UNIVERSITI PUTRA MALAYSIA

ELECTROENCEPHALOGRAPHIC RESPONSES TO LOW AND HIGH VOLTAGE OF ELECTROEJACULATOR USED FOR SEMEN COLLECTION IN BULLS

NADHIRAH BINTI YAHAYA

ELECTROENCEPHALOGRAPHIC RESPONSES TO LOW AND HIGH VOLTAGE OF ELECTROEJACULATOR USED FOR SEMEN COLLECTION IN BULLS

NADHIRAH BINTI YAHAYA

FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA
SERDANG, SELANGOR.
2020/2021

It is hereby certified that we have read this project entitled "Electroencephalographic Responses to Low and High Voltage Electroejaculator Used for Semen Collection in Bulls", by Nadhirah binti Yahaya and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project.

DR. UBEDULLAH KAKA

DVM (Pak), MSc (Pak),Ph.D (UPM),

Lecturer,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

ASSOC. PROF. DR. NURHUSIEN YIMER DEGU

DVM (Ethiopia),Ph.D (UPM),

Lecture,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-supervisor)

PROF. DR. GOH YONG MENG

DVM (UPM), Ph.D (UPM),

Lecturer,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-supervisor)

DEDICATION

I would like to dedicate this paper to my parents and my family for supporting my dream and ambitions; and giving moral support when I am in conflict with myself throughout my studies. Besides that, for my dear self because you have achieved one of your ambitions through blood, sweat and tears throughout those years.

ACKNOWLEDGEMENT

A special gratitude to Allah SWT for giving me chances to experience this fruitful project and blessed me with kind people to guide and help. I would like to thank my parents for being supportive throughout the years achieving this point of life.

Besides that, I am grateful for being supervised by my supervisor who was really helpful and supportive throughout the project. Thank you to my co-supervisors and Dr Shan for the consultation and advices. Theriogenology laboratory staffs and Bull unit, TPU staffs for bearing with my incompetent skills on the procedure for the first time.

Finally, thank you to my friends (Bosco, Yusrina and Faizatul), DVM2020 and DVM2023 for helping me on field. Not to forget my batch mates (DVM2021) who indirectly gave hands to me throughout this project.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRAK

Abstrak daripada kertas projek yang dikemukan kepada Fakulti Perubatan Veterinar, Universiti Putra Malaysia bagi memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek.

RESPONS ELEKTROENSEFALOGRAM TERHADAP VOLTAN TINGGI DAN RENDAH ELEKTROEJAKULATOR SEMASA PEMUNGUTAN AIR MANI DARIPADA LEMBU JANTAN.

Oleh

Nadhirah Yahaya

Penyelia: Dr. Ubedullah Kaka

Penyelia bersama: Prof. Madya Dr. Nurhusien Yimer Degu dan Prof. Dr. Goh Yong Meng

Elektroejakulasi(EE) merupakan salah satu prosedur rutin untuk mengutip air mani bagi program pembiakan haiwan ternakan dan penilaian kesuburan lembu jantan. Tiada kesimpulan yang kukuh dilaporkan mengenai rasa sakit terhasil daripada EE. Electroensefalografi (EEG) adalah alat yang objektif untuk menilai tahap kesakitan berdasarkan penyelidikan sebelum ini. Walaubagaimanapun, tiada kajian dilaporkan bagi menilai tahap kesakitan prosedur EE dengan menggunakan EEG. Oleh itu, kajian ini telah direka bagi membandingkan respons EEG dan perubahan sikap lembu jantan terhadap voltan rendah(≤6) dan tinggi (>6) elektroejakulator. Terdapat lapan ekor lembu jantan berumur 2 tahun dengan anggaran berat badan 200kg telah digunakan dalam kajian ini. Dua pelekat elektrod EEG dilekapkan pada kepala lembu jantan Respons EEG dan perubahan sikap lembu jantan direkod semasa sebelum EE, ketika

EE dan 20 minit selepas EE yang berdurasi 5 minit. Data EEG dianalisis secara luar talian selepas tamat kajian. Keputusan keseluruhan menunjukkan parameter EEG meningkat secara signifikan ketika EE berbanding ketika sebelum EE, kemudian parameter ini menurun menuju ke permulaan respons EEG selepas 20 minit EE tamat. Frekuensi pertengahan yang merupakan indikasi kesakitan telah meningkat secara signifikan ($p=0.0001$) ketika EE telah dilihat dalam haiwan kumpulan voltan rendah(47.14 ± 6.67) jika dibandingkan haiwan kumpulan voltan tinggi (32.66 ± 4.53). Namun begitu, lembu jantan menunjukkan perubahan sikap bagi kedua-dua kumpulan seperti tulang belakang membongkok, keluar air liur dan pergelutan semasa prosedur mengutip air mani. Secara tuntas, kenaikan respons EEG dan perubahan sikap semasa EE telah mengemukakan bahawa penggunaan EE untuk mengutip air mani adalah sakit kepada lembu jantan. Kajian masa depan diusul untuk menyatukan keputusan kajian ini dan analisis hormon tekanan bersama EEG.

Kata kunci: elektroejakulasi, respons elektroensefalografi , kesakitan, perubahan sikap, lembu jantan

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, University Putra Malaysia in partial fulfilment of the course VPD 4999 – Project.

ELECTROENCEPHALOGRAPHIC RESPONSES TO LOW AND HIGH VOLTAGE OF ELECTROEJACULATOR USED FOR SEMEN COLLECTION IN BULLS

By

Nadhirah Yahaya

Supervisor: Dr. Ubedullah Kaka

Co-supervisors: AP Dr. Nurhusein Yimer Degu & Prof. Dr. Goh Yong Meng

Electroejaculation (EE) is one of the routine procedure used to collect semen for breeding programme and bulls soundness evaluation at the farm. No concrete conclusion regarding pain produced by EE has been reported. Electroencephalography (EEG) is an objective tool to evaluate pain in recently reported studies. No EEG study on evaluating pain associated with EE has been reported. Therefore, this study was designed to compare the EEG responses and the behaviour changes to low (≤6) and high (>6) voltage of the electro-ejaculator in bulls. A total of eight bulls of 2 years age and average of 200kg body weight were used in this study. Bulls were attached with two EEG adhesive electrodes on their head and the EEG and behaviour were recorded for 5 minutes, before EE (baseline), during EE and 20 minutes after EE. Analysis of EEG was performed offline after the end of experiments. The results revealed that

EEG parameters increased significantly during EE compared to baseline and returned towards baseline 20 minutes after EE. Median frequency, an indicator of pain increased significantly compared to baseline in both high and low voltage groups. Furthermore, significant increases (p=0.0001) was observed in low voltage group (47.14±6.67) animals compared to the animals in the high voltage group (32.66±4.53). Total power also increased significantly (p=0.0001). The bulls in both groups showed behavioural changes such as arched back, salivation and struggling during the EE for semen collection. Overall, the increment of EEG responses and behavioural changes during EE suggests that the semen collection using EE is painful. Future studies to consolidate the findings of this study with the stress hormone analysis along with EEG are recommended.

