final report

Project code: B.AWW.0257

Prepared by: Kelly Stanger and Andrew Fisher, University of Melbourne
Nikki Kells and Craig Johnson, Massey University
Tristan Jubb, Livestock Services, Bendigo

Date published: 21/08/2018

PUBLISHED BY
Meat and Livestock Australia Limited
Locked Bag 1961
NORTH SYDNEY NSW 2059

Evaluation of different intravenous methods of euthanasia for ruminant livestock to enable the collection of brain samples for disease surveillance.

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Executive summary

Transmissible spongiform encephalopathies (TSE) are rare, but fatal diseases associated with degeneration of the brain. These diseases are commonly referred to as 'mad cow disease' or 'scrapie' in cattle and sheep, respectively. Australia is free of TSE and the risk of introduction is deemed negligible, but active, on-farm surveillance is required to demonstrate this 'disease-free' status and ensure continued access to international markets.

TSE surveillance requires post-mortem, microscopic examination of brain material collected from animals exhibiting signs of neurological disease. Euthanasia by captive bolt or firearm can render the brain unsuitable for collection of samples and so injectable barbiturates such as pentobarbitone are commonly used in this situation. Barbiturate residues remain in the carcass for many years and can cause the death of carnivorous and scavenging species that consume the carcass, all of which makes carcass disposal problematic. Euthanasia of heavily sedated animals using saturated KCl (SS-KCl) or saturated $MgSO_4$ (SS-$MgSO_4$) has been proposed as a low risk alternative, but scientific studies to evaluate whether these methods are humane have been lacking.

This study evaluated the physiological, behavioural and nociceptive responses of heavily sedated sheep, that were euthanased with either pentobarbitone (n=10), SS-KCl (n=11) or SS- $MgSO_4$ (n=10). The potential for conscious perception of pain was evaluated by measuring cortical brain activity using electroencephalography (EEG). Physiological variables including the time until end of rhythmic breathing and cardiac arrest, as well as behavioural responses were also recorded (kicking and paddling).

In this study, there was no evidence of nociception associated with the euthanasia with pentobarbitone, SS-KCl or SS-$MgSO_4$. Based on this, all methods were deemed humane, however, the use of SS-$MgSO_4$ rather than SS-KCl is recommended.

Although no conscious perception of pain was evident for any group, animals euthanased with SS-KCl consistently displayed severe, behavioural response to this agent. These behaviours were observed after the animal was unconscious and included sustained kicking, paddling and tetanic convulsions which are visually unappealing and pose a threat to the safety of personnel involved in the process.

A smaller volume of SS-$MgSO_4$ (1-2ml/kg) was required to achieve brain death (permanent isoelectric EEG waveform) compared with SS-KCl (1-7ml/kg). The volumes required are expected to be larger in a field setting because EEG analysis is not available and infusion should continue until clinical death has been confirmed (i.e. loss of reflex activity).

The use of SS-$MgSO_4$ instead of pentobarbitone decreases the risk of secondary poisoning of carnivorous animals. Saturated salts are extremely unlikely to cause any adverse reactions or death of animals consuming contaminated carcasses. Similarly, the risk of secondary poisoning associated with xylazine is also very low, because experimental models show very high tolerance for ingestion of xylazine.

The recommendations for field veterinarians are as follows. SS-$MgSO_4$ is a safe, rapid, humane and practical method of euthanasing livestock that enables preservation of brain material. This method could also be used where a fire arm or captive bolt is not available. The SS-$MgSO_4$ must only be administered intravenously to an animal that is heavily sedated with xylazine. The infusion should continue until death has been confirmed.

Table of contents

1 Background

Transmissible spongiform encephalopathies (TSE) are caused by an abnormal accumulation of proteins (prions) in the brain that causes damage to the central nervous system (Radostits et al., 2007). These diseases are more commonly referred to as 'mad cow disease' or 'scrapie' in cattle and sheep or goats, respectively. Animals typically develop this disease following the consumption of animal protein feeds ('swill feeding') that contain this prion protein, but this practice has since been banned. Affected animals develop neurological signs that may include behavioural changes, incoordination, excitability or progressive weakness (Radostits et al., 2007).

Australia is free of TSE and the risk of introduction is deemed negligible, but maintenance of this 'disease free' status requires active, on-farm surveillance. A national TSE surveillance project has been developed to comply with the international standards set by the World Organisation for Animal Health (Anon, 2017). There are currently no tests to diagnose TSE in the live animal and so the program requires post-mortem examination of the brains from a number of sheep and cattle each year that have shown signs of neurological disease.

The diagnosis or exclusion of TSE as a cause of neurological disease requires microscopic examination of the intact brain stem or spinal cord. Commonly, firearms or captive bolts are used to euthanase livestock on farm, but these methods are unsuitable for TSE surveillance because they cause extensive damage to the brain. Consequently, veterinarians typically use an injectable barbiturate anaesthetic (e.g. pentobarbitone) in this situation, but this poses a threat to companion and scavenging animals due to the potentially lethal residues which remain in the carcass. Secondary poisoning through the ingestion of such carcasses have caused the death of carnivorous animals, including farm dogs and wildlife species, up to 2 years after an animal had been euthanased with a barbiturate (Payne et al., 2015, Kaiser et al., 2010). Therefore, barbiturates should only be used when a carcass can be disposed of immediately, commonly by deep burial, which potentially limits the number of livestock producers that can be involved in surveillance efforts.

An alternative, cheap and safe, but relatively unresearched method of euthanasia for farm animals is the administration of saturated salt solutions such as magnesium sulphate (SS-$MgSO_4$) or potassium chloride (SS-KCl). These agents, particularly SS-$MgSO_4$ (Epsom salts) in solution, were one of the first anaesthetics and euthanasia agents described for use in large animals (Riebold et al., 1982), and do not pose the same residue risks as the barbiturate agents. These agents have been used in the field by large animal veterinarians to euthanase animals that have been heavily sedated with an alpha-2 agonist, such as xylazine (Jubb, T. Pers Comm). Field observations indicate that the transition to death using SS-$MgSO_4$ is smooth, occurs rapidly with few or no tetanic spasms, and no other overt signs to indicate that the animal perceives pain or is distressed. Fewer field observations have been reported for SS-KCl. Transition to death is rapid, but tetanic spasms and involuntary muscular activity are often reported.

