



## Histological evaluation of sheep ovarian tissue after laparoscopic partial ovariectomy



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### ABSTRACT

In the present study, there was assessment of the damage to tissue caused by partial laparoscopic ovariectomy using bipolar forceps in sheep. Fragments of ovaries of six sheep were removed using bipolar forceps by making a transverse section in the middle third of the organ *via* three-portals that were made using laparoscopy. The fragments were subjected to standard histological examinations and the lesions attributed to the procedure were investigated using an optical microscope and Image J software. The results were assessed using an analysis of variance and the Tukey test. All the laminae had minimal tissue damage. The mean amount of highly damaged tissue was 1.8%, and of partially damaged tissue was 5.6%. The mean total area of healthy tissue in the fragments was 94.4%. The results of the study indicate this procedure can be conducted with very little tissue damage occurring. The use of this procedure, therefore, can be incorporated in future reproductive studies without altering the functions of the *in situ* ovarian tissues.

### 1. Introduction

There is an ongoing need to develop less invasive procedures for use in animals, because pain can affect animal welfare and lead to production problems (Fitzpatrick et al., 2006). Conventional surgical procedures can harm animals, leading to problems such as adhesions and fibroses (Piatek et al., 2015) that in turn necessitate longer surgical operations and consequently greater pain is associated when surgeries occur when these conditions prevail (Teixeira et al., 2011a). Laparoscopic procedures can reportedly

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reduce surgical trauma, because in addition to involving shorter surgery times these procedures are associated with less pain, less postoperative stress, and result in more efficient recovery from surgery (Teixeira et al., 2011a), allowing the animal to return to production more quickly (Bouré, 2005).

Ovariectomy is performed in several different species and can involve total or partial organ removal. Surgeries are often conducted for the removal of ovarian tumors. Surgeries also occur for production purposes (Carvalho et al., 2010) with preservation of reproductive tissues so that animals on which surgeries are performed can continue reproduce after the procedures are conducted (Dittrich et al., 2014; Courbiere et al., 2016). Surgeries of this type can also be conducted for therapeutic purposes in the case of biopsy transplants aimed at restoring fertility after cancer treatment (Dittrich et al., 2015). The first techniques described for preserving or for fertility recovery after ovarian surgeries, were surgeries performed for testicular germ-cell transplants (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). The preservation of female reproductive capacity also occurs when using ovarian tissue transplantation (Pacheco and Oktay, 2017; Leonel et al., 2018), cryopreservation (Yao et al., 2017; Vatanparast et al., 2018), and when there is a combination use of both techniques (Donnez et al., 2013; Campbell et al., 2014). Many obstacles to the development of this technique, however, remain.

Ovariectomies using endo-surgery commonly involve removal of the entire organ, or there is not sufficient care taken to avoid damage of the remaining *in situ* tissue during surgery which often results in significant tissue damage. There are not reports in the literature regarding the possible damage to tissue in cases of partial removal of ovarian tissue. This information is important because the removal of ovarian fragments is sometimes recommended for biopsies, culture, cryopreservation, or transplantation. It is essential that ovarian fragments collected using surgical procedures contain preserved primordial follicles because if this is not the case the surgeries did not result in a positive outcome.

In the present study, tissues collected when there was partial ovariectomies performed using laparoscopy with bipolar forceps were assessed to evaluate the percentage of tissue damage caused by imposing the technique. Furthermore, there was assessments of how much of the remaining *in situ* tissue was suitable for use when other reproductive or diagnostic techniques were conducted.

## 2. Materials and methods

### 2.1. Animals and ethics

The study was approved by the Animal Ethics and Welfare Committee of Pará Federal University, Brazil (protocol number 7816250216). Six pubescent Santa Inês sheep were used. The sheep were less than 3 years old and had not undergone any previous reproductive surgical procedures. Ewes were maintained at the university's veterinary hospital as a group in 16 m<sup>2</sup> pens, where they received daily rations of elephant grass as forage, commercial feed (Presence® sheep maintenance brand), mineral supplementation, and water *ad libitum*. Prior to intervention the sheep were adapted to the pens for a period of up to 30 days. A partial ovariectomy was performed by trained veterinarians with there being adaptations of procedures described by Teixeira et al. (2011b) and Barros et al. (2015) (adapted).