Keywords: electroejaculation, electroencephalographic response, pain, behavioural changes, bulls

1.0 INTRODUCTION

Electroejaculation (EE) is the most common method used to collect semen due to its flexibility, convenience and easily adaptable to cattle handling facilities (Palmer, 2005). This technique does not require cattle for mounting and is safe for the person in charge. In humans EE has been reported to be painful without anaesthesia (Ohl,1993, Damian and Ungerfield, 2011). Thus, it is assumed to be painful in animals. Recently, a modified method of EE has been reported (Baiee et al., 2018) that proved to minimize discomfortness compared to conventional EE during semen collection. This modified method uses gradual increase in the voltage of electroejaculator by staging. Baiee et al (2018) explained there are three stages depending on the number of electrical impulses per stage. In his study, for the first stage once the EE switched on, the electrical stimulus were increased from zero until three volt and then EE immediately was switched off, after that immediately switched on to begin with stage two from zero until six volt. The third stage, the stimulation were from zero until ejaculation.

Animals are not as expressive as human being when experience pain or discomfort. Even though the bulls show less sign of discomfort as they are said as stoic animals,(Gleerup, 2017), their pain perception should not be neglected. To date, very few studies have attempted to assess pain in response to EE (Stafford,1995; Mossure et al., 1998; Falk et al., 2001; Etson et al 2004; Palmer 2005; Damian and Ungerfield, 2011; Whitlock et al., 2012; Boussena et al., 2013; Pagliosa et al., 2015; Baiee et al., 2018) and yet the results are inconclusive. These studies used subjective tools to evaluate the pain such as hormones, heart rate, substance P and behaviour that produced discrepant result as it can increase or change as a result of stress handling.

Recently, electroencephalography (EEG) has been reported to be an objective tool to assess pain in various studies of animal species such as dogs (Kaka et al.2015,2016) , sheep (Sylvester et al. 2002), horses (Murrell et al. 2003,2005), red deer (Johnson et al. 2005,2006), goats(Sabow et al. 2016,2017) and cattle (Small et al. 2019; Imlan et al.2020) .

EEG is a neurophysiological technique to measure electrical potential of the neurone directly from the brain via attachment of electrodes at the head. So far, no study has been reported to evaluate pain using EEG in bulls during EE. Therefore, this study was designed with following objectives:

1) To evaluate pain in response to low and high voltage of EE in bulls.
2) To compare behavioural changes to low and high voltage of EE in bulls.

2.0 LITERATURE REVIEW

2.1 Anatomy and Physiology of Male reproductive system

A mature bull's reproductive organs include a pair of testes, vesicular glands, bulbourethral glands, a prostate gland and a fibroelastic penis, which incorporates a sigmoid flexure (Amann, 1986; Chenoweth & Kastelic, 2007). A testes is made of spermatic cord, an epididymis and a deferent duct which its point of highest development is an ampulla (Dyce, 1971; Chenoweth & Kastelic, 2007). Scrotum is a layer of skin that encapsulate the testes; which plays important role in testicular thermoregulation (Chenoweth & Kastelic, 2007).

Bovine semen consists of spermatozoa suspended in seminal plasma that are derived from testes, epididymides and accessory gland (Chenoweth & Kastelic, 2007). Tubular transport system consists of the convoluted and straight seminiferous tubules, rete testis, efferent ductules, epididymis, ductus deferens and urethra (Nabors & Linford, 2015). This transport system transports spermatozoa from the testicles, also allows maturation and storage of spermatozoa besides providing fluid to assist movement of the spermatozoa. Intromission process is when the bull erects its penis and ejaculate the sperm to achieve the transfer of sperm to the cow from the bull (Nabors & Linford, 2015).

2.2 Bull maturity

Brito (2014) defined puberty in general term as the process by which bull becomes capable of reproducing; that involves development of gonads and secondary sexual organs and development of the ability to breed. However, for research purposes he defined puberty in bulls as an event while most researchers defined bull achieved sexual maturity when it ejaculates semen containing more than 50 million sperm with 10% of it is motile (Wolf, 1965; Brito,2014). In addition, Brito (2014) said the puberty age is influenced by management, genetics and nutrition. He concluded *Bos indicus* bulls reached sexual maturity at later age compare with *Bos taurus*. Spermatogenesis efficiency of *Bos indicus* reaches adult levels at 2.5-3.5 years of age (Brito,2014; Macmillan & Halfs, 1968; Killian & Amann, 1972).

2.3 Electro-ejaculation

Stafford (1995) concluded electro-ejaculation (EE) is the only available practical method to use for semen collection in untrained domestic ruminants for artificial vagina method. Semen collection is important and commonly carried out in farm to evaluate the semen quality and for artificial insemination procedure. EE involves stimulation by electrical current of emission; a sympathetic response, erection and ejaculation; both controlled by sacral parasympathetic nerves (Stafford,1995; Ball,1986; Ganong,1986).

A probe is inserted into the rectum to stimulate the emission, erection and ejaculation. This intra-rectal electrical probe transmits oscillating current of either pulse-wave or sine-wave form in domestic animals (Carter, 1990; Stafford, 1995). In addition, Ball(1986) and Stafford(1995) stated in their studies that in bulls the current produced by probe used may be altered and stimulation started from lower to higher voltage (maximum 13-16 v (Palmer,2005)) until ejaculation occur with a continuous stimulation. Palmer also reported in year 2005 that the intensity of the voltage is steadily increased and held for 1-2 second with each successive stimulation followed by 0.5-1 second of rest. Latter rectal probes were designed to avoid unnecessary stimulation of nerves dorsal to the rectum. Applicable to most circumstances, probes with size 6.5-7.5 cm is suffice to stimulate ejaculation. However, older and often larger bulls require probes with size of 9cm for better contact to the rectal mucosa and enhanced response to electrical stimulation (Palmer,2005).

Baiee et al. (2018) experimented on two methods of EE stimulation. Method I is the EE stimulation using automatic mode until ejaculation while Method II is EE stimulation by gradual increase into 3 stages using automatic mode. They found that Method II gave minimal signs of discomfort compared to Method I. The stimulation will be stopped when the bulls reached ejaculation.

2.4 Pain in response to electro-ejaculator stimulation

Adcock and Tucker (2018) defined pain as a multifaceted phenomenon that can be classified into acute nociceptive pain, inflammatory pain and neuropathic pain. They have discussed pain that occur through husbandry practices, including electro-ejaculation procedure for collection of semen and placed EE under stimulus that cause acute nociceptive pain in cattle. Skin and deep tissue contains a specialized class of sensory neurons that detects noxious stimuli (Adcock & Tucker, 2018). However, pain recognition remains a fundamental hurdle and currently there is no gold standard method to quantify pain in either man or animals (Murrell & Johnson, 2006). Sneddon & Gentle (2000) also suggested that since there is no specific measurable parameter indicative of pain, the measurement and evaluation of animal welfare and pain is difficult and subjective.

Electroejaculation (EE) is reported to be painful in human without anaesthesia (Ohl, 1993; Damian and Ungerfeld, 2010) and thus is assumed to be painful in animals. Many studies have been done to evaluate pain associated with EE using different evaluating tools such as behaviour changes (Stafford, 1995; Whitlock et al., 2012; Baiee et al., 2018), cortisol concentration (Falk et al., 2001), substance P (Whitlock et al., 2012), heart rate (Mosure et al., 1998; Boussena et al., 2013) and blood progesterone (Falk et al., 2001; Etson et al., 2004; Whitlock et al., 2012). However, these studies produced discrepant results, which could be due to the tools used such as hormones and heart rate that also increased due to the stressful handling of animals.

