

## Oocyte recovery and in vitro embryo production in cows treated with a single dose of follicle-stimulating hormone

Thiago Marquez FERNANDES<sup>1</sup> , Paulo Roberto ADONA<sup>1,\*</sup> , Samuel GUEMRA<sup>1</sup> ,  
Tiago Henrique Camara DE BEM<sup>2</sup> , Moysés dos Santos MIRANDA<sup>3</sup> 

<sup>1</sup>Animal Health and Production, Unopar University, Araçongas, Parana, Brazil

<sup>2</sup>Department of Veterinary Medicine, University of Sao Paulo, Pirassununga, Sao Paulo, Brazil

<sup>3</sup>LabTronVet - Veterinary Medicine, Federal University of Pará, Castanhal, Parana, Brazil

Received: 26.07.2019 • Accepted/Published Online: 14.12.2019 • Final Version: 10.02.2020

**Abstract:** Cows were treated with follicle-stimulating hormone (FSH) in a single dose and then subjected to ovum pick-up (OPU) for evaluation of oocyte recovery and in vitro fertilization (IVF). Cows underwent OPU followed by application of FSH (200 mg) on day 2 and new OPU on day 3. This methodology was repeated 3 times sequentially. In FSH-free the cows (control), OPU was performed at intervals of one week. The number of oocytes recovered was higher in the control cows ( $22.2 \pm 1.5$ ) compared to the treated cows ( $16.9 \pm 1.4$ ;  $P < 0.05$ ). There was no difference between the control ( $13.2 \pm 0.6$ ) and the treated ( $11.2 \pm 1.1$ ) cows in the number of viable oocytes ( $P > 0.05$ ). There was no difference between the control cows ( $47.1 \pm 4.2\%$ ) and the treated cows ( $45.3 \pm 7.7\%$ ) with respect to the percentage of embryos ( $P > 0.05$ ). There were no differences in pregnancy for the blastocysts between the control and the treated cows ( $50.0 \pm 2.1$  and  $47.3 \pm 2.5\%$ ;  $P > 0.05$ ), respectively. In conclusion, the single-dose FSH protocol did not significantly alter the evaluated results of viable oocytes, in vitro production of embryos, or pregnancy percentage. However, there is a possibility of several consecutive OPU/IVF sessions every three days.

**Key words:** Bovine, cryopreservation, ovum pick-up, pregnancy

Ovaries are structures related to the production and secretion of hormones and other molecules that are essential to reproductive performance [1–3]. Pools of follicles are recruited continuously throughout the reproductive life of bovines but only one follicle advances to ovulation and this one is considered to be the dominant follicle. The other follicles gradually become atretic during the phase of follicular growth [2,4].

The production of gonadotropins [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)] occurs in the pituitary gland. FSH stimulates follicular development and estrogen secretion in the ovary [1,5]. During the formation of the granulosa cell layer, FSH receptors are expressed in the oocyte-cumulus complex, and therefore the follicular development becomes dependent on the presence of FSH [1,6]. Growth and follicular dominance are related to plasma concentrations of FSH, and a reduction in plasma FSH levels interrupts the growth of the subordinate follicles, which later enter atresia and inhibit the recruitment of new follicles, reducing the quantity and quality of the recruited follicles [2]. In cattle, these ovarian processes are interesting

in respect to research on reproductive biotechniques, especially for the development of new ovarian stimulation protocols that promote follicular growth, thus allowing the collection of a greater number of oocytes or embryos.

The manipulation of plasma FSH levels could be an alternative method to maximize oocyte recovery via ovum pick-up (OPU), delaying the dominant follicle selection, preventing atresia of subordinate follicles, and increasing the mean diameter of follicles by making them available for OPU [7]. The response to exogenous FSH, however, may be influenced by factors such as variation in the concentration of FSH administered, the breed (*Bos taurus indicus* or *Bos taurus taurus*), the method of application, the frequency of administration, and the interaction of FSH with LH [8–11]. The Nelore breed (*Bos taurus indicus*) differs from other breeds as these cows produce more follicles per wave of follicular growth and are more sensitive to FSH treatment, and they stand out regarding the in vitro production of embryos [11,12]. These peculiarities of the Nelore breed may have implications for the success of FSH treatments for manipulation of follicular development.

