LAIS MENDES VIEIRA

Exogenous gonadotropin supplementation to increase in vitro embryo production in Holstein donors

São Paulo

2016

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Suplementação exógena com gonadotrofinas para aumentar a produção in vitro de embriões em doadoras Holandesas

Tese apresentada ao Programa de Pós-Graduação em Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Departamento:

Reprodução Animal

Área de concentração:

Reprodução Animal

Orientador:

Prof. Dr. Pietro Sampaio Baruselli

De acordo:_____

Orientador

São Paulo
2016

Obs: A versão original se encontra disponível na Biblioteca da FMVZ/USP

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T.3385
FMVZ

Vieira, Lais Mendes
Exogenous gonadotropin supplementation to increase in vitro embryo production in Holstein donors / Lais Mendes Vieira. -- 2016.
77 f. : il.

Título traduzido: Suplementação exógena com gonadotrofinas para aumentar a produção *in vitro* de embriões em doadoras Holandesas.

Tese (Doutorado) - Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Reprodução Animal, São Paulo, 2016.

Programa de Pós-Graduação: Reprodução Animal.

Área de concentração: Reprodução Animal.

Orientador: Prof. Dr. Pietro Sampaio Baruselli.

1. Bovino. 2. Embrião. 3. FSH. 4. OPU-PIVE. 5. Superestimulação. I. Título.

CERTIFICADO

Certificamos que o Projeto intitulado "Suplementação exógena com gonadotrofinas para aumentar a produção in vitro de embriões em doadoras Holandesas", protocolado sob o CEUA nº 7738051015, sob a responsabilidade de **Pietro Sampaio Baruselli** *e equipe; Lais Mendes Vieira* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 16/02/2016.

We certify that the proposal "Exogenous gonadotropin supplementation to increase in vitro embryo production in Holstein donors", utilizing 303 Bovines (303 females), protocol number CEUA 7738051015, under the responsibility of **Pietro Sampaio Baruselli** *and team; Lais Mendes Vieira* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of São Paulo (CEUA/FMVZ) in the meeting of 02/16/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de nov/201 a mar/201 Área: Reprodução Animal

Procedência:	Animais provenientes de estabelecimentos comerciais				
Espécie:	Bovinos	sexo: Fêmeas	idade: a	N:	303
Linhagem:	Holandesa		Peso: a		

Resumo: Nos últimos anos, o uso da aspiração folicular (OPU) e a produção in vitro de embriões (PIVE) em rebanhos bovinos tem aumentado em todo o mundo, sendo o Brasil líder mundial na produção desses embriões. A OPU-PIVE é uma biotecnologia que permite a rápida multiplicação de indivíduos baseado na genética da doadora de oócito e do doador de sêmen. No entanto, algumas peculiaridades das fêmeas Bos taurus de leite (população de folículos antrais e qualidade de oócitos) foram determinadas como fatores responsáveis pela reduzida eficiência da técnica (OPU-PIVE) entre estes rebanhos. Dadas as dificuldades de estabelecer programas de OPU-PIVE com alta eficiência em vacas leiteiras, quatro estudos serão realizados envolvendo diferentes tipos de tratamentos de superestimulação folicular em doadoras da raça Holandesa lactantes e não-lactantes, com o objetivo de otimizar o desempenho desses animais na técnica em questão. O primeiro estudo visa aumentar a PIVE de doadoras raça Holandesa (lactantes e não-lactantes) após serem submetidas ao tratamento superestimulatório tradicional com quatro aplicações de pFSH (hormônio folículo estimulante porcino) a cada 12 h. Para este efeito, 30 doadoras (15 em lactação e 15 vacas não lactantes) receberão o protocolo de sincronização da onda de crescimento folicular [protocolo a base de benzoato de estradiol (BE) e progesterona (P4)] e serão submetidas ao tratamento de superestimulação em um design cross-over. Após o primeiro estudo, outros três experimentos serão realizados (Exp. 2: n = 23 novilhas da raça Holandesa, Exp. 3: n = 90 vacas não lactantes da raça Holandesa e Exp. 4: n = 160 vacas não lactação da raça Holandesa) para avaliar o efeito de diferentes diluentes para o pFSH ou hormônios folículo estimulantes, visando superestimular as doadoras com apenas uma única injeção. Em geral, o presente estudo avaliará o efeito da superestimulação no perfil plasmático de FSH, na proporção do tamanho dos folículos presentes no momento da OPU e a produção in vitro de embriões após os tratamentos.