2.5 Electroencephalography

Electroencephalography (EEG) is a neurophysiological technique to read electrical potential of the brain produced by neurones (Kumar & Bhuvaneswari, 2012;Murrell & Johnson, 2006). EEG is reported as an objective, non-invasive and stress free method suitable for measurement of nociception (Kaka et al., 2016)

It is widely used in human medicine to detect brain problem such as epilepsy, Alzheimer, seizure disorder, autism and chronic pain (Kumar & Bhuvaneswari, 2012). While in animals EEG is used as a device to detect pain and nociception in many studies (Murrell & Johnson, 2006). Electrodes placement in humans are on the scalp of the head and in animals are at the head (Murrell & Johnson, 2006; Kaka et al.,2015); for example cattle at the middle of the frontal bone and at the mastoid process.

Usage of EEG to evaluate pain and the efficiency of anaesthesia and analgesic drugs have been reported in studies of various species (Murrell and Johnson, 2006) such as in dogs (Kongara et al., 2010; Kaka et al., 2015 & 2016), horses (Murrell et al., 2003 & 2005), cattle (Gibson et al., 2007, 2009a,2009b ; Zulkifli et al., 2014; Small et al., 2018; Imlan et al., 2020), sheep (Sylvester et al., 2002; Johnson et al., 2009; Sanchez-Barrera et al., 2014; Harris et al., 2020) and goats (Sabow et al., 2016 & 2017). Many studies have been reported in horses (Miller et al., 1995b; Otto et al., 1996; Murrell et al., 2003, 2005b; Haga & Dolvik, 2005; Murrell & Johnson, 2006) because this species faced many complications in anaesthesia (Johnston et al., 1995; Brodbelt, 2005; Murrell & Johnson, 2006).

In human pain studies, most commonly stimuli used were thermal, mechanical and electrical stimuli. In cattle, few studies have reported pain evaluation using EEG, for dehorning (Gibson et al., 2007; Johnson et al., 2012), slaughtering (Gibson et al., 2009a; Johnson et al., 2012), and stunning (Johnson et al., 2012) procedure. However, there is no study reported to evaluate pain for electroejaculation procedure in animal species instead.

3.0 METHODOLOGY

3.1 Management of Bulls

This project was conducted following the animal ethics guidelines of the Research Policy of Universiti Putra Malaysia (UPM/IACUC/AUP-U031/2020). A total of 8 bulls with average age of 2 years, kept in Taman Pertanian University were used in this study. These bulls were fed with *Brachia Decumbens* grass and palm kernel cake with ad-libitum drinking water provided.

3.2 Preparation of Bulls

The bulls were drived from the pen to chute one by one and were restrained properly with minimal stress prior conducting the procedure.

3.3 Experimental Procedure

Electroencephalography

Each of the bulls were attached with two Kendall™ (Covidien 11c, 15 Hampshire Street, Mansfield 02048 USA) conductive adhesive hydrogel foam electrodes at middle of frontal bone and another one at the mastoid process after shaving hairs and cleaning with 70% alcohol. The electroencephalographic signal were recorded for five minutes, before the EE procedure (baseline),during EE procedure and 20 minutes after EE procedure. The signals were sampled at rate 2kHz, and raw EEG was re-sampled with low pass filter of 200 Hz into delta frequency (0.1 to 4 Hz), theta frequency (4.1 to 8 Hz), alpha frequency (8.1 to 12 Hz) and beta frequency (12.1 to 20 Hz).

Prior to EEG analysis, the raw EEG recordings was resampled at 1024 Hz and only frequencies between 0.1 and 30 Hz were obtained to minimize the presence of artifacts. Analysis of the EEG data was performed offline after the completion of experiments using the Chart Spectral Analysis Function (Chart 5.0TM software). Possible interferences from concurrent electrocardiograph signals were digitally removed from the raw EEG recordings using the Chart 5.0TM software prior to analysis. Signals were then processed for consecutive non-overlapping 1-second epoch yielding 60 epochs per minute. Root mean square (RMS) for each of alpha, beta, delta, theta waves, median frequency (MF) and total power (Ptot) before and after EE was calculated. Median frequency (MF or F50) is the frequency below which 50% of the total power of the EEG and total power (Ptot) is the total area under the power spectrum curve.

Figure 1 Position of the electrode at the middle of the frontal bone and the mastoid process

Electroejaculation

A lubricated probe 51 mm in diameter and 330 mm in length with 3 electrodes distributed longitudinally and ventrally was inserted into the bull´s rectum, once ready. Electrical stimulation was stimulated from the electroejaculator (Electrojac 6;

Neogen® Corporation, Lansing, IM48912; L24290812) to the probe with voltage increment as a staged (Baie et al., 2018). The voltage was set into low voltage(≤6) and high voltage(>6). The cut-off point of this experiment was at point of ejaculation or 12 volts.

Behaviour changes recording (Discomfort signs)

The discomfort signs were recorded before EE (baseline), during EE and 20 minutes after relaxation by the same person throughout the experiment. Videos of the behaviour were also recorded from side and front view to ease the evaluation of the behaviour.

3.4 Statistical analysis

The data from EEG recording was analysed using SAS system, version 9.1(SAS Inst. Inc., Cary., NC). One-way ANOVA was used to analyse the data and significant difference among mean result was subjected to post hoc Tukey's multiple comparison test. All results were presented as Mean ± Standard error(SE). Data for signs of discomfort were analysed using IBM SPSS Statistics, Version 25 non-parametric test; Man-Whitney test (discomfort signs compared with treatment group) and Kruskal-Wallis test (discomfort signs compared with time) followed by Bonferonni-Dunnet's multiple comparison test for statiscally significant difference result. The strength of correlation between mean of MF and discomfort signs were tested using Spearman's Correlation Test.

4.0 RESULT

Table 1 and **Table 2** shows EEG result before, during EE and after 20 minutes after EE procedure for Low group treatment (≤6V). Results show that alpha, beta, delta and theta (**Table** 1) MF and Ptot (**Table 2**) increased significantly(p=0.0001) during EE compared to baseline and the values returned to baseline twenty minutes after the procedure (relaxation).

Table 1 Mean ± SE(μV) of alpha, beta, delta and theta waves of Low group

Parameter	Time			P value
	Baseline	EE	Relax	
Alpha	$1.1520^{b} \pm 0.1107$	$2.1902^{a} \pm 0.6988$	$1.1645^{b} \pm 0.1077$	0.0001
Beta	$1.9945^{b} \pm 0.3643$	$5.0151^{a} \pm 1.6195$	$2.0594^{b} \pm 0.2359$	0.0001
Delta	$4.6061^{b} \pm 0.5374$	$11.2986^{a} \pm 4.8013$	$3.9897^{b} \pm 0.1759$	0.0001
Theta	$1.5161^{b} \pm 0.1657$	$2.2922^{a} \pm 0.5841$	$1.3721^{b} \pm 0.0775$	0.0002

[a,b] Difference across the row with different superscript differed significantly at $p<0.05$.EE= Electroejaculation

Table 2 Electroencephalogram total power (Ptot) (μV) and median frequency (MF) (Hz) values (Mean ± SE) of Low group

Parameter	Time			P value
	Baseline	EE	Relax	
Ptot	7.9217[b] ± 0.8495	31.5368[a] ± 2.7459	7.223[b] ± 0.4123	0.0001
MF	13.4275[b] ± 1.4595	47.1436[a] ± 6.6765	16.895[b] ± 0.6813	0.0001

[a,b] Difference across the row with different superscript differed significantly at $p<0.05$.EE= Electroejaculation

Table 3 and **Table 4** shows EEG result before, during EE and after 20 minutes after EE procedure for High group treatment(>6V). Results show that alpha, beta, delta and theta (**Table 3)** MF and Ptot (**Table 4**) increased significantly (p=0.0001) during EE compared to baseline and the values returned to baseline twenty minutes after the procedure (relaxation).