\* Correspondence: paulo\_adona@yahoo.com.br

Approximately 80% of the Brazilian cattle herd belong to the Nelore breed, which is the base of the productive chain in the country. Because of its importance and the need to develop a simplified follicular overstimulation protocol, the objective of the present study was to examine oocyte-donor Nelore cows treated with single doses of FSH and subjected to OPU after 24 h in three consecutive procedures every three days, to evaluate the effects of FSH on oocyte recovery, in vitro embryo production, and pregnancy rates.

We selected 12 cows of the Nelore breed aged between four and six years, cycling normally (presence of corpus luteum), and with a body condition score greater than 3.0 (scale of 1–5). The donors were also selected if they had follicular populations of more than six follicles per ovary. The groups of donors (6 control and 6 treated cows) were established, presenting similar follicular population among themselves. The donors were kept in a pasture of *Brachiaria brizantha*, with access to water and mineral salt ad libitum. The experiment was approved by the relevant committee of the university and was performed in accordance with animal welfare and ethics (No. 004/17).

The OPU interval and the dose of FSH were based on the studies of Chaubal et al. [13] and Vieira et al. [14], but with adaptations aimed at simplifying the protocol. Initially (day 0), follicles larger than 3 mm in diameter were removed two days (day 2) prior to intramuscular injection of 200 mg of FSH (Folltropin-V, Bioniche Animal Health, Inc., USA) in a single dose. Another OPU procedure (day 3) was performed 24 h after application of FSH. These procedures were repeated three consecutive times at 3-day intervals for each FSH/OPU. For the control group (FSH-free cows), OPU was performed at 1-week intervals. The OPU procedure was based on the study of De Almeida et al. [15], using a convex transducer (5–12 MHz) coupled with an ultrasound device (DP-2200Vet, Mindray Medical International Ltd., China). Oocytes aspirated from cows were selected in a petri dish (Corning, 430167) using a stereomicroscope (SMZ645, Nikon Instruments Inc., USA). Viable oocytes with more than one layer of cumulus cells with homogeneous cytoplasm were selected and sent to the in vitro embryo production laboratory in TCM 199-HEPES (M7528) medium supplemented with 10% fetal bovine serum and 50 µg/mL gentamicin. The oocytes were matured in TCM 199 (M4530) supplemented with 10% fetal bovine serum, 0.2 µM pyruvate, 100 µM cysteamine, 50 µg/mL gentamicin, 0.5 µg/mL (FSH, Folltropin-V), and 5.0 µg/mL (LH, Lutropin). Maturation was performed in petri dishes (Corning, 430166), in 100 µL of medium drops, under mineral oil for 22 h in a cell culture incubator (Forma, 3110, Thermo Fisher Scientific Inc., USA) at 38.5 °C in an atmosphere containing 5% CO<sub>2</sub> [15].

Frozen Nelore semen was prepared according to the density gradient technique using BoviPure (Nidacon International AB, Sweden). In vitro fertilization was carried out in Tyrode's albumin, lactate, and pyruvate medium with 2 mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine, and 20 µg/mL heparin. Oocytes and spermatozoa ( $2 \times 10^6$  cells/mL) were co-incubated in 100 µL drops of the medium under mineral oil in petri dishes (Corning, 430166) for 18 h. In vitro culturing was performed in a synthetic oviduct fluid (SOF) medium at 38.5 °C in an atmosphere of 5% CO<sub>2</sub> [16]. Only class I blastocysts (seven days after in vitro fertilization) were transferred or cryopreserved.