Despite the potential benefits of using saturated salt solutions in heavily sedated animals, the method has not been widely utilised or promoted in Australia because the American Veterinary Medical Association (AVMA) does not advocate the procedure (Leary et al., 2013). An expert panel representing the AVMA produce guidelines for the euthanasia of animals, which serves as a reference for euthanasia drug use guidelines in Australia. For a euthanasia agent to be advocated for use by the

AVMA, it must cause smooth loss of consciousness of an animal prior to cardiac and respiratory arrest. The animal must experience minimal pain and distress and the agent must be delivered in a way that ensures the safety of the personnel involved.

The current euthanasia guidelines recommend that SS-$MgSO_4$ and SS-KCl are only used to euthanase unconscious livestock. The animal should be in a surgical plane of anaesthesia, which is characterised by loss of consciousness, loss of muscle reflex response and loss of response to noxious stimuli (Leary et al., 2013). Currently, deep sedation using an alpha-2 agonist (e.g. xylazine) followed by the administration of either SS-KCl or SS-$MgSO_4$ is not a recognised method of humane euthanasia, because the alpha-2 agonist does not induce general anaesthesia (Shearer, 2014, Dewell et al., 2014). Although xylazine causes heavy sedation, provides some analgesia (pain relief), muscle relaxation and often induces a state that resembles general anaesthesia, it does not render an- animal completely unconscious (Evers et al., 2006).

To date, there are no published studies that have directly evaluated the perception of pain or distress associated with euthanasia using SS-$MgSO_4$ or SS-KCl in livestock that were heavily sedated with xylazine. The observational evidence reported by field veterinarians in Australia strongly suggests that euthanasia with SS-$MgSO_4$ following heavy sedation with xylazine results in a rapid and smooth death. However, the absence of observed adverse behaviours does not necessary equate to the absence of perceived distress by an animal. Therefore, a scientifically rigorous, objective evaluation of the potential for distress associated with these methods was needed to determine whether they are humane.

Recently, the measurement of brain activity by electroencephalography (EEG) has been used to evaluate the perception of pain in animals, particularly in relation to killing methods for meat supply (Murrell and Johnson, 2006, Gibson et al., 2009a). The validity of EEG to evaluate conscious perception of pain is well established for both humans and animals (Chang et al., 2001, Chen et al., 1989, Gibson et al., 2009a, Murrell and Johnson, 2006, Rault et al., 2014). This method of analysis measures nociception by the cerebral cortex, with painful stimuli causing a change in the components of frequency including F50, F95 and Ptot. The Ptot is the total area under the power spectrum curve and the F50 and F95 is the frequency below which 50% and 95% of the power of EEG is located, respectively. These parameters are indirect measures of pain, but F50 and Ptot in particular are strongly correlated with noxious stimulation (Murrell, Johnson 2005, Gibson). Whilst F95 has been also been correlated with nociception in some studies, the response is variable. Changes in F95 are reliably associated with depth of anaesthesia. In the context of this study, F95 was included as an index of sedation following xylazine administration.

The aim of this project was to use EEG analysis in conjunction with behavioural and physiological assessment of heavily sedated animals that are euthanased with saturated salt solutions, to provide scientific evidence of the capacity of these methods to cause a humane death.

2 Project objectives

The objectives of this study were to;

- To identify a practical and humane method for the euthanasia of livestock for TSE surveillance that maintains the integrity of the brain.

- To identify a practical method of euthanasia that helps to minimises the risk of secondary poisoning of carnivorous animals following the ingestion of euthanased livestock.
- To evaluate the behavioural responses and brain activity (EEG) of animals euthanased with either pentobarbitone (gold standard), SS-KCl or SS-$MgSO_4$ under heavy sedation with xylazine to evaluate the humanness of each method
- To generate objective, scientific data to support the use of the most appropriate method of euthanasia that facilitates the collection of intact brain samples for TSE surveillance

3 Methodology

3.1 Preparation of saturated salt solutions

At 30°C, the saturation point for KCl and $MgSO_4$ is 37.2 g and 39.7 g respectively (Rumble, 2017). A stock solution of each saturated solution was made prior to the start of the trial, based on the approximate solubility when water temperature is 30°C. When the temperature of a saturated salt solution decreases, excess salts precipitated out of fluid and settled at the bottom of the solution. Only the fluid above the precipitate layer (supernatant) was used for intravenous (IV) infusion.

The stock solutions were prepared as follows;

- SS-KCl - 2kg of 97% Potassium Chloride (KCl) was added to 5L of water
- SS-$MgSO_4$ – 4kg of Epsom salts ($MgSO_4$.7H2O) was added to 5L of water

At the beginning of each trial day, these stock solutions were vigorously shaken to resuspend the salt and achieve saturation at the relative daily temperature of the fluid. The solutions were then left to settle for 20 minutes and only the supernatant decanted for intravenous administration.

3.2 Animals and housing

Thirty-two, mixed age cull ewes were sourced from Cutty's Livestock Services in Dannevirke and were treated with an anthelmintic drench prior to transportation. Animals were in good health at the time of purchase and were in moderate to poor body condition (Score 2.5 ± 0.3 out of 5)(Anon, 2011). Three days prior to the start of the trial, animals were transported approximately 50km to Massey University at Palmerston North, New Zealand. Animals were housed outdoors in paddocks with a pasture base of brown top (*Agrostis* species) and clover pasture (2300 kg DM/ha) and had *ad lib* access to water.

Ewes were allocated one of the three treatment groups (section) using a random number generator and processed over five trial days. At the start of each trial day, the test group of ewes was moved to indoor pens that adjoined the study facility (3x3m)

Immediately prior to euthanasia, a single animal was moved from the indoor pen and a clinical examination was conducted. Clinical assessment included auscultation of the heart and lungs, a record of body temperature, live weight (±0.5 kg, Tru-Test) and estimation of body condition score (Anon, 2011).