Local do experimento:

São Paulo, 19 de julho de 2016

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

São Paulo, 02 de setembro de 2016
CEUA N 7738051015

Ilmo(a). Sr(a).
Responsável: Pietro Sampaio Baruselli
Área: Reprodução Animal
Pietro Sampaio Baruselli (orientador)

Título da proposta: "Suplementação exógena com gonadotrofinas para aumentar a produção in vitro de embriões em doadoras Holandesas".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ/USP

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 02/setembro/2016) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Houve um erro de digitação na data de vigência da proposta. Dessa forma, para manter as informações corretas no certificado, solicito a atualização dessas informações. Todas as demais informações, bem como o cronograma do projeto foram mantidos.".

Comentário da CEUA: "O pesquisador confirma o período de vigência da proposta e pede sua correção, uma vez que o certificado saiu com um erro de digitação. Onde lê-se: de nov/201 a mar/201 Leia-se: 11/ 2015 a 3/2016.".

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

FOLHA DE AVALIAÇÃO

Autor: VIEIRA, Lais Mendes

Título: **Exogenous gonadotropin supplementation to increase in vitro embryo production in Holstein donors**

Tese apresentada ao Programa de Pós-Graduação em Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências

Data: ___/___/____

Banca Examinadora

Prof. Dr.:_____

Instituição:_____Julgamento:_____

Prof. Dr.:_____

Instituição:_____Julgamento:_____

Prof. Dr.:_____

Instituição:_____Julgamento:_____

Prof. Dr.:_____

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Prof. Dr.:_____

Instituição:_____Julgamento:_____

Agradecimento
Acknowledgment

Aos meus **pais** que sempre estiveram e estão ao meu lado, me apoiando e confiando nas minhas decisões ao longo da vida. Agradeço pela educação que nos deram e o constante e incansável amor de todos os dias. Obrigada, Mommy e Daddy! Vocês são mais do que exemplos, sem vocês eu não estaria aqui hoje.

Aos meus **irmãos, Taís e Roberval Jr.,** por acreditarem na trajetória da irmã caçula de vocês. Mesmo sendo algo totalmente diferente da realidade de vocês, sempre tentaram compreender o caminho e me apoiaram em todos os momentos. Muito obrigada. Agradeço também aos irmãos que ganhei algo longo da vida, **Célia** e **Tony,** cunhados mais do que queridos. Vocês alegram e energizam a família, obrigada por me darem a oportunidade de compartilhar esse momento com vocês.

Ao meu namorado, amigo e companheiro **João.** Esse sem dúvida merece os meus mais profundos agradecimentos. Ele é a "linha de frente", o primeiro a enfrentar a luta. Independentemente do dia, dias maravilhosos, bons, tranquilos, ruins, cansativos, exaustivos, era ele que tinha que estar com toda a paciência, carinho e amor para comemorar junto ou me confortar. E ele sempre fez com extrema sabedoria e sensatez. Obrigada pela compreensão e por sempre estar ao meu lado, me apoiando e encorajando a seguir. Obrigada, amor!

Ao **Prof. Pietro**, exemplo de profissionalismo e dedicação, sem dúvida uma das pessoas que mais me ensinou na vida. Os ensinamentos eram constantes, desde ensinamento técnico, mas acima de tudo, ensinamentos da vida, ética, postura, profissionalismo, tranquilidade e sabedoria. Sem dúvida, parte da minha carreira e amadurecimento pessoal se deve aos seus valiosos exemplos e conselhos. Obrigada Professor pela orientação, confiança, oportunidades, paciência e encorajamento. Com certeza meu eterno agradecimento e meu eterno Orientador.

Ao meu **grande amigo Carlos Alberto Rodrigues (Carlão)**, pois sem ele hoje eu não estaria aqui. Foi ele que me mostrou que a experiência acadêmica poderia ser muito diferente do que eu imaginava, e eu agradeço todos os dias por ter tido essa incrível experiência durante esses últimos anos. Agradeço o constante aprendizado, as constantes orientações e as inúmeras oportunidades que me foram concedidas. Obrigada por ter acreditado e confiado em mim e no meu trabalho. Digo que tenho o meu pai biológico, Roberval, e o meu pai "veterinário", Carlão. Muito obrigada!!