Cryopreservation was performed in PBS with 20% FBS, 1.5 M ethylene glycol, 50 µg/mL gentamicin, and 100 µM cysteamine. During the dehydration step (10 min), the embryos (three) were packaged in straws (0.25 mL) and placed in Freeze Control equipment (CryoLogic Pty. Ltd., Australia) set to –6 °C. Seeding was carried out on the embryo column and the freezing curve was 0.5 °C/min to –30° C, and then they were stored in liquid nitrogen. Defrosting of straws was performed by keeping them in air for 10 s and then immersing them in water at 36 °C for 1 min. The embryos remained in SOF medium in a cell culture incubator for 2 h and the embryos with cell expansion (rehydrated), as visualized by stereomicroscope, were transferred.

For embryo transfer and pregnancy diagnosis, the recipients (½ Nelore and ½ Brown Swiss) in the follicular phase (cyclic) were synchronized with an intravaginal progesterone implant according to the procedure previously described by Marques et al. [17]. The transfer was made by the transcervical method for recipients with corpus luteum of  $\geq 18$  mm in diameter. Pregnancy was diagnosed by transrectal ultrasonography (DP-2200Vet) 60 days after the transfer. The Mann–Whitney U test was used to determine the effects of exogenous FSH in Nelore donors with respect to the frequencies of total and viable oocytes, blastocysts, cryopreservation, and pregnancy. Values of  $P < 0.05$  were considered significant.

In total, 400 oocytes were recovered from the control cows and 305 from the FSH-treated animals. The mean number of oocytes recovered (Figure 1a) was significantly higher in the control cows ( $22.2 \pm 1.5$ ) than in those treated with FSH ( $16.9 \pm 1.4$ ;  $P < 0.05$ ). The mean number of viable oocytes selected was 238 for the control and 201 for the FSH-treated animals. There were no significant differences ( $P > 0.05$ ) between the control and FSH-treated cows for the mean number ( $13.2 \pm 0.6$  and  $11.2 \pm 1.1$ , respectively) or the mean percentage ( $59.5 \pm 3.7\%$  and  $65.9 \pm 2.1\%$ , respectively) of viable oocytes (Figure 1b and 1c).

The number of embryos produced (class I) from viable oocytes was 112 for control cows and 91 for FSH-treated

animals. There was no significant difference between the control and FSH-treated cows ( $P > 0.05$ ) in relation to the average percentage of embryos ( $47.1 \pm 4.2\%$  and  $45.3 \pm 7.7\%$ , respectively) or the mean number of embryos ( $6.2 \pm 0.8$  and  $5.1 \pm 0.6$ , respectively) produced in vitro in both groups. (Figure 2a and 2b).

The blastocyst rehydration rates (cryopreserved) did not differ among donors of the control group and those treated with FSH ( $P > 0.05$ ). There was also no significant variation in the pregnancy rates (Table) between groups with cryopreserved and fresh blastocysts or between the control and the FSH-treated donors ( $P > 0.05$ ).

In vitro production of bovine embryos is one of several biotechnologies widely used to accelerate the production of genetically superior cows and OPU is the first step towards better use of genetic material from donors. In conjunction with biotechniques, the manipulation of plasma FSH concentrations may be an alternative method

for maximizing oocyte recovery via OPU. In the present study, however, the animals that were treated with FSH had a mean oocyte recovery lower than that of the control. Despite this reduction in oocyte numbers, three OPU procedures were carried out in a period of nine days. If these OPU procedures (every three days) were carried out without FSH treatment, the reduction in oocyte numbers would probably be much more significant [18]. One of the factors that may have contributed to the lower oocyte recovery in cows treated with FSH is probably the greater sensitivity of the Nelore breed (*Bos taurus indicus*) to the effects of exogenous FSH [11,19]. Previous studies, however, are variable in their results regarding oocyte recovery with FSH administration. This may be due to differences in the breed of animals, age of the donors, and/or the protocols of FSH administration used. For example, in almost all of the previous studies, three or more doses of FSH were administered on consecutive days, which differs from