The animal was then restrained and wool was clipped from the neck and sections of the head to facilitate intravenous catheter and electrode placement. At this time, brain activity as measured by electroencephalography (EEG) was recorded for 1-2 minutes (base-line EEG, section 3.5.2).

A high dose of xylazine (0.4mg/kg) was then administered into the muscle of the neck to induce heavy sedation. Following this, the ewe was moved into a smaller pen (1x3m) for approximately 10 minutes or until it displayed behaviour consistent with heavy sedation. An animal was deemed heavily sedated when its general activity level was reduced (stationary, head directed down), it became voluntarily recumbent or would become recumbent when light pressure was applied to the hip. Once a state of heavy sedation had been achieved, the animal was transferred into the study area and placed in lateral recumbency (on its side) on an elevated, material stretcher.

At this time and to minimise discomfort, 0.5ml of local anaesthetic (2% lignocaine hydrochloride) was administered subcutaneously (SC) at the site of catheterization. An 18-gauge catheter was then placed into the jugular vein and secured in place with suture material.

Electrodes were fitted and brain activity of the heavily sedated animal was recorded for 1-2 minutes (post-sedation EEG, section). The animal was then euthanased according to its treatment allocation.

3.3 Electroencephalogram (EEG) recording

Subcutaneous 27-gauge 0.5-inch stainless steel needle electrodes (Ambu; Ballerup, Denmark) were used to record the electroencephalogram bilaterally. A five-electrode montage was used with inverting electrodes positioned parallel to the midline over the left and right zygomatic processes, non-inverting electrodes positioned over the left and right mastoid processes and a common earth electrode located caudal to the poll on the left side of the neck (Mayhew and Washbourne, 1990).

3.4 Treatments

Ewes were allocated to one of the following treatment groups using a random number generator;

1. **Pento:** Xylazine (0.4mg/kg IM) sedation followed by euthanasia by intravenous barbiturate overdose (Pentobarbitone, 150mg/kg IV)
2. **SS-KCl:** Xylazine (0.4mg/kg IM) sedation followed by euthanasia by intravenous administration of SS-KCl solution (administered IV to effect).
3. **SS-$MgSO_4$:** Xylazine (0.4mg/kg IM) sedation followed by euthanasia by intravenous administration of SS-$MgSO_4$ solution (administered IV to effect).

3.4.1 Euthanasia by pentobarbitone (Pento)

The results obtained from this treatment served as 'control' group for comparative assessment of SS-KCl and SS-$MgSO_4$ treatments.

The lethal dose of pentobarbitone was calculated at 150mg/kg as per the manufacturer's recommendations (Pentobarb 500, Provet). The calculated volume was administered directly from a syringe into the indwelling jugular catheter over a 60-second period.

3.4.2 Euthanasia by saturated salt solution (SS-KCl or SS-$MgSO_4$)

Approximately 1000 ml of the relevant saturated salt solution was transferred into an empty IV fluid bag which was attached to a 2.2-m IV giving set. The IV fluid bag was then positioned approximately 850 mm above the catheter site to facilitate fluid flow by gravity. After post-sedation baseline brain activity (EEG) had been recorded (section), the saturated salt solution was administered via the indwelling jugular catheter.

The weight of the fluid bag was recorded prior to infusion and after euthanasia to determine the amount of saturated salt that was administered for euthanasia. The time from start until end of infusion was also recorded.

The rate of fluid infusion was then calculated for each animal (total volume administered (ml)/total infusion time (min)). The total volume required to cause death was calculated retrospectively using the rate of infusion and the time until permanent isoelectric EEG waveform was observed.

3.5 Measurements

3.5.1 Behavioural and physiological measurements

A video camera was used to continuously record the behaviour of each sheep immediately prior to and during the IV infusion of the euthanasia agent. The time from the start of infusion to observable behavioural responses were recorded verbally and then matched retrospectively to the EEG trace. The presence of rhythmic breathing and involuntary muscular activity were assessed visually, and audible heart sounds were assessed by auscultation.

Upon completion of the study, all video recordings were reviewed and behavioural responses associated with the movement artefacts on the raw EEG traces were classified using a behavioural ethogram (Table 1a and 1b).

Table 1a: Classification and definition of adverse behavioural responses observed in sheep being euthanased with either pentobarbitone, saturated potassium chloride or saturated magnesium sulphate.

Behaviour	Definition
Muscle twitch	Fasciculations of single muscles or muscle groups on the limbs or body
Head extension	Extension of the head and neck from the normal sedated position
Spinal flexion	Flexion of the spine from the normal sedated position
Muscle contraction	Obvious contraction of single muscles or muscle groups on the limbs or body
Kicking	Kicking movements made by one limb at a time
Paddling	Repeated kicking movements by multiple limbs at the same time
Tetanic spasms/convulsions	Spasms or convulsions of the entire body, involving multiple limbs and the back/neck

Table 1b: Classification and definition of the duration of adverse behavioural responses observed in sheep being euthanased with either pentobarbitone, saturated potassium chloride or saturated magnesium sulphate.

Duration classification	Definition
Brief	<2 seconds, with a gap of >2 seconds before any further behaviour of the same type
Intermediate	2-5 seconds, with a gap of >2 seconds before any further behaviour of the same type
Prolonged	>5 seconds, with a gap of >2 seconds before any further behaviour of the same type

For the purposes of sufficient data for meaningful analysis, behavioural and duration data were further classified as a mild, moderate or severe adverse response (Table 2).

Table 2: The classification of visual behaviours that occurred in response to the intravenous administration of either pentobarbitone or a saturated salt solution (SS-KCl or SS-$MgSO_4$) to sheep, heavily sedated with xylazine (0.4mg/kg IM).

