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Effect of FSH Stimulation with Pluronic® F127 on the Quality of Oocytes Collected by Follicular Aspiration in *Bos taurus indicus* Heifers

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Abstract

In bovine *in vitro* embryo production (IVP), FSH has been used to improve the quality and developmental capacity of oocytes to reach the blastocyst stage. The objective of this study was to evaluate in *Bos taurus indicus* heifers the effect of FSH incorporated in Pluronic® F127 on the quality of oocytes collected by follicular aspiration. A total of 30 cyclic Brahman heifers were selected and assigned into one of three experimental groups (control group, FSH-SC and FSH-PF127). Heifers in the FSH-SC group received a single dose of 50 mg FSH subcutaneously and heifers in the FSH-PF127 group were treated with 50 mg FSH incorporated in Pluronic® F127. The rate of viable oocytes was higher for the FSH-PF127 group compared to the control and FSH-SC groups. The administration of a single dose of 50 mg of FSH in Pluronic® F127 influenced the oocyte quality and the rate of viable oocytes of Brahman breed heifers.

KEYWORDS
Biopolymers, Bovine, Ovum pick-up, Superstimulation

INTRODUCTION

In *in vitro* bovine embryo production (IVP) associated with the ultrasonography-guided follicular aspiration (UPO) technique, FSH has been used to improve the quality and developmental capacity of oocytes and blastocyst production (Merton *et al*., 2003; Blondin *et al*., 2012). A study conducted in heifers and Gyr cows using FSH prior to UPO at doses of 100 and 140 mg, respectively, obtained a high number of viable oocytes and embryos produced (Elliff *et al*., 2018). The results of a later study by Fernandes *et al*. (2020) conducted in Nelore cows administering 200 mg FSH at a single dose, 24 hours before UPO, did not indicate positive effect of FSH on the number of oocytes retrieved and blastocyst production rate.

Variability in results has led to superstimulation protocols in IVP programs to improve efficiency and simplicity by using a single dose of FSH released slowly and over several days. In this sense, the incorporation of biodegradable polymers is an alternative to improve hormone stability, decrease animal handling, and increase ease of use (Bó *et al*., 2018). Ongaratto *et al*. (2020) and Vieira *et al*. (2016) incorporated FSH into hyaluronan using Bos taurus taurus cows and found that cows treated with the biopolymer presented stable plasma FSH concentrations and an increase in the proportion of medium and large follicles, while blastocyst rates were not affected.

Another alternative for the administration of peptide hormones can be through Pluronic® F127 (PF127), a biodegradable, non-toxic and stable copolymer with a unique property of reversible thermal gelation. Below 4 ºC its natural state is liquid and at body temperature (37 ºC) it transforms into a semi-solid gel (Wenzel *et al*., 2002).

Therefore, the objective of this study was to evaluate the effect of ovarian stimulation in *Bos taurus indicus* heifers with the application of FSH incorporated in Pluronic® F127 in a single dose on the quality of oocytes collected by follicular aspiration.

MATERIALS AND METHODS

The present study was developed out in accordance with the laws and regulations of Colombia, established by resolution 8430 of 1993, which provides for biomedical research with animals. The study was approved by the Ethics and Bioethics Committee of the University of Santander.

Animal selection and location

A total of 30 cyclic Brahman (*Bos taurus indicus*) heifers were selected, with average weight of 380±27.5 kg, 26.6±2.8 months of age and body condition status of 4.0±0.4 on the scale of 1 to 5 (Ayres *et al*., 2009). All animals were maintained on pastures based on *Brachiaria decumbens* and *Brachiaria brizantha*, with mineral supplementation (60 g/day/ heifers) and water *ad libitum*. The study was conducted under tropical conditions of temperature 33 °C and average annual rainfall of 1360 mm.

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Experimental design

Selected heifers were randomly assigned into one of three experimental groups (control group, FSH-SC and FSH-PF127). To remove the animal effect on the results, all females underwent the three treatments. Each experimental group underwent two repetitions and the interval between aspirations was 30 days. Prior to each follicular aspiration, the animals were submitted to a new hormonal protocol for the synchronization of the follicular wave.

