Effect of single dose follicle stimulating hormone on follicular aspiration, *in vitro* fertilization and pregnancy rate

Efeito do hormônio folículo estimulante em dose única na aspiração folicular, fertilização in vitro e taxa de prenhez

Mayara Ellen Bardi de Moraes[1]; Paulo Roberto Adona[1] ; Samuel Guemra[1];
Tiago Henrique Camara de Bem[2]; Moysés dos Santos Miranda[3]

[1] Universidade Norte do Paraná, Curso de Medicina Veterinária, Arapongas – PR, Brazil
[2] Universidade de São Paulo, Faculdade de Zootecnia e Engenharia de Alimentos, Departamento de Medicina Veterinária, Pirassununga – SP, Brazil
[3] Universidade Federal do Pará, Faculdade de Medicina Veterinária, LabTronVet, Castanhal – PA, Brazil

ABSTRACT

The present study evaluated Brangus cows treated with single doses of follicle stimulating hormone (FSH) subjected to follicular aspiration after 24 h to assess oocyte recovery, *in vitro* fertilization and pregnancy rate. Follicles exceeding 3 millimeters in diameter were aspirated, 200 mg of FSH was administered 2 days later, and a new ovum pickup was performed 24 h afterward. These methods were performed 3 times every 3 days. In control, follicular aspirations occurred at intervals of 1-week without FSH administration o. The aspirated oocytes were evaluated, submitted to *in vitro* fertilization and the embryos were transferred to the recipients. The average recovery of oocytes was higher (p<0.05) in control cows (12.4±1.8) than in treated cows (9.4±1.3). There was no difference (p>0.05) in the mean percentage of viable oocytes (52.0±3.9 and 62.7±4.7%) or the mean percentage of embryos (41.4±4.8 and 41.5±4.2%) among control and treated cows, respectively. The mean percentage of pregnancy did not differ (p>0.05) for control cows (43.8±2.7%), and treated cows (40.9±6.8%). In conclusion, FSH treatment did not improve oocyte recovery, *in vitro* fertilization, and pregnancy percentage. However, there is possibility of several consecutive ovum pickup every t3 days, concentrating the *in vitro* fertilization and the pregnancy percentage.

Keywords: Bovine. Follicle. Ovum pickup.

RESUMO

O presente estudo avaliou vacas Brangus tratadas com doses únicas de hormônio folículo estimulante (FSH) submetidas a aspiração folicular após vinte e quatro horas, para avaliação da recuperação oocitária, fertilização *in vitro* e taxa de prenhez. Folículos superiores a três milímetros de diâmetro foram aspirados, 200 mg de FSH foram administrados dois dias depois e uma nova aspiração folicular foi realizada 24 horas após. Esses métodos foram efetivados três vezes a cada três dias. No controle, as aspirações foliculares ocorreram em intervalos de uma semana sem administração de FSH. Os oócitos aspirados foram avaliados, submetidos à fertilização *in vitro* e os embriões foram transferidos em receptoras. A recuperação média dos oócitos foi superior (p<0,05) nas vacas controle (12,4±1,8) do que nas vacas tratadas (9,4±1,3). Não houve diferença (p>0,05) na porcentagem média de oócitos viáveis (52,0±3,9 e 62,7±4,7%) ou na porcentagem média de embriões (41,4±4,8 e 41,5±4,2%) entre vacas controle e vacas tratadas, respectivamente. A porcentagem média de prenhez não diferiu (p>0,05) para as vacas controle (43,8±2,7%) e as tratadas (40,9±6,8%). Em conclusão, o tratamento com FSH não melhorou a recuperação de oócitos, a fertilização *in vitro* e o percentual de prenhez. No entanto, existe a possibilidade de várias aspirações foliculares consecutivas a cada três dias, concentrando a fertilização *in vitro* e o percentual de prenhez.

Palavras-chave: Aspiração follicular. Bovinos. Folículos.

