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Establishment of an anesthetic protocol for semen collection by electroejaculation in six-banded armadillos (*Euphractus sexcinctus* Linnaeus, 1758)

[*Estabelecimento de um protocolo anestésico para coleta de sêmen por eletroejaculação em tatus-peba (Euphractus sexcinctus Linnaeus, 1758)*]

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ABSTRACT

The aim was to verify the effects of different anesthetic protocols used during electroejaculation (EEJ) in six-banded armadillos (*Euphractus sexcinctus*). Four sexually matured animals were physically restrained and subjected to semen collection by the EEJ following three treatments: The control group consisted of no use of anesthesia; in the others, the anesthetic combinations xylazine/ketamine/propofol or butorphanol/ ketamine/propofol were administered. For each group, twelve procedures were conducted for EEJ. Semen was evaluated for volume, color, aspect, motility, sperm concentration, morphology, viability, and functional membrane integrity. The highest efficiency (100% ejaculates) was achieved when the control group was used; the xylazine/ketamine/propofol association provided only 11 ejaculates from a total of 12 attempts (91.6% efficiency), while only 4 ejaculates (33% efficiency) were obtained with butorphanol/ketamine/propofol ($P<0.05$). Both protocols provided rapid induction and relaxation enough to perform the EEJ. In the use of butorphanol/ketamine/propofol, the animals recovered at 16.5±1.5min, a time shorter than in the use of xylazine/ketamine/propofol protocol, 20.7±1.0min ($P>0.05$). The semen volume and sperm concentration obtained in the use of xylazine/ketamine/propofol association were significantly higher than those verified for butorphanol/ketamine/propofol protocol. In conclusion, the xylazine/ketamine/propofol association is indicated for anesthesia of six-banded armadillos submitted to EEJ.

Keywords: semen, Edentate, Xenarthra, electroejaculation

RESUMO

*Objetivou-se verificar os efeitos de diferentes protocolos anestésicos usados durante a eletroejaculação (EEJ) em tatus-peba (*Euphractus sexcinctus*). Quatro animais sexualmente maduros foram contidos fisicamente e submetidos à coleta de sêmen por EEJ, seguindo três tratamentos: o grupo controle consistiu do não uso de anestesia; nos outros, foram administradas combinações anestésicas de xilazina/cetamina/propofol, ou butorfanol/cetamina/propofol. Para cada grupo, foram conduzidos 12 procedimentos de EEJ. O sêmen foi avaliado para volume, cor, aspecto, motilidade, concentração de espermatozoides, morfologia, viabilidade e integridade funcional da membrana. A mais alta eficiência (100% de ejaculados) foi alcançada quando o grupo controle foi utilizado; a associação de cetamina/xilazina/propofol forneceu apenas 11 ejaculados de um total de 12 tentativas (de eficiência 91,6%), enquanto apenas quatro ejaculados (eficiência de 33%) foram obtidos com butorfanol/cetamina/propofol ($P < 0,05$). Ambos os protocolos forneceram rápida indução e relaxamento suficientes para executar a EEJ. Na utilização de butorfanol/cetamina/propofol, os animais se recuperaram em 16,5±1,5min, um tempo mais curto do que no uso de xilazina/cetamina/protocolo de propofol, 20,7±1,0min ($P>0,05$). O volume de sêmen e a concentração espermática obtidos no uso da associação xilazina/cetamina/propofol foram significativamente maiores do que os verificados para o protocolo butorfanol/cetamina/propofol. Em conclusão, a associação de cetamina/xilazina/propofol é indicada para anestesia de tatus-peba submetidos à EEJ.*

Palavras-chave: sêmen, Edentata, Xenarthra, eletroejaculação

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INTRODUCTION

The *Euphractus sexcinctus* Linaeus, 1758, popularly known as six-banded armadillo, seems to be resistant to environmental alterations induced by humans, being classified as a stable population (International…, 2015). Therefore, the studies conducted in this species could help us understand the sperm physiology of other threatened xenarthrans such as the giant armadillo (*Priodontes giganteus*) and the pink fairy armadillo (*Chlamyphorus truncatus*), and are also allowing the adaptation of reproductive techniques to conserve them (Santos *et al.,* 2011).