A querida **Harumi** por todo apoio durante esses anos de VRA. Parceria profissional e nos esportes, foram inúmeros relatórios, conversas, bate-papos. Parabéns pela sua eficiência e exemplo profissional, aprendi muito com você!

A minha querida **amiga e companheira de apto Jú (Gleyci)**. Tudo começou por acaso, mas resultou em uma amizade sincera e duradoura!! Obrigada pelos inúmeros momentos de alegria, descontração, discussões, reflexões, gargalhadas, enfim... obrigada pela sua amizade! Aprendi e aprendo muito com você!!

Aos **amigos do grupo,** Mili, Cecília, Bruna Guerreiro, Bruno Gonçalves, Badá, Marcos e Rômulo. A diversidade potencializa o nosso crescimento, obrigada pelo constante aprendizado e as incansáveis ajudas durante todo doutorado. Vocês são dez!!

Aos meus amigos, **Manoel Sá Filho, Alexandre Souza, José Nélio, Alessandra Ambrósio e Robertinha** que nunca mediram esforços para me ensinar. Sou eternamente grata a todos os momentos em que pararam o que tinham que fazer para muitas vezes me explicar desde as coisas mais simples do mundo da reprodução até grandes discussões do mundo da estatística. Obrigada pela paciência, pelos conselhos, sugestões e discussões. Vocês são exemplos profissionais.

Ao meu amigo **Márcio Mendanha** que mesmo dos bastidores sempre está por perto querendo saber se estava tudo bem, se tudo está caminhando conforme o planejado. Obrigada pela amizade e pelos inúmeros bate-papos. Muito obrigada!

Agradeço ao **Dr. Gabriel Bó e Dr. Mapletoft** que desde o primeiro contato nunca hesitaram em ajudar. Grande parte do mérito desse trabalho se deve a experiência e sabedoria de vocês. Muito obrigada pela imensa colaboração, paciência e constante atenção.

A todos da **fazenda Sata Rita – Agrindus**, em especial, ao Roberto Jank, por terem disponibilizado os animais da fazenda que este estudo pudesse ser realizado; Beco, Thiago, Micky, Laércio, obrigada pelo companheirismo e pelo enorme apoio.

Ao **Fininho** por todo apoio técnico durante os projetos, parceria e enorme paciência durante a condução dos trabalhos, mesmo diante de imprevistos.

A todos da **fazenda São José – Bela Vista**, em especial, ao Luiz Gustavo e a Kelly, por terem disponibilizado o serviço, os animais e o laboratório da fazenda para que este estudo pudesse ser realizado; Nivaldo e Zetinho, obrigada pelo companheirismo e pelo enorme apoio.

RESUMO

VIEIRA, L. M. **Suplementação exógena com gonadotrofinas para aumentar a produção *in vitro* de embriões em doadoras Holandesas.** [Exogenous gonadotropin supplementation to increase in vitro embryo production in Holstein donos]. 2016. 77 f. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

Nos últimos anos, o emprego da aspiração folicular (OPU) e produção *in vitro* de embriões (PIVE) têm crescido mundialmente nos rebanhos bovinos. A OPU-PIVE viabiliza a rápida multiplicação do material genético, utilizando tanto a base genética da fêmea como do macho. No entanto, fêmeas de leite *Bos taurus* apresentam algumas peculiaridades, como menor população de folículos antrais e inferior qualidade oocitária, as quais foram apontadas como fatores responsáveis pela reduzida eficiência da técnica nesses rebanhos. Frente às dificuldades para o estabelecimento de programas de OPU-PIVE eficientes em vacas de leite *Bos taurus*, três experimentos foram conduzidos envolvendo diferentes tipos de tratamentos superestimulatórios em doadoras de oócitos lactantes e não lactantes. O primeiro estudo teve como objetivo aumentar a PIVE em doadoras da raça Holandesa lactantes e não lactantes submetidas ao tratamento tradicional de superstimulação com doses decrescentes de FSH porcino (FSHp) a cada 12 h, previamente à OPU. Nesse estudo, as doadoras (n = 15 vacas lactantes e n = 15 não lactantes da raça Holandesa) receberam protocolo para sincronização da emergência da onda folicular [protocolo a base de benzoato de estradiol (BE) e progesterona (P4)] e foram submetidas aos tratamentos de superestimulação em um delineamento experimental *cross-over*. Os outros dois estudos (n = 23 novilhas da raça Holandesa e n = 72 vacas não lactantes da raça Holandesa) foram desenvolvidos para avaliar diferentes diluentes para o FSHp com o objetivo de viabilizar a superestimulação com apenas uma aplicação de FSHp. No geral, os estudos avaliaram o efeito da superestimulação no perfil plasmático de FSH, na proporção de folículos classificados como pequenos (<6mm), médios (6-10mm) ou grandes (>10mm), conforme diâmetro folicular, na PIVE e no estabelecimento gestacional após transferência de embriões. A superestimulação aumentou a proporção de folículos médios, aumentou a competência oocitária e resultou em maior produção de blastocistos por sessão de OPU. A aplicação única de FSHp associado ao diluente de liberação lenta (ácido hialurônico), resultou em semelhante área sob a curva de FSH, semelhante proporção de folículos pequenos, médios e grandes e semelhante PIVE comparado ao tratamento superestimulatório tradicional com doses de FSHp administradas a cada 12h. Independentemente do diluente e da dose de FSHp utilizada, o tratamento superestimulatório

