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# Ovum Pick-up Technique in Recently Weaned Ewe Lambs Subjected to Ovarian Stimulation

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## ABSTRACT

***Background*:** The purpose of this study was to evaluate the effectiveness and possible complications of the laparoscopic ovum pick-up (LOPU) technique and to optimize ovarian stimulation in recently weaned ewe lambs.
***Materials, Methods & Results*:** Thirty-six healthy lambs, aging 4-8 weeks, were used in this study and divided into six different groups of six animals. Intravaginal implants of medroxyprogesterone were used for follicular wave induction, and parenteral equine chorionic gonadotropin (eCG) and follicle stimulating hormone (FSH) were administered for ovarian stimulation. Ewes from group GCN did not receive any treatment. In group GCI, the animals received only intravaginal implants. For other groups, ovarian stimulation was carried out as follows: in Group G80U, ewes were treated with a single dose of 80 mg of FSHp; in Group G80M, ewes received 4 administrations of 20 mg FSH, in Group G160U, they were treated with a single dose of 160 mg FSH; and in Group G160M they received 4 administrations of 40 mg FSH). Quantitative and qualitative assessment of retrieved oocytes were carried out after LOPU. *In vitro* maturation (IVM) of oocytes was also quantitative and qualitatively analyzed. Surgical procedure was divided into 6 stages and overall and stage surgical times were assessed. Post-operative pain and plasma fibrinogen were also assessed. The mean overall surgical time was 24.3 ± 5.2 min. Post-operative pain scores and plasma fibrinogen did not differ significantly from baseline values ($P > 0.05$). The number of oocytes recovered from GCN, GCI, G80U, G80M, G160U and G160M were 1.1 ± 1.6, 1.1 ± 1.8, 4.6 ± 3.2, 2.2 ± 0, 8, 5.8 ± 3.1 and 3.0 ± 2.0, respectively, and the rates of viable oocytes retrieved were 57.0%, 14.2%, 51.7%, 18.0%, 70.2% and 33.3%, respectively.
***Discussion*:** In spite of the risk of anesthesia, the incidence of regurgitation and rumen distention was low, which were reported as mild complications. The risk of reduced lung volume, hypoxia, increased intrathoracic and vascular pressure and increased cardiac stress were managed using assisted ventilation and short duration of the surgical procedure. No iatrogenic damage to the viscera occurred. The blind insertion of the first trocar without previous pneumoperitoneum as performed in the current study was safe and optimized surgery. Adhesion formation was not observed in the current study. The formation of adhesion could have been prevented by careful manipulation of the reproductive tract and removal of clots from the ovarian surface with 0.9% NaCl solution. The excellent recovery of the animals and the absence of mild/severe pain were attributed to the minimally invasive nature of the laparoscopic procedure. The average number of visualized and aspirated follicles, retrieved oocytes and the oocyte recovery rates for G80U and G160U were similar to those reported by studies in adult sheep. An extremely low IVM rate has been reported in sheep. In the current study, the use of 160 mg of FSH in a single dose (G160U) obtained similar cytoplasmic and nuclear maturation results to those observed in adult sheep. It is thus suggested that weaned ewe lambs may require high concentrations of exogenous FSH to produce a higher number of better quality follicles and to achieve superior *in vitro* maturation rates. Thus, the groups treated with a single-douse of 80 mg of FSH or on a fractional administration regimen possibly did not achieve sufficient serum levels for adequate follicular growth. The best results including IVM were observed for the G160U group, which showed promising commercial viability.

**Keywords:** follicular puncture, laparoscopy, pre-pubertal sheep.

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## INTRODUCTION

The sheep industry has grown worldwide in the last decades and reproductive biotechnologies, such as *in vitro* production (IVP), are considered to be the main reason for such increase [12,35]. Ovum pick-up (OPU) maximizes the production of embryos and improves the rate of pregnancies in comparison to other conventional breeding techniques. It can be applied to acyclic or seasonal anestrous ewes, pregnant ewes and even ewe lambs. Moreover, OPU contributes to the development of a wide variety of emerging biotechnologies, such as cloning and transgenesis [2,4,6,18].