Classification	Duration	Behaviour
Mild	Brief	Muscle twitch Muscle contraction Head extension Spinal flexion
Moderate	Intermediate	Mild responses lasting 2-5 sec Kicking or paddling <5s duration
Severe	Prolonged	Moderate responses with duration >5s Tetanic spasms/convulsions

3.5.2 Electroencephalography

The EEG signals were amplified using isolated differential signal amplifiers (Iso-Dam isolated biological amplifier, World Precision Instruments, Sarasota FL, USA) and recorded with a gain of 1000 and band-pass of 1.0–500 Hz. The EEG signal was digitised at a rate of 1 kHz (Powerlab 16/30, ADInstruments Ltd, Sydney, Australia) and analysed off-line at the conclusion of the experiment.

EEG activity was recorded on three occasions;

i. *Base-line EEG:* Recorded prior to the administration of any medication.
ii. *Post-sedation EEG:* Recorded after xylazine was administered to effect.
iii. *Infusion EEG:* Recorded from the start of infusion until permanent isoelectric waveform could be confirmed in real time.

3.5.3 Clinical death

An animal was pronounced 'clinically dead' once rhythmic breathing had stopped, audible heart sounds were absent, there were no recognisable EEG waveforms, and jaw tone, tongue tone and corneal reflex were absent. The infusion of the saturated salt solution continued until respiration and heart contraction was absent and permanent isoelectric EEG waveform activity was evident.

3.6 Statistical analysis

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

In the event that right cortex data were unsuitable for analysis (i.e. electrode displacement, extensive artefact in a single channel), data from both the left and right hemispheres were recorded. However, only data from the right cerebral cortex were analysed because previous studies using a similar model have demonstrated equivalence in spectral EEG between hemispheres (Murrell and Johnson, 2006).

Raw EEG data were inspected manually and any epochs (time periods) containing movement artefacts, or that were overscale, underscale or out of range, were excluded from further analysis. The EEG recorded immediately following the start of infusion with the euthanasia solution (*infusion EEG*) was compared to baseline *post sedation EEG*. This data was subsequently classified and interpreted using methods described previously by Gibson et al (2009a), Sutherland et al (2016) and Newhook & Blackmore (1982).

Five different categories were identified;

i) **Normal** – amplitude and frequency similar to that of baseline
ii) **Transitiona**l – amplitude less than 50% of baseline EEG with marked change in frequency component
iii) **Low frequency high amplitude (LFHA)** – increased low frequency activity with a concurrent increase in amplitude
iv) **Burst suppression** – active or transitional EEG interspersed with periods of isoelectric EEG lasting for 0.5 seconds or more
v) **Isoelectric** – a stable trace with amplitude less than 12.5% of baseline, consisting of background noise with little or no low-frequency component.

The total power (Ptot), median frequency (F50) and 95% spectral edge frequency (F95) were calculated for consecutive 1-second epochs, using purpose-written software (Spectral Analyser, CB Johnson, Massey University, Palmerston North, NZ, 2002). Fast Fourier Transformation was applied to each epoch, yielding sequential power spectra with 1 Hz frequency bins.

3.6.1 Effect of xylazine on EEG activity

To determine the effect of xylazine on the *base-line EEG*, the mean F50, F95 and Ptot were calculated over 30–60 consecutive seconds of artefact-free EEG *base-line EEG* and *post-sedation EEG*. The exact length of the EEG segments varied between individuals depending on the degree of general movement artefact (non-sedated sheep) or respiratory movement artefact (sedated sheep) in the EEG. Paired T-tests were used to compare means before and after xylazine sedation. The distribution of differences in F50, F95 and Ptot were all found to follow a normal distribution, thereby justifying the use of parametric tests.

3.6.2 Comparison of real-time, clinical endpoints

The effect of treatment on the latency to cessation of rhythmic breathing and detectable heart-beat, along with the appearance of transitional and isoelectric EEG waveforms, was evaluated using generalised linear models that included treatment and day as fixed effects and sheep as a random

effect. Where a significant treatment effect was found, Tukey-Kramer post-hoc tests were performed to identify group differences.

3.6.3 Nociception associated with euthanasia

To test for evidence of nociception following administration of euthanasia agents, the mean F50, F95 and Ptot were calculated and compared for three consecutive non-overlapping 15-second periods *(infusion EEG)*: immediately preceding the start of infusion (P1), from 1–15 seconds after start of infusion (P2) and from 16–30 seconds after start of infusion (P3). Beyond this period EEG was not suitable for spectral analysis, due to the appearance of transitional or isoelectric waveforms in some individuals. A mixed model was used to compare mean F50, F95 and Ptot between periods. The model used a first-order autoregressive correlation structure and included treatment and day as fixed effects, sheep as a random effect and period as a repeated measure. Where significant interaction effects were identified, post-hoc pairwise comparisons were performed on the variables of interest (periods within treatment) and resultant p-values manually corrected for multiple comparisons.

Plots of standardised residuals versus predicted values were evaluated to test the assumption of normally distributed within-group errors. The distribution of residuals for all EEG variables were found to approximate a normal distribution and were therefore considered suitable for parametric analysis.

3.7 Animal ethics

All procures were conducted in accordance with the Massey University, Animal Ethics Committee application MUAE 1811.

4 Results

A total of 31 animals were enrolled in this trial (10 Pento; 11 SS-KCl; 10 SS-MgSO4). The average live weight of animals in the Pento, SS-KCl and SS-$MgSO_4$ groups were 45.8 ± 4.4 kg, 48.7 ± 6.0 kg and 47.7 ± 7.3kg, respectively. The average body condition score for animals in the Pento, SS-KCl and SS-$MgSO_4$ groups were 2.5 ± 0.3, 2.5 ± 0.3 and 2.6 ± 0.3, respectively. There was no significant difference in live weight or body condition score of animals between treatment groups.

4.1 Effect of xylazine on EEG activity

Data from 26 sheep were included in the analysis, with five sheep excluded due to excessive amounts of artefact in the EEG. There was a significant reduction in F50 and F95 between the *base-line EEG* and *post-sedation EEG* waveforms, but no significant change in Ptot (Table 3).

