The Old School, Brewhouse Hill, Wheathampstead,
Hertfordshire AL4 8AN, UK
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Assessment of aversion and unconsciousness during exposure to carbon dioxide at high concentration in lambs

P Rodríguez[†‡], A Dalmau[†], X Manteca[‡], H Litvan[§], EW Jensen[#] and A Velarde*[†]

[†] IRTA, Animal Welfare Subprogram, IRTA, Veïnat de Sies, s/n 17121, Monells, Spain
[‡] UAB, School of Veterinary Science, 08193 Bellaterra, Spain
[§] UAB, Department of Cardiac Anaesthesia and Postoperative Intensive Care, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain
[#] Center for Research in Biomedical Engineering, Technical University of Catalonia, Barcelona, Spain
* Contact for correspondence and requests for reprints: antonio.velarde@irta.es

Abstract

*The most widely used stunning method in sheep is electrical. However, in lambs, this method leads to rupture of the blood vessels, provoking ecchymoses. In pigs (*Sus scrofa*), the use of CO_2-stunning systems has increased in popularity due to positive effects on meat quality and animal welfare (movement of animals in groups). The aim here was to assess the effectiveness of a progressive exposure to 90% carbon dioxide (CO_2) in inducing unconsciousness in lambs (*Ovis aries*) through changes in the middle latency auditory-evoked potentials (MLAEP) of the central nervous system (CNS), blood parameters (pH, carbon dioxide partial pressure (pCO_2), oxygen partial pressure (pO_2), oxygen saturation ($SatO_2$) and bicarbonate (HCO_3^-), behaviour (head-shaking, sneezing, gasping and gagging) and physiological reflexes (corneal reflex, breathing and sensitivity to pain). Fourteen male lambs of the Ripollesa breed, weighing between 19 and 25 kg, were progressively exposed to an atmosphere of 90% CO_2 over 66 s. All blood parameters changed between 23 and 43 s after the onset of the immersion. The MLAEP did not decrease significantly until after 48 s exposure to CO_2, suggesting an absence of auditory-evoked brain activity. Before that, lambs exhibited head-shaking and sneezing starting at 10.6 (± 0.77) s, and gasping starting at 20.6 (± 1.36) s. After exposure, all animals showed absence of breathing and sensibility to pain, and 36% of them absence of corneal reflex. The pH and pCO_2 recovered basal values at 90 and 120 s, respectively, after the end of the exposure. The burst suppression index (BS%) and the A-Line ARX index (AAI) recovered basal levels at 116 and 159 s, respectively. Exposure to CO_2 at high concentration induces effective stunning in sheep for a period of 124 s. However, during exposure, the animals exhibited signs of aversion and breathlessness.*

Keywords: *animal welfare, auditory-evoked potential, aversion, carbon dioxide, sheep, stunning*

Introduction

At present, the most widely used methods for stunning lambs (*Ovis aries*) in commercial slaughterhouses are electrical and, to a small extent, mechanical stunning. Electrical stunning is elicited by passing electrical current through the brain, with intensity high enough to induce an epileptiform state, characterised by tonic and clonic seizures, and the instantaneous loss of brain functionality (Velarde *et al* 2003) or through the brain and body to induce cardiac arrest. However, it also produces an increase in muscle activity and blood pressure which may cause haemorrhages (ecchymoses and petechiae) and bone fractures in the carcases (Petersen *et al* 1986; Vergara & Gallego 2000). These defects in carcase quality have an economic impact for the meat industry.

In pigs, carbon dioxide (CO_2) stunning decreases the incidence and extent of haemorrhages in the carcase (Velarde *et al* 2001). The lower incidence of haemorrhages is probably due to the decrease in the muscle contraction during CO_2 exposure compared to electrical stunning. CO_2 is a gas that, when inhaled at high concentrations, induces hypercapnic hypoxia and changes in blood parameters. Among these changes, an increase of carbon dioxide partial pressure (pCO_2) and bicarbonate concentration (HCO_3^-) and a decrease of blood pH, oxygen partial pressure (pO_2) and oxygen saturation ($SatO_2$) have been described by Lomhort (1998), Martoft *et al* (2001) and Rodríguez *et al* (2008). In consequence, the pH of the cerebrospinal fluid (CSF) decreases and the animal loses consciousness (Gregory 1987; Raj & Gregory 1996; Raj 1999). In this regard, normal pH of CSF is 7.4 and a state of analgesia and anaesthesia are induced at pH 7.1 and 6.8, respectively (Eisele *et al* 1967). In commercial conditions, pigs are immersed into the gas following a concentration gradient, so that, the deeper the cage is lowered, the higher the CO_2 concentration, until it reaches 80–90% CO_2 concentration in atmospheric air. This system has a number of welfare advantages compared with electrical stunning. It precludes the need for animal restraint thereby allowing group stunning, which reduces handling stress prior to stunning

(Velarde *et al* 2000; EFSA 2004). Nevertheless, it has also been criticised since the loss of consciousness is not immediate (Raj & Gregory 1995), and is dependent upon the CO_2 concentration and the speed at which animals are lowered towards the bottom of the pit, where the highest CO_2 concentration is achieved (Troeger & Woltersdorf 1991; Raj & Gregory 1996). During CO_2 exposure, pigs (*Sus scrofa*) show signs of aversion, such as retreat attempts, head-shaking, sneezing, breathlessness, freezing, escape attempts, gasping and vocalisations (Manning & Schwartzstein 1995; Lambooij *et al* 1999; Raj 1999; Holst 2001). The main causes of aversion and distress are the irritation of the nasal mucosal membranes and lungs (Manning & Schwartzstein 1995) and the severe respiratory distress (Raj & Gregory 1996) induced by the inhalation of CO_2 at high concentrations. Although carbon dioxide in high concentrations has been also used for the euthanasia of small laboratory animals, such as rodents and rabbits (*Oryctolagus cuniculus*), exposure to the gas has been recognised as often triggering severe aversive reactions during most experimental investigations (Leach *et al* 2001). Based on comparative physiology, these effects should be similar in lambs. However, to our knowledge, no studies on the aversiveness of exposure to high concentration of CO_2 in lambs have been published.