### 2.2. Preoperative preparation and anesthetic protocol

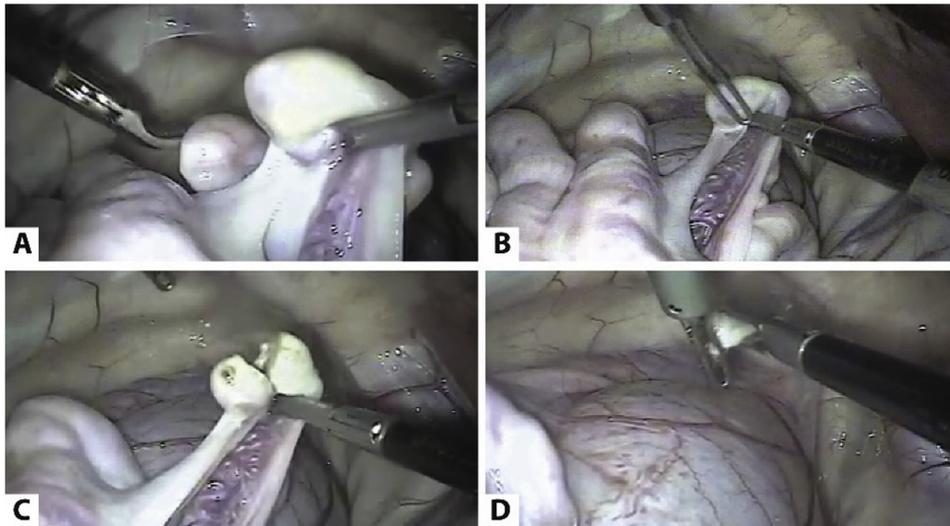
After not allowing water and food consumption for 36 h, the animals were pre-medicated with 0.1 mg/kg xylazine hydrochloride (intramuscular; Xilazin® Syntec, Brazil). After 10 min, anesthetic induction was performed with 6 mg/kg of propofol (intravenous; Propovan® Cristalia, Brazil). After induction, the animals were intubated using an 8-mm cuffed endotracheal tube to provide 100% oxygen, and there was isoflurane-based inhalation anesthesia used (Isoforine® Cristalia, Brazil.).

### 2.3. Laparoscopic partial ovariectomy for biopsy

Laparoscopy was performed through three portals. The first procedure was conducted using the open technique (Bouré, 2005). A pneumoperitoneum was induced and maintained using 5 L/min of CO<sub>2</sub> infusion into the peritoneal cavity and the intra-abdominal pressure was maintained between 5 and 8 mm Hg. A rigid endoscope was placed in the first trocar (10 mm) to video-assist the placement of the other portals.

The second 5-mm trocar was positioned laterally to the left side and caudally, using video-assistance. The same strategy was used for the third portal which was also 5 mm and was placed in the right antimere. Babcock forceps were inserted through the second portal and a second pair of Babcock forceps was inserted through the third portal which facilitated manipulation of the uterus, uterine horns, ovarian bursae, and the individual ovaries.

Once grasped, the ovary was positioned to enable visualization (Fig. 1a). Cutting and bipolar coagulation forceps (Tripol PowerBlade®, Wem & Vivamed, Ribeirão Preto, São Paulo, Brazil) insertion occurred through the second portal. The organ was secured in the middle with the bipolar forceps (Fig. 1b), and intraabdominal removal of half of the ovary was performed (Fig. 1c). The cutoff button was pressed 1 s after the coagulation pedal was activated. The ovarian fragment was totally severed from the remaining tissue (Fig. 1d) then withdrawn through one of the laparoscopic portals. Lastly, the pneumoperitoneum was reversed, and the closure of the surgical portal incisions was performed.



**Fig. 1.** Videolaparoscopic image of the abdominal cavity of the sheep, showing: (A) Ovary selected for partial ovariectomy; (B) Ovary before cauterization with bipolar forceps; (C) Ovary after partial ovariectomy with bipolar forceps without hemorrhages and (D) Removal of completely missing ovarian fragment.

#### 2.4. Histological analysis

After partial ovariectomy the fragments were immersed in 10% formalin solution for processing. The samples underwent histological preparation in the laboratory, during which tissues were clarified in xylose and embedded in paraffin for sectioning and staining with hematoxylin-eosin (HE). Selected photomicrographs of each sample were analyzed using Image J software (Collins, 2007), and histological measurements were taken and compared. The lengths of these areas (in millimeters) were analyzed based on the extent of damage in relation to the total length of the tissue.