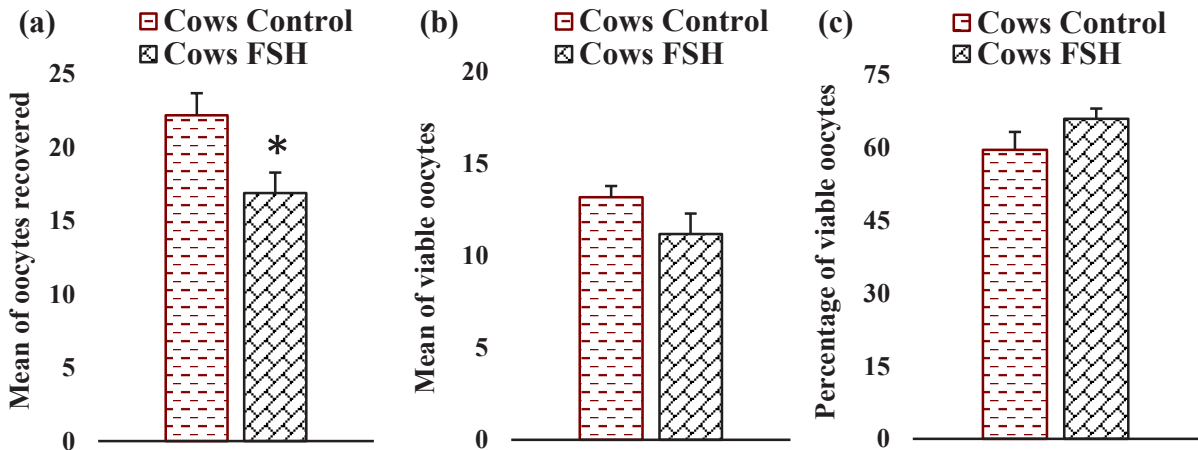


Figure 1. Mean of oocytes recovered (a), mean of viable oocytes (b), and percentage of viable oocytes (c) from cows treated with follicle-stimulating hormone (FSH) or not (control). \* $P < 0.05$ .

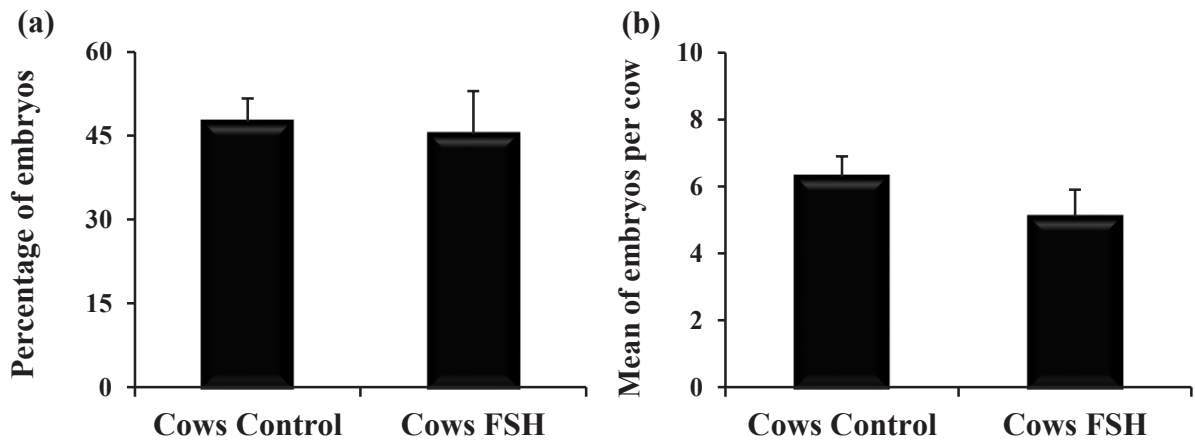


Figure 2. Mean percentage of in vitro embryo production (a) and mean number of embryos per cow (b) of cows treated with follicle-stimulating hormone (FSH) or not. Seven days after in vitro fertilization.