The depth and duration of unconsciousness from CO_2 gas stunning depends on gas concentration, exposure time and the animal (Atkinson *et al* 2012). To ensure good animal welfare, the stun should ensure unconsciousness is induced for a sufficient duration to include not only the stun-to-stick interval but also the time taken for brain death to occur due to sticking. The duration of unconsciousness determines the maximum stun-to-stick interval. It is, therefore, imperative for animal welfare that unconsciousness is closely monitored, and animals re-stunned when necessary.

Electroencephalograms (EEG) or electrocorticograms (ECoGs) are widely used to record the spontaneous and evoked electrical activity in the brain to ascertain the state of consciousness and sensibility following stunning (Hoenderken 1983; Forslid 1987). From the different methodologies to record brain activity, the middle latency auditory-evoked potentials (MLAEP) have been suggested as the most precise indicator of the level of consciousness (Thornton *et al* 1989) and have been used to evaluate changes in neural activity and assess depth of anaesthesia in humans (Litvan *et al* 2002), rats (*Rattus norvegicus*) (Jensen *et al* 1996, 1998) and pigs (Martoft *et al* 2002; Rodríguez *et al* 2008). From the MLAEP, the A-line ARX index (AAI) and the burst suppression index (BS%) can be estimated to assess unconsciousness during states of anaesthesia (Jensen 1999; Litvan *et al* 2002; Rodríguez *et al* 2008). The AAI is a numerical index, ranging from 0 to 60 that quantifies MLAEP variations of amplitude and latency. Higher values are related with awareness, while decreases of AAI indicate a gradual loss of consciousness, an AAI index below 40 is indicative of deep unconsciousness (Jensen 1999). The BS% indicates the percentage of iso-electric activity of the EEG during the preceding 30 s and ranges from 0 to 100 (Litvan *et al* 2002).

The aim of this study was to assess the effectiveness of a progressive exposure to 90% CO_2 and the duration of unconsciousness using AAI, BS%, blood gas parameters, behaviour and physiological reflex in lambs. The duration of unconsciousness will determine the maximum stun-to-stick interval.

Materials and methods

Study animals

Fourteen male lambs of the Ripollesa breed with a mean (± SEM) live weight of 22.4 (± 2.03) kg were used. The sample size was based on calculations carried out for a previous study using the same methodology, which was designed to achieve a power TYPE I error of 0.025 and TYPE II error of 0.80. Three days before the start of the study, the lambs were transported from the farm of origin to the IRTA facilities, and housed in two pens (1.80 × 2.20 m; length × width) provided with straw. These pens were adjacent to the slaughterhouse facilities. Water and food were available *ad libitum* and animals were fasted 12 h prior to the experimental procedure.

Experimental procedure

The study was carried out during three consecutive weeks. Each day, from Monday to Friday, two lambs were exposed individually to 90% CO_2 in a CO_2 Dip-Lift apparatus (BUTINA Aps, Copenhagen, Denmark). This system consists of a 195 × 61 × 90 cm (length × width × height) cage which was lowered into a 260-cm deep well pre-filled with CO_2 (Carburos Metálicos SA, Barcelona, Spain). The required CO_2 concentration of 90% was supplied through an inlet valve at the bottom of the well, and monitored continuously via sensors fitted on the wall 50 cm above floor level. When the CO_2 concentration dropped below the pre-set value of 90% a valve released sufficient gas to restore the original setting before shutting off. According to a previous study (Dalmau *et al* 2010), with 90% CO_2 at the bottom of the well, the CO_2 concentration decreased to 80, 55 and 20% at 110, 160 and 210 cm above the bottom of the pit, respectively. The CO_2 exposure cycle lasted 66 s, and consisted of the first 23 s (during which the cage was lowered), the following 20 s (while the cage remained stationary at the bottom of the pit at the highest CO_2 concentration), and the final 23 s (during which the cage was raised). The floor of the crate was perforated to facilitate gas distribution within the crate.

Each lamb to be stunned was placed in sternal recumbency in a net restrainer in order to minimise discomfort to the animal. The restrained animal's limbs were approximately 10 cm above the ground. Blood gases, auditory-evoked potentials and behavioural measures were recorded during the 10 min prior to CO_2 exposure, during exposure to CO_2, and for a further 10 min after exposure. After the CO_2 exposure, the presence of rhythmic breathing, corneal reflex and sensibility to pain were monitored.