Table 3 Mean ± SE(μV) of alpha, beta, delta and theta waves of High group

Parameter	Time			P value
	Baseline	EE	Relax	
Alpha	1.1544[b] ± 0.1572	2.5994[a] ± 0.1865	1.1297[b] ± 0.1551	0.0001
Beta	1.9544[b] ± 0.3438	4.2006[a] ± 0.3438	2.0459[b] ± 0.4327	0.0001
Delta	4.4374[b] ±	15.6186[a] ±	4.4206[b] ±	0.0001

Theta	1.2209[b] ± 0.6626 0.0835	2.7004[a] ± 6.4102 0.3678	1.2784[b] ± 0.6576 0.0837	0.0001

[a,b] Difference across the row with different superscript differed significantly at p<0.05.EE= Electroejaculation

Table 4 Electroencephalogram total power (Ptot)(μV) and median frequency (MF)(Hz) values (Mean ± SE) of High group

Parameter	Time			P value
	Baseline	EE	Relax	
Ptot	8.0755[b] ± 0.4602	25.0752[a] ± 6.9345	7.223[b] ± 0.4123	0.0001
MF	13.0372[b] ± 1.0090	32.6661[a] ± 4.5307	13.7685[b] ± 2.4011	0.0001

[a,b] Difference across the row with different superscript differed significantly at p<0.05.EE= Electroejaculation

Table 5 shows comparison of median frequency (MF) across the group treatment. There is difference in MF across the time at p=0.0001 and post hoc test shows the MF are significantly different from baseline, during EE for Low voltage group(≤6V) and during EE for High voltage group(>6V). From the result, we can also see that the MF of Low voltage group are higher than the high voltage group treatment. This shows that the bulls in the Low voltage group treatment have low tolerance to nociceptive.

Table 5 Differences in electroencephalogram total power (Ptot)(µV) and median frequency (MF)(Hz) values (Mean ± SE) when compared between group treatment.

Parameter	Group treatment			P value
	Time			
	Baseline	EE Low	EE High	
MF	13.4275[c] ± 1.4595	47.1436[a] ± 6.6765	32.6661[b] ± 4.5307	0.0001

[a,b,c] Difference across the row with different superscript differed significantly at $p<0.05$.EE Low= Electroejaculation Low voltage group treatment. EE High= Electroejaculation High voltage group treatment.

Figure 2 and **Figure 3** shows distribution of arc back reaction across the time. Based on **Figure 2**, there is no arc back during baseline for Low voltage group(≤6V). However, during EE all of the bulls showed arc back. After 20 minutes of relaxation, no arc back was observed . **Figure 3** shows percentage of bulls that reacted arc back in High voltage group(>6V). All bulls show arched back during EE. For baseline and after 20 minutes relaxation there were no arc back observed.

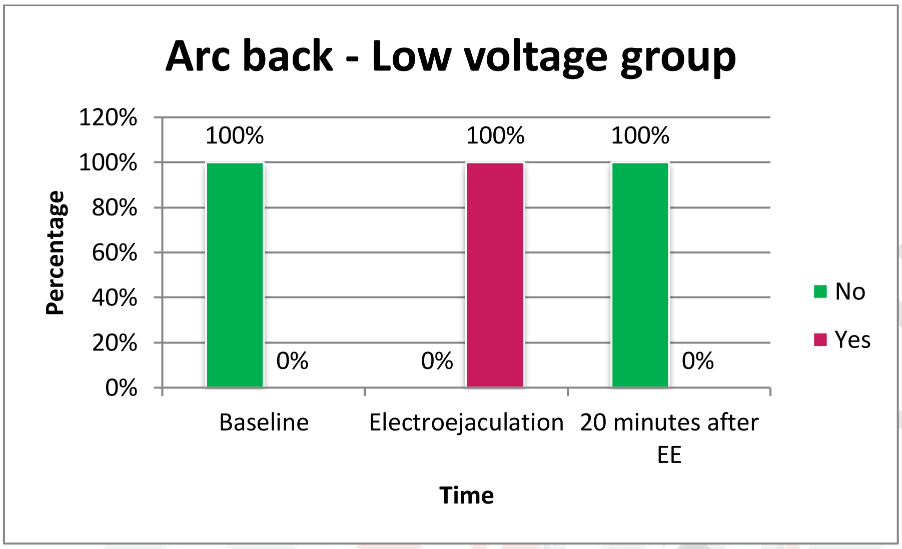

Figure 2: Percentage of bulls showing arc back behaviour in Low voltage group

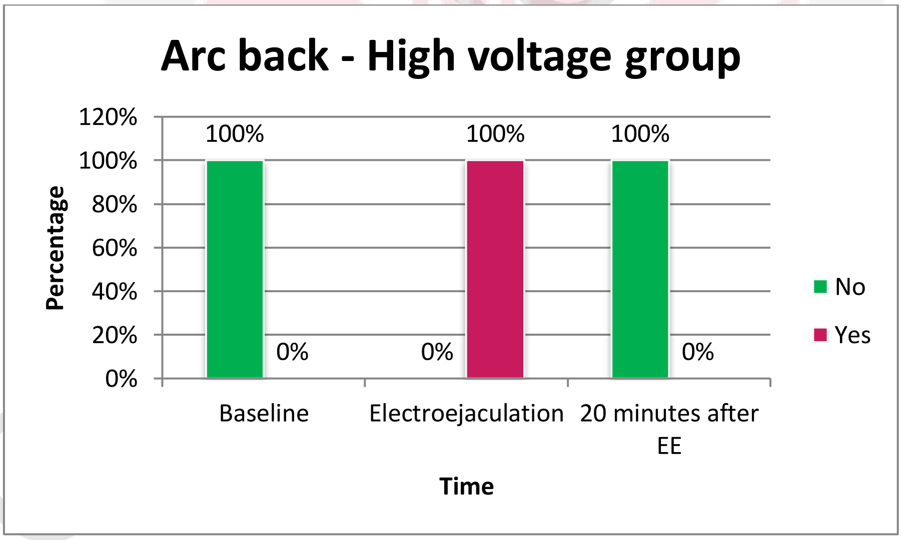

Figure 3: Percentage of bulls showing arc back behaviour in High voltage group

Figure 4 shows percentage of bulls showing struggling behaviour during EE in low voltage group(≤6V). All bulls show struggling behaviour during EE.