The high efficiency of laparoscopic ovum pick-up (LOPU) associated to hormonal ovarian stimulation protocols is attributed to the minimal invasiveness of such surgical procedure, which allows several repeated follicular aspirations to be performed at short intervals. Even though this technique has been employed for some years [33, 36,40], the surgical stages of LOPU and their complications have not been meticulously assessed, nor have their relationship to postoperative pain been analyzed. Moreover, there is great disagreement about the best time to perform oocyte retrieval and which is the most efficient ovarian stimulation protocol. Thus, the aim of this study was to evaluate the LOPU technique and to establish the optimal protocol for ovarian stimulation using eCG (Equine Chorionic Gonadotropin) and FSH (Follicle Stimulating Hormone) in recently weaned ewe lambs.

## MATERIALS AND METHODS

### *Animals*

The current study was approved by the Ethics Committee for Animal Use from the Faculty of Agrarian and Veterinary Sciences, University of São Paulo State, Brazil (Universidade Estadual Paulista - UNESP). The ethical principles of the European Commission for experiments involving animals (Directive 83/609EEC) were also followed.

Thirty-six 4 to 8 weeks old Santa Ines ewe lambs with average body condition score of 3.0 (1 to 5 scaling system [20]) and average weight of 12.3 ± 3.4 kg were used. Prior to the study, animals were subjected to a general clinical examination and systemic evaluation of the reproductive tract and were considered to be healthy.

Animals were randomly distributed into six groups (n = 6): two control groups, of which received no hormonal stimulation for follicular wave induction or ovarian stimulation (GCN) and one which received follicular wave induction alone (GCI); two groups receiving 80 mg FSH1, as a single dose injection (G80U) or as 4 applications of 20 mg FSH on a 12-h interval (G80M); and two groups receiving 160 mg FSH, as a single dose injection (G160U) or as 4 applications of 40 mg FSH on a 12-h interval (G160M).

In all animals, except for those of the GCN group, follicular wave was induced prior to ovarian stimulation by 60 mg medroxyprogesterone acetate1 intravaginal implants (MAP Progespon™)[1], inserted on a random day of the estrous cycle (Day 0) and which remained intravaginaly for 6 days. On Day 5, 300 IU of eCG[1] was administered intramuscularly. This protocol was used to induce a follicular wave 36 h after the withdraw of the progesterone releasing device and to induce new follicular waves 12 h after that initial wave [33]. Ovarian stimulation was performed 48 h after progesterone sponge removal using 300 IU eCG alone or combined to FSH according to the experimental group.

In order to eliminate the variable *moment of LOPU*, a previous evaluation was carried out using two groups of four animals equally weaned and of the same age as those used in this study. These animals were subjected to LOPU and B-mode ultrasound 48 h after the start of ovarian stimulation and 60 h after the start of the protocol.

### *Laparoscopy for ovum pick-up (LOPU)*

Following 36 h of food and water fasting, animals were premedicated with 2 mg/kg tramadol[2] intramuscularly and after 15 min, anesthesia was induced using an intravenous bolus of 6 mg/kg propofol[2]. Anesthesia was maintained using isoflurane[2] vaporized in 100% oxygen, in a semi-closed system, following endotracheal intubation with 6 mm cuffed tubes.

The abdomen was aseptically prepared for surgery and 0.4 mL of lidocaine hydrochloride (Lidovet™)[3] was injected (0.2 mL subcutaneously and 0.2 mL intramuscularly) at the trocar incision sites.