Correspondence to:
Paulo Roberto Adona
Universidade Norte do Paraná, Curso de Medicina Veterinária.
Av. Gaturamo, Rodovia PR 218
CEP: 86702-670, Arapongas – PR, Brasil
e-mail: paulo_adona@yahoo.com.br

Introduction

The recovery of oocytes via follicular aspiration associated with *in vitro* fertilization is a biotechnique used to accelerate the production of animals with superior characteristics. Embryos produced *in vitro* correspond to more than 40% of the embryos produced in the world (Monteiro et al., 2017; Perry, 2014). This interest in the *in vitro* fertilization is due to several factors, such as improvements in the *in vitro* culture system, acceleration of the production of animals with superior characteristics, and the possibility of using sexed semen, enabling the production of livestock of a defined sex (Gimenes et al., 2015; Monteiro et al., 2017; Pontes et al., 2010).

Despite advances in *in vitro* fertilization, several factors may still limit the achievement of success of this biotechnology, such as: variations in the number of ovarian follicles between animals or breeds (*Bos indicus* and *Bos taurus*), and the management techniques for *in vivo* production of embryos (Baruselli et al., 2007; Gimenes et al., 2015; Monteiro et al., 2017; Pontes et al., 2010). There is still no conclusive biological explanation to explain the variability between animals in the amount of oocytes aspirated via ovum pickup (OPU) (Pontes et al., 2010). However, due to the advances in *in vitro* fertilization, there is an increasing interest in races, until now little explored in the use of biotechniques (Guemra et al., 2014). The enhancement of hormonal treatment that promotes ovarian stimulation adjusted to support follicular development may allow collection of a more significant number of oocytes per animal, optimizing the production of embryos *in vitro* (Pontes et al., 2010; Vieira et al., 2014). This allows a substantial development in the production of composite breeds used for dairy or cutting, considering their adaptability and productivity (Guemra et al., 2014; Pontes et al., 2010; Vieira et al., 2014). Controlling the plasma concentration of follicle stimulating hormone (FSH) may be an option to elevate oocyte recovery in different pure or synthetic races.

Follicular development is related to the transient increase of serum FSH that promotes the emergence of small follicles (Gupta et al., 2014). However, the decreasing concentration of serum FSH disrupts the development of the subordinate follicles that enter atresia and interrupts the selection of new subordinate follicles. A single follicle continues development (growth) to ovulatory size with an increase in the amount of estradiol synthesis, since the subordinate follicles lose the ability to synthesize estradiol and die from follicular atresia (Adams et al., 2008; Gupta et al., 2014; Nasser et al., 1993).

A strategy to increase oocyte recovery by OPU may be via the application of exogenous FSH in oocyte donors. This transient increase in serum FSH may delay the advance of the dominant follicle, atresia of the other follicles (subordinate), and increased size of the ovarian follicles making them accessible to OPU (Ginther et al., 2017; Mihm et al., 1997). However, this transient increase in exogenous FSH may be influenced by a number of factors, ranging from changes in the administered concentration to the frequency of administration, and the interactions with the luteinizing hormone (LH) (Kelly et al., 1997). Another critical factor that must be considered concerns the reproductive peculiarities of the *Bos indicus* and *Bos taurus* breeds (Baruselli et al., 2007).

Crossing between animals *Bos taurus* and *Bos indicus* is a strategy used to improve productivity and rusticity of animals in subtropical areas (Lamy et al., 2012). These crosses in cattle generally outperform the pure parental breeds in subtropical conditions due to heterosis (Burrow, 2015). The Brangus breed, originating from the genetics of Angus animals crossed with Zebu, produces more rustic animals with good adaptability and fertility, and may be an option for subtropical areas. Despite their value, there are few studies in the literature about the use of assisted reproductive biotechniques using Brangus cows. In this context, the present study aimed to evaluate Brangus cows, treated with FSH in single doses and follicular aspiration 24 h after FSH treatment, with new doses every 3 days, to determine the number of oocytes aspirated, *in vitro* fertilization and rate of pregnancy.

Material and Methods

Selection of donor cows

Twelve Brangus cows (5/8 Nellore and 3/8 Angus) were selected (estrous cycle) in the follicle development phase, containing more than 10 follicles in the ovaries (>3 mm diameters) and with a body score equal to or greater than 3 (scale 1-5). The cows were organized in groups (treated with FSH and control without FSH) with a similar follicular population and kept in *Brachiaria brizantha* grass, with availability to water and mineral salt ad libitum.