Table 3 Comparison of the median frequency (F50), 95% spectral edge frequency (F95) and total power (Ptot) of the sheep EEG before and after administration of 0.4 mg/kg xylazine to effect

	Mean difference	95% CI Mean	Std. Err.	DF	t-value	Pr>|t|
F50	4.83	3.17–6.50	0.81	25	5.98	<0.0001
F95	3.17	2.29–4.04	0.43	25	7.43	<0.0001
Ptot	-0.61	-4.05–2.83	1.67	25	-0.36	0.7190

4.2 Comparison of real-time, clinical endpoints

The required volume of saturated salt solution to reach the onset of permanent isoelectric EEG waveform was 150ml (range: 39-358ml) and 50ml (range: 38-68ml) for SS-KCl and SS-$MgSO_4$, respectively. This equated to approximately 1-7ml/kg and 1-2ml/kg live weight for SS-KCl and SS-$MgSO_4$, respectively.

Data were available from 30/31, 27/31, 29/31 and 29/31 sheep for time to end of rhythmic breathing, end of heart beat, onset of transitional EEG and onset of isoelectric EEG, respectively. Results of statistical comparison of endpoints between treatments are shown in Table 4.

Table 4: Comparison of times to end of rhythmic breathing (ERB), end of detectable heart beat (EHB), onset of transitional EEG (Trans) and onset of permanent isoelectric EEG (Iso) in sheep euthanased with either sodium pentobarbitone (Pento), potassium chloride (SS-KCl) or magnesium sulphate (SS-$MgSO_4$)

Variable	Treatment	n	Ave time sec (SE)	DF	F value	Pr > F
ERB	Pento	9	32 (1.5)	2	43.73	<0.0001[a, b]
	SS-KCl	11	16 (1.3)			
	SS-$MgSO_4$	10	16 (1.4)			
EHB	Pento	8	138 (7.9)	2	56.75	<0.0001[a,b,c]
	SS-KCl	9	24 (7.4)			
	SS-$MgSO_4$	10	60 (7.1)			
Trans	Pento	8	29 (4.6)	2	2.69	0.087
	SS-KCl	11	53 (8.9)			
	SS-$MgSO_4$	10	43 (5.5)			
Iso	Pento	9	41 (13.3)	2	26.22	<0.0001[a,c]
	SS-KCl	10	165 (12.6)			
	SS-$MgSO_4$	10	64 (12.6)			

[a]p<0.05 for least square means treatment effect between Pento and SS-KCl; [b]p<0.05 for least square means treatment effect between Pento and SS-MgSO4, [c]p<0.05 for least square means treatment effect between SS-KCl and SS-MgSO4

Rhythmic breathing and audible heart sounds were lost significantly more quickly for animals euthanased with either SS-KCl or SS-$MgSO_4$, compared with those euthanased with Pento. These results were comparable between the SS-KCl and SS-$MgSO_4$ groups.

There was no effect of treatment on the time taken to the onset of transitional EEG waveform, with all animals reaching this point within 109 seconds from the start of infusion (range: 10 to 109 sec).

The onset of a permanent isoelectric EEG waveform occurred most quickly in the pentobarbitone group, followed by the SS-$MgSO_4$ group. Animals in the SS-KCl group took significantly more time to reach this point than those in the Pento ($p<0.001$) and SS-$MgSO_4$ groups ($p<0.001$). The pentobarbital and $MgSO_4$ groups did not differ ($P=0.430$).

4.3 Nociception associated with euthanasia

4.3.1 Characterisation of raw EEG following start of infusion

The results of EEG classification for all individuals, grouped by treatment, are illustrated in Fig. 1. Where movement artefact occurred (yellow bars), it was not possible to classify the EEG. Periods of normal EEG indicate compatibility with conscious awareness (grey bars). Transitional EEG represents a state that is probably not compatible with awareness (blue bars), whereas the occurrence of burst suppression (red bars), low-frequency-high-amplitude (green bars), or isoelectric EEG (black bars) are considered incompatible with conscious awareness (Gibson et al., 2009b, Newhook and Blackmore, 1982, Sutherland et al., 2016).

A total of 62 movement artefacts were identified on the EEG traces (yellow bars, Fig. 1). Of these, 57 were associated with an adverse behavioural response as identified by review of video recordings (Table 5; 1 Pento; 48 SS-KCl; 8 SS-$MgSO_4$). The other five were movement artefact associated with electrode placement.

Table 5: A summary of the number of adverse behavioural responses in sheep euthanased with either sodium pentobarbital (Pento), potassium chloride (SS-KCl) or magnesium sulphate (SS-$MgSO_4$).

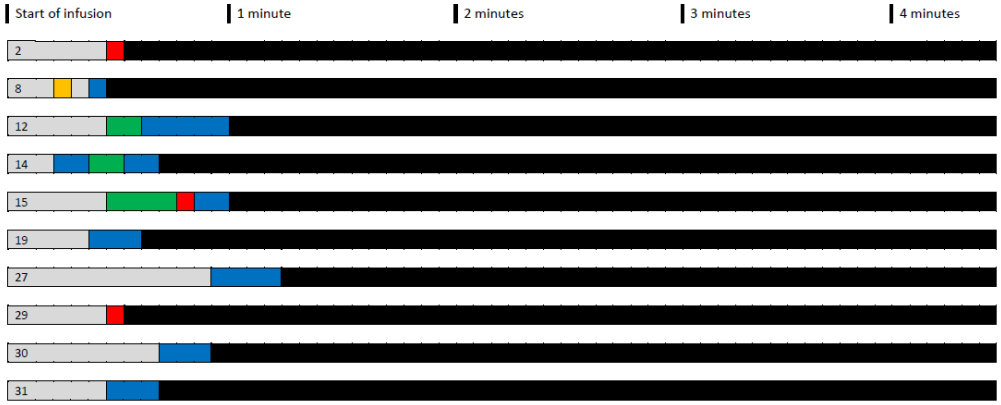
Treatment	No. of adverse behavioural responses (%)		
	Mild	*Moderate*	*Severe*
Pento	1 (100)	0 (0)	0 (0)
SS-KCl	23 (48)	12 (25)	13 (27)
SS-$MgSO_4$	6 (75)	2 (25)	0 (0)

[a]Mild response: <2sec duration, behaviours included muscle twitch, muscle contraction, head extension, spinal flexion; [b]Moderate response: 2-5sec duration, include mild responses of longer duration, or kicking or paddling for <5s; [c]Severe response: >5sec duration, included sustained moderate responses and tetanic spasms/convulsions.