resultou em maior produção de blastocistos por sessão de OPU comparado às vacas não tratadas com FSHp. Adicionalmente, semelhante taxa de estabelecimento gestacional foi observada, independentemente do tratamento utilizado na doadora. Portanto, os dados obtidos no presente estudo permitem concluir que independentemente do tipo de tratamento (diluente para FSHp ou dose utilizada de FSHp), a superestimulação previamente ao processo de OPU, aumentou o desenvolvimento de embriões *in vitro*, número de blastocistos por sessão de OPU e resultando em semelhante taxa de estabelecimento gestacional após transferência de embriões.

Palavras-chave: Bovino. Embrião. FSH. OPU-PIVE. Superestimulação.

ABSTRACT

VIEIRA, L. M. **Exogenous gonadotropin supplementation to increase in vitro embryo production in Holstein donos**. **[**Suplementação exógena com gonadotrofinas para aumentar a produção *in vitro* de embriões em doadoras Holandesas]. 2016. 77 f. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

In the last years, the use of *ovum pick-up* (OPU) and *in vitro* produced embryo (IVEP) technology has worldwide increased in cattle herds. The OPU-IVEP enable a rapid individual multiplication based on the female and male donor genetic. However, *Bos taurus* dairy females present some peculiarities, as reduced antral follicle population and lower oocyte quality, and these factors have been awarded as responsible to the reduced technique efficiency among these herds. Given the difficulties of establishing OPU-IVEP programs with high efficiency in *Bos taurus* dairy cows, three studies were carried out, involving different types of superstimulation treatments in lactating and non-lactating Holstein donors. The first study aimed to increase IVEP in lactating and non-lactating Holstein dairy donors submitted to the traditional twice-daily porcine FSH (pFSH) superstimulation treatment prior to the OPU. For this purpose, donors (n = 15 lactating and n = 15 non-lactating Holstein cows) received the follicular wave synchronization protocol [estradiol benzoate (EB) and progesterone (P4) based protocol] and were submitted to superstimulation treatment in a *cross-over* design. Other two experiments were performed (n = 23 Holstein heifers and n = 72 non-lactating Holstein cows) to evaluate different pFSH diluents to enable superstimulation with a single injection. In general, these studies evaluated the effect of superstimulation on the plasmatic FSH profile, proportion of follicles sizes previous to OPU (small: <6mm; medium: 6-10mm; and large follicles: >10mm), on *in vitro* embryo production and pregnancy establishment after the produced embryo transfer. The superstimulation treatment improved the proportion of medium sized-follicles, improved oocyte competence and resulted in greater amount of blastocyst per OPU session. A single injection of pFSH combined with a slow release carrier (hyaluronan), resulted in similar FSH area under curve, proportion of follicles sizes previous to OPU and *in vitro* embryo production compared to the twice-daily superstimulating treatment. Regardless pFSH diluent and dose, superstimulating hormone resulted in greater number of blastocysts per OPU session compared to the non-pFSH treated donors. Additionally, similar pregnancy establishment was observed, regardless embryo donor treatment. Therefore, with the present data, we can conclude that regardless treatment type (pFSH diluent or dose), the superstimulation procedure prior to the OPU, enhanced *in*

vitro embryo development, increased the number of blastocyst per OPU session and resulted in similar pregnancy establishment after embryo transfer.