OPU from donors

At day zero (Day 0), ovaries with follicles greater than 3 mm in diameter were aspirated to start the study. After 48 h (day 2) in the FSH-treated cow group, a single 200 mg dose of FSH (Folltropin-V, Bioniche Animal Health USA, Inc.) was applied intramuscularly and 24 h later (Day 3), a new OPU was performed. Three consecutive treatments were performed every 3 days (Figure 1). In cows without hormonal treatments, follicular aspirations are usually performed at intervals of 1 week or more. However, follicular aspiration performed more than once per week significantly reduces the number of oocytes recovered. According to this explanation, untreated cows (control) were submitted to OPU once a week. A convex transducer (5-12 MHz) coupled to the DP-2200Vet ultrasound (Mindray Medical International Ltd., China) was used to perform the OPU according to Guemra et al. (2014).

Selection of oocytes aspirated

Most chemical items were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), unless otherwise specified. After each OPU, the oocytes were selected and counted using a filter and a gridded search dish all-in-one (Bioniche Animal Health Canada, ECE051) and a stereomicroscope (Nikon Instruments Inc., USA, SMZ1000). Only oocytes classified as viable (one or more layers of cumulus cells and homogeneous cytoplasm) were sent for *in vitro* fertilization (laboratory) in 1.5 mL tubes (Corning Incorporated, USA, 430487) with 1 mL H199 (Sigma, M7528) with 10% FBS, 50 µg/mL gentamycin, 100 µM cysteamine and 0.2 µM pyruvate (Guemra et al., 2014).

In vitro maturation

After approximately 4 h the *in vitro* maturation of the oocytes was done in B199 (Sigma, M4530) supplemented with 10% FBS, 5.0 µg/mL luteinizing hormone and 0.5 µg/mL follicle stimulating hormone (Bioniche Animal Health USA, Inc.), 100 µM cysteamine, 0.2 µM pyruvate and 50 µg/mL gentamicin. The oocytes were matured in petri dishes (Corning, 430166) in 100 µL media droplets under mineral oil for 22 h in a CO_2 incubator (Thermo Fisher Scientific Inc., USA, Forma Series II 3110) at 38.5 °C under an atmosphere of 5% CO_2 in the air (Adona et al., 2016).

In vitro fertilization

Cryopreserved semen (Brangus) was processed using the density gradient methodology (90% and 45%) with Percoll (Annes et al., 2017) and the *in vitro* fertilization was carried out in a Tyrode's albumin, lactate and pyruvate (TALP) medium supplemented with epinephrine (250 µM) penicillamine (2 µM), hypotaurine (1 µM), and 20 µg/mL heparin (Parrish, 2014). Oocytes and spermatozoa ($2x10^6$ cells/mL) were cultured for 18 h in 100 µL medium (TALP) drops under mineral oil in petri dishes (Corning, 430166). Embryonic
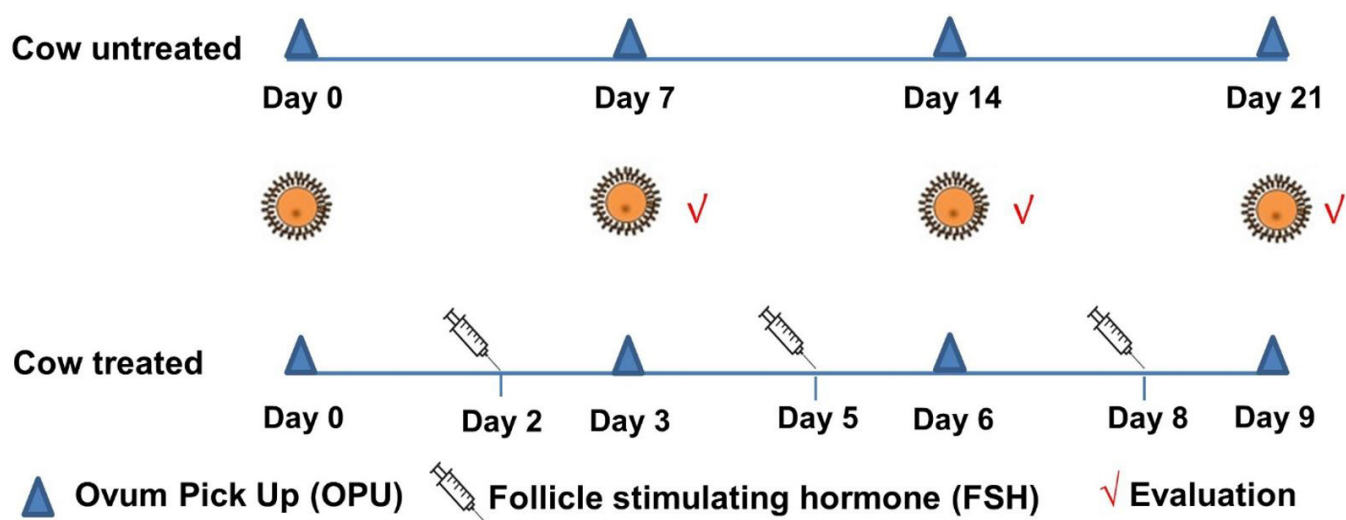