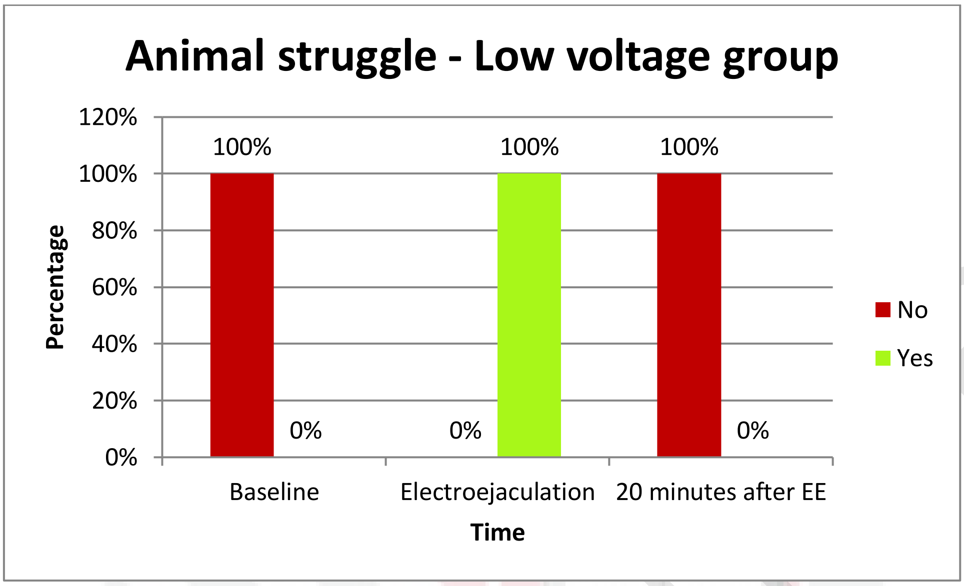

Figure 4: Percentage of bulls showing struggling behaviour in Low voltage group

Figure 5 shows percentage of showing struggle in high voltage group(>6V) . For baseline, 25% of bulls show struggling compared in the Low voltage group. All bulls show struggling during EE procedure.

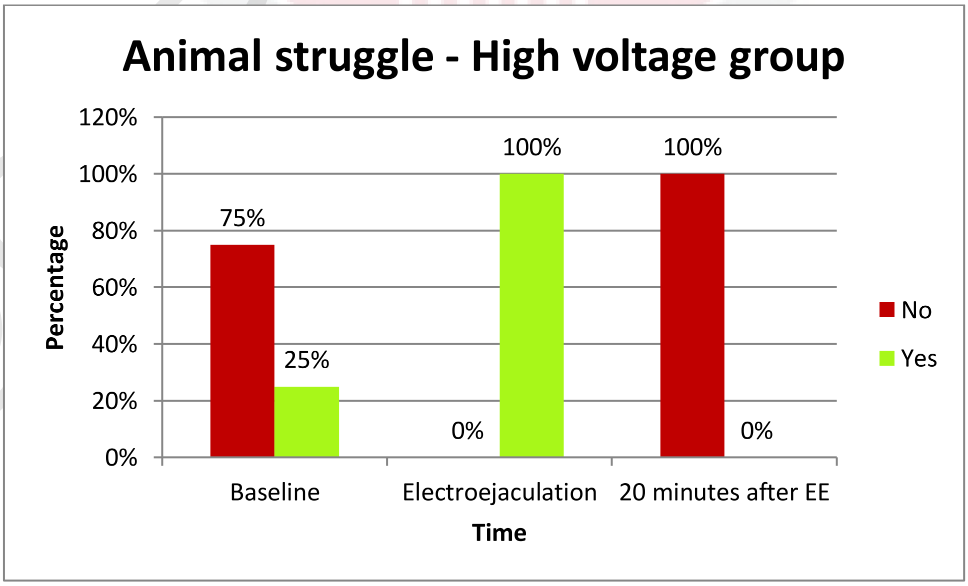

Figure 5: Percentage of bulls showing struggling behaviour in High voltage group

Figure 6 shows head position of bulls during EE procedure for low group(≤6V). About 25% were having head down or up during EE stimulation and 75% of the bulls having head down first the head up as voltage increased. **Figure 7** has opposites result compared to **Figure 6**. **Figure 7**, for high voltage group(>6V), only 25% were having head down and up and 75% having head up or down as voltage increased.

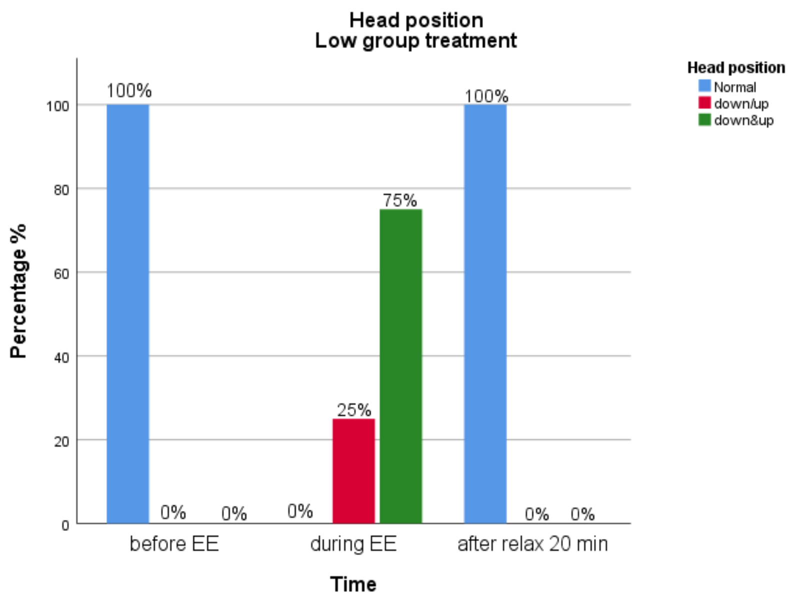

Figure 6: Percentage of bulls showing head position behaviour in Low voltage group

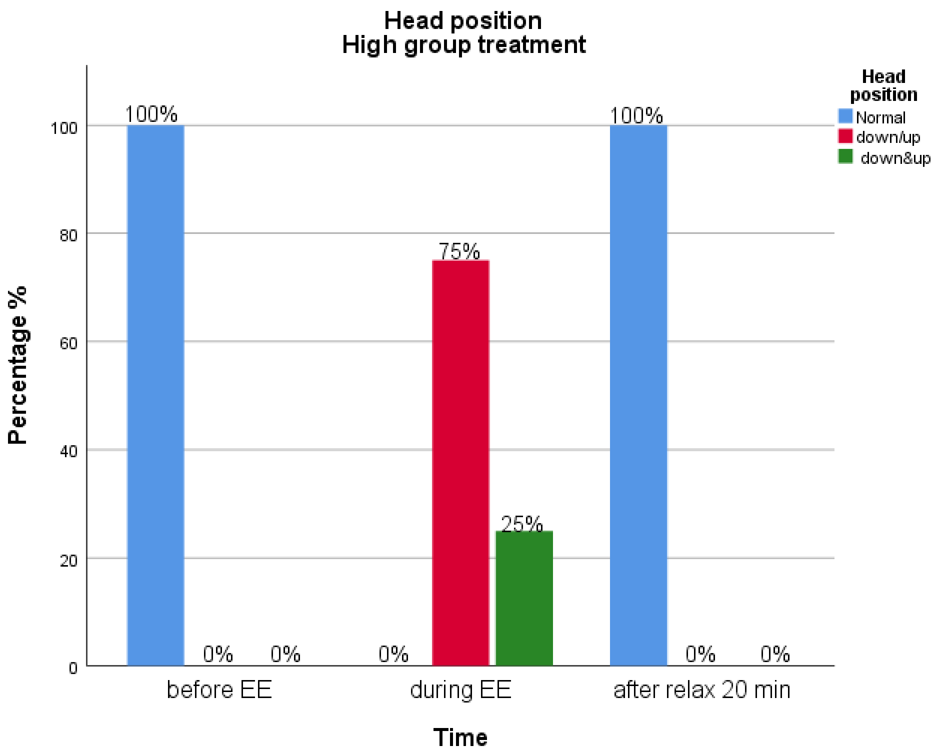

Figure 7: Percentage of bulls showing head position behaviour in High voltage group