Keywords: Bovine. Embryo. FSH. OPU-IVEP. Superstimulation.

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SUMÁRIO

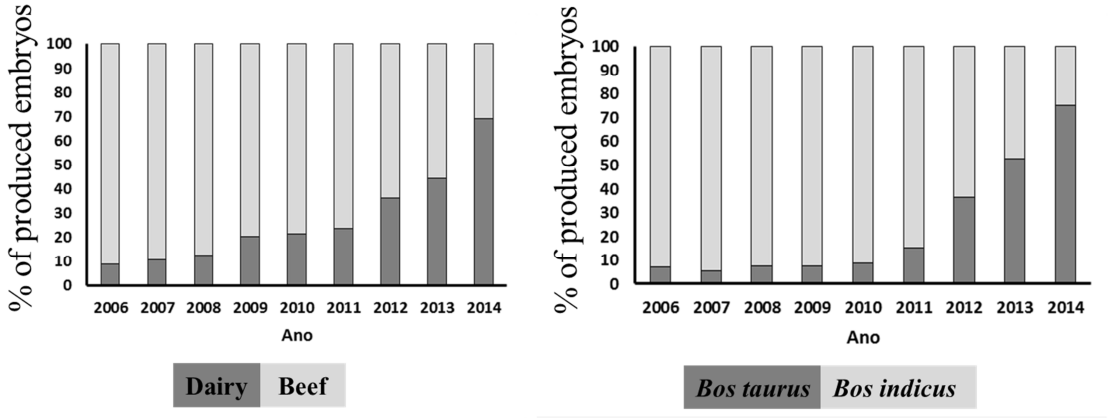
SUMMARY

1 GENERAL INTRODUCTION

In the last 20 years, the worldwide bovine embryo production market underwent significant changes regarding the prevalence of the technology used to produce embryos. From 2000 to 2012 the *in vitro* embryo production (IVEP) method went from 17.4% to 38.8% of representativeness compared to the *in vivo* technology (IETS, 2013). Moreover, this global increase was related to the remarkable improvement in the Brazilian IVEP market. Brazil enlarged over twenty times the bovine IVEP from 2000 (12,500 embryos) to 2014 [348,468 embryos; (SARTORI et al., 2016)].

Considering the *in vitro* embryos produced in Brazil, in 2014, 70.2% (244,686 embryos) were obtained from dairy donors (SARTORI et al., 2016). For the first time, the number of IVEP produced from dairy breeds exceeded the number of embryos produced from beef breed. And, interestingly, the increased observed in IVEP market was also related to the increased in IVEP among *Bos taurus* donors (Figure 1; VIANA et al. 2015[1]). Out of 244,686 embryos produced in dairy cows, 229,727 came from *Bos taurus* donors (VIANA et al. 2015[2]).

Figure 1 – Brazilian *in vitro* embryo market. Adapted from Viana et al. unpublished data



Fonte: Vieira, L. M. (2016).

[1,2] VIANA J.H.M., FIGUEIREDO A.C.S. Produção e transferência de embriões bovinos em 2014: reflexos de um ano de turbulências. 2015, dados não publicados.

Regarding *Bos taurus* dairy donors, these animals are usually represented by Holstein cows with high yield production and therefore have several physiologic and metabolic peculiar characteristics. Considering the OPU and IVEP procedures, antral follicle population [AFP; (GUERREIRO et al., 2014)] and oocyte competence (FERREIRA et al., 2011) have been cited as important factors to obtain a successful and efficient OPU-IVEP outcome. In this context, previous studies have already reported lower AFP in *Bos taurus* females compared to *Bos indicus*, hampering the IVEP efficiency (PONTES et al., 2010; BATISTA et al., 2014; BATISTA et al., 2015). Additionally, lactating dairy cows have peculiar metabolic system, linked to nutrition (WILTBANK et al., 2006) and modified endocrine profiles (SARTORI et al., 2004), which has been associated with a suboptimal fertility, with compromised oocyte and embryo quality (SARTORI et al., 2002); also negatively associated to IVEP (FERREIRA et al., 2011). Therefore, several researchers initiated studies aiming to enhance the OPU-IVEP outcomes in this challenging animal category.