Figure 1 – Synthetized delineation of the experimental representation. Consecutive OPU protocol for treated (with 200 mg FSH) and untreated Brangus cows.
Adapted figure: Silva et al. (2019).

development was performed in synthetic oviduct fluid (SOF) medium at 38.5°C at a 5% CO_2 atmosphere in the air (Holm et al., 1999; Sovernigo et al., 2017).

Embryo transfer and pregnancy diagnosis

Recipient cows (Nellore ½ and Brown Swiss ½) were synchronized with intravaginal progesterone implants according to the methodology described by Marques et al. (2015). Embryos (Class I) were transferred by the transcervical method in cows with a *corpus luteum* of ≥18 mm in diameter. The procedure for assessing pregnancy was by transrectal ultrasonography, 60 days after blastocyst transfer.

Statistical analysis

Variables for retrieved oocytes, viable oocytes, embryos and pregnancy were analyzed applying the non-parametric chi-square (X^2) test. Values of $p < 0.05$ were assessed as significant.

Results

Oocyte recovery

A total of 223 oocytes were recovered in the control cows and 169 in FSH-treated cows in 3 OPU sessions. The average recovery of oocytes (Figure 2A) is higher ($p < 0.05$) in control cows (12.4 ± 1.8) than in cows treated with FSH (9.4 ± 1.3).

The total number of viable oocytes selected was 116 for control cows, and 106 for FSH-treated cows. There are no significant differences ($p > 0.05$) in the mean number (6.4 ± 1.2 and 5.9 ± 1.1) or the mean percentage (52.0 ± 3.9 and 62.7 ± 4.7%) of viable oocytes between control (without FSH), and FSH-treated cows (Figure 2B, C).

In vitro fertilization

From the viable oocytes, 48 embryos were produced *in vitro* from control cows and 44 embryos from cows treated with FSH. The mean percentage of embryos (41.4 ± 4.8 and 41.5 ± 4.2%) or mean number of embryos per cow (2.7 ± 0.8 and 2.4 ± 0.6) are not significantly different ($p > 0.05$) between control (without FSH) and FSH-treated cows (Figure 3A, B).

Pregnancy rate

Embryos produced *in vitro* for the control cows (48) and FSH-treated cows (44) were transferred to recipient cows. Twenty-one positive pregnancies were reported for control and 18 for FSH-treated cows. The mean percentage of pregnancies for the transferred embryos (*in vitro*) (Figure 4) are not significantly different ($p > 0.05$), between
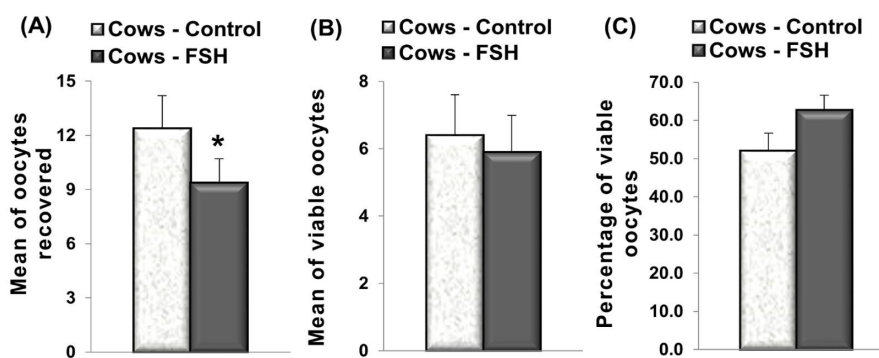