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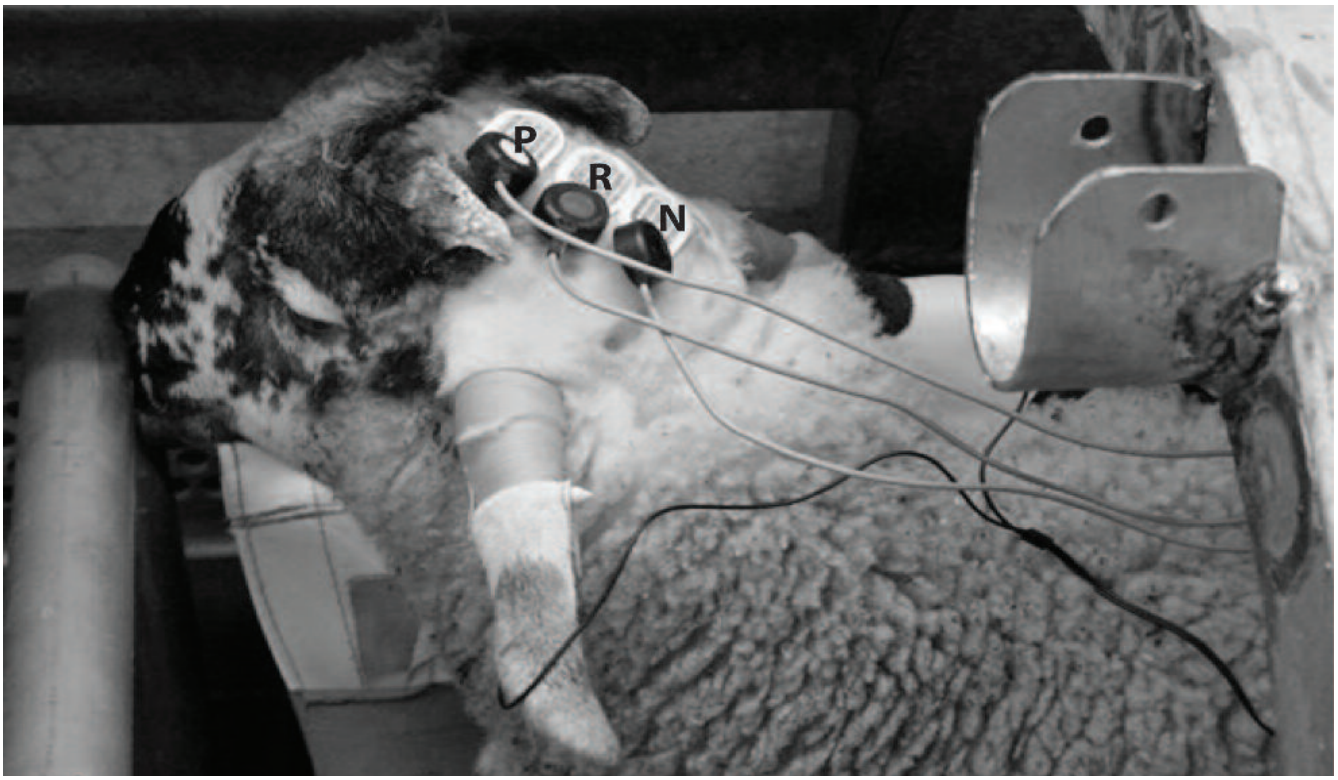
Figure 1



Position of the electrodes: positive (P), negative (N) and reference (R) on the head of a lamb in order to record the MLAEP.

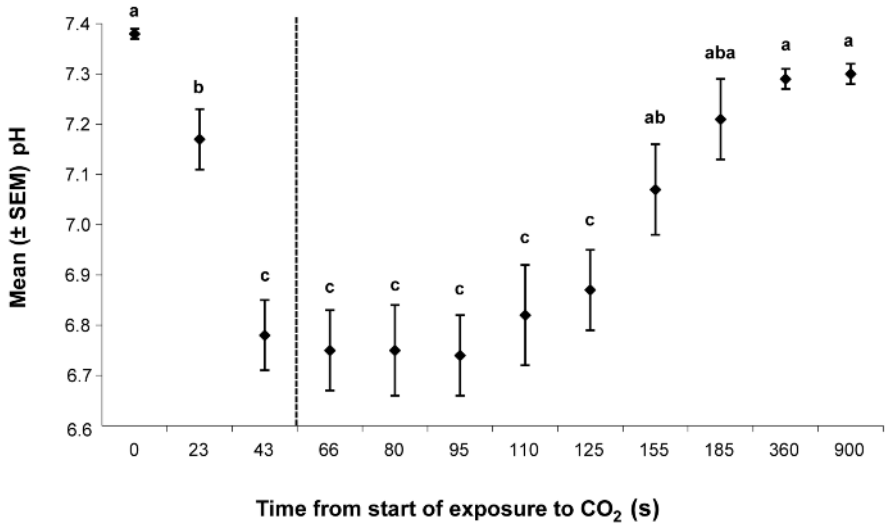
Blood gases

Blood samples were taken with an 18 G × 10 cm long catheter (Vygon®, Valencia, Spain) placed in the carotid artery, through a puncture in the lateral region of the neck, and fixed with suture thread in 12 lambs. Prior to each lamb being immersed in the well, the catheter was connected to an extended catheter (300 × 0.25 cm; length × internal diameter) (Vygon, Spain). In each lamb, a total of 12 blood samples were collected. The basal sample was taken immediately prior to cage descent. During CO_2 exposure, three more samples were taken: i) when the lamb reached the bottom of the well (23 s); ii) when the lamb began its ascent (43 s); and iii) at the end of the exposure (66 s). During recovery, eight more samples were collected at 15, 30, 45, 60, 90, 120, 295 and 835 s after the end of CO_2 exposure. Prior to taking the blood samples, the residual blood of the catheter was removed. Blood samples were preserved in ice in 2-ml syringes with heparin and analysed, 1 h after collection, using gas testing equipment (ECOSYS II-Eschweiler Compact BGA, Holzkoppelweg, Germany) for the determination of pH, pCO_2, pO_2, $SatO_2$ and HCO_{3-} levels.

Middle latency auditory-evoked potential (MLAEP)

The MLAEP was recorded in all 14 lambs through three surface electrodes (Blue sensor wet gel electrodes, AMBU, Denmark) placed at various points on the shaved skull. The positive electrode was placed on the intersection of the frontal and parietal sutures at the midline. The negative electrode was placed on the dorsal part of the occipital bone, and the reference electrode was placed equidistant between the positive and the negative electrodes (Figure 1). Afterwards, the impedance was measured. Values below 3 KOhms (KΩ) were considered adequate for recording. In case the impedance was higher, the skull was shaved again and the electrodes replaced. Lambs were then fitted with headphones that emitted a bilateral 2 ms click train (intensity 70 dB sound pressure limit) with a sampling frequency of 880 Hz and a repetition frequency of 9 Hz. The electrodes were connected to an AEP monitor (ALARIS AEPPTM Danmeter, Odense, Denmark) to record the EEG signal. The MLAEP were extracted from the EEG, between 20 and 80 ms after each auditory stimulus, using an autoregressive model with an exogenous input (ARX) adaptive method (Jensen *et al* 1998). The A-Line ARX Index (AAI) and the burst suppression (BS%) were calculated from the extracted MLAEP wave (Jensen 1999; Litvan *et al* 2002). The AEP monitor displayed a new value on the display every second but the processing time was still based on the ARX model. A moving time average was applied in this version of the AEP monitor which means that the total delay, already included in the calculation, was 6 s. For the data analysis, the basal data recorded during 10 min before the CO_2 exposure were averaged and compared to individual 1-s values during exposure to CO_2 and until 10 min afterwards.