Animals were placed in dorsal recumbency followed by a 45º head-down tilt (Trendelenburg position). A small skin incision was made 10-15 cm cranial to the udder and 5 cm to the right of the midline for blind insertion of the first trocar (5 mm) with insuffla-

tion valve. $CO_2$ pneumoperitoneum was established and intra-abdominal pressure set to 5 mmHg, using a 5 L/min insufflation rate. A 5 mm 0º angled laparoscope connected to a video camera and a fiber optic cable was inserted through the first trocar for a brief abdominal exploration. Subsequently, the second trocar (5 mm) was inserted symmetrically to the first one using the video-assisted technique. The third trocar (5 mm) was placed on the midline, 20 cm cranial to the udder. The rigid endoscope was placed in the midline trocar, in order to create the required triangulation. Two 5 mm Babcock laparoscopic forceps were inserted through the first and second trocars for atraumatic manipulation of the uterus, fallopian tubes, ovarian bursa and ovaries. The gonads were stabilized by gentle grasping of the mesovarium so as to avoid collateral damage.

Prior to follicular aspiration, the ovaries were examined and the number of follicles of 2 to 8 mm in diameter was recorded. The aspiration needle was inserted in the cavity through the abdominal wall near the ovary and the multiple punctures performed by moving the ovaries in different positions using the atraumatic forceps. The needle was initially placed parallel to the ovarian surface, which allowed the puncture of follicles at the gonad's edge. If such positioning was not possible, the punctures were then performed perpendicularly.

Once inserted into the follicle, the needle was carefully moved to ensure that all contents were aspirated. The vacuum system was set to the maximum aspiration pressure of 50 mmHg. A single lumen aspiration circuit consisting of an 18G short bevel needle connected to a 50 cm long cannula attached to a collection tube (50 mL) through a silicone stopper, was used. Vacuum was produced by a vacuum pump (500 BRS)[4] equipped with a sphygmomanometer. Prior to oocyte aspiration, ovaries were rinsed with phosphate-buffered saline (PBS) containing heparin and, at the end of this procedure, approximately 2 mL of the medium was left within the aspiration circuit to retrieve the oocytes.

At the end of the aspirations, ovaries were rinsed with 10 mL of sterile normal saline to remove clots from the ovarian surface in order to avoid the formation of postoperative adhesions. The trocars were withdrawn, the pneumoperitoneum was completely drained and skin sutures were performed using 2-0 nylon thread in an interrupted horizontal mattress (Wolf) pattern. No suture was applied to the muscular or subcutaneous layers. The surgical wound was treated with povidone-iodine followed by the application of a repellent ointment around the wound.

*Intraoperative assessment*

The intraoperative period was divided into the following stages: beginning of surgery (BS), primary trocar insertion (T1), secondary and tertiary trocar insertion (T2/3), manipulation and aspiration of the first ovary (MA1), manipulation and aspiration of the second ovary (MA2), pneumoperitoneum drainage and skin suture (S). Complications, technical concerns, time for completion of each step and total surgical time were recorded.

*Postoperative assessment*

Following surgery, animals were placed in a clean and quiet environment and observed until they were able to stand up unassisted. Behavioral pain assessment was evaluated during the first 6 h after surgery [24,34]. The evaluation consisted of a score for kyphosis, spontaneous movements and food intake. Animals scored 0 (no signs of pain), 1 (minimal/mild pain) or 2 (severe pain) for each of three variables evaluated. The final score consisted of the sum of the scores obtained for each variable.

Systemic inflammatory response and surgical wound healing were also evaluated. Blood was sampled by jugular puncture immediately after and at 2, 4, 6, 8, 10, 12, 14 and 16 days following laparoscopy to measure plasma fibrinogen (mg/dL), using a manual refractometer.

A quarter (n = 9) of the operated animals were subjected to a second exploratory laparoscopy in order to assess whether any lesions or adhesions were present on the internal reproductive organs following the LOPU procedure.

*Ovum pick up and* in vitro *maturation analysis*

The number of visible follicles (VF), aspirated follicles (AF) and retrieved oocytes (RO) were recorded and the average of these values and the oocyte retrieval rate (percentage of RO / AF - RR) compared amongst groups.