On day 0 (Day 0; AM), all heifers received a progesterone (P4) intravaginal device (Sincrogest®, Ouro Fino, Brazil), 2 mg IM of estradiol benzoate (Sincrodiol®, Ouro Fino, Brazil) and 150 µg IM of D-cloprostenol (Sincrocio®, Ouro Fino, Brazil). On Day 4 (AM), females in the control group did not receive gonadotropin-based treatment, however, heifers in the FSH-SC group received a single dose of 50 mg FSH subcutaneously (SC) (Folltropin®, Bioniche Animal Health, Belleville, Ontario, Canada) and heifers in the FSH-PF127 group were treated with 50 mg FSH incorporated in PF127 (Basf, Mount Olive, NJ) administered subcutaneously as a single dose. On Day 6 (PM) the P4 device was removed and UPO was performed. The interval between the application of the single dose of FSH and UPO for the FSH-SC and FSH-PF127 groups was 54 h, period that promotes oocyte development (Nivet *et al*., 2012).

Preparation of Pluronic® F127

For the preparation of PF127, the methodology proposed by Wenzel *et al*. (2002) was applied - with some modifications. It was used 20% of PF127 and diluted in 30% of D-PBS (D-PBS®, Vitrocell, Brazil); after this preparation, 50 mg of FSH were added and submitted to mechanical agitation with Vortex for 3 minutes. The mixture was stored at 4 ºC for a period of 12 hours remaining in liquid state. After this time, the compound was administered subcutaneously to the animals while maintaining the cold chain.

Ultrasound-guided follicular aspiration

UPO was performed on those follicles with a diameter ≥ 3 mm and were classified into three groups: small follicles (≤ 5 mm), medium follicles (5 to 9 mm) and large follicles (≥ 9 mm). Disposable needles of 20 "G" (WTA-Vet, Brazil) were used, coupled to a Teflon line with negative pressure of 70 mm /Hg. Prior to aspiration, epidural anesthesia (0.2 mg/kg of Roncaina®, Ropsohnlab, Colombia) was applied between the last sacral vertebra and the first coccygeal, and the follicles of both ovaries were counted with the help of an ultrasound (Mindray, DP 2200 VET, China) equipped with a 7.5 MHz frequency microconvex transducer and coupled to a transvaginal guide for follicular aspiration.

A conical tube of 50 mL with 5 mL of D-PBS was used, supplemented with 0.1% heparin (Liquemine®, Roche, Brazil) and 1% fetal bovine serum (Gibco BRL, Grand Island, NY) in each animal for the collection of follicular fluid and oocytes. Once the follicular fluid was collected, it was transferred to an EmCon filter (Agtech, USA) adding 100 mL of D-PBS for the removal of clots and cells. The remaining structures COCs (cumulus-oocyte complexes) were washed in TCM 199 buffered with HEPES (TCM-199; Gibco BRL, Grand Island, NY) plus 10% fetal bovine serum (Gibco BRL, Grand Island, NY), 16 µg/mL sodium pyruvate and 83.4 µg/mL amikacin (Instituto Bioquimico, Rio de Janeiro, Brazil). Viable oocytes for *in vitro* maturation were considered grade I (three or more cumulus cell layers), II (two cumulus cell layers) and III (three cumulus cell layers), classified according to the number of cumulus cells and homogeneous appearance of the cytoplasm. The classification of COCs was performed as described by Wright (1998).

Maturation *in vitro*

Viable COCs were matured for 24 hours in TCM 199 medium supplemented with HEPES (25 mM), 10 % SFB, 1.0 µg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, Canada), 50 µg/mL hCG (Profasi™, Serono, São Paulo, Brazil), 1.0 µg/mL estradiol, 16 µg/mL sodium pyruvate, ITS (5 ug/mL insulin-transferrin-selenium), 83.4 µg/mL amikacin and covered with sterile mineral oil (Sigma-Aldrich Co, USA). Atmospheric conditions were 38.7 ºC, 6 % CO_2, 5 % O_2 and 89 % Nitrogen.