Figure 2



Mean (± SEM) pH values before, during and after exposure to 90% CO_2 for 66 s. Dotted vertical line indicates the end of the CO_2 exposure. Values with differing superscripts differ significantly ($P < 0.05$).

Animal behaviour and physiological reflexes

The behaviour of the lambs before and during the exposure to the CO_2 was recorded in all 14 lambs using two video cameras (Sony Colour CCD AVC 565, Circontrol, Barcelona, Spain) placed in the roof of the cage. The first camera recorded a dorsal view of the animal and the second a cranial view of the face of the animal. Each animal was marked individually on the back. The behavioural parameters scored were:

- Head-shaking and sneezing — lambs showing lateral movement followed by sneezing (Manning & Schwartzstein 1995; Holst 2002);
- Gasping — occurred when lambs raised their head and opened their mouths (Lambooij *et al* 1999);
- Gagging — lambs performed low frequency inhalations and, sometimes, emitted sounds similar to snoring (Raj 1999);
- Vocalisation — shouts or snores emitted by the animal (EFSA 2004); and
- Muscular excitation — repeated muscular movement of the whole body, including head movement upwards (Rodríguez 2008).

All recording times were synchronised with the time at which the lambs began their descent into the well.

During exposure to the gas, increased respiration rate was evaluated from the beginning until its complete disappearance. After CO_2 immersion, the presence of corneal reflex (by palpating the cornea with a pencil), breathing (as indicated by the breathing sound and the movements of the flanks) and sensibility to pain (as indicated by withdrawing its head as response to a nose prick with a hypodermic needle) were monitored every 5 s until all reflexes were recovered.

After the end of the experimental day, the lambs were euthanised by exposure to 90% CO_2 for 7 min and subsequently exsanguinated. At the time of the study, the exposure to high concentration of CO_2 was considered a legal stunning method in sheep, and therefore the decision was taken to use it as the method of euthanasia.

Statistical analysis

The MLAEP data (AAI and BS%) and blood parameters (pH, pCO_2, pO_2, $SatO_2$ and HCO_3) were assessed by means of linear mixed models with repeated measures (PROC MIXED) of the Statistical Analysis System (SAS; SAS Institute Inc, Cary, NC, USA; 1999-2001). In all cases, the variables were submitted to symmetrical composition of covariance structure (CS), 'animal' being the repeated subject. When the variance analysis showed significant differences ($P < 0.05$), the comparisons of the least square mean values (LSMEANS) was adjusted to the Tukey multiple comparison test. The residual maximum likelihood was used as a method of estimation. The LSMEANS of fixed effects were used when analysis of variance indicated differences. Significance was held at $P < 0.05$. In the case of the physiological reflexes (increased respiration rate, corneal reflex, breathing and sensibility to pain) and behavioural data (head-shake with sneezing, gasping and gagging) only a descriptive analysis was carried out.

The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of IRTA.

Results

Blood gas

The mean (± SEM) blood pH prior to CO_2 exposure was 7.38 (± 0.01). During gas inhalation, the blood pH decreased and differed significantly (n = 121; $P = 0.003$; $F_{12, 82} = 3.56$) from the basal pH, 23 s from the start of CO_2 exposure (7.17 [± 0.06]) through to 90 s post-inhalation recovery at 7.07 (± 0.09) (Figure 2). The pH value reached its lowest level 30 s after the end of immersion, with an average value of 6.74 (± 0.08). Average basal pCO_2 was 35.0 (± 1.92) mm Hg. It increased during CO_2 inhalation and differed significantly (n = 121; $P < 0.0001$; $F_{12, 82} = 26.46$) from the basal value 23 s after the onset of