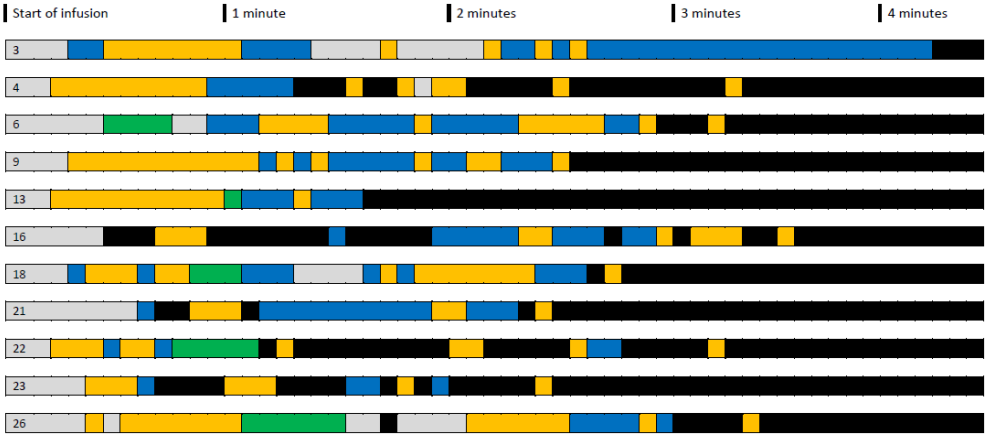
Animals in the Pento group exhibited the least number of adverse responses to euthanasia (n=1), followed by SS-$MgSO_4$ (n=8) and then SS-KCl (n=48). Animals in the SS-KCl demonstrated the most number of adverse behaviours per animal (4.3±1.4).

Nine of the 11 animals in the SS-KCl groups exhibited severe behavioural responses to euthanasia which included sustained kicking, paddling, spinal flexion and/or tetanic convulsions. No severe behavioural responses were noted in the SS-$MgSO_4$ or Pento groups. There were few behavioural responses noted for the SS-$MgSO_4$ group and a majority of these were classified as mild (Table 5).

a) Pentobarbitone



b) SS-KCl



c) SS-$MgSO_4$

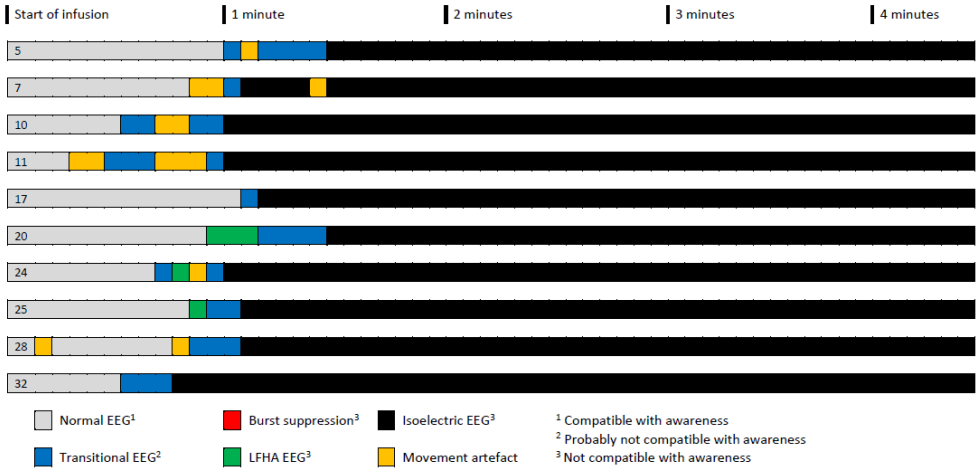


Fig. 1. Electroencephalography (EEG) activity of sheep that were euthanasia via intravenous infusion of either pentobarbitone (a), saturated KCl (b) or saturated $MgSO_4$ (c) following heavy sedation with xylazine (0.4mg/kg IM).

4.3.2 EEG response to infusion of euthanasia agents

Data from 27 sheep were included in the analysis (n=11 KCl, n=9 MgSO4, n=7 Pento). Data from four sheep (n=1 $MgSO_4$, n=3 Pento) were excluded due to the presence of extensive respiratory artefact in all three periods.

There was no evidence of nociception associated with euthanasia with Pento, SS-KCl or SS-$MgSO_4$ (Table 6). Although a significant Period X Treatment effect was identified (Table 6), post-hoc pairwise comparisons between periods within treatment revealed no significant interaction effects for F50, F95 or Ptot. There was a tendency for F50 to be slightly lower in period 3 than period 1 for sheep euthanased with $MgSO_4$ (t=-2.86, P=0.060) and F95 to be higher in period 3 compared with period 2 in sheep euthanased with pentobarbital (t=-2.86, P=0.061), but this was not significant.

Table 6 Effect of treatment (n=3; pentobarbital, potassium chloride or magnesium sulphate), period (n=3; 15 seconds prior to infusion, 0–15 seconds and 16–30 seconds after start of infusion) and day of testing (n=5) on the mean median frequency (F50), 95% spectral edge frequency (F95) and total power (Ptot) of the sheep EEG.

	Period		Treatment		Day		Period x Treatment	
Variable	F value	Pr>F	F value	Pr>F	F value	Pr>F	F value	Pr>F
F50	1.04	0.389	0.6	0.427	0.68	0.469	3.71	0.007
F95	0.06	0.941	0	0.995	1.23	0.328	3.29	0.021
Ptot	1.47	0.243	1.40	0.269	1.91	0.149	2.94	0.032

5 Discussion

The primary aim of this study was to determine if euthanasia using saturated salt solutions was a humane method of euthanasia of livestock that were heavily sedated with xylazine. Previous reports have indicated that this method of euthanasia is inhumane because xylazine does not induce a state of unconsciousness and animals were likely to perceive pain or experience distress (Leary et al., 2013, Shearer, 2014). Results from the present study indicate that although xylazine did not induce a state of unconsciousness, there was no evidence to suggest animals consciously perceived pain associated with the administration of SS-KCl or SS-$MgSO_4$ when compared with the Pento group.