**Table.** Pregnancy rates from fresh and cryopreserved embryos transferred from cows treated with follicle-stimulating hormone (FSH) or not.

Treatments	Total N	Rehydrated/Cryo N/N (% $\pm$ SD)	Pregnancy N/N (% $\pm$ SD)	Pregnancy/Fresh N/N (% $\pm$ SD)
Donors - control	112	25/42 (59.5 $\pm$ 5.3)	10/25 (40.0 $\pm$ 6.0)	35/70 (50.0 $\pm$ 2.1)
Donors - FSH	91	24/36 (66.7 $\pm$ 14.0)	10/24 (41.7 $\pm$ 3.6)	26/55 (47.3 $\pm$ 2.5)

Mean percentage (%) and standard deviation ( $\pm$ SD) of the mean from three replicates. Cryopreserved (Cryo), seven days after in vitro fertilization.

the present study, in which only a single dose of FSH was administered and 24 h later OPU was performed [13,14,20]. Results on the effects of single-dose administration of FSH at short intervals and OPU for oocyte retrieval are scarce, and in addition, previous studies describe protocols with other variables and with different breeds (e.g., Holstein and Angus), differing greatly from this study [13,14,21]. As already mentioned and according to Baruselli et al. [11], there are some important physiological differences between *Bos taurus indicus* and *Bos taurus taurus* animals that affect the rates of ovarian overstimulation, resulting in variation between studies [11,19].

As already mentioned, under FSH treatment, there was significant reduction in the total mean of recovered oocytes, but this same effect was not observed in the percentage of viable oocytes. This similarity (viable oocytes) was also observed in a previous study despite the differences between the breed of animals and FSH administration protocol [20]. The present study also exhibited a similar result to another study in the percentage of viable oocytes with respect to the Nelore breed (*Bos taurus indicus*) [22]. Despite the similarity observed in the percentage of viable oocytes among donors (control group and group treated with FSH), FSH is involved in increasing the diameter of the follicles and may also contribute to better organization of the oocyte cytoplasm, contributing to the quality of the oocytes recovered for use in programs of in vitro production of embryos [20,23].

No significant difference was observed in the percentage of embryos between the groups, probably owing to the similarity in the percentage of viable oocytes between the control and treated animals. This similarity in the

percentage of embryos was possibly due to the quality of oocytes subjected to in vitro fertilization, which was similar for both groups, since the selected oocytes exhibited more than one layer of compact cumulus cells and homogeneous cytoplasm. FSH has been related to the acquisition of oocyte developmental competence, both in vivo and in vitro, in addition to the regulation of somatic cellular functions for follicular development [8,23], but this increase in embryo production rate was not observed in this or in another previous study [20]. However, there have been studies that reported that in vitro embryo production rate is affected by intrinsic oocyte quality, where in vitro embryo quality is compromised by the in vitro culture system [24,25]. Based on this assumption, as the percentage of viable oocytes between the groups was similar, the percentage of embryos produced in vitro can also be similar between the groups.

In this study, the properties of in vitro culture and cryopreservation systems were identical between treatments, and the different methods (cows treated with FSH or not) did not significantly influence the survival of the embryos during cryopreservation or the pregnancy rates of either cryopreserved embryos or fresh embryos. The absence of significant differences in pregnancy rates between treatments can be attributed to the similar quality of the embryos. Results similar to those found in the present study have been reported in the literature, with different breeds or protocols for obtaining oocytes [22,26].

In conclusion, the single-dose FSH protocol did not significantly alter the evaluated results of viable oocytes, in vitro production of embryos, and pregnancy percentage. However, there is a possibility of several consecutive OPU/IVF sessions every three days.