About 75% of bulls showed salivation during EE procedure in low voltage group(≤ 6V) (**Figure 8**). Meanwhile, in high voltage group(>6V) 100% of the bulls had salivation during EE procedure (**Figure 9**).

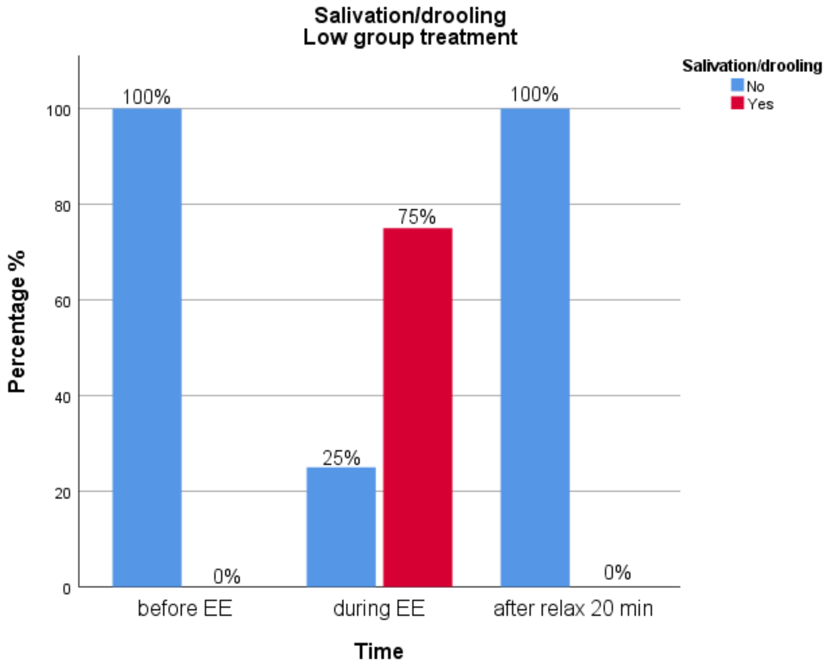

Figure 8: Percentage of bulls showing salivation/drooling in Low voltage group

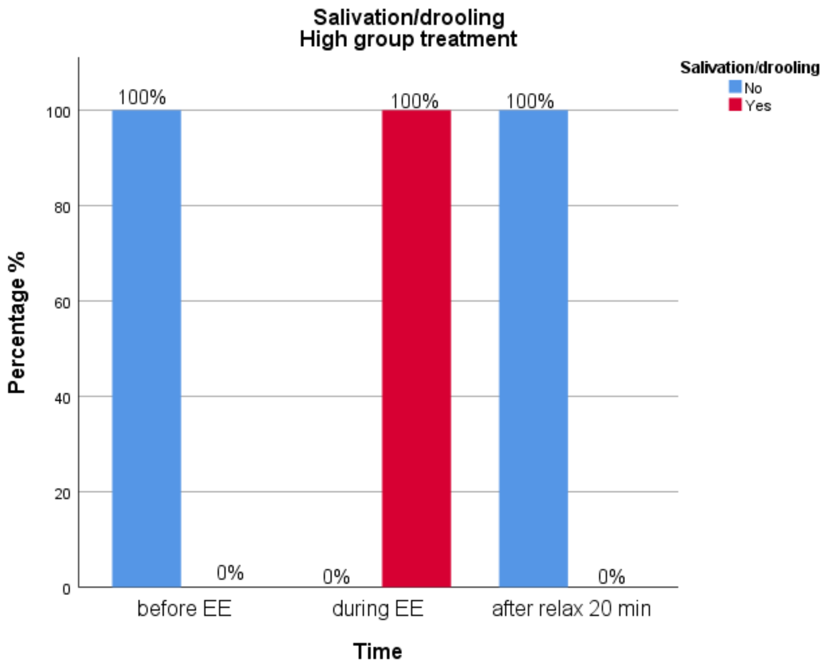

Figure 9: Percentage of bulls showing salivation/drooling in Low voltage group

Table 6 reveals there is strong correlation between Mean Ptot and Mean MF with arched back, animal struggle, , head position and salivation/drooling with 0.847, 0.735, 0.829, 0.844 and 0.784; 0.702, 0.704, 0.767, 0.798 and 0.683 respectively.

Table 6 Strength of correlation between total power (Ptot) and median frequency(MF) with behaviour changes.

Parameter	Arc back	Animal struggle	Head position	Salivation/Drooling
Mean Ptot	0.847*	0.735*	0.844*	0.784*
Mean MF	0.702*	0.704*	0.798*	0.683*

*Correlation significant at the level 0.01 level (2-tailed). MF=Median frequency

In general, result showed that the electrical stimulation from the electroejaculator caused a change in EEG responses and the behaviour for low and high voltage groups treatment.

5.0 DISCUSSION

Result of this study revealed that the EEG parameters of pain increased significantly during EE stimulation compared to baseline in both groups. Median Frequency (MF) and Total power (Ptot) changes are associated with noxious stimulation (Murrell et al 2003,2005; Haga and Ranheim, 2005). Kaka et al. (2015 & 2016) also found that MF and Ptot of EEG increase in response to noxious stimulus.. EEG directly measures the changes in the electrical activity of the cerebral cortex, which perceives pain (Johnson, 2007). This change in EEG spectrum is known de-synchronization of the EEG in response to noxious stimuli. Similar de-synchronization in EEG during noxious stimulation was also observed in minimal anaesthesia model that was applied to the other species like deer, sheep and rats (Slyvester et al., 2002; Woodbury et al., 2005). Thus, the results of this study are in line with previous studies in various species of animals and suggest that EE stimulation caused painful experience to the bulls.

Animals tend to show signs of discomfort and stress when they are subjected to any unfamiliar object or condition (Baiee et al., 2018). The discomfort signs that was reported in previous studies such as recumbent and arc back (Baiee et al. 2018), jumping and kicking (Small et al., 2019) and extension of hind limb (Wells et al., 1966). In this study, we recorded arched back, salivation, lying down, head moving up and down; and struggling when exhibited by the bulls in response to EE stimulation. Palmer (2005) has mentioned that intense muscle contraction, struggling, vocalization and occasionally recumbency is an indicative of discomfort during EE stimulation in bulls. Animal tends to show signs of discomfort and stress when they are subjected to any unfamiliar object or condition (Baiee et al., 2018). In this study, no vocalization were observed; Palmer (2005) has proposed that absence of vocalization does not mean there is no pain, and vocalization differences between individuals may be associated with EE technique. Painful procedure can be indicated with the ability to elicit vocalization which is consider as acute reaction, even though vocalization may not be expected since not all animal vocalizes in response to pain, unless it is severe pain (Palmer, 2005).