Aware of the *in vitro* embryo market positioning and knowing the need to increase the efficiency of IVEP in *Bos taurus* dairy donors, the present study evaluated different simplified superstimulation treatments aiming to recover oocytes suitable for IVEP procedures followed homogeneous follicular growth.

2 HYPOTHESES

All hypotheses are described below and illustrated in Figure 2.

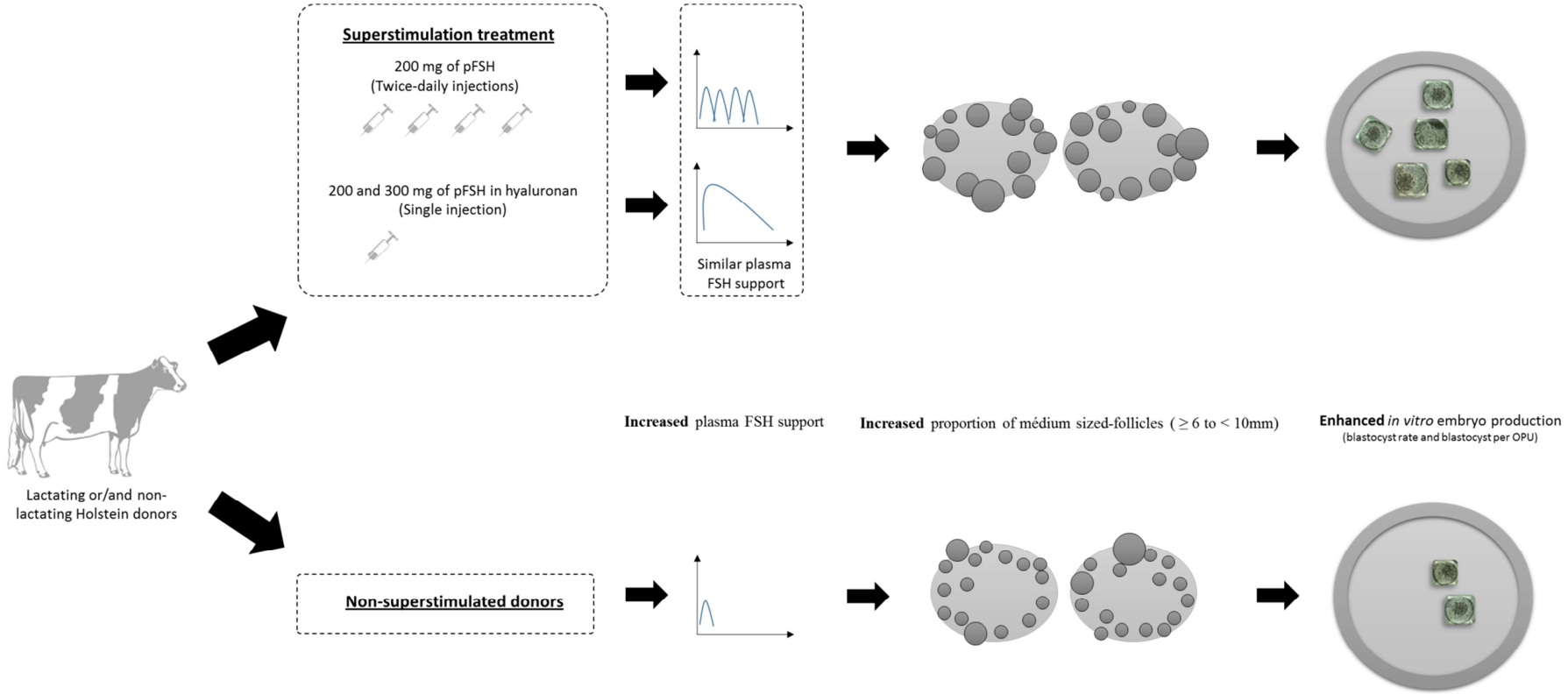
2.1 The superstimulation with four (twice-daily) pFSH injections in synchronized donors (lactating and non-lactating cows) increase medium sized-follicles and thereby enhance *in vitro* embryo production.

2.2 Regardless treatment group, synchronized non-lactating donors present greater *in vitro* embryo production compared to lactating Holstein cows.

2.3 A single injection of 200 or 300 mg of pFSH combined with hyaluronan present similar plasma FSH support compared to the traditional treatment (four decreasing doses, 12 h apart).