Statistical analysis

For the evaluation of follicular size, according to the treatment applied, a difference of proportions test was performed. In the evaluation of the other variables, a completely randomized block (heifers) statistical design was applied, using the analysis of variance with blocks, and a posteriori contrast applying Fisher's LSD test. When the assumptions of normality and homoscedasticity were not met, the Friedman test was used (again including heifers as a block) with their respective a posteriori tests. The significance level was 5%. R software version 4.1.1 was used.

RESULTS

Follicles were classified into three groups: small follicles (≤ 5 mm), medium follicles (5 to 9 mm) and large follicles (≥ 9 mm) (Table 1). The proportion of small follicles was significant (P < 0.00001) for the control group relative to the FSH-SC and FSH-PF127 groups. The proportion of medium follicles was similar in the FSH-SC and FSH-PF127 groups, however, the control group had fewer follicles of the same diameter. This same proportion was observed for large follicles (≥ 9 mm) (Fig. 1).

Tab. 1. Oocytes (mean±SEM) classified as grade I, II or III in Brahman heifers treated without previous treatment (control), with a single subcutaneous injection of FSH or a single subcutaneous injection of FSH diluted in 20% de Pluronic F127 prior to OPU.

	Control	FSH-SC	FSH-PF127	P – *value*
Oocytes grade I	1.70±0.58	1.90±0.57	3.00±0.67	0.97
Oocytes grade II	2.20±0.66	1.70±0.53	2.40±0.59	0.33
Oocytes grade III	0.90±0.63[a]	2.60±0.61[b]	5.60±0.69[c]	<0.0001

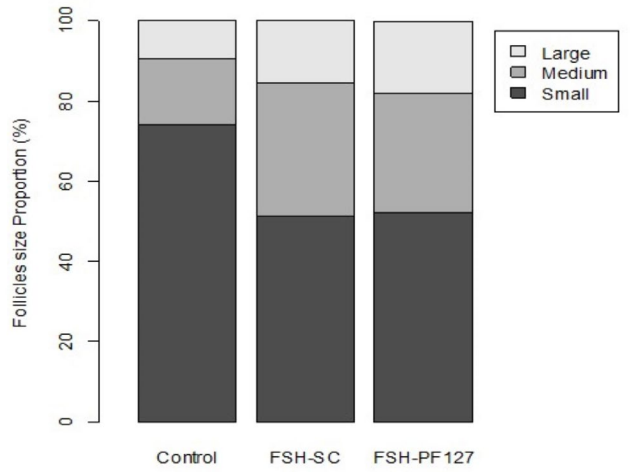
The antral follicular population for the control and FSH-PF127 groups was similar (P > 0.05), however differences were observed between these two groups in relation to the FSH-SC group (P = 0.010) (Fig. 2).

A total of 170 oocytes were retrieved from the control group, 202 from the FSH-SC group and 438 from the FSH-PF127 group in 2 UPO sessions per group. The number of oocytes retrieved from the FSH-PF127 group was higher compared to the control and FSH-SC groups (P = 0.001) (Fig. 3).

Regarding the quality of grade, I (P = 0.97) and II (P = 0.33) oocytes, no effect of FSH was observed. However, the highest number of grade III oocytes (P < 0.0001) was presented by the FSH-PF127 group in relation to the control and FSH-SC groups (Tab. 1).

Finally, the rate of viable oocytes was higher (P < 0.0001) for females treated with FSH-PF127 compared to heifers treated

without FSH (control) and with FSH-SC (Fig. 4).



Follicles size	P – *value*		
	Control - FSH-SC	Control - FSH-PF127	FSH-PF127 - FSH-SC
Small	< 0.00001	< 0.00001	0.7425
Medium	< 0.00001	< 0.00001	0.2032
Large	0.003814	< 0.00001	0.2894

Fig. 1. Proportion of small (≤ 5 mm), medium (5 to 9 mm) and large (≥ 9 mm) follicles of *Bos taurus indicus* heifers subjected to superstimulation with FSH applied prior to follicular aspiration.

