 ± 1.3 seconds	Yes
Frequency of calibration of the equipment			No
Quality of the gas	CO_2 source	Provider Praxair (name and location of the manufacturer not provided)	Yes
	Gas composition of the atmosphere		No
	Humidity and temperature		No

Parameter	Component	Description presented in study (Villagrá and Gómez) (all specifications should be in internationally recognised units)	Is the information required by the EFSA guidance present? (yes, no or not applicable)
Temperature of the gas		16.4 ± 0.6 °C	Yes

(a): Provide information on mean or median and range and standard deviation or interquartile range of the detailed parameter.
(b): Referring to the legal parameter 'duration of exposure.'
(c): In the case of simple stunning.

Table 12: Study in the commercial abattoir. Information provided by the submitted study in relation to the onset and duration of unconsciousness and insensibility (Section 3.2.1 of EFSA guidance (EFSA AHAW Panel, 2013a))

Parameter	Information provided (Villagrá and Gómez)	Is the information required by the EFSA guidance present? (yes, no or not applicable)
Start and end of EEG measurement	No	Not applicable
EEG measurement	No	Not applicable
EEG recording analysis	No	Not applicable
EEG results	No	
ABM to detect onset of unconsciousness	Gagging, rhythmic breathing, corneal reflex, vocalisation, righting reflex. Two experimental groups were used: with and without sticking The times recovering the different signs were shown for rabbits stunned without sticking: following exposure to 80 % CO_2 for 200 seconds, mean time for the first gagging was about 45 seconds, whereas rhythmic breathing was achieved at 73 ± 14.87 seconds. The righting position of the animals was seen in a mean time of 277.4 ± 93.01seconds. Following exposure to 90 % CO_2 for 200 seconds, only one animal recovered from the stunning, showing initial gasping at 43 seconds after exiting the CO_2 tunnel and total recovery at 372 seconds The times recovering the different signs were shown for rabbits stunned with sticking: when 80 % CO_2 was applied for 200 seconds, 3 out of 300 rabbits (1 %) showed signs of recovery but only rhythmic breathing. One of them was breathing until the moment of bleeding, when it lost rhythmic breathing immediately; the second one presented rhythmic breathing each 3 seconds until 25 seconds post bleeding, and the third one presented rhythmic breathing until 22 seconds post bleeding. It has to be remarked that the corneal reflex was not recovered in any of the animals When 90 % CO_2 was applied for 200 seconds, 0 out of 300 rabbits showed signs of recovery of consciousness	Yes

ABM, animal-based measure.

Table 13: Study under slaughterhouse conditions: information provided by the submitted study in relation to animal based measures (ABMs) associated with pain, distress and suffering during the induction of unconsciousness (section 3.2.1 of EFSA guidance (EFSA AHAW Panel, 2013a))

Response type	Groups of ABMs	Information provided in the study (Villagrá and Gómez)	Is the information required by the EFSA guidance present? (yes, no or not applicable)
Behaviour	Vocalisation	Yes	Yes
	Postures and movements	Righting reflex	Yes
	General behaviour	Gagging, rhythmic breathing	Yes
		Stunning with sticking: using 80 % CO_2 for 200 seconds resulted in 3 of 300 rabbits showing signs of recovery. If 90 % CO_2 for 200 seconds was applied, no signs of recovery of consciousness were observed	
		Stunning without sticking: using 80 % CO_2 12 out of 100 rabbits showed signs of recovery	
Physiological response	Hormone concentration	No	No
	Blood metabolites	No	No
	Autonomic responses	Corneal reflex	Yes
Neurological response	Brain activity	No	No

ABBREVIATIONS

ABM	animal-based measure
AHAW Panel	EFSA Panel on Animal Health and Welfare
CO_2	carbon dioxide
EC	European Commission
EFSA	European Food Safety Authority
ECG	electrocardiography
ToR	terms of reference provided by the European Commission