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Pattern of follicular development in sheep subjected to ovarian superstimulation after follicular ablation by laparoscopic ovum pick-up

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ABSTRACT: *The aim of this study was to determine the pattern of follicular development in ewes following ablation of all follicles associated with ovarian superstimulatory treatment. After confirmation of reproductive cyclicity using ultrasonography and progesterone levels, five adult ewes of undetermined breed received a vaginal pessary containing 60mg of medroxyprogesterone, maintained for 10 days, with the application of 100µg cloprostenol on the fourth day. On the tenth day, 300UI equine chorionic gonadotropin (eCG) was applied together with 80mg FSH, and all visible follicles were aspirated by laparoscopic ovum pick-up (LOPU). Following LOPU, ovarian structures were assessed using sonography every 8 hours for a period of 64 hours, and the number and diameter of small (<2.5mm), medium-sized (2.5-4.5mm), and large (>4.5mm) follicles were recorded. The average diameter of small follicles reduced from 5.6±1.5mm initially to 0.8±1.3mm by 56 hours. Population size of the medium-sized follicles during the observation period followed a parabolic distribution wherein the theoretical maximum size emerged at 34.6 hours with no difference at ($P>0.05$) 24 (4.6±1.5) and 48 hours (4.8±2.3). It was concluded that in ewes subjected to ovarian superstimulation immediately following LOPU, follicular development is characterized by a new wave of follicle growth with a predominance of medium-sized follicles (2.5-4.5mm) between 24 and 48 hours, and a theoretical population maximum occurred at 34.6 hours.*

Key words: follicular dynamics, superstimulation, ultrasound, ewes.

Padrão de desenvolvimento folicular em ovelhas submetidas à superestimulação ovariana após ablação folicular por laparoscopia

RESUMO: *O objetivo deste estudo foi determinar o padrão do desenvolvimento folicular em ovelhas após a ablação de todos os folículos, associado a tratamento superestimulatório ovariano. Após confirmação da ciclicidade reprodutiva por avaliação ultrassonográfica e dosagem de progesterona, cinco ovelhas adultas, sem raça definida, ao início do tratamento, receberam pessário vaginal contendo 60mg de acetato de medroxiprogesterona. Após quatro dias, foi administrado 100µg cloprostenol sódico e, no décimo dia, realizou-se a remoção do pessário e aspiração de todos os folículos ovarianos visíveis, guiada por laparoscopia, além da administração de 300UI eCG associada a 80mg FSH. Ao final do tratamento, a cada 8 horas, foi realizada avaliação ultrassonográfica das estruturas ovarianas por um período de 64 horas, onde o número e o diâmetro de folículos pequenos (<2,5mm), médios (2,5 a 4,5mm) e grandes (>4,5mm) foram contabilizados. O número de folículos pequenos, que inicialmente apresentava média 5,6±1,5, foi reduzido para 0,8±1,3 a partir de 56 horas, folículos grandes somente foram vistos a partir de 32 horas (0,2±0,4); a população de folículos médios, durante o período de observação, apresentou uma distribuição parabólica cujo vértice, considerado o ponto teórico de maior concentração, foi às 34,6 horas, não havendo diferença ($P>0,05$) nessa população de folículos, entre 24 e 48 horas (4,6±1,5 vs 4,8±2,3). Conclui-se que, em ovelhas submetidas à superestimulação ovariana iniciada imediatamente após remoção de todos os folículos visíveis à laparoscopia, o desenvolvimento folicular é caracterizado pela emergência de nova onda de crescimento, com predomínio de folículos médios (2,5 a 4,5mm) entre 24 e 48 horas, sendo o ponto teórico de maior ocorrência às 34,6 horas.*

Palavras-chave: dinâmica folicular, superestimulação, ultrassom, ovelhas.

INTRODUCTION

Multiple factors such as age (ARMSTRONG, 2001), aspiration technique, follicle diameter (COGNIE et al., 1996, 1998; KHATIR et al., 2007), oocyte diameter (DURINZI et al., 1995), presence of corpus luteum (WANIA et al., 1999), and superstimulatory treatment (BALDASSARRE et al., 1996, 2002), can influence the recovery rate and quality of cumulus

oophorus complexes (COCs) obtained from animals. These factors can also influence the rate of oocyte maturation and the rate of *in vitro* blastocyst production.

During *in vitro* embryo production, better recovery rates and oocyte utilization are observed when the follicles collected from ewes are larger than 3mm in diameter (COGNIE et al., 1998). However, although large follicles are more easily visualized, the high viscosity of their

contents may contribute to decreased recovery rates (BALDASSARE et al., 1994a; SENEDA et al., 2001).