Initial studies showed that electroejaculation (EEJ) without using anesthesia is effective for obtaining semen from conscious six-banded armadillo; however, the animals manifested high stress levels showing intense averseness and vocalization (Serafim *et al.,* 2010; Santos *et al.,* 2011; Sousa *et al., 2013*). The establishment of an adequate anesthetic protocol could ensure immobilization and reduce pain and stress associated to EEJ, with a minimal interference in the semen quality, as reported for coatis (*Nasua nasua* – Barros *et al.,* 2009) and peccaries (*Pecari tayacu* – Souza *et al.,* 2009). Some factors such as the drug efficiency, safety, availability and costs, the existence of an antagonist (Silva *et al.,* 2004), and its influence on the ejaculatory response (Barros *et al.,* 2009) should be evaluated when choosing an anesthetic protocol. Special attention should be addressed to the last factor because anesthesia is suggested to impair sperm motility and concentration, and also promote urine contamination in the Southern three-banded armadillo, *Tolypeutes matacu*s (Herrick *et al.,* 2002).

Several drugs and combinations have been used for EEJ in mammals. The combination of xylazine and ketamine produces relatively safe and reliable short-term anesthesia, being widely used for wild carnivores EEJ (Deem, 2004). The butorphanol tartrate is a morphinan-type synthetic opioid analgesic (Wagver, 1999) reported for EEJ in the rhinoceros (Agil *et al.,* 2008). In addition, the propofol, an intravenous anesthetic agent that causes the stimulation of muscular relaxation, and contributes to the ejaculatory process (Souza *et al.,* 2009), is reported for EEJ in humans (Chung *et al., 1998*), cats (Chatdarong *et al.,* 2006), and peccaries (Souza *et al.,* 2009).

It is known that an anesthetic agent, alone or in combination, has not always exhibited the same response when used for different species or even among same individuals (Barros *et al.,* 2009). The aim of this study was to evaluate the effects of xylazine/ketamine/propofol or butorphanol/ketamine/propofol combinations on semen collection by EEJ in captive six-banded armadillos.

MATERIALS AND METHODS

The UFERSA ethics committee approved the animal care procedures adopted (Process no. 23091.002754/2011-99). A total of 4 sexually mature, six-banded male armadillos, weighing 3.0 ± 0.2kg and aging approximately 3.5 years old, were used in this experiment. The animals were captured from their natural habitat, being kept in captivity at the Center of Multiplication of Wild Animals from UFERSA, Brazil (5°10'S – 37°10'W; temperature range, 27 – 29°C). The individuals were housed in individual cages, and exposed to 11h natural photoperiod conditions. They were fed with dog chow pellets and fruits once a day, with free access to water.

Three restraint protocols for EEJ were tested in this experiment. The control group consisted of not using anesthesia; the mechanical restraint was conducted by two operators using appropriate gloves, as previously reported by Serafim *et al.* (2010). In the second protocol, the animals were mechanically restrained and further pre-medicated with an intramuscular administration of xylazine (1mg/kg; Rompun, Bayer, São Paulo, Brazil) and ketamine (7mg/kg; Ketalar, Pfizer, São Paulo, Brazil), followed by an intravenous administration of 5mg/kg propofol (Propovan, Cristalia, Fortaleza, Brazil) in their bolus. The last protocol consisted of the mechanical restraint added to a pre-medication with an intramuscular administration of butorphanol (0.4mg/kg; Torbugesic-SA, Zoetis, São Paulo, Brazil) and ketamine (7mg/kg), also followed by propofol administration.

Each animal was randomly subjected to three electroejaculatory sections for each protocol, totalizing 12 procedures per treatment. If animals showed signs of awakening, 1/4 of the respective

propofol dosage was administered. Armadillos were kept under fluid therapy (0.9 % physiological saline solution), and their body temperature, pulse, and respiratory frequency were monitored. The regaining of consciousness, together with the expression of normal behavior, was interpreted as an onset of anesthetic recovery.

The electroejaculatory sections were conducted at a 15-day interval. For the procedure, armadillos were fasted for 12h before the experiments began. In each occasion, they were placed in a dorsal recumbency (Fig. 1A), and the pubic region was cleaned. The same electroejaculation procedure was used for all animals and lasted for approximately 15min (Serafim *et al.,* 2010). An electroejaculator (Fig. 1B; Eletrojet®, Eletrovet, São Paulo, SP, Brazil) connected to a 12V source was used to deliver three stimulation cycles, with a 5min interval between successive cycles. The first cycle consisted of 10 stimuli of 2, 3, and 4 mA, in succession; the second was 10 stimuli of 3, 4, and 5 mA, sequentially; and the third was 10 stimuli of 5 and 6 mA. The probe of the electroejaculator presented 12.5cm for length, 1.0cm for diameter, and two longitudinal electrodes. Approximately 8cm of the probe was inserted into the rectum of each armadillo, which ejaculated into plastic tubes (Fig. 1C).

Figure 1. Procedure for semen collection in six-banded armadillo *Euphractus sexcinctus*: (A) *E. sexcinctus* anesthetized (arrow head) with penile exposure (arrow); (B) The electroejaculator (Eletrojet®, Eletrovet, São Paulo, SP, Brazil) and the rectal probe presenting longitudinal electrodes (Bar = 1cm); (C) Penis (black arrow) being conducted to plastic tubes (white arrow) for semen collection.