Retrieved oocytes were stocked in collection medium (PBS supplemented with 10 IU/mL heparin at 36°C) and, in the laboratory, the aspirated liquid was carefully transferred to Petri dishes and observed under

a stereomicroscope at 40X magnification. Once visualized, oocytes were placed in a plate containing 300 to 500 μL of washing medium (TCM 199, antibiotics, pyruvate, fetal bovine serum and HEPES buffered saline solution) and classified according to their quality into stages I, II, III or degenerated [22,26]. Only oocytes in stages I to III with bright cytoplasmic staining and uniform aspect were considered viable.

Oocytes received three 100 to 200 μL droplets of washing medium before being incubated in 100 μL droplets of maturation medium under mineral oil in a 5% $CO_2$ humidified chamber at 39ºC, for 24 h [9]. Oocytes were transferred to a drop of Hoechst 33342 dye diluted in glycerol, placed between a slide and cover slip, sealed with varnish and analyzed under microscopy to determine the stage of nuclear and cytoplasmic maturation. *In vitro* maturation rates were compared between groups.

Classification of nuclear maturation stages was based on the morphology of the DNA and the stage of chromosome condensation, in: (1) mature oocytes (MII) with metaphase axis by the ooplasm periphery and expulsion of the first polar body (PB), (2) immature oocytes (MI) in which no first polar body could be seen and (3) degenerated/indeterminate oocytes (D/ID) in which nuclear development could not be determined or did not have evident chromatin [39].

Classification of cytoplasmic maturation was based on cortical granules distribution within the oocyte cytoplasm, as follows: immature (I), with cortical granules distributed throughout the cytoplasm; incomplete cytoplasmic maturation (ICM) but in a transitional stage of maturity, with cortical granules dispersed in the cytoplasm and in the peripheral region of the oocyte near the plasma membrane; and complete cytoplasmic maturation (CCM), with cortical granules dispersed only in the periphery of the oocyte, near the plasma membrane [1,28].

*Statistical analysis*

Data were subjected to normality test and are expressed as mean ± standard deviation (SD). Intraoperative stages, number of visible and aspirated follicles and the number of oocytes retrieved were subjected to *One-Way* ANOVA and Tukey test ($P < 0.05$). Quantity and quality of oocytes, oocyte retrieval and *in vitro* maturation rates are expressed in absolute and relative (percentage) values and were subjected to analysis of variance (GLM) and Tukey test ($P < 0.05$) using the statistical program SAS™.

## RESULTS

The 36-h fasting allowed for appropriate manipulation and visualization of the abdominal cavity besides preventing the reflux of ruminal contents in most surgeries. Poor visualization occurred in only 2 animals (5.5%), due to craniocaudal distension of the rumen and reflux of rumen fluid was observed in only 3 (8.3%) animals.

The Trendelenburg positioning caused respiratory depression in 33.3% of the animals, however, such complication was easily managed using assisted ventilation. The mean overall surgical time for the procedure was 24.3 ± 5.2 min and the time elapsed in each surgical stage is shown in Figure 1. Ovarian manipulation and follicular aspiration times (MO1 and MO2) were greater than those of other surgical stages ($P < 0.05$).

The system used for ovum pick up, which allowed a maximum pressure of a 50 mmHg, was found to be efficient for oocyte retrieval with mean numbers of visualized and aspirated follicles and retrieved oocytes of 10.8 ± 5.2, 8.1 ± 3.4 and 5.8 ± 3.1, respectively. Bleeding from follicular puncture was minimal. Rinsing the ovaries aided in clot removal, thus minimizing the chances of adhesions. None of the animals sampled for second look laparoscopy (25%) showed any damage caused by the first procedure.

All animals experienced uneventful recovery from anesthesia and were able to stand up unassisted and with no signs of pain (0 ± 0.5) immediately after the procedure.

The plasma fibrinogen values observed were within the normal limits for sheep (100 to 500 mg/dL [19]) and no significant difference was observed between the stages assessed ($P > 0.05$), as shown in Figure 2.