BALDASSARE et al. (1994b) obtained better recovery rates in follicles larger than 3mm (83.8 %) than in those smaller than 3mm (63.4%). RODRIGUEZ et al. (2006) obtained a higher proportion of high-quality COCs from the puncture of follicles with a diameter between 3 and 5mm, using vacuum pressures of around 10mL min⁻¹ H₂O.

Ovarian superstimulation in ewes prior to aspiration is extremely important for improving oocyte recovery rates, obtaining better-quality COCs, and increasing cell yield (BALDASSARE et al., 1996; COGNIE et al., 2003).

The development of techniques for ovarian superstimulation in ewes is limited by the wide variation in responses to gonadotropin stimulation, among other causes, the presence of dominant follicles at the initiation of treatment is a major interfering factor (TERVIT et al., 1992; BALDASSARE et al., 1994), since response to FSH is inversely proportional to the presence of follicles larger than 5mm (BREBION & COGNIE, 1989; COGNIE, 1999).

Since follicle diameter before aspiration can influence the COCs recovery rate and *in vitro* maturation, the objective of this study was to evaluate the pattern of follicular growth in ewes subjected to follicle aspiration via laparoscopic ovum pick-up (LOPU), followed by hormonal superstimulation. The ultimate aim was to determine the time following treatment at which the population of follicles with diameters between 2.5 and 4.5mm is largest with a view to identify the optimal harvest time for downstream applications.

MATERIALS AND METHODS

Animals and trial location

The study was conducted in spring, at the “Biotechnology Laboratory Applied to Reproduction of Sheep and Goats” and the Laboratório de Endocrinologia do Departamento de Reprodução Animal e Radiologia Veterinária, (FMVZ), Universidade Estadual Paulista (UNESP), in Brazil, Botucatu-SP, located at latitude 22°53' south and longitude 48°26' west, at an altitude of 804m.

Five adult undetermined breed were selected by assessment and confirmation of adequate physical condition and clinical and

reproductive status. During the trial period, they received water and mineral salt *ad libitum* and a specific and complete pelleted ration for the maintenance of adult ewes.

The ovarian cycle was detected by quantification of plasmatic progesterone and by sequential ultrasound evaluation every 24 hours for 7 days in order to identify the presence of the corpus luteum.

Follicle ablation

After determination of ovarian cyclicity, the ewes received an intravaginal pessary containing 60 mg of medroxyprogesterone acetate (Progespon®, Zoetis Indústria de Produtos Veterinários Ltda., Brazil), which was maintained for 10 days with the application of 100µg cloprostenol (Ciosin®, MSD Saúde Animal, Brazil) on the fourth day. After removal of the intravaginal pessary, LOPU was performed for all visible follicles in both ovaries.

Animals were fasted for 24 hours and prohibited access to water for 12 hours; they received 30.000UI benzathine penicillin kg⁻¹ before the procedure. General anesthesia was induced using a mixture of ketamine (5.0mg kg⁻¹) and xylazine hydrochloride (0.2mg /kg⁻¹). Epidural anesthesia was induced at the site of catheter insertion with 4mL of 2% lidocaine without vasoconstrictor following regional block with 2% lidocaine with vasoconstrictor.

Ewes were restrained in a cradle in the standard position for laparoscopic artificial insemination, the Trendelenburg position, with a 45° inclination. Shaving of the abdominal region was antiseptically prepared with 2% chlorhexidine antibacterial solution and 70% ethanol.

A trocar (5mm) with a valve to inflate the cavity was inserted into the left paramedian retro-umbilical region, creating the pneumoperitoneum with a pressure ranging from 5 to 8mmHg. Following inflation, the optical probe was introduced.

Two more trocars were inserted into the right paramedian retro-umbilical region; these had a smaller diameter to allow for insertion of atraumatic forceps to manipulate the uterus, ovaries, fallopian tubes, and LOPU system.

Laparoscopy equipment comprised a line, an aluminum stopper, a stem, and a needle for follicular aspiration (WTA – Tecnologia Aplicada). Vacuum pressure used was 40mmHg, generated by a Digital Vacuum Pump BV 003d (WTA – Tecnologia Aplicada).

At the trocar insertion sites, separate interrupted stitch sutures were performed using nylon 0. Subsequently, terramycin and hydrocortisone spray were applied to the site (Terracortril®, Pfizer Saúde Animal, Brazil).

Superstimulatory treatment and ultrasonic monitoring of ovarian activity

Following LOPU, 80mg of FSH (Folltropin® V, Bioniche Animal Health Product, Canada) was administered together with 300UI eCG (Equine Chorionic Gonadotropin - Novormon® - Schering-Pough).