The administration of a high dose of xylazine caused significant reduction in brain wave activity as determined by an increase in F50 and F95. This result was expected, with the changes in EEG activity representative of heavy sedation, but not general anaesthesia or loss of conscious awareness. This result is consistent with a previous study that evaluated the level of consciousness of cattle that were administered a high dose of xylazine (Dewell et al., 2014).

Previous studies using EEG to evaluate animal response to noxious stimuli have demonstrated that conscious perception of pain is reflected by a significant and transient (60-90 sec) increase in F50 and F95, and an overall decrease in Ptot (cite). In the present study, there was no significant difference in EEG frequency (P50, P95, Ptot) between SS-KCl, SS-$MgSO_4$ or Pento groups. This indicates that although animals were not in a state of unconsciousness at the time of infusion with saturated salt solution, there was no measurable cortical nociception response detected.

It was not possible to determine if this result was associated with the analgesic or antinociceptive properties of xylazine (Riviere and Papich, 2008) or whether the infusion of the saturated salt solutions did not evoke a nociceptive response. Further studies could examine the antinociceptive properties of xylazine in sheep using a minimal anaesthetic model.

Although there was no evidence of nociception for any treatment group, clinical end-point and behavioural data supports the preferential use of SS-$MgSO_4$ over SS-KCl. Although there was no cortical recognition of pain, animal euthanised with SS-KCl consistently exhibited severe adverse behaviours during infusion. These responses often included violent kicking, leg paddling and sustained spinal flexion or convulsion after conscious EEG activity had ceased. No severe behavioural responses were observed in the SS-$MgSO_4$ group.

The severe adverse behavioural responses observed for the SS-KCl group occurred after conscious awareness was lost and therefore had no direct impact on the animals' welfare. However, with increasing scrutiny and awareness of animal welfare practices, visually unappealing responses as observed when administering SS-KCl should be avoided where superior methods of euthanasia are available. Additionally, violent and unpredictable muscle spasms could pose a serious threat to the safety of personnel administering the SS-KCl, particularly if cattle are being euthanased with this method.

The disparity in clinical end-point and behavioural responses between SS-KCl and SS-$MgSO_4$ are likely associated with differences in their modes of action. Potassium chloride has a key role in regulating the resting membrane potential of cells and affects membrane excitability, particularly of cardiac, muscle and nerve cells (cite). Excess KCl causes hyperexcitability of cells which results in cardiac arrest and increased muscle contractile activity (Riviere and Papich, 2008, Grimm et al., 2015). This change in the cellular membrane potential is likely to be responsible for the skeletal muscle hyperexcitability and spasmic activity noted in this group. In contrast, $MgSO_4$ has neuromuscular blockage activity and is viewed as a muscle relaxant and central nervous system depressant (Riviere and Papich, 2008, Grimm et al., 2015). An overdose has depressive rather than excitatory effects and often results in complete CNS depression and respiratory arrest. Although no differences in the humaneness of SS-KCl or SS-$MgSO_4$ were evident, the difference in behavioural responses alone are significant enough to support to preferential use of SS-$MgSO_4$.

In addition to the collection of intact brain samples, the purpose of this study was also to identify a practical method of euthanasia that minimises the risk of secondary poisoning of carnivorous animals following the ingestion of euthanased livestock. In this respect, some of the major advantages of saturated salt solutions are that they are not controlled substances, they are easily obtained, transported and can be prepared in the field (Leary et al., 2013).

In this study, a 50 kg sheep required 50-350 ml of SS-KCl or 50-100 ml of SS-$MgSO_4$ solution infused before permanent isoelectric waveform was observed (brain death). This result was unexpected because previous field observations reported that larger volumes of SS-$MgSO_4$ were needed to euthanase an animal, compared with SS-KCl. This difference may be associated with the retrospective calculation used in this study to determine the volume of saturated salt required to reach isoelectric endpoint, rather than the observation of clinical death in a field setting. Although the smaller and more consistent volumes of SS-$MgSO_4$ required to euthanase a sheep may be advantageous in a field

setting, the recommendation of in-field use is that infusion with saturated salt solution continues until clinical death is confirmed.

Although residues in carcasses were not specifically measured in this study, the risk of secondary poisoning associated with consumption of carcasses containing SS-KCl or SS-$MgSO_4$ is deemed negligible. The AVMA guidelines currently acknowledge that one of the advantages of SS-KCl and SS-$MgSO_4$ is that the risk of secondary poisoning is reduced and when used in accordance with their recommendations, may be a good choice of euthanasia where carcass disposal is not possible (Leary et al., 2013).

The risk of secondary poisoning associated with high doses of xylazine are also deemed negligible. Previous studies have reported that there were no treatment-related adverse effects in beagle dogs that were fed 0.3 to 3mg/kg per day for 1 to 13 weeks (Chamberlain and Brynes, 1998). Based on these studies, a 20kg dog could safely consume 60mg of xylazine each day for 13 weeks with no adverse response. In the present study, a 50kg sheep received 0.4mg/kg of xylazine which equated to a total dose of 20mg. By extrapolation, a 20kg dog could therefore safely consume three, 50kg sheep treated with xylazine (0.4mg/kg). Xylazine has been used in avian species at relatively high doses (1-2mg IM) with only light sedative effects (Riviere and Papich, 2008), but little is known about the risk oral toxicity for other carnivorous wildlife species. This warrants further investigation.

6 Conclusions/recommendations

There was no evidence of conscious pain perception associated with euthanasia with either SS-KCl or SS-$MgSO_4$. As such, both are deemed humane methods to euthanase livestock that have been heavily sedated with xylazine. However, based on consistent and severe adverse, post-mortem behavioural responses observed during the administration of SS-KCl, SS-$MgSO_4$ is the preferred euthanasia agent.