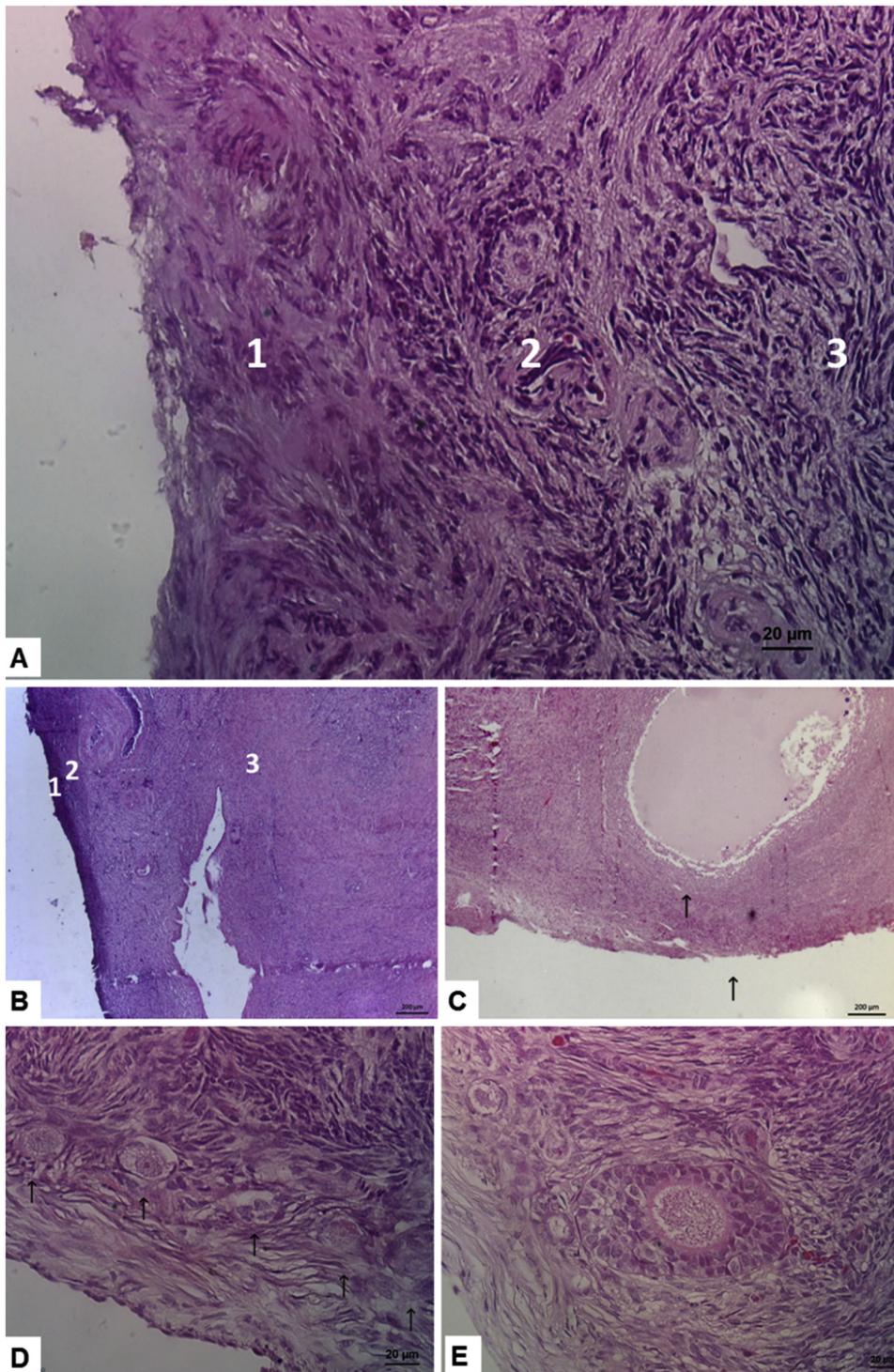
The analyzed tissues were classified as highly damaged, partially damaged, or unaltered (Fig. 2a). The highly damaged classification was recorded when there was a coagulation necrosis region corresponding to the area in immediate contact with the bipolar forceps during the procedure, in which the function of the cells was completely impaired. The partially damaged classification was recorded when there was a region with swelling and intercalated cell degeneration with necrotic coagulation cells (area of intersection between totally injured and completely healthy tissue). Tissues were classified as unaltered if there was no impairment to the structure of the cells. To evaluate the state of preservation and the feasibility of the use of the tissue for reproductive techniques, the total number of preantral follicles in a single histological section (3–5 $\mu$ m) of the fragment removed from each sheep was determined. The lengths of these regions were measured and compared with the total size of the tissue on the slide. Each damaged portion of the tissue was also characterized descriptively in terms of its histological characteristics.