Ejaculates were immediately evaluated for color and aspect. The volume was determined by micropipettes (5 – 200µL). Sperm motility and vigor (strength of the sperm flagellum beating, 0 – 5 scale) were analyzed through light microscopy (× 100 and × 400). The sperm concentration (sperm × $10^6 mL^{-1}$) was determined using a Neubauer counting chamber. A slide stained with brome-phenol blue was prepared for the analyses of sperm viability and morphology under light microscopy (400 × and 1000 ×), counting 200 cells per slide. For the evaluation of sperm membrane functionality, a hypo-osmotic swelling test (Santos *et al.,* 2011) was performed immediately after the semen collection, using a citric acid and fructose hypo-osmotic solution (50mOsm/L). A total of 200 cells were evaluated through phase-contrast microscopy (× 400), and the presence of swollen coiled tails indicated a functional sperm membrane.

Data were expressed as mean ± standard error of the mean (SEM) and checked for normality by Shapiro–Wilk test, and for homoscedasticity by Levene's test using the univariate procedure of the Statistical Analysis System software (Statistical Analysis System, 1994). Data were analyzed by General Linear Model (GLM). The effect of different restraint protocols on seminal parameters was analyzed by Tukey test. To evaluate the efficiency of restraint protocols for semen obtaining, the Fisher's test was applied. For all statistical analysis, a significant difference of 5% was considered.

RESULTS

A total of 36 EEJ procedures were conducted for armadillo semen collection, being 12 for each restraint protocol. The highest efficiency, 100% ejaculates (12/12), was achieved without using anesthesia. In this treatment, all the animals were aversive to the electroejaculatory procedure and vocalized intensively, trying to run away during the procedure.

The xylazine/ketamine/propofol combination provided 11 ejaculates, showing a 91.6 % efficiency (11/12). Such results were significantly higher than those obtained with butorphanol/ketamine/propofol combination, which provided only 33.3% efficiency (4/12). Using this combination, one of the 4 animals did not ejaculate in any attempts.

Both anesthetic protocols provided rapid induction and relaxation enough to perform semen collection. Muscular contractions of the hind legs were observed in all of the protocols whenever electrical stimulation was performed. Penile erection was observed only in four procedures by using the butorphanol/ketamine/propofol combination, before semen collection, which occurred during the second phase (3 – 5 mA) of the EEJ procedure. With regard to the recovering time from anesthesia, it was significantly shorter in the use of butorphanol/ketamine/propofol, 16.5±1.5min, than in the use of xylazine/ ketamine/propofol, 20.7±1.0min (P>0.05).

All the ejaculates were translucent-white, viscous, and contained sperm. Urine contamination was not observed in any samples. Several sperm were agglutinated, resulting in head-to-head adhesions arranged in rouleaux formation. A pH closer to 9.0 was verified for all samples. The effects of restraint protocols on semen characteristics are reported in Tab. 1. Semen samples obtained with all treatments, in general, presented a good quality.

Table 1. Semen characteristics (Mean ± SEM) of captive six-banded armadillos (*Euphractus sexcinctus),* collected by electroejaculation without previous anesthesia, or using xylazine/ ketamine/ propofol or butorphanol/ketamine/propofol anesthetic protocols

Semen characteristics	Control (using no anesthesia) (n=12)	Xylazine/ketamine + propofol (n=11)	Butorphanol/ketamine + propofol (n=4)
Volume (µL)	279.1±48.6a	267.2±67.8a	43.7±18.1b
Sperm Motility (%)	65.8±7.1a	56.4±7.5a	60.0±15.5a
Vigor (0-5)	1.4±0.1a	1.3±0.1a	1.0±0.0a
Viability (%)	84.9±4.2a	47.7±6.2b	26.0±1.3b
Sperm concentration ($x10^6$ sperm/ml)	37.8±7.3ab	65.0±55.6a	6.7±3.7b
Functional membrane integrity (%)	59.6±5.9a	26.8±5.9b	21.2±2.6b
Morphology			
Normal (%)	89.2±1.48b	69.1±7.3a	74.7±4.3a
Primary defects (%)	0.4±0.18a	1.9±1.8a	0.0±0.0a
Secondary defects (%)	10.3±1.5b	26.0±3.9a	25.4±4.2a

Within a row, means without a common superscript differ (P< 0.05).