Ultrasonographic evaluation of ovarian structures was performed over a period of 64 hours, with an interval of 8 hours between observations. An ultrasound B - mode scanner (Prosound 2 VET; Aloka Co. Ltd., Japan) equipped with a linear transducer operating at 7.5MHz (UST-660-7.5; Aloka Co. Ltd., Japan) was used. Diameter and growth pattern of all detectable follicles was recorded and they were classified as small (<2.5mm), medium-sized (2.5 to 4.5mm), or large (>4.5mm).

Statistical analyses

Data were analyzed using analysis of variance (ANOVA) and Dunn's multiple comparison test at 5% probability. Data were plotted on a scatter plot, and the trend lines of the distributions were established. Where possible, the equation that best defined the phenomenon was calculated. The point of greatest concentration of follicles with diameters between 2.5 and 4.5mm was established using the theoretical model.

RESULTS

All ewes demonstrated ovarian activity via ultrasonographic assessment, assessed by detection of the by corpus luteum or by observation of ovulation during the ultrasound examinations carried out in the period before LOPU. This was confirmed by progesterone concentrations detected in paired samples and all ewes giving plasma readings equal to or greater than 1ng/mL at least at one time point.

Follicle size was measured using ultrasound monitoring throughout the follicular growth period following the completion of LOPU and superstimulatory treatment. Follicles were categorized into populations of small, medium-sized, and large follicles according to their diameter (Table 1).

The smallest number of small follicles (<2.5mm) was observed 56 hours after the completion of LOPU ($P<0.05$). The largest population of medium follicles (2.5 to 4.5mm) was observed between 24 and 48 hours after LOPU ($P<0.05$). The incidence of large follicles (>4.5mm) remained stable throughout the observation period ($P>0.05$).

The population size of medium-sized follicles (Figure 1) followed a parabolic distribution throughout the observation period, represented by the equation $y=-0.0071x^2+0.4908x-2.2214$. The theoretical population maximum, represented by the vertex at the peak of the parabola, occurred at 34.6 hours ($X_v=-0.4908/[-0.0071]$).

DISCUSSION

Follicular development after treatment exhibited typical wave behavior (Figure 1), a group of small follicles initially observed 8 hours after LOPU increased in size, giving rise to the population of medium-sized follicles. Some of these came into atresia with only few large follicles remaining, in accordance with the pattern of follicular dynamics of the estrous cycle (GINTHER et al., 1994; RAVINDRA et al., 1994; LOPEZ-SEBASTIAN et al., 1997). However, number of structures was higher than is generally observed following superstimulatory treatment.

Follicles with small diameters are difficult to visualize and aspirate. Large follicles, in addition to having lower rates of recovery following aspiration owing to the high viscosity of follicular fluid, have aged or over-mature oocytes, and cumulus cells with low adherence, increasing the likelihood of loss during aspiration (BALDASSARE, 1995; SENEDA et al., 2001).

Therefore, determining the time at which the population of medium-sized follicles is largest is very important for obtaining the optimal number of high-quality COCs for *in vitro* embryo production (COGNIÉ et al., 1998; RODRIGUEZ et al., 2006; KARAMI-SHABANKAREH & MIRSHAMSI, 2012). The population maximum was determined using a theoretical model, and was represented by the peak of the parabolic distribution, with the highest proportion of follicles with diameters between 2.5 and 4.5mm appearing at 34.6 hours.

Superstimulation of ovaries is more successful in the absence of large follicles (BREBION et al., 1992; COGNIÉ, 1999), the removal creates an ideal environment for ovarian

Table 1 - Mean \pm SD number of small ($\emptyset < 2.5\text{mm}$), medium-sized ($\emptyset = 2.5$ to 4.5mm), and large ($\emptyset > 4.5\text{mm}$) follicles observed in ultrasound scans performed at 8-hour intervals following laparoscopic ovum pick-up (LOPU) and ovarian superstimulation in ewes.

Follicles	Hours after LOPU							
	8	16	24	32	40	48	56	64
Small	5.6 ^a \pm 1.5	3.2 ^{ab} \pm 1.1	4.0 ^a \pm 1.2	1.0 ^{ab} \pm 1.2	1.0 ^{ab} \pm 1.2	1.4 ^{ab} \pm 1.5	0.8 ^b \pm 1.3	0.2 ^b \pm 0.4
Medium	1.6 ^{bc} \pm 1.14	3.4 ^{bc} \pm 1.14	4.6 ^{ab} \pm 1.52	7.0 ^a \pm 1.73	7.0 ^a \pm 2.45	4.8 ^{ab} \pm 2.28	1.6 ^{bc} \pm 1.5	0.8 ^c \pm 0.8
Large	0.0 \pm 0	0.0 \pm 0	0.0 \pm 0	0.2 \pm 0.4	0.6 \pm 0.8	0.4 \pm 0.5	2.0 \pm 1.2	1.4 \pm 0.8

a, b, c Different letters in the same row indicated differences in values ($P < 0.05$) in the different moments of observation \emptyset = follicle diameter.