The best time to perform LOPU proved to be 48 h after initiation of follicular stimulation, when a greater number of retrievable follicles (2-8 mm in diameter) were present. The mean number of visualized follicles, retrieved oocytes and retrieval rates are shown in Table 1. There was no significant difference ($P < 0.05$) in the number of viable oocytes between groups G80U and G160U. The frequency of the different stages of oocytes quality is detailed in Table 2.

The number of oocytes retrieved from G80U and G160U was greater than that of other groups ($P < 0.05$). The best results for oocyte maturation based on nuclear and cytoplasmic maturation were observed in G160U ($P < 0.05$), as shown in Table 3.

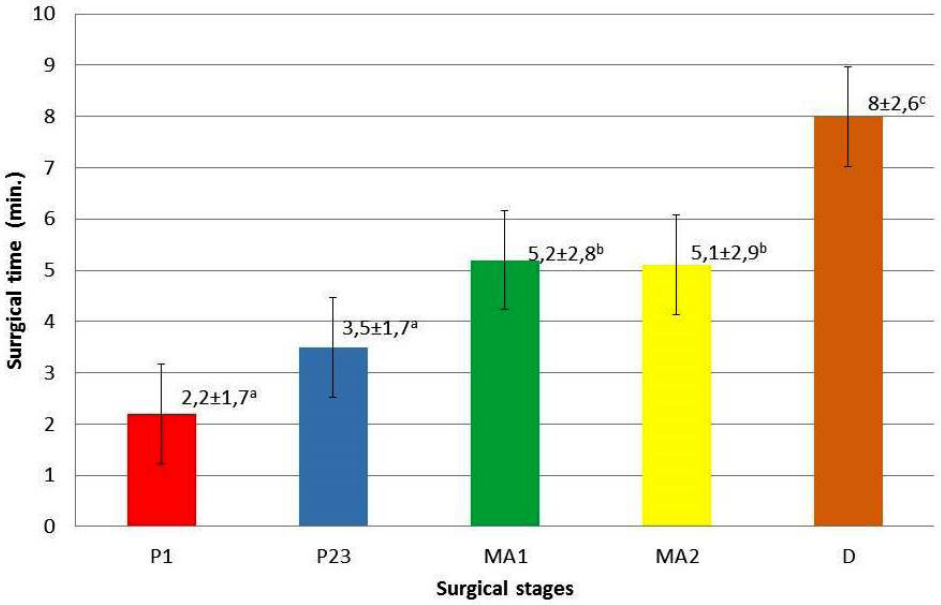


**Figure 1.** Surgical time elapsed during each stage of the LOPU procedure. Same letters mean $P > 0.05$ and different letters $P < 0.05$. T1: the first laparoscopic trocar insertion; T2/3: insertion of the second and third laparoscopic trocars; MA1: manipulation and aspiration of the first ovary; MA2: manipulation and aspiration of the second ovary; D: time elapsed from final Inspection of the abdominal cavity to skin suture.