In present study, the bulls showed arched back, struggling and salivation during EE stimulation. These behaviour expressed by the bulls explained that bulls were in discomfort when electrical stimulation was applied. The arched back behaviour shown during EE stimulation in this study, have also been reported by Baiee et al. (2018) who found that 100% of the bulls tested showed arching of the back during EE stimulation. In the present study, both muscle spasm and struggling were observed during EE stimulation, some of the bulls expressed moderate reaction, but some shows more intense reaction. No bulls were observed recumbent during EE stimulation, this is in contrast with finding by Baiee et al. (2018) observed 16.67% of recumbency during EE stimulation. This happened may be due to EE technique or observation of recumbency by the assessors.

6.0 CONCLUSION AND RECOMMENDATION

From the findings of this study it is concluded that EE procedure was painful to the bulls as indicated by EEG and behaviour indicators. Future studies to consolidate the findings of this study with the stress hormone analysis along with EEG are recommended.

7.0 REFERENCES

Adcock, S. J., & Tucker, C. B. (2018). Painful procedures: When and what should we be measuring in cattle?. In *Advances in Cattle Welfare* (pp. 157-198). Woodhead Publishing.

Amann, R.P. (1986) How a bull works. Proceedings of the 11th NAAB Technical Conference on A.I. and Reproduction, Milwaukee, pp 6–18.

Ball L.(1986). Electroejaculation. In: Klemm WR (ed). *Applied Electronics for Veterinary Medicine and Animal Physiology*. Pp 395- 441. CCThomas.

Bath, G.F. (1998). Management of pain in production animals. Applied Animal and Behavioural Science, 59: 9-18.

Baiee, F., Haron, W., Yusoff, R., Ariff, O., Yimer, N., Hammadi, S., ... & Kaka, A. (2018). Modification of Electro-Ejaculation Technique to Minimise Discomfort during Semen Collection in Bulls. *Pakistan Journal of Zoology*, *50*(1).

Boussena, S., Bouaziz, O., Dehim, M.L., Hireche, S., Aimeur, R., Kasouia, R. 2013. The effects of electroejaculation on some physiological parameters (rectal temperature, respiratory and cardiac rates) in Ouled Djellal breed. Slovakian Journal of Animal Science, 46, 1, 16-21.

Brito, L. F. (2014). Bull development: sexual development and puberty in bulls. In: *Bovine Reproduction*. Hopper, R.M. (ed). ABS Global, Wisconsin, USA, pp 41-57.

Brodbelt, D. (2005) The confidential enquiry into perioperative small animal fatalities (cepsaf), a study of complications in small anaesthesia in the UK.

Paper presented at the Voorjaarsdagen International Veterinary Congress, Amsterdam.

Carter, P. D., Hamilton, P. A. & Duffy, J.H.(1990) Electroejaculation in goats. Australian Veterinary Journal 67, 91-93.

Chenoweth, P. J. and Kastelic, J. P. (2007). Clinical reproductive physiology and endocrinology of bulls. Youngquist, R. S and Threlfall, W. R. (Ed.). *Current Therapy in Large Animal Theriogenology* (2nd ed., pp. 221-228).

Dyce KM and Wensing CJG (1971). *Essentials of bovine anatomy.*Philadelphia: Lea & Febiger.

Damián, J. P., & Ungerfeld, R. (2011). The stress response of frequently electroejaculated rams to electroejaculation: hormonal, physiological, biochemical, haematological and behavioural parameters. *Reproduction in domestic animals*, *46*(4), 646-650.

Etson C.J., Waldner C.L., Barth A.D. 2004. Evaluation of a segmented rectal probe and epidural anesthesia for electro-ejaculation of bulls. Canadian Veterinary Journal, 45 (3), 235-240.

Falk AJ, Waldner CL, Cotter BS, Gudmundson J, Barth AD. 2001. Effects of epidural lidocaine anesthesia on bulls during electroejaculation. Canadian Veterinary Journal, 42 (2), 116-120.

Ganong WF.(1981) Review of Medical Physiology.pp 34.5. Lange Medical Publications.

Gibson, T. J., Johnson, C. B., Stafford, K. J., Mitchinson, S. L., & Mellor, D. J. (2007). Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal*, *55*(4), 152-157.

Gibson, T.J, Johnson, C.B., Murrell, J., Chambers, J., Stafford, K. & Mellor, D., 2009a. Components of electroencephalographic responses to slaughter in halothane anaesthetised calves: Effects of cutting neck tissues compared with major blood vessels. N. Z. Vet. J. 57, 84-89.

Gibson, T, Johnson, C., Murrell, J., Hulls, C., Mitchinson, S., Stafford, K., Johnstone, A. & Mellor, D., 2009b.Electroencephalographic responses of halothane anaesthetised calves to slaughter by ventral-neck incision without prior stunning. N. Z. Vet. J. 57, 77-83.

Gibson, T. J., Oliveira, S. E. O., Dalla Costa, F. A., & Gregory, N. G. (2019). Electroencephalographic assessment of pneumatically powered penetrating and non-penetrating captive-bolt stunning of bulls. *Meat science*, *151*, 54-59.

Gleerup, K. B., Forkman, B., Otten, N. D., Munksgaard, L., & Andersen, P. H. (2017). Identifying pain behaviours in dairy cattle. *WCDS Adv. Dairy Technol*, *55*, 231-239.

Harris, C., White, P. J., Mohler, V. L., & Lomax, S. (2020). Electroencephalography Can Distinguish between Pain and Anaesthetic Intervention in Conscious Lambs Undergoing Castration. *Animals, 10*(3), 428.

Haga, H. A., & Ranheim, B. (2005). Castration of piglets: the analgesic effects of intratesticular and intrafunicular lidocaine injection. *Veterinary anaesthesia and analgesia*, *32*(1), 1-9.

Haga, H. A., & Dolvik, N. I. (2005). Electroencephalographic and cardiovascular variables as nociceptive indicators in isoflurane-anaesthetized horses. *Veterinary anaesthesia and analgesia*, *32*(3), 128-135.

Imlan, J. C., Kaka, U., Goh, Y. M., Idrus, Z., Awad, E. A., Abubakar, A. A., ... & Sazili, A. Q. (2020). Effects of Slaughter Knife Sharpness on Blood Biochemical and Electroencephalogram Changes in Cattle. *Animals*, *10*(4), 579.

Johnston, G., Taylor, P., Holmes, M. & Wood, J. (1995) Confidential enquiry of perioperative equine fatalities (CEPEF-1): preliminary results. Equine Veterinary Journal, 27, 193–200.

Johnson, C. B., Wilson, P. R., Woodbury M.R., Caulkett, N. A. (2005). Comparison of analgesic techniques for antler removal in halothane-anesthetized red-deer(Cervus elaphus): electroencephalographic responses. Veterinary Anesthesia and Analgesia, 36: 273 – 279.