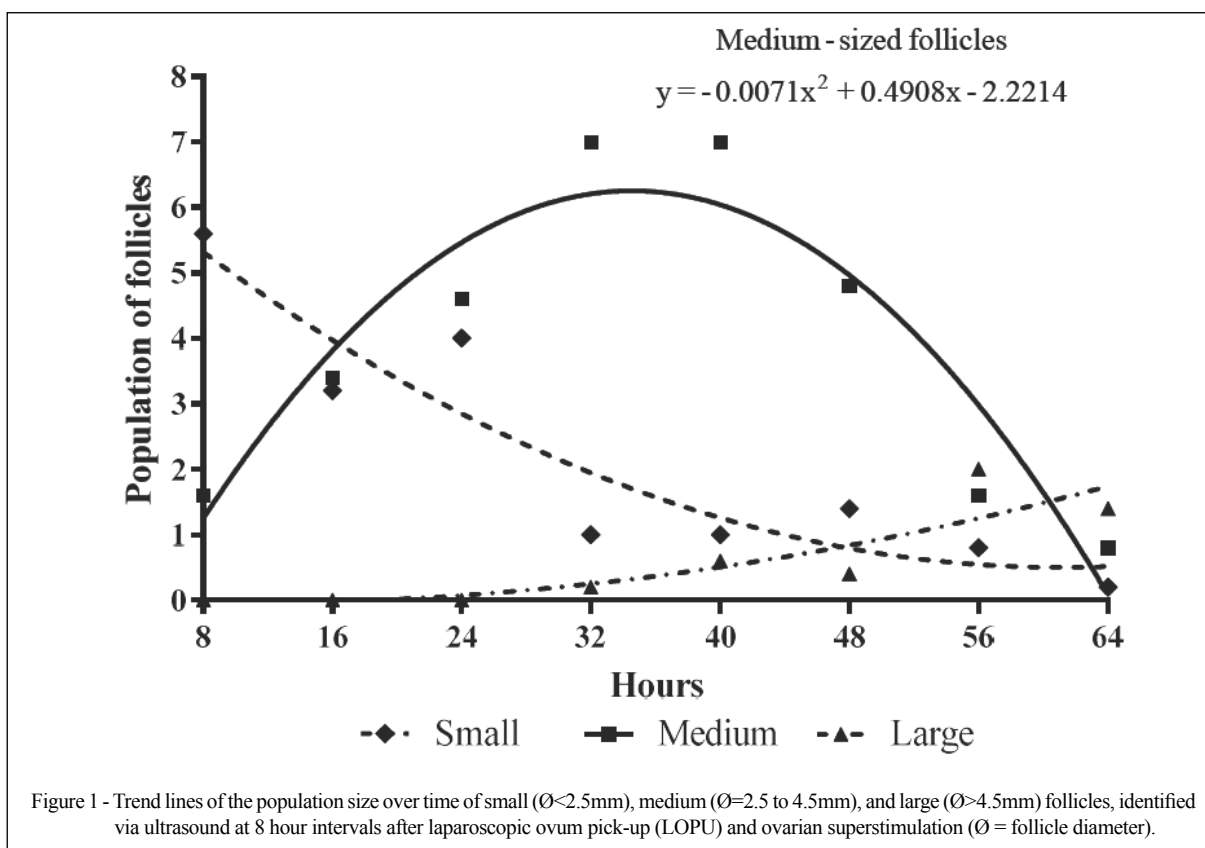
response to these treatments. In this study, follicular ablation via LOPU proved effective in controlling the ovarian follicular population following removal of the largest follicles, including those with aged oocytes and large diameters. Large follicles did not redevelop in the 24 hours following LOPU.

Furthermore, follicular ablation via LOPU facilitates greater control of the follicle population than is possible using other techniques, such as GnRH agonists and antagonists (DUFOUR et al., 2000; GONZALEZ-BULNES et al., 2002; COGNIE et al., 2003; LOPEZ-ALONSO et al., 2005) or progestogens associated with estrogen (TAKADA et

al., 2004). It also allowed the use of oocytes obtained during the follicular ablation procedure, increasing the number of recovered structures.

CONCLUSION

LOPU is an efficient method for the aspiration of the visible population of ovarian follicles. This technique, when associated with ovarian superstimulation, triggers a wave of follicular growth with a large proportion of medium-sized follicles between 24 to 48 hours; this population reaches a maximum theoretical point at 34.6 hours.



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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The study, protocol 105/2011, has been certified according to the Ethical Principles in Animal Experimentation and was approved by the Ethics Committee on Animal Use (CEUA) FMVZ-Unesp-Botucatu, on May 13, 2011.

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