Figure 2 – Mean of total oocytes recovered (A), mean of viable oocytes (B) and percentage of viable oocytes (C) for treated (follicle stimulating hormone - FSH) and untreate3d cows. *Significant difference ($p < 0.05$).
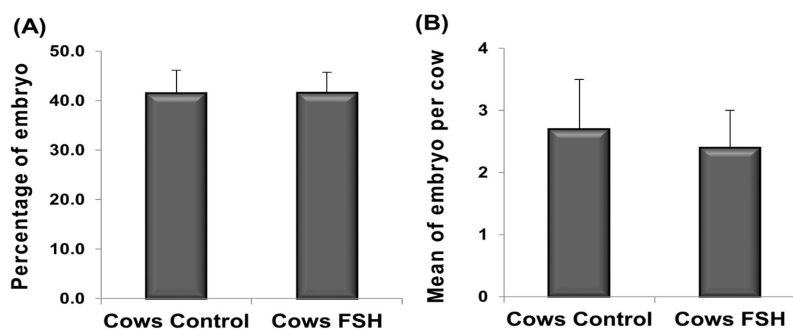

Figure 3 – Mean percentage of blastocysts in vitro production (A) and mean of blastocysts per cow (B) for treated (follicle stimulating hormone - FSH) and untreated cows.
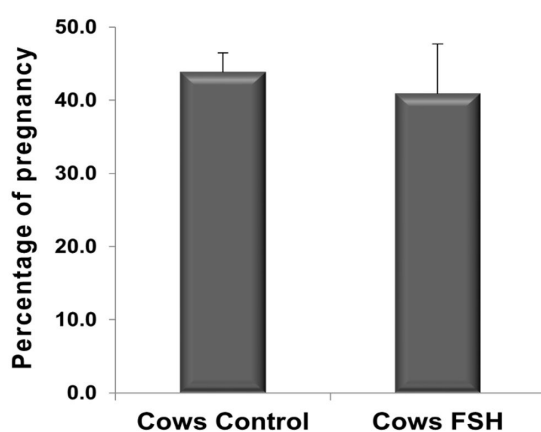
Figure 4 – Mean percentage of pregnancies for treated (follicle stimulating hormone - FSH) and untreated cows.

control (without FSH) cows (43.8 ± 2.7%) and FSH-treated cows (40.9 ± 6.8%).

Discussion

Hormone stimulation with FSH before OPU can be used to increase follicular population and oocyte quality for the *in vitro* production of embryos (Aller et al., 2010; Goodhand et al., 1999). However, in the present study, the administration of FSH before OPU produced a lower recovery in the total number of oocytes in hormone-treated Brangus cows. This lower rate in total oocyte retrieval in oocyte donor cows treated with FSH may question the overall benefits of superstimulation (Vieira et al., 2014). However, studies show discordant results in the recovery of oocytes with FSH stimulation before OPU. These disagreements between studies may be related to cow breeds, FSH administration protocols, and other causes, such as the age of the cows, genetics, climate and nutrition (Chaubal et al., 2007; Goodhand et al., 2000; Ptak et al., 2003; Silva et al., 2017; Vieira et al., 2014; Vieira et al., 2016).