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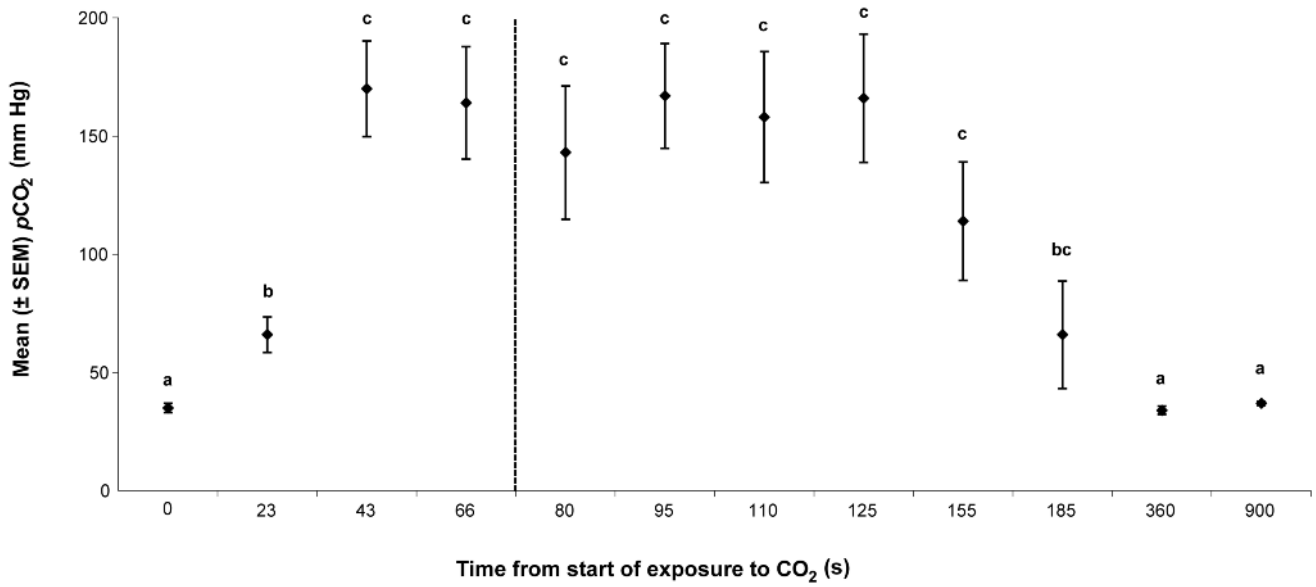
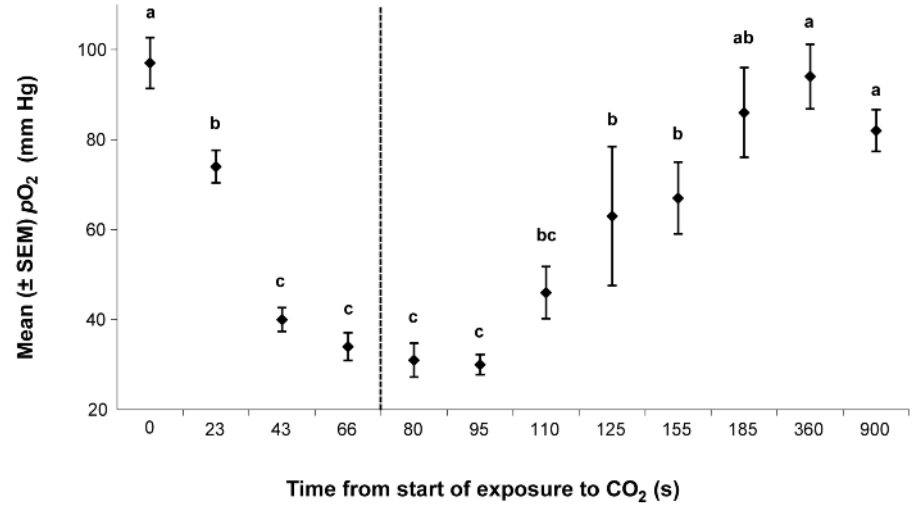
Figure 3



Mean (± SEM) pCO_2 values before, during and after exposure to 90% CO_2 for 66 s. Dotted vertical line indicates the end of the CO_2 exposure. Values with differing superscripts differ significantly ($P < 0.05$).

Figure 4



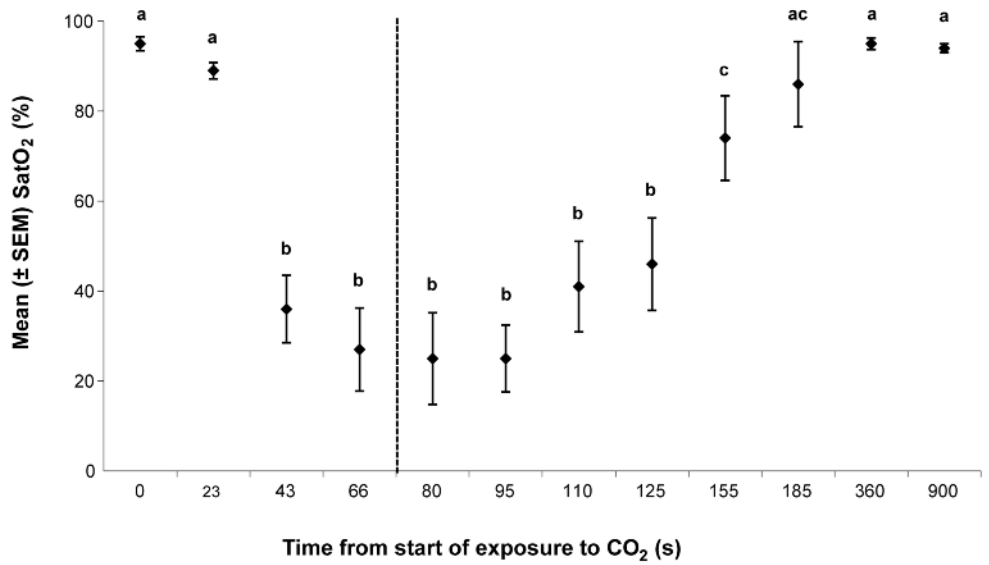
Mean (± SEM) pO_2 values before, during and after exposure to 90% CO_2 for 66 s. Dotted vertical line indicates the end of the CO_2 exposure. Values with differing superscripts differ significantly ($P < 0.05$).

the CO_2 exposure through to after 120 s post-inhalational recovery (Figure 3). The pCO_2 peaked in value, 43 s into gas immersion, at 170.0 (± 20.25) mm Hg. Basal pO_2 and $SatO_2$ values were 97.0 (± 5.66) mm Hg and 95.0 (± 1.54)%, respectively. Both decreased during the immersion and differed significantly (n = 121; $P = 0.0381$; $F_{12, 81} = 1.97$ and n = 121; $P = 0.0003$; $F_{12, 80} = 3.60$, respectively) from basal values from 23 and 43 s after the onset of exposure to CO_2, through until 120 s of post-inhalational recovery (Figures 4 and 5). The pO_2 reached its lowest value (31.1 [± 3.06] mm Hg) 30 s after the end of the CO_2 exposure, and the $SatO_2$ 15 s after the onset of the exposure, with a value of 25.0 (± 10.19)%. Finally, average basal $HCO_{3\_}$ concentration was 19.0 (± 1.37) mmol L^{-1} (Figure 6). It increased during the immersion, being significantly different (n = 121; $P = 0.0030$; $F_{12, 74} = 2.84$) from basal values at 43 s after the onset of the CO_2 exposure through until 120 s after the end of exposure.