2.4 A single injection of 200 or 300 mg of pFSH combined with hyaluronan in synchronized donors (non-lactating cows) increase medium sized-follicles and thereby enhance *in vitro* embryo production, being comparable to the traditional treatment (four decreasing doses, 12 h apart) and greater than the non-superstimulated donors.

Figure 2 - Hypothetical model design.



Fonte: Vieira, L. M. (2016).

3 OBJECTIVES

3.1 Evaluate *in vitro* embryo production in synchronized donors (lactating and non-lactating cows) superstimulated with four (twice-daily) pFSH injections.

3.2 Evaluate plasma FSH profile after single injection of 200 or 300 mg of pFSH combined with hyaluronan in Holstein heifers.

3.3 Evaluate *in vitro* embryo production and pregnancy establishment in synchronized donors (non-lactating cows) superstimulated with a single injection of 200 or 300 mg of pFSH combined with hyaluronan.

4 SUPERSTIMULATION PRIOR TO THE OVUM PICK-UP TO IMPROVE IN VITRO EMBRYO PRODUCTION IN LACTATING AND NON-LACTATING HOLSTEIN COWS

ABSTRACT

The present study evaluated the efficacy of superstimulation with pFSH (Folltropin®) prior to the ovum pick-up (OPU) on *in vitro* embryo production (IVEP) in lactating and non-lactating Holstein donors. A total of 30 Holstein cows (15 lactating and 15 non-lactating) was blocked by lactation status to one of two groups (Control or pFSH), in a cross-over design. On a random day of the estrous cycle, all cows received an intravaginal progesterone device and 2.0 mg IM of estradiol benzoate (Day 0). Cows in the Control group received no further treatment, while cows in the pFSH group received a total dosage of 200 mg of pFSH on Days 4 and 5 in four decreasing doses 12 h apart (57, 57, 43 and 43 mg). On Day 7, the progesterone device was removed and OPU was conducted in both groups (40 h after the last pFSH injection in the pFSH-treated group). There was no difference between groups (P = 0.92) in the numbers of follicles that were aspirated per OPU session (17.2 ± 1.3 vs. 17.1 ± 1.1 in Control and pFSH-treated cows, respectively); however, pFSH-treated cows had a higher (P < 0.001) percentage of medium-sized follicles (6 to 10 mm) at the time of the OPU (55.1%; 285/517) than Control cows (20.8%; 107/514). Although recovery rate was lower (60.0%, 310/517 vs. 69.8%, 359/514; P = 0.002), pFSH-treated cows had a higher blastocyst production rate (34.5%, 89/258 vs. 19.8%, 55/278; P < 0.001) and more transferable embryos per OPU session were produced in the pFSH group (3.0 ± 0.5 vs. 1.8 ± 0.4; P = 0.02). Regardless of treatment, non-lactating cows had a higher blastocyst rate (41.9%, 106/253 vs. 13.4%, 38/283; P = 0.001) and produced more transferable embryos per OPU session (3.5 ± 0.5 vs. 1.3 ± 0.3; P = 0.003) than lactating cows. Thus, superstimulation of Holstein donors with pFSH prior to OPU increased the efficiency of IVEP. In addition, non-lactating donors had higher percentage of *in vitro* blastocyst development and produced more embryos per OPU session than lactating cows.

Keywords: Bovine. Holstein. *In vitro* embryo production. Oocyte competence.

4.1 INTRODUCTION

The success of dairy operations is related to increased genetic gain, reproductive efficiency and milk yield per cow. Among the reproductive biotechnologies, *in vitro* embryo production (IVEP) has been considered an important alternative to rapidly enhance genetic progress through the female lineage in dairy cattle. However, oocyte quality has been considered an important factor (LEROY et al., 2008a; SARTORI; BASTOS; WILTBANK, 2010; BARUSELLI et al., 2012; LEROY et al., 2012) contributing to the low fertility reported for high producing lactating dairy cattle (WALSH; WILLIAMS; EVANS, 2011). In addition, regardless the significant variation within animal (MERTON et al., 2003; PONTES et al., 2009), IVEP efficiency has been reported to be lower in lactating Holstein cattle as compared to Holstein heifers (FERREIRA et al., 2011) and beef cattle (RATTO et al., 2011). Therefore, further studies are required to evaluate alternative strategies to improve