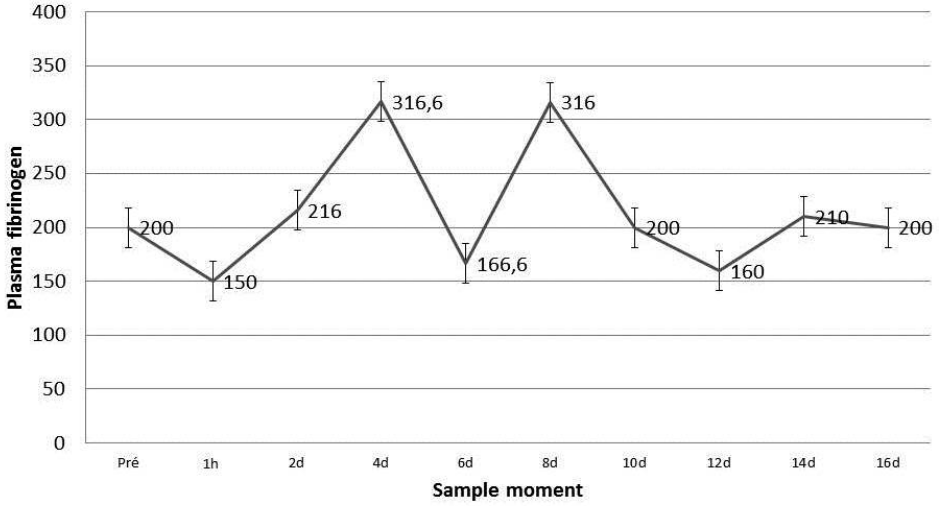


**Figure 2.** Plasma fibrinogen in prepubertal sheep subjected to LOPU, showing no change compared to baseline for sheep (100-500 mg/dL; Jain, [19]).

**Table 1.** Average number ± SD of visualized follicles (VF), aspirated follicles (AF), oocytes retrieved (OR) and retrieval rate (RR) in the different groups of ewe lambs subjected to follicular wave induction and ovarian stimulation prior to LOPU procedure.

| | GCN | GCI | G80U | G80M | G160U | G160M |
|---|---|---|---|---|---|---|
| VF | 10.5 ± 4.2ª | 8.1 ± 5.4ª | 11.4 ± 3ª | 10.8 ± 5.1ª | 10.8 ± 5.2ª | 9.4 ± 5.3ª |
| AF | 5.6 ± 1.9ª | 7.3 ± 4.5ᵇ | 9.4 ± 1.6ᵇ | 7.8 ± 2.4ᵇ | 8.1 ± 3.4ᵇ | 9.0 ± 2.6ᵇ |
| OR | 1.1 ± 1.6ª | 1.1 ± 1.8ª | 4.6 ± 3.2ᵇ | 2.2 ± 0.8ª | 5.8 ± 3.1ᶜ | 3.0 ± 2.0ª |
| RR(%) | 19.60ª | 15.0ª | 48.9ᶜ | 37.5ᵇ | 71.6ᵈ | 33.3ᵇ |

Different letters indicate significant difference ($P < 0.05$) between observations.

**Table 2.** Percentages and absolute values (in parenthesis) of the different stages of oocyte quality and the total number of viable oocytes in the different groups of ewe lambs subjected to follicular wave induction and ovarian stimulation prior to LOPU procedure.

| | GCN | GCI | G80U | G80M | G160U | G160M |
|---|---|---|---|---|---|---|
| GI | 28.5(2)$^{a}$ | 14.2(1)$^{a}$ | 34.4(7)$^{b}$ | 9.0(1)$^{a}$ | 54.0(20)$^{c}$ | 20(3)$^{a}$ |
| GII | 28.5(2)$^{a}$ | 0(0)$^{a}$ | 17.3(4)$^{b}$ | 9.0(1)$^{a}$ | 16.2(6)$^{c}$ | 13.3(2)$^{a}$ |
| GIII | 14.2(1)$^{a}$ | 14.2(1)$^{a}$ | 34.4(7)$^{b}$ | 36.3(4)$^{a}$ | 18.9(7)$^{c}$ | 46.6(7)$^{a}$ |
| Deg | 28.5(2)$^{a}$ | 71.4(5)$^{a}$ | 17.3(4)$^{b}$ | 45.4(5)$^{a}$ | 10.8(4)$^{c}$ | 20(3)$^{a}$ |
| Total VO | 61.2(5)$^{a}$ | 28.4(2)$^{a}$ | 86.1(18)$^{b}$ | 54.3(6)$^{a}$ | 89.1(33)$^{c}$ | 79.9(12)$^{a}$ |
| Total of retrieval oocytes | (7)$^{a}$ | (7)$^{a}$ | (22)$^{b}$ | (11)$^{a}$ | (37)$^{c}$ | (15)$^{a}$ |

Stage I (GI), II (GII), III (GIII) and degenerated (Deg) oocytes. VO: Viable oocytes. Different letters indicate significant difference ($P < 0.05$) between observations.