Johnson C.B., Sylvester SP, Stafford KJ, Mitchinson SL, Ward RN, Mellor DJ (2009): Effects of age on the electroencephalographic response to castration in lambs anaesthetized with halothane in oxygen from birth to 6 weeks old. Veterinary Anaesthesia and Analgesia 36, 273–279.

Johnson, C. B., Gibson, T. J., Stafford, K. J., & Mellor, D. J. (2012). Pain perception at slaughter. *Animal Welfare*, *21*(S2), 113-122.

Kaka, U., Hui Cheng, C., Meng, G. Y., Fakurazi, S., Kaka, A., Behan, A. A., & Ebrahimi, M. (2015). Electroencephalographic changes associated with antinociceptive actions of lidocaine, ketamine, meloxicam, and morphine administration in minimally anaesthetized dogs. *BioMed research international*, *2015*.

Kaka, U., Goh, Y. M., Chean, L. W., & Chen, H. C. (2016). Electroencephalographic changes associated with non-invasive nociceptive stimulus in minimally anaesthetised dogs. *Polish Journal of Veterinary Sciences*.

Killian G, Amann R.(1972). Reproductive capacity of dairy bulls. IX.Changes in reproductive organ weights and semen characteristics of Holstein bulls during the first thirty weeks after puberty.*J Dairy Sci*;55:1631–1635

Kongara, K., Chambers, J. P., & Johnson, C. B. (2010). Electroencephalographic responses of tramadol, parecoxib and morphine to acute noxious electrical stimulation in anaesthetised dogs. *Research in Veterinary Science*, *88*(1), 127-133.

Kumar, J. S., & Bhuvaneswari, P. (2012). Analysis of Electroencephalography (EEG) signals and its categorization–a study. *Procedia engineering*, *38*, 2525-2536.

Macmillan K & Hafs H.(1968). Gonadal and extra gonadal sperm numbers during reproductive development of Holstein bulls. *J Anim Sci*, 27:697–700.

Miller, S., Short, C. & Ekstro¨m, P. (1995b). Quantitative electroencephalographic evaluation to determine the quality of analgesia during anesthesia of horses for arthroscopic surgery. American Journal of Veterinary Research, 56, 374–379.

Mosure, W. L., Meyer, R. A., Gudmundson, J., & Barth, A. D. (1998). Evaluation of possible methods to reduce pain associated with electroejaculation in bulls. *The Canadian Veterinary Journal*, *39*(8), 504.

Murrell, J. C., Johnson, C. B., White, K. L., Taylor, P. M., Haberham, Z. L., & Waterman–Pearson, A. E. (2003). Changes in the EEG during castration in horses and ponies anaesthetized with halothane. *Veterinary Anaesthesia and Analgesia*, *30*(3), 138-146.

Murrell, J. C., White, K. L., Johnson, C. B., Taylor, P. M., Doherty, T. J., & Waterman-Pearson, A. E. (2005). Investigation of the EEG effects of intravenous lidocaine during halothane anaesthesia in ponies. *Veterinary Anaesthesia and Analgesia*, *32*(4), 212-221.

Murrell, J. C., Johnson, C. B. (2006). Neurophysiological techniques to assess pain in animals. J. vet. Pharmacol. Therap. 29, 325–335.

Ohl, D. A. (1993). Electroejaculation. *The Urologic Clinics of North America*, *20*(1), 181-188.

Nabors B., Linford, R. 2015. Anatomy of reproductive system of the bull. In*: Bovine Reproduction*. Hopper, R.M. (ed). Jonh Wiley & Sons Inc., Ames-Iowa, USA, pp 5-10.

Otto, K. A., Voigt, S., Piepenbrock, S., Deegen, E., & Short, C. E. (1996). Differences in quantitated electroencephalographic variables during surgical stimulation of horses anesthetized with isoflurane. *Veterinary Surgery*, *25*(3), 249-255.

Palmer, C. W. (2005). Welfare aspects of theriogenology: investigating alternatives to electroejaculation of bulls. *Theriogenology*, *64*(3), 469-479.

Pagliosa, R. C., DeROSSI, R., Costa, D. S., & Faria, F. J. (2015). Efficacy of caudal epidural injection of lidocaine, xylazine and xylazine plus hyaluronidase in reducing discomfort produced by electroejaculation in bulls. *Journal of Veterinary Medical Science*, *77*(11), 1339-1345.

Sneddon, L. U. (2018). Comparative physiology of nociception and pain. Physiology, 33(1), 63-73.

Sneddon, L. U., & Gentle, M. J. (2000). Pain in farm animals in Animal Welfare and Animal Health.

Sánchez-Barrera, I. C., Albarracin, W., & Rojas, M. J. (2014). Electroencephalographic spectrum power of sheep's brain after stunning. *Journal of Applied Animal Research*, *42*(1), 73-76.

Sabow, A. B., Goh, Y. M., Zulkifli, I., Sazili, A. Q., Kaka, U., Ab Kadi, M. Z. A., ... & Adeyemi, K. D. (2016). Blood parameters and electroencephalographic responses of goats to slaughter without stunning. *Meat science*, *121*, 148-155.

Sabow, A. B., Goh, Y. M., Zulkifli, I., Sazili, A. Q., Ab Kadir, M. Z. A., Kaka, U., ... & Ebrahimi, M. (2017). Electroencephalographic responses to neck cut and exsanguination in minimally anaesthetized goats. *South African Journal of Animal Science*, *47*(1), 34-40.

Stafford, K. J. (1995). Electroejaculation: a welfare issue. *Surveillance*, *22*(2), 15-17.

Zulkifli, I., Goh, Y. M., Norbaiyah, B., Sazili, A. Q., Lotfi, M., Soleimani, A. F., & Small, A. H. (2014). Changes in blood parameters and electroencephalogram of cattle as affected by different stunning and slaughter methods in cattle. *Animal Production Science*, *54*(2), 187-193. http://dx.doi.org/10.1071/AN12128

Sylvester, S. P., Johnson, C. B., Stafford, K. J., Ward, R. N., & Mellor, D. J. (2002). Effects of age on pain sensation following castration in lambs. In *Proc NZ Physiol Soc* (Vol. 21, p. 72).

Wells, M. E., Philpot, W. N., Musgrave, S. D., Jones, E. W., & Brock, W. E. (1966). Effect of method of semen collection and tranquilization on semen quality and bull behavior. *Journal of Dairy Science*, *49*(5), 500-503.

Whitlock, B. K., Coffman, E. A., Coetzee, J. F., & Daniel, J. A. (2012). Electroejaculation increased vocalization and plasma concentrations of cortisol and progesterone, but not substance P, in beef bulls. *Theriogenology*, *78*(4), 737-746.

Wolf F, Almquist J, Hale E. Prepuberal behavior and puberal characteristics of beef bulls on high nutrient allowance. *J Anim Sci* 1965;24:761–765

Woodbury, M. R., Caulkett, N. A., Johnson, C. B., & Wilson, P. R. (2005). Comparison of analgesic techniques for antler removal in halothane-anaesthetized red deer (Cervus elaphus): cardiovascular and somatic responses. *Veterinary Anaesthesia and Analgesia*, *32*(2), 72-82.