#### 2.5. Post-operative analysis

After full recovery, the sheep were returned to the pens in which they were maintained pre-surgery, where their pain in the first 6 h was evaluated using a behavioral scale described by Teixeira et al. (2011a). In cases where sheep had behavioral responses indicating they were in pain, rescue analgesia was performed using flunixin meglumine in the amount of 2 mg/kg which was administered intravenously (Banamine® MSD Animal Health, Brazil). Sixty days after the surgery all sheep underwent a further exploratory laparoscopy to macroscopically examine possible lesions in the internal genitourinary tract and the formation of adhesions potentially caused by the previous surgical procedures. After 1 year, ultrasonography monitoring was performed for 16 days to evaluate the functioning of the remaining half ovary. The follicles were classified as small (2–4 mm), medium (5–7 mm), or pre-ovulatory (> 8 mm).

#### 2.6. Statistical analysis

The length of the removed tissue and the highly damaged, partially damaged, and unaltered tissue lengths were recorded in millimeters and percentages, and summarized as means and standard deviations. The pain assessment values were also summarized as means and standard deviations, as were the preantral follicle counts. The values for measurements of the total removed tissue length and the highly damaged, partially damaged, and unaltered tissue lengths were tested for normality of distribution and subjected to statistical analysis with the Friedman test using the BioEstat 5.0 statistics software (Ayres et al., 2007).



**Fig. 2.** Histological sections of sheep ovary after biopsy with bipolar forceps showing (A) - (1) highly damaged cells in the surgical margin, (2) partially damaged tissue and (3) unaltered tissue; (B) Damage tissue proportion in (1) highly damaged tissue representing 1.8%; (2) partially damaged tissue representing 5.6% and (3) unaltered tissue representing 94.4% of the fragment, and (C) the bipolar clamp section performed close to the preovulatory follicles (small arrows) and (D) and (E) presence of preantral follicle preserved (small arrows).

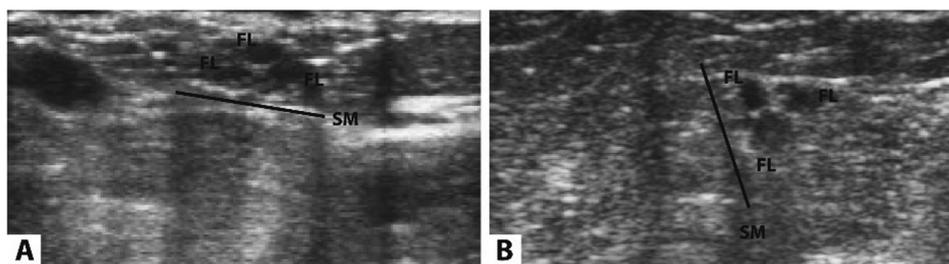


Fig. 3. Ultrasonography 1 year after partial ovariectomy in sheep using bipolar forceps – (A) and (B) Half ovary with surgical margin (SM) and presence of follicles (FL).

### 3. Results

There were no unforeseen circumstances that arose during the time the procedures was conducted, and no hemorrhaging during the sectioning and coagulation of ovarian tissue (Fig. 1c). The mean total length of the biopsy was  $5.721 \text{ mm} \pm 1.924 \text{ mm}$ . The mean length of highly damaged tissue was  $0.106 \text{ mm} \pm 0.066 \text{ mm}$ , and the mean length of partially damaged tissue was  $0.324 \text{ mm} \pm 0.153 \text{ mm}$ . The area of highly damaged tissue corresponded to a mean of 1.8% of the total ovarian fragment that was collected ( $P < 0.05$ ), and the partially damaged tissue corresponded to a mean of 5.6% ( $P < 0.05$ ) of the tissue fragment, thus there was a mean of 94.4% of unaltered tissue in the analyzed fragments (Fig. 2b).

In the surgical margin, the tissue had considerably more damage, with retracted nucleus cells and coagulative necrosis (highly damaged; Fig. 2a and b). There was no highly damaged tissue in two sheep in which there was only partially damaged and unaltered tissue. The partially damaged tissue was characterized by swollen cells intercalated with unaltered cells. In two sheep, bipolar clamp sectioning was performed close to the preovulatory follicles, and morphology of these follicles was altered as a consequence of the surgery to only a minor extent (Fig. 2c).