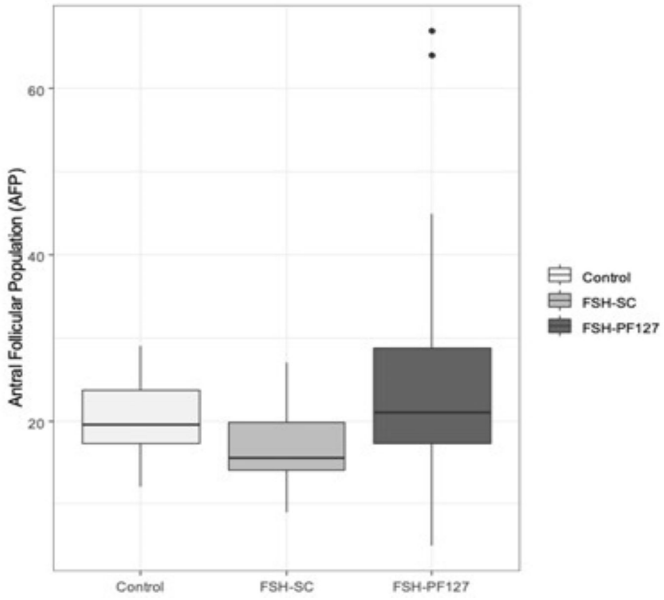


Fig. 2. Antral follicular population of *Bos taurus indicus* heifers subjected to superstimulation with FSH applied prior to follicular aspiration.

DISCUSSION

In the present study it was evidenced that the application of FSH prior to UPO increased the proportion of medium follicles (5 to 9 mm). These results are consistent with those presented by Egashira *et al.* (2019); Da Silva *et al.* (2017) and Vieira *et al.* (2016). The pathway by which FSH is considered to stimulate follicular growth is through the synthesis of insulin-like growth factor type 1, a factor mediated by protein kinase A present in medium-sized follicles (Yu *et al.*, 2003). In addition, FSH contributes to steroidogenesis and granulosa cell mitogenesis (Spicer and Aad, 2007), which stimulates oocyte developmental capacity (Sirard, 2012; Nivet *et al.*, 2012).

One study reported, Gyr heifers subjected to 100 mg of FSH in decreasing doses, a greater number of viable oocytes that were be submitted to IVP (Elliff *et al.*, 2018). These results were like those obtained with females treated with FSH-PF127, which presented a greater number of viable oocytes in relation to the control and FSH-SC groups.

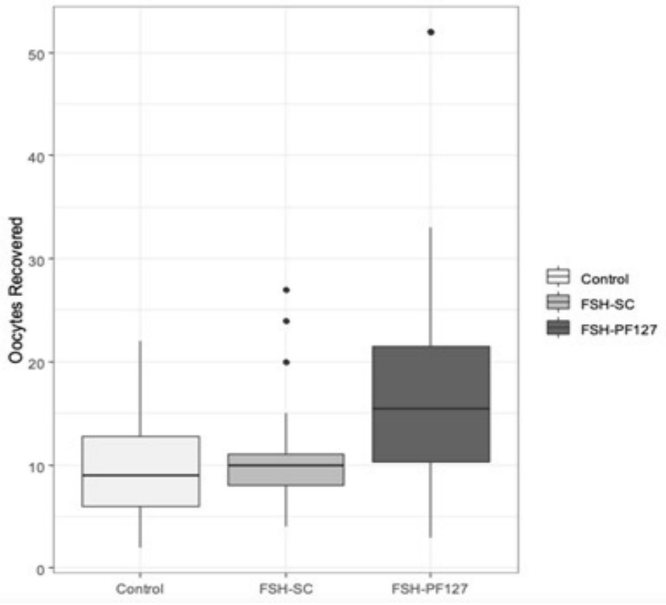


Fig. 3. Number of oocytes recovered from *Bos taurus indicus* heifers subjected to superstimulation with FSH applied prior to follicular aspiration.