**Table 3.** Percentage and absolute values of the different stages of nuclear and cytoplasmic maturation observed in the different groups of ewe lambs subjected to follicular wave induction and ovarian stimulation prior to LOPU procedure.

| | GCN | GCI | G80U | G80M | G160U | G160M |
|---|---|---|---|---|---|---|
| Nuclear maturation | | | | | | |
| MII | 0(0)$^{a}$ | 0(0)$^{a}$ | 16.6(3)$^{a}$ | 33.3(2)$^{b}$ | 63.6(21)$^{c}$ | 46.1(6)$^{b}$ |
| MI | 0(0)$^{a}$ | 0(0)$^{a}$ | 27.7(5)$^{b}$ | 33.3(2)$^{b}$ | 18.1(6)$^{a}$ | 30.7(4)$^{b}$ |
| D/NI | 100(5)$^{a}$ | 100(2)$^{a}$ | 55.5(10)$^{c}$ | 33.3(2)$^{b}$ | 18.1(6)$^{a}$ | 23.0(3)$^{a}$ |
| Cytoplasmic maturation | | | | | | |
| MCC | 0(0)$^{a}$ | 0(0)$^{a}$ | 11.1(2)$^{a}$ | 33.3(2)$^{b}$ | 69.6(23)$^{c}$ | 46.1(6)$^{b}$ |
| MCI | 20(1)$^{a}$ | 0(0)$^{a}$ | 38.8(7)$^{b}$ | 16.6(1)$^{a}$ | 15.1(5)$^{a}$ | 23.0(3)$^{a}$ |
| I | 80(4)$^{a}$ | 100(2)$^{a}$ | 50(9)$^{c}$ | 50(3)$^{c}$ | 15.1(5)$^{a}$ | 30.7(4)$^{b}$ |

MII: matured oocytes. MI: immature oocytes. D/NA: degenerated oocytes/not amenable to determination. MCC: complete cytoplasmic maturation. MCI: incomplete cytoplasmic maturation. I: immature. Different letters indicate significant difference ($P < 0.05$) between observations.

## DISCUSSION

Ruminal reflux has been a commonly reported complication in sheep undergoing laparoscopy, even after fasting for 72 and 24 h for food and water, respectively. Pneumoperitoneum and intravenous anesthesia using ketamine and acepromazine have been considered to be the causes of such findings [5]. Even though a different anesthetic protocol was used, regurgitation and caudal rumen distention were observed in our study. However, the incidence of these complications was low and they were not considered to be severe during the peri or postoperative periods.

Reduced lung volume, hypoxia, increased intrathoracic and vascular pressure and increased cardiac effort have been reported as disorders caused by patient positioning and by pneumoperitoneum [8,13,23]. Such complications, however, were not harmful for the animals in the current study due to the use of assisted ventilation and short duration of the surgical procedure.

No accidents occurred in the present study, and the blind insertion of the first trocar was not an obstacle that would have increased the time of surgery due to the training and cohesion of the surgical team. That potential intraoperative accidents was reported, especially laceration of viscera during trocar insertion, can occur [32]. Therefore, the use of the Veress needle, as recommended in other studies [14], was not employed in our study. The mean surgical time observed in the present study was similar to the 35 min [10] and the 18-20 min [40] in follicular aspirations performed by laparoscopy in goats and sheep, respectively. In laparoscopic liver biopsies in sheep and horses in 23 and 21 min, respectively [14,30], and 120-150 min to perform laparoscopic ovariectomy in cattle [7]. As demonstrated by the above studies, the laparoscopic technique facilitates the execution of different procedures within an adequate surgical time. However, it should be noted that surgical training was essential in achieving such goals.

The time elapsed in each surgical stage ($P < 0.05$) are detailed in Figure 1. The insertion of the trocars (stages T1 to T2/3) was quicker than the manipulation of the ovaries and follicle aspiration (MA1 and MA2). The final surgical stage (S) was the longest one [33]. As previously mentioned, the main difficulty of the whole procedure involved the MA1 and MA2 stages. However, the distension of the abdominal cavity due to the pneumoperitoneum, as well as image magnification by the video system, made the procedure simpler and resulted in rapid execution of the surgeries.