MLAEP

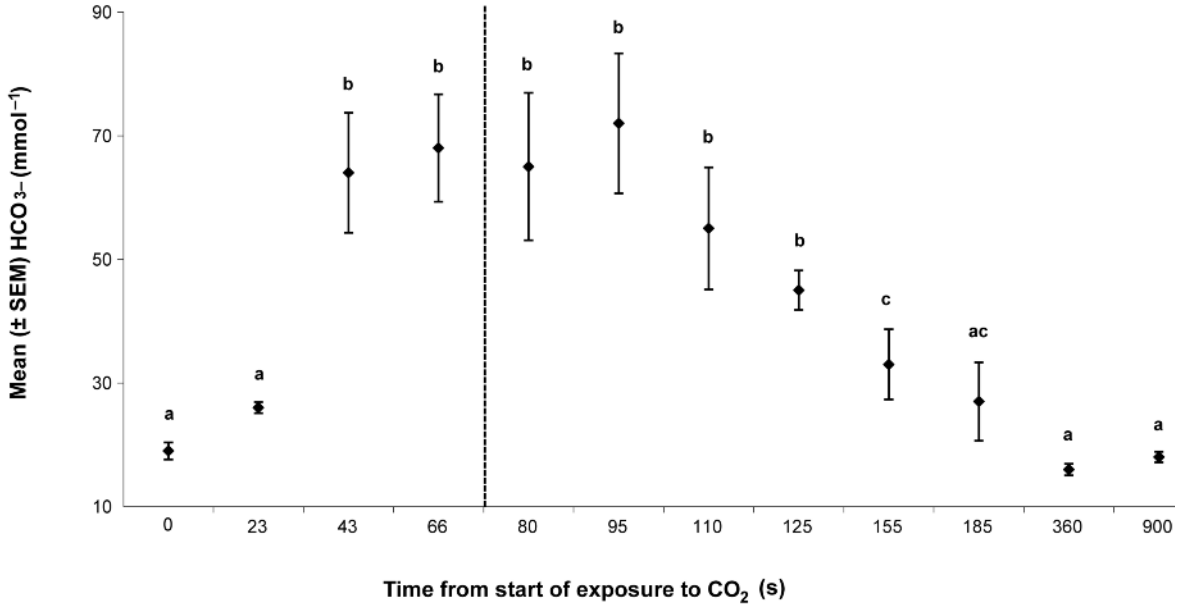
Average AAI and BS% indices during induction to unconsciousness and recovery are shown in Figure 7. The mean basal AAI index was 59.8 (± 0.11). It began to decrease

Figure 5



Mean (± SEM) percentage $SatO_2$ before, during and after exposure to 90% CO_2 for 66 s. Dotted vertical line indicates the end of the CO_2 exposure. Values with differing superscripts differ significantly ($P < 0.05$).

Figure 6



Mean (± SEM) HCO_{3-} values before, during and after exposure to 90% CO_2 for 66 s. Dotted vertical line indicates the end of the CO_2 exposure. Values with differing superscripts differ significantly ($P < 0.05$).

approximately 44 s (58.6 ± 1.78) after the start of induction, and by 48 s the AAI differed significantly (n = 17,969; $P < 0.0001$; $F_{668, 17,000} = 69.27$) from the basal value with a reading of 50.2 (± 1.78). The AAI continued to decrease after the end of the CO_2 cycle, reaching its lowest value 66 s after the end of the exposure, with a value of 15.3 (± 1.96). The AAI value then began to gradually increase but remained significantly different ($P < 0.05$) from basal levels until 159 s after the end of immersion. The AAI decreased below 40 from 52 s after the start of induction through until 124 s after the end of immersion.

The BS% basal value was 0. It began to increase at 27 s (3.1 [± 2.33]) of the CO_2 exposure, and showed significant differences (n = 17,969; $P < 0.0001$; $F_{668, 17,000} = 64.75$) at 46 s (12.8 [± 2.33]). It continued to increase until 116 s after the end of exposure. It reached the highest values 11 s after the end of exposure (64.8 [± 2.40]).

Animal behaviour and physiological reflexes

During exposure to CO_2, 93% of the lambs (13/14) exhibited head-shakes and sneezing, starting on average at 10.6 (± 0.77) s after beginning the exposure. Thereafter, 42% of the animals (6/14) showed gasping (20.6 [±1.36] s).

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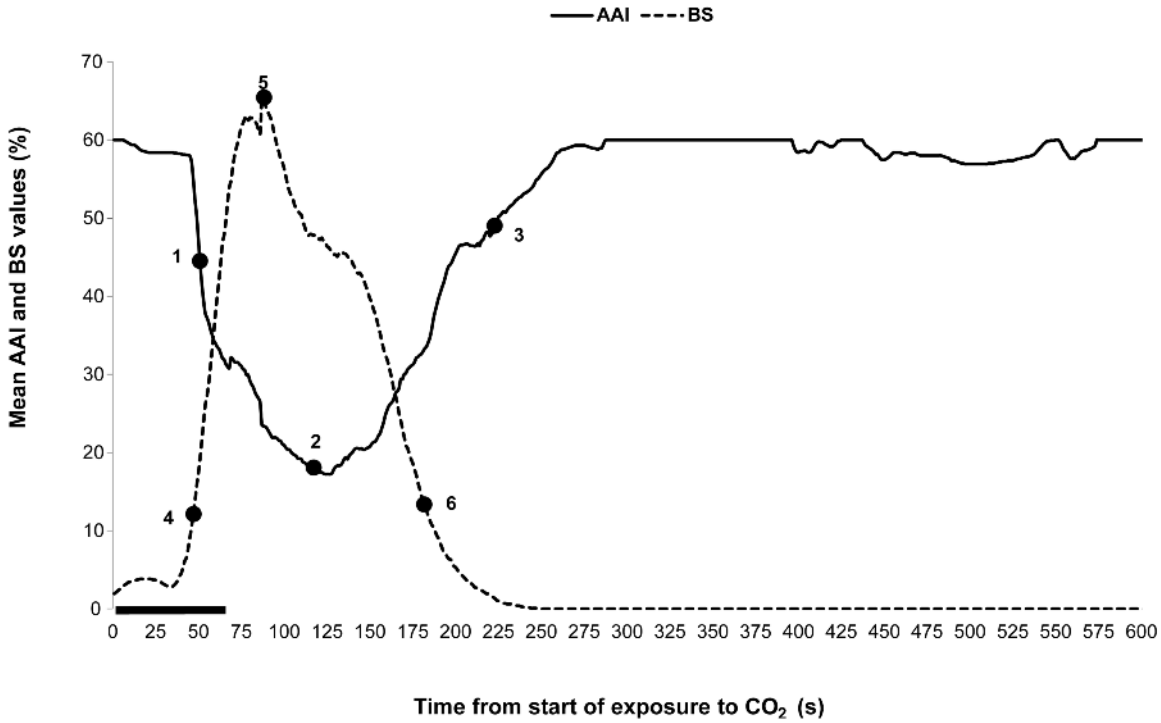
Figure 7