When brain material is required or when captive bolt or firearms are not available, intravenous infusion with SS-$MgSO_4$ is a rapid, safe, humane and practical means of euthanasia. Prior to SS-$MgSO_4$ infusion, animals should be given a high dose of xylazine. This method poses minimal risk to scavenging animals by secondary poisoning and requires no specialised equipment.

7 Key messages

When brain material is required or when a firearm or captive bolt is not available, the administration of SS-$MgSO_4$ to livestock that are heavily sedated with xylazine provides a safe, reliable, humane and practical means of euthanasia. The risks associated with secondary poisoning of carnivorous animals is deemed negligible. The infusion of SS-$MgSO_4$ should be continued until death has been confirmed.

8 Bibliography

ANON. 2011. *Condition Scoring of Sheep* [Online]. http://www.lifetimewool.com.au/conditionscore.aspx. [Accessed June 29 2018].

ANON 2017. National TSE Surveillance Project (NTSESP) - National Guidelines for Field Operations. Australia: Animal Health Australia.

CHAMBERLAIN & BRYNES 1998. The regulatory status of xylazine for use in food-producing animals in the United States. *Journal of Veterinary Pharmacology and Therapeutics,* 21**,** 322-329.

CHANG, P. F., ARENDT-NIELSEN, L., GRAVEN-NIELSEN, T., SVENSSON, P. & CHEN, A. C. N. 2001. Different EEG topographic effects of painful and non-painful intramuscular stimulation in man. *Experimental Brain Research,* 141**,** 195-203.

CHEN, A. C. N., DWORKIN, S. F., J., H. & GEHRIG, J. 1989. Topographic brain measures of human pain and pain responsivity. *Pain,* 37.

DEWELL, R., BERGAMASCO, L., KELLY, C., SHEARER, J. & DEWELL, G. 2014. Clinical study to assess the level of consciousness/general anesthesia following the administration of high doses of xylazine hydrochloride in cattle. Animal Industry Report: AS 660, ASL R2848.

EVERS, A. S., CROWDER, C. M. & BALSER, J. R. 2006. General anaesthetics. *In:* BRUNTON, L. L., LAZO, J. S. & PARKER, K. L. (eds.) *Goodman and Gillman's the pharmacological basis of therapeutics.* New York: McGraw-Hill Medical Publishing Division.

GIBSON, T. J., JOHNSON, C. B., MURRELL, J. C., HULLS, C. M., MITCHINSON, S. L., STAFFORD, K. J., JOHNSTONE, A. C. & MELLOR, D. J. 2009a. Electroencephalographic responses of halothane-anaesthetised calves to slaughter by ventral-neck incision without prior stunning. *New Zealand Veterinary Journal,* 57**,** 77-83.

GIBSON, T. J., JOHNSON, C. B., MURRELL, J. C., MITCHINSON, S. L., STAFFORD, K. J. & MELLOR, D. J. 2009b. Electroencephalographic responses to concussive non-penetrative captive-bolt stunning in halothane-anaesthetised calves. *New Zealand Veterinary Journal,* 57**,** 90-95.

GRIMM, K. A., ROBERTSON, S. A., LAMONT, L. A., TRANQUILLI, W. J., GREENE, S. A., ROBERTSON, S. A., GRIMM, K. A., LAMONT, L. A., TRANQUILLI, W. J. & GREENE, S. A. 2015. *Veterinary Anesthesia and Analgesia,* Hoboken, USA, John Wiley & Sons, Incorporated.

KAISER, A., MCFARLAND, W., SIEMION, R. & RAISBECK, M. 2010. Secondary pentobarbital poisoning in two dogs: a cautionary tale. *Journal of Veterinary Diagnostic Investigations,* 22**,** 632-634.

LEARY, S., UNDERWOOD, W., ANTHONY, R., CARTNER, S. & COREY 2013. AVMA Guidelines for the Euthanasia of Animals. University of Tennessee.

MAYHEW, I. G. & WASHBOURNE, J. R. 1990. A method of assessing auditory and brainstem function in horses. *British Veterinary Journal* 146**,** 509-18.

MURRELL, J. C. & JOHNSON, C. B. 2006. Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics,* 29**,** 325-335.

NEWHOOK, J. C. & BLACKMORE, D. K. 1982. Electroencephalographic studies of stunning and slaughter of sheep and calves: Part 1, The onset of permanent insensibility in sheep during slaughter. *Meat Science,* 6**,** 221-233.

PAYNE, J., FARRIS, R., PARKER, G., BONHOTAL, J. & SCHWARZ, M. 2015. Quantification of sodium pentobarbital residues from equine mortality compost piles. *Journal of Animal Science,* 93**,** 1824-9.

RADOSTITS, O., GAY, C., HINCHCLIFFE, K. & CONSTABLE, P. 2007. *Veterinary Medicine - A textbook of the diseases of cattle, horses, sheep, pigs and goats,* United Kingdom, Elsevier Limited.

RAULT, J.-L., HEMSWORTH, P., CAKEBREAD, P., MELLOR, D. & JOHNSON, C. 2014. Evaluation of microwave energy as a humane stunning technique based on electroencephalography (EEG) of anaesthetised cattle. *Animal welfare,* 23**,** 391-400.

RIEBOLD, T., GOBLE, D. & GEISER, D. 1982. *Large animal anesthesia : principles and techniques,* Iowa, USA, Iowa State University Press.

RIVIERE, J. & PAPICH, M. G. 2008. *Veterinary pharmacology and therapeutics - 9th edition,* USA, Iowa State University Press.

RUMBLE, J. 2017. *CRC Handbook of Chemistry and Physics, 98th Edition*, Taylor & Francis Group.

SHEARER, J. 2014. Euthansia of cattle. *XXVIII World Buiatrics Congress.* Cairns, Australia.

SUTHERLAND, M. A., WATSON, T. J., JOHNSON, C. B. & MILLMAN, S. T. 2016. Evaluation of the efficacy of a non-penetrating captive bolt to euthanase neonatal goats up to 48 hours of age. *Animal Welfare,* 25**,** 471-479.