Physiological differences between bovine breeds (cows) are widely described in the literature. These particularities may have implications for the success of treatments that manipulate follicular development (Bó et al., 2003; Sartori et al., 2016). However, more studies are necessary to evaluate synthetic cows (crosses between *Bos indicus* and *Bos taurus*) as donors of oocytes with the application of FSH and OPU, especially for the Brangus breed. However, despite the decrease in the number of oocytes aspirated in the Brangus cows treated with FSH, there is the possibility of several consecutive OPU in close periods (every 3 days), optimizing donor cows and the rates of pregnancy derived from the *in vitro* fertilization.

Decrease in the number of total oocytes aspirated in FSH-treated Brangus cows did not directly affect

the number or rate of viable oocytes among the donors evaluated. However, FSH may have favored uniformity in the number of viable oocytes. According to some studies, FSH is implicated in the follicular development and arrangement of the oocyte cytoplasm determining its quality (Silva et al., 2017; Ginther et al., 2016). This similarity regarding viable oocytes was also observed in other publications that found that the percentage of viable oocytes did not undergo significant changes even in races and protocols of FSH application different from those used in this study (Chaubal et al., 2006; Silva et al., 2017; Vieira et al., 2014). The percentages of viable oocytes in this study, regardless of the treatment of the cows, were superior to that observed in another study that used Brangus cows in the OPU procedure for *in vitro* embryo production (Guemra et al., 2014). These different results, even within the same breed (Brangus), may be due to the heterogeneity of the synthetic breeds, FSH administration protocols and other factors like age, climate, nutrition, and animal genetics, as previously mentioned (Chaubal et al., 2006; Ptak et al., 2003).

The results of *in vitro* fertilization are evaluated in terms of the quality of the blastocysts and the pregnancy percentage. Low competence in the *in vitro* fertilization has been attributed to the lower efficiency of oocytes from growing follicles (anthrax) and submitted to *in vitro* production of embryos (Merton et al., 2003; Sirard & Blondin, 1996). However, in spite of the distinctions in the protocols used for obtaining oocytes between cows treated and not with FSH, the percentage of *in vitro* fertilization was similar for both groups. These results suppose that the treatment of donors did not significantly alter the intrinsic quality of the oocytes, the results of the *in vitro* fertilization being equivalent to other researches in different races (Silva et al., 2017; Ribeiro et al., 2011). Another cause that plays a crucial function in the *in vitro* fertilization is oocyte-linked cumulus cells that may negatively affect the outcome of *in vitro* fertilization (Macaulay et al., 2014; Zhang et al., 1995). However, the methods for classification of viable oocytes for *in vitro* fertilization in both groups were the same, homogeneous cytoplasm with more than one layer of compact cumulus cells. This similarity in the percentage of embryos is possibly due to the quality of the oocytes submitted to *in vitro* fertilization, which was similar for both groups. However, FSH has been related to oocyte development capacity, both in vivo and *in vitro*, in addition to control of somatic cellular activities for follicular growth (Sirard et al., 2007), but a relevant increase in the *in vitro* fertilization in FSH-treated animals was not observed in this or other studies (Silva et al., 2017; Ribeiro et al., 2011).

Rizos et al. (2002) mentioned that the quality of blastocysts is compromised by the *in vitro* culture system and may affect pregnancy rates. However, in this study, the properties of the *in vitro* culture system were identical between groups, and they did not interfere with the rate of development of the embryos. Furthermore, the different treatments, cows treated or not with FSH, did not significantly influence the percentages of blastocysts and pregnancies. The absence of relevant variation in the pregnancy percentage of embryos produced *in vitro* for treated or non-FSH-treated cows can be characterized by the equivalent quality of the embryos transferred. The results observed in the present study are similar to those verified in the literature, with distinct breeds and protocols for obtaining oocytes (Guemra et al., 2014; Sanches et al., 2016).

In conclusion, single dose FSH protocol in Brangus donors did not improve oocyte recovery (in general terms), *in vitro* fertilization, and pregnancy percentage. However, there is the possibility of several consecutive OPU in close periods, optimizing donor cows and the rates of pregnancy derived from the *in vitro* fertilization.

Conflicts of Interest

The authors declare no conflicts of interest.

Ethics Statement

The use of cows for the experiment was submitted and approved by the Unopar Ethics Committee (nº 004/17) following the guidelines of Animal Experimentation.

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