AAI and BS% mean values during exposure and recovery in lambs immersed in 90% CO_2 for 66 s. The times at which the AAI (1) and BS% (4) showed differences with basal levels, the minimum AAI (2) and maximum BS% (5) and the times at which AAI (3) and BS% (6) returned to basal levels are show. Black line indicates CO_2 exposure.

All animals showed increased respiration rate which began, on average, at 22.8 (± 0.93) s and lasted, on average, until 37.3 (± 0.90) s. Gagging was observed in 86% of lambs (12/14), which began, on average, at 60.8 (± 1.59) s. No animals showed vocalisation or muscular excitation during the CO_2 exposure.

At the end of exposure, 36% of animals (5/14) had no corneal reflex. The reflex returned in these animals 31.0 (± 2.38) s later. The outstanding 64% (9/14) of animals that had a positive corneal reflex at the end of immersion lost it 19.0 (± 1.41) s later before regaining it at 50.8 (± 3.80) s after the end of gas immersion. Animals recovered breathing and sensitivity to pain at 69.3 (± 6.04) and 815.8 (± 46.9) s after the end of gas immersion, respectively.

Discussion

The study was carried out before Regulation 1099/2009 (EC 2009) started to be applied. Under the previous Directive 93/119/EC (EC 1993), the exposure to high concentration of CO_2 in sheep was not forbidden, and some slaughterhouses in Spain used this stunning method. This method has some welfare advantages compared with electrical stunning, as it precludes the need for animal restraint, thereby allowing group stunning, which reduces handling stress prior to stunning. In the New Regulation, the prohibition of the gas method in sheep is due to the lack of scientific literature on the welfare consequences of this method in this species. This manuscript aimed to fill this gap and provide scientific evidence on the effect of exposure to high concentrations of CO_2 on the welfare of sheep.

Animals were recovered after exposure to CO_2 to assess the duration of unconsciousness that can be only determined if the animals are not bled and recovery is allowed. According to the EFSA (2013), studies under controlled laboratory conditions should determine the duration of unconsciousness and insensibility using EEG. The loss and recovery of consciousness in lambs exposed for 66 s to 90% CO_2 was assessed by means of behavioural parameters, blood parameters, middle latency auditory-evoked potentials and physiological reflexes. The analysis of blood parameters during the exposure to high CO_2 concentrations has been used to monitor unconsciousness in pigs (Lomholt 1998; Rodríguez *et al* 2008). CO_2 immersion leads to an increase in blood pCO_2 and HCO_{3-} and decrease in pH, pO_2 and SpO_2. When the inhaled CO_2 reaches the blood, part of it is transported within the haemoglobin and the rest is combined with water to form carbonic acid (H_2CO_3; Murillo 1995). This acid dissociates into hydrogen (H^+) and bicarbonate ion (HCO_{3-}), decreasing the blood pH (Capaul 1995). In lambs, our results show that inhalation of 90% CO_2 leads to respiratory acidosis at 23 s, when the blood pH decreases significantly from basal levels. As a consequence, the pH of cerebrospinal fluid (CSF) bathing the brain decreases, inducing gradual loss of consciousness and sensibility through inhibition of spontaneous brain activity (Gregory 1987).

The BS% and AAI values changed during exposure to CO_2 with significant differences from basal levels at 46 and 48 s, respectively. According to these results, when lambs were

exposed to 90% CO_2, the depressive effect of CO_2 was not immediate (Forslid 1992; Raj & Gregory 1995), and the loss of brain functionality did not appear until 46 s of induction. Deep unconsciousness, indicated by an AAI lower than 40, occurred from 52 s after the start of induction. Such a decrease in brain activity occurred after the changes in pH, pCO_2 and pO_2 at 23 s and in SpO_2 and HCO_{3-} at 43 s. These changes in the pCO_2 induced hypercapnic hypoxia and consequently a decline in the CSF pH that provoked depression of brain activity and the loss of consciousness in the animal. In our study, lambs showed deep unconsciousness when blood pH dropped below 6.78. Arterial pH paralleled the pH alteration in CSF, indicating an acute effect of hypercapnia in the brain when the blood pH lowered 6.78 (Martoft *et al* 2003). During the CO_2 exposure, 93% of the lambs exhibited head-shake and sneezing, when brain activity is not depressed and lambs are still conscious, indicating aversion to the carbon dioxide concentration. These results are consistent across a variety of species, such as chickens (*Gallus gallus domesticus*), turkeys (*Meleagris gallopavo*), pigs and mink (*Neovison vison*) (Raj & Gregory 1995, 1996; Raj 1996; Cooper *et al* 1998), which have shown that they perceive carbon dioxide as being aversive. This aversion to CO_2 has been reported to be greater than motivation to feed (in a CO_2 atmosphere) after overnight fasting in pigs, poultry and rats (Raj & Gregory 1995; Raj 1996; Kirkden *et al* 2005). It also agrees with Cantieni (1976) who found that the majority of the pigs tested preferred to go without water for 72 h rather than endure exposure to carbon dioxide again. The aversive effect of 90% CO_2 is due to stimulation of highly sensitised CO_2 nociceptors in the nasal mucosae and lungs (Peppel & Anton 1993), where the presence of CO_2-sensitive chemoreceptors has been described (Manning & Schwartzstein 1995). Additionally, carbon dioxide induces severe respiratory distress causing hyperventilation and a sense of breathlessness during the induction phase prior to loss of consciousness (Gregory *et al* 1990; Danneman *et al* 1997).