## References

- Gervasio CG, Bernuci MP, Silva-de-Sa MF, Rosa ESAC. The role of androgen hormones in early follicular development. *ISRN Obstetrics and Gynecology* 2014; 10: 1-10. doi: 10.1155/2014/818010
- Adams GP, Jaiswal R, Singh J, Malhi P. Progress in understanding ovarian follicular dynamics in cattle. *Theriogenology* 2008; 69 (1): 72-80. doi: 10.1016/j.theriogenology.2007.09.026
- Moussa M, Farouk SM. Ovarian stimulation induces high expression of interleukin-1 $\beta$  and disrupts the histological features of the fallopian tube. *Turkish Journal of Veterinary and Animal Sciences* 2019; 43 (2): 186-196. doi: 10.3906/vet-1710-52

4. Ginther OJ, Siddiqui MA, Baldrighi JM, Wolf CA. Systemic effect of follicle-stimulating hormone and intraovarian effect of the corpus luteum on complete regression vs recovery of regressing wave-2 follicles in heifers. *Domestic Animal Endocrinology* 2016; 55: 51-59. doi: 10.1016/j.domaniend.2015.11.002
5. Shimizu T. Molecular and cellular mechanisms for the regulation of ovarian follicular function in cows. *Journal of Reproduction and Development* 2016; 62 (4): 323-329. doi: 10.1262/jrd.2016-044
6. Bao B, Garverick HA. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *Journal of Animal Science* 1998; 76 (7): 1903-1921. doi: 10.2527/1998,761903x
7. Ginther OJ, Siddiqui MA, Baldrighi JM, Araujo ER. An intraovarian mechanism that enhances the effect of an FSH surge on recovery of subordinate follicles in heifers. *Domestic Animal Endocrinology* 2017; 59: 67-74. doi: 10.1016/j.domaniend.2016.10.003
8. Zacarias TA, Kawamoto TS, Scaliante Junior JR, Silva Guimaraes AL, Franco MM et al. Hormonal stimulation in 4 to 7 months old Nelore (*Bos taurus indicus*) females improved ovarian follicular responses but not the *in vitro* embryo production. *Theriogenology* 2018; 118: 130-136. doi: 10.1016/j.theriogenology.2018.05.039
9. Sartori R, Bastos M, Baruselli P, Gimenes L, Ereno R et al. Physiological differences and implications to reproductive management of *Bos taurus* and *Bos indicus* cattle in a tropical environment. *Society of Reproduction and Fertility Supplement* 2010; 67: 357-375.
10. Kaya A, Güneş E, Memili E. Application of reproductive biotechnologies for sustainable production of livestock in Turkey. *Turkish Journal of Veterinary and Animal Sciences* 2018; 42 (3): 143-151. doi: 10.3906/vet-1706-66
11. Baruselli PS, Gimenes LU, Sales JNS. Reproductive physiology of *Bos taurus* and *Bos indicus* females. *Revista Brasileira de Reprodução Animal* 2007; 31: 205-211. doi: 10.1016/j.theriogenology.2016.04.016
12. Silva-Santos KC, Santos GM, Siloto LS, Santos JT, Oliveira ER et al. The correlation between the number of antral follicles and ovarian reserves (preantral follicles) in purebred *Bos indicus* and *Bos taurus* cows. *Animal Reproduction Science* 2014; 151 (3): 119-125. doi: 10.1016/j.anireprosci.2014.10.016
13. Chaubal SA, Ferre LB, Molina JA, Faber DC, Bols PE et al. Hormonal treatments for increasing the oocyte and embryo production in an OPU-IVP system. *Theriogenology* 2007; 67 (4): 719-728. doi: 10.1016/j.theriogenology.2006.07.022