In this study, a group of heifers were treated with a single dose of FSH applied subcutaneously and a second group was incorporated FSH in Pluronic® F127. Both treatments were efficient in increasing the number of oocytes recovered, improving oocyte quality, and increasing the rate of viable oocytes. It was observed that the oocyte quality grade I and II was not affected in any of the treatments; however, grade III oocytes of heifers treated with FSH in PF127 were higher in relation to the control and FSH-SC groups. These results are divergent from those reported by Da Silva *et al.* (2017), where treatment with FSH did not alter oocyte quality. However, a meta-analysis study found that FSH administration prior to UPO in Bos taurus taurus females, grade I plus grade II oocytes were higher relative to animals not treated with FSH (Sarwar *et al.*, 2020).

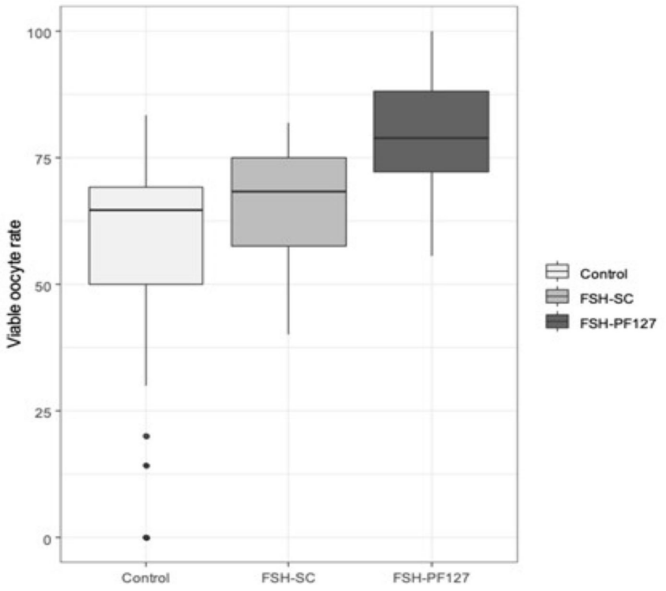


Fig. 4. Viable oocyte rate of *Bos taurus indicus* heifers subjected to superstimulation with FSH applied prior to follicular aspiration.

Another study indicated that exogenous application of FSH increases mRNA transcription of granulosa cells, which activates molecular mechanisms of response to LH and increases markers of oocyte competence in granulosa cells (Dias *et al.*, 2018). According to Sasson *et al.* (2003) and Grieshaber *et al.* (2003) FSH treatments increase the expression of genes involved with the

reorganization of the microtubules of the oocyte cytoskeleton and the synthesis of actin, tubulin, kinesin, and tropomyosin. This contributes to a better reorganization of the cytoplasm and improves oocyte quality.

The administration of gonadotrophins in UPO-PIV programs and in superovulation protocols require the application of two doses of intramuscular FSH per day, management that triggers stress in the animals and possibly errors in the administration of the treatments (Bó *et al*., 2018). In this context, FSH-based hormonal protocols have been simplified in recent years: FSH was reduced to a single dose (Sakaguchi and Nagano, 2020) and the route of administration was modified with the application subcutaneously or using biodegradable polymers such as polyvinylpyrrolidone (Chasombat *et al*., 2013), aluminum hydroxide gel (Egashira *et al*., 2019) and Hyaluronan (Vieira *et al*., 2016).

In *Bos taurus indicus* information is very limited about the use of FSH by unconventional routes prior to UPO. However, in Bos taurus taurus it was demonstrated that the administration of a single dose of FSH was efficient in the superovulation of the treated females (Hiraizumi *et al*., 2015). It is important to highlight in this study and based on the available literature that Pluronic® F127 was used for the first time, in which FSH was incorporated in reduced doses (50 mg), which represents a new approach in the use of biopolymers for the optimization of UPO-PIV programs in cattle.

CONCLUSION

With these results it is concluded that the administration of a single dose of 50 mg of FSH using a biodegradable polymer such as Pluronic® F127 influence the follicular population, the size of the follicles, the oocyte quality, and the rate of viable oocytes of Brahman breed heifers.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in the conduct of the study.

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