All the removed fragments had preserved preantral follicles (Fig. 2d and e), even in tissue areas close to the surgical margin. The mean number of preserved follicles in the fragments was  $32.8 \pm 26.8$ . None of the sheep required rescue analgesia. In the immediate post-operative period the sheep ate and ambulation was normal, and there were no symptoms of discomfort. Sixty days after surgery the remaining ovarian tissue had little or no adhesions and all indications were that follicular and corpora lutea development were unaffected by the surgical procedures. One year after the surgery the residual ovary halves *in situ* remained functional. There were small, medium, and pre-ovulatory follicles (Fig. 3a and b), and corpora lutea present that indicated the surgical procedures had no effect on ovarian follicular and corpora lutea development and occurrence of ovulation.

### 4. Discussion

Ovariectomy using bipolar forceps is commonly performed in dogs and cats (Gauthier et al., 2014; Spillebeen et al., 2017), and has also been reported in sheep (Barros et al., 2015). There, however, have been no studies investigating the morphology of ovarian tissues following partial ovariectomy or biopsies using this technique. While many techniques are used to perform ovariectomies or biopsies in animals, if the surrounding tissue is to be preserved it is important to use cutting instruments that do not cause harm. Accordingly, bipolar forceps are not normally recommended for this procedure as it is believed the use of these instruments can cause thermal trauma (Courbiere et al., 2016; Katić and Dupré, 2017). Notably, however, in the present study a mean of only 1.8% of the tissue collected had thermal trauma damage. The results of the present study indicate that the protocol used allowed for preservation of the preantral follicles, theoretically enabling the subsequent use of these techniques for *in vitro* fertilization.

All the laminae had tissue damage, but with a negligible amount of organ function impairment. In previous studies where there was use of the techniques that were utilized in the present study where there were ovariectomy procedures using bipolar forceps, the tissue sectioning was performed away from the ovaries to avoid high energy dissipation in the adjacent tissues (Teixeira et al., 2011b; Barros et al., 2015). In the present study, however, even on the surface of the ovary where sectioning occurred, there was only damage to a narrow strip of tissue, possibly as a result of simultaneous blood coagulation at the surgical margin.

Removal of the entire organ is common in studies involving the use of the ovaries for transplants or cryopreservation (Campbell et al., 2014; Leonel et al., 2018), to avoid bleeding or tissue damage. The results of the present study indicate that it is possible to perform a partial ovariectomy while preserving the tissue and maintaining the typical tissue histological and follicular characteristics. Furthermore, the *in situ* tissue of the ovary on which surgery was performed remained functional when evaluated after 60 days and 1 year.

In the present study, the use of bipolar forceps induced little adhesion formation after partial ovariectomy, which is a significant result. The use of conventional ovariectomy procedures often lead to a large number of adhesions or at least the formation of fibrous bands that bind the adjacent tissues (Piatek et al., 2015) and in some cases may even cause abnormalities in the uterine region (Gauthier et al., 2014). The maintenance of ovarian functions after the surgery in the present study as evidenced by the presence of preovulatory follicles that were identified using ultrasonography confirms that the technique has considerable potential in the preservation and maintenance of fertility after its use. This renders the technique viable for use in patients with ovarian failure caused by diseases, surgical procedures, or therapeutic procedures that impair reproductive efficiency resulting in fertility problems (Donnez

et al., 2013) such as ovarian cysts and endometriosis, and even chemotherapy and radiotherapy in cancer treatment.

The absence of surgical complications such as hemorrhages or pain is also an important characteristic when conducting biopsy procedures, and indicates that such techniques can be more widely used for biopsies of other organs (Petre et al., 2012), especially those that are highly vascularized as are the ovaries. The use of the protocol for the present study was effective because there were no hemorrhagic episodes during the procedure. Similar to other studies where there was use of such procedures, there was not discomfort of the animals post-surgery.

In the present study, there was use of the partial ovariectomy technique using bipolar forceps to facilitate the surgical procedure. After conducting the surgery, there was no hemorrhaging or formation of adhesions, and there were negligible amounts of tissue lesioning and impaired reproductive tissue function. The results of the current study may prompt future studies involving cell culture and the use of ovarian tissues for grafting, transplantation, *in vitro* fertilization, and cryopreservation.

### Conflict of interest

There is no conflict of interest.

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