Gasping was also exhibited by 42% of the lambs before loss of consciousness (at 21 s), and occurred at the same time that pCO_2 increased and pO_2 and blood pH decreased compared to basal levels. Gasping is a rudimentary respiratory activity occurring through the mouth, and is associated with breathlessness during the inhalation of CO_2 (Raj & Gregory 1996; Llonch *et al* 2012). Afterwards, hypercapnia increased respiration rate (from 22.8 [± 0.93] to 37.3 [± 0.90] respiratory movements per min) at 23 s of CO_2 exposure, provoking respiratory distress (Raj & Gregory 1996). Other reflexes and behaviours described in pigs, such as muscular excitation, convulsions, and vocalisations (Raj & Gregory 1995; EFSA 2004; Velarde *et al* 2007) were not observed in lambs. Lambs do not vocalise in painful or fearful situations (Kiley 1972; EFSA 2004) and their behavioural response to aversive stimuli (such as high concentration of CO_2 exposure) is different to those shown by pigs, where 89% of the animals vocalised (Rodríguez *et al* 2008). These results will confirm that both species react differently to exposure to high concentrations of CO_2, but in both species the reaction is associated with aversion and breathlessness, expressed by means of head-shaking with sneezing, gasping and increased respiration rate.

In this study, lambs were exposed to 90% CO_2. In a study carried out in pigs, Velarde *et al* (2007) concluded that aversion increases when the CO_2 concentration increases from 70 to 90% due possibly to increased irritation of the nasal mucosal membranes and more severe hyperventilation. Conversely, a decrease in the concentration of carbon dioxide increased the time to loss of consciousness and therefore lengthened the perception of the aversive stimulus. If higher concentrations are used for rapid induction of anaesthesia, it needs to be assumed that this may be more aversive.

The current study compared the behaviour of lambs before and during the exposure of the CO_2, but not during the procedure without being exposed to CO_2. The last comparison would allow differentiation between aversion due to the descending movement of the stunning box and handling process and aversion due to CO_2 exposure. However, for the most part, the measured variables assessed are quite specific to the gas exposure.

In the commercial slaughterhouse, gas-stunning systems might have certain animal welfare advantages compared with electrical stunning. Animals are stunned in groups with the minimum amount of restraint and handling stress (EFSA 2004). However, these results showed that exposure to 90% CO_2 does not induce immediate loss of consciousness and lambs experienced aversion during exposure to the gas. Under practical conditions, the absence of corneal reflex, breathing and sensibility to pain is used to assess unconsciousness (Holst 2001; Velarde *et al* 2003; Rodríguez *et al* 2008). After the CO_2 exposure, all lambs showed absence of breathing and sensibility to pain when the AAI and BS% differed significantly from basal levels. Animals recovered breathing (69.3 [± 6.04] s) and sensibility to pain (815.8 [± 46.90]), immediately after the AAI and BS% returned to basal levels.

The corneal reflex has been described as the last one to disappear during induction of unconsciousness with CO_2 and the first to reappear during recovery (Holst 2001). In our study, only 36% did not have a corneal reflex after the exposure to CO_2, the others lost it after the exposure to the CO_2 while the AAI was continuing decreasing and the BS% increasing, confirming the post-induction effect of CO_2 on the corneal reflex in lambs, as stated by Panella *et al* (2008) and Rodríguez *et al* (2008) in pigs. On the other hand, all animals recovered the corneal reflex before the AAI and BS% reached minimum and maximum values, respectively. This casts an element of doubt over the effectiveness of the corneal reflex in assessing the consciousness of animals immersed in high concentrations of CO_2. Other authors, such as Forslid (1987) and Panella *et al* (2008), suggested there was great variability in the presence of the corneal reflex between animals after CO_2 exposure.

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The study reveals that after 66 s of exposure to 90% CO_2, it takes 70 s to recover breathing, and 159 s to return to a normal level of brain activity. However, in order to avoid pain and stress during slaughter, the lamb should be stuck while in deep anaesthesia. In our study, this occurred during the 124 s after the end of immersion, when the AAI and blood pH increased to 40 and 7.2, respectively. This would suggest that when inhalation of CO_2 finished, blood pH recovered normal levels earlier than CSF. In the present study, the AAI index reaches its lowest value of 15.3 at 66 s and, afterwards, began to increase gradually. In this instance we suggest this time of 66 s to be the maximum stun-stick interval to avoid pain and suffering during slaughter.

Animal welfare implications and conclusion

According to the MLAEP, the loss of consciousness when a commercial dip-lift stunning system is used occurs, on average, at 48 s of exposure to 90% CO_2 by volume in atmospheric air. During this time, lambs exhibited head-shake and sneezing, gasping and increased respiration rate. The fact that these behaviours occur when the animal is conscious is evidence that induction of CO_2 anaesthesia is not immediate and lambs may suffer from fear, pain and/or stress during immersion into gas. The presence of these behaviours clearly indicates aversion to exposure to an atmosphere with a high concentration of CO_2. This aversion might be similar to that experienced by other species, such as pigs, where high concentration of CO_2 is commercially used. At commercial slaughterhouses, the efficiency of the stunning should be routinely monitored to prevent animals recovering consciousness before death, as this will cause pain and suffering (EFSA 2004). According to blood parameters and MLAEP, the onset of breathing is the most reliable physiological reflex to be used in practice for assessing recovery of consciousness after CO_2 stunning in lambs.

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