DISCUSSION

When EEJ was conducted in conscious six-banded armadillos, animals were aversive and emitted a "piglike squeal" similar to that manifested by individuals under a frightening situation (Clark, 1951), a behavior not observed when the anesthetic protocols were adopted. Vocalization is an easily observable indicator of distress and pain, useful in determining the most painful components of individual procedures (Watts and Stookey, 2000). Electroejaculation without anesthesia in humans is painful (Ohl, 1993) and, therefore, it may be assumed that it is painful to animals as well (Wendy *et al.,* 1998).

As a counterpoint, the efficiency of electroejaculation associated to the anesthetic protocols was lower than that verified in conscious armadillos. The efficiency, however, was dependent of the anesthetic combination employed, being the best results achieved in the use of xylazine/ketamine/propofol combination. The interference of anesthetic protocol on the EEJ efficiency was previously verified for chinchillas (*Chinchilla lanigera)*, in which 100% and 60% efficiency were described for conscious and anesthetized animals, respectively (Busso *et al.,* 2005).

When armadillos were pre-medicated with xylazine/ketamine, ejaculates were efficiently obtained (91.6 %) but penile erections were not observed. It is known that alpha-adrenergic agonists such as xylazine or medetomidine may enhance the emission of semen once muscles involved in semen emission have alpha-adrenergic innervation (Sjöstrand, 1965; Knight, 1974). On the other hand, when butorphanol–ketamine combination was used for armadillo's pre-medication, penile erection was observed, but a low efficiency was achieved (33.3 %) for semen collection. A similar observation was reported by Busso *et al.* (2005) when using ketamine for EEJ in chinchillas. In this species, penile erection was always evident even in animals that did not ejaculate. Authors suggest that ketamine exerts a more powerful inhibitory effect on the sympathetic than on the parasympathetic outflow in this species, a fact also noticed in rats (Chanacham and McGrath, 1976). This could be an explanation not only for the results observed in the armadillos used in this study, but also for the fact that the same drugs associated with distinct ones may present divergent results.

Even though recovering time was shorter when butorphanol/ketamine was employed ($P< 0.05$), we assume that both associations provided a quick recovery from anesthesia in six-banded armadillos. In the armadillo *Tolypeutes matacus*, the use of xylazine/ketamine combination promoted a recovery of 36.6 ± 11.8min (Osozco, 2011), which is higher than that found for *E. sexcinctus* in present study. Certainly, factors such as the species and the use of different ketamine doses – 30.9mg/kg (Osozco, 2011) could contribute to the discrepancy in the recovering times, thus justifying the requirement for the adequate species-specific establishment of pharmacological restraint protocols (Juvenal *et al.,* 2008). Moreover, such protocols should also be compatible with the objectives of immobilization, considering that animals must return to the physiological normality as quickly as possible (Green, 1982), especially in the case of xenarthras that present unique physiologic and metabolic characteristics.

Armadillo's semen characteristics found in the present study were similar to those previously reported by Serafim *et al.* (2010). In general, no effect of restraint protocol was verified on sperm motility or kinetic rating ($P> 0.05$), and values higher than 60% motile sperm without forward progression were achieved. The absence of sperm progression could be associated to the great viscosity of ejaculates and the formation of sperm rolleaux, as previously reported for the six-banded (Santos *et al.,* 2011) and the nine-banded armadillos (Schmidt, 1990). The use of anesthetic protocols containing butorphanol is reported for decreasing the *in vitro* sperm motility in humans (Xu *et al.,* 2013), but this side effect was not verified *in vivo* for armadillos. However, the semen volume, as well as the sperm concentration, was significantly lower when using butorphanol/ketamine combination in comparison to the xylazine/ketamine use. In fact, butorphanol can cause an intracellular inhibition of adenylate cyclase, thus there were closing of influx membrane calcium channels and opening of membrane potassium channels (Gear *et al., 1999*). It is known that drugs that blockage the calcium channels can reduce the contractility of the vas deferens and seminal vesicle (Kiguti and Pupo, 2012), thus affecting the semen volume or sperm concentration.

A negative effect on sperm viability, functional membrane integrity, and morphology was found using both anesthetic combinations for EEJ in armadillos. It is known that EEJ promotes osmotic alterations on ejaculates due to the overstimulation of accessory glands (Dooley and Pineda, 1986). We hypothesize that the use of anesthetic associations in armadillos could increase these osmotic alterations that impair the sperm membrane, interfering on its viability, and functional membrane integrity with also increasing the secondary morphological defects. However, further studies should be conducted in order to investigate if these alterations could impair sperm fertility.

CONCLUSION

In conclusion, we indicate the use of xylazine/ketamine/propofol combination for anesthesia of six-banded armadillos submitted to the EEJ procedure. This association provides a more efficient ejaculate achievement than butorphanol/ketamine/propofol combination, but both provide adequate semen parameters and an excellent recovering time from the anesthesia.

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