14. Vieira LM, Rodrigues CA, Castro Netto A, Guerreiro BM, Silveira CRA et al. Efficacy of a single intramuscular injection of porcine FSH in hyaluronan prior to ovum pick-up in Holstein cattle. *Theriogenology* 2016; 85 (5): 877-886. doi: 10.1016/j.theriogenology.2015.10.036
15. de Almeida Barros FD, Adona PR, Guemra S, De Bem THC, Monzani PS et al. *In vitro* embryos production of oocytes selected based on follicular size and by brilliant cresyl blue staining. *Semina Ciências Agrárias* 2019; 40 (4): 1523-1534. doi: 10.5433/1679-0359.2019v40n4p1523
16. Sovernigo TC, Adona PR, Monzani PS, Guemra S, Barros F et al. Effects of supplementation of medium with different antioxidants during *in vitro* maturation of bovine oocytes on subsequent embryo production. *Reproduction in Domestic Animals* 2017; 52 (4): 561-569. doi: 10.1111/rda.12946
17. Marques MO, Morotti F, da Silva CB, Junior MR, da Silva RC et al. Influence of category--heifers, primiparous and multiparous lactating cows--in a large-scale resynchronization fixed-time artificial insemination program. *Journal of Veterinary Science* 2015; 16 (3): 367-371. doi: 10.4142/jvs.2015.16.3.367
18. De Roover R, Feugang JM, Bols PE, Genicot G, Hanzen C. Effects of ovum pick-up frequency and FSH stimulation: a retrospective study on seven years of beef cattle *in vitro* embryo production. *Reproduction in Domestic Animals* 2008; 43 (2): 239-245. doi: 10.1111/j.1439-0531.2007.00873.x
19. Barros CM, Nogueira MF. Embryo transfer in *Bos indicus* cattle. *Theriogenology* 2001; 56 (9): 1483-1496. doi: 10.1016/S0093-691X(01)00648-3
20. da Silva JCB, Ferreira RM, Maturana Filho M, Naves JR, Santin T et al. Use of FSH in two different regimens for ovarian superstimulation prior to ovum pick up and *in vitro* embryo production in Holstein cows. *Theriogenology* 2017; 90: 65-73. doi: 10.1016/j.theriogenology.2016.11.016
21. Silva BLMD, Adona PR, Guemra S, Monzani PS, Miranda MdS. Ovum pick up: cows treated with single doses of follicle stimulating hormone. *Journal of Agricultural Science* 2019; 11 (10): 231. doi: 10.5539/jas.v11n10p231
22. Guemra S, da Silva Santo E, Zanin R, Monzani PS, Sovernigo TC et al. Effect of temporary meiosis block during prematuration of bovine cumulus-oocyte complexes on pregnancy rates in a commercial setting for *in vitro* embryo production. *Theriogenology* 2014; 81 (7): 982-987. doi: 10.1016/j.theriogenology.2014.01.026
23. Dias FCF, Khan MIR, Sirard MA, Adams GP, Singh J. Transcriptome analysis of granulosa cells after conventional vs long FSH-induced superstimulation in cattle. *BMC Genomics* 2018; 19 (258): 1-11. doi: 10.1186/s12864-018-4642-9
24. De Bem T, Adona PR, Bressan FF, Mesquita LG, Chiaratti MR et al. The influence of morphology, follicle size and Bcl-2 and Bax transcripts on the developmental competence of bovine oocytes. *Reproduction in Domestic Animals* 2014; 49 (4): 576-583. doi: 10.1111/rda.12325
25. Lopera-Vasquez R, Hamdi M, Maillo V, Gutierrez-Adan A, Bermejo-Alvarez P et al. Effect of bovine oviductal extracellular vesicles on embryo development and quality *in vitro*. *Reproduction* 2017; 153 (4): 461-470. doi: 10.1530/REP-16-0384
26. Sanches BV, Lunardelli PA, Tannura JH, Cardoso BL, Pereira MH et al. A new direct transfer protocol for cryopreserved IVF embryos. *Theriogenology* 2016; 85 (6): 1147-1151. doi: 10.1016/j.theriogenology.2015.11.029