Adhesion formation in small ruminants following subsequent follicular aspirations using laparoscopy [10,16,34]. Such complications were not observed in the current study. Other studies did not report postoperative adhesion formation in ewes submitted to nine laparoscopic ovum pick up sessions in seven-day intervals. Adhesion formation can be prevented by careful manipulation of the reproductive tract and removal of clots from the ovarian surface with 0.9% NaCl solution. The excellent recovery of the animals and the absence of mild/severe pain were attributed to the minimally invasive nature of the laparoscopic procedure [33].

Plasma fibrinogen values were within the normal limits for sheep (100 to 500 mg/dL) with no significant differences being found at any in the study [19]. Such findings suggest that the laparoscopic approach may cause minimal postoperative inflammation and acute phase response. However, even though fibrinogen levels remained within the normal limits, there was a slight increase at 72 to 96 h ($P > 0.05$). The increase in plasma fibrinogen between the different stages was similar to those reported in sheep [17,27] and in cattle [15] undergoing orchiectomy. The increase in orchiectomy was observed in 48-72 h. Also it was noted an increase above the ceiling, at 96 h after collection of ruminal fluid using trocar in Santa Inês ewes [15,25].

The LOPU technique, using vacuum, proved favorable for oocyte retrieval. The average number of visualized and aspirated follicles, retrieved oocytes and the oocyte recovery rates for G80U and G160U were similar to those reported by studies in adult sheep, who was obtained 13.24 ± 2.0, 11.27 ± 3.03, 5.79 ± 2.3 and 51.69% in VF, AF, RO and RR, respectively. Furthermore, the results obtained in this study were similar to those reported by Baldassarre and Karatzas [3] of 13.4 follicles/ewe, and Basso *et al*. [4] of 14.3 follicles/ewe. Oocyte recovery rate ranged from 40 to 90%, as previously observed by others [11,18,29]. Small caliber needles (21 G and 22 G) can damage the oocytes at the time of follicular aspiration, however, such complication did not occur in the current study as an 18 G needle was used [2].

Previous studies using young sheep showed that follicular aspiration did not influence weight gain or early puberty. The best results reported on the number of aspirated follicles per session were 9 to 15 follicles/ewe [36, 38]. In prepubertal goats, on the other hand, the number of oocytes retrieved was almost two fold greater than those in adults, and a similar rate of IVM was observed in both age groups [21].

An extremely low IVM rate has been reported in sheep [37]. In the current study, the use of 160 mg of FSH in a single dose (G160U) obtained similar cytoplasmic and nuclear maturation results to those observed in adult sheep [3,33]. It is thus suggested that weaned ewe lambs may require high concentrations of exogenous FSH to produce a higher number of better quality follicles and to achieve superior in vitro maturation rates. Thus, the groups that received 80 mg of FSH administration on a single dose or fractional applications possibly did not achieve the sufficient serum levels necessary for adequate follicular growth.

## CONCLUSIONS

This study demonstrated that the LOPU technique can be successfully used in recently weaned ewe lambs, aged four to eight weeks old, and does not result in significant complications during the intraoperative period. Such technique allows for rapid execution of the procedure and good recovery of animals after the procedure, especially due to its minimally invasive nature.

The protocol for ovarian stimulation using a single dose of 160 mg FSH, subsequent to follicular wave induction with eCG, was considered efficient and resulted in favorable rates of retrieved oocytes and IVM.

Notably, laparoscopic ovum pick up for *in vitro* production of embryos requires further studies. However, results of both evaluation of surgical technique and ovarian stimulation protocols showed a great progress in such reproductive biotechnique.

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***Ethical approval*.** The current study was approved by the Ethics Committee for Animal Use from the Faculty of Agrarian and Veterinary Sciences, University of São Paulo State, Brazil (Universidade Estadual Paulista - UNESP) protocol no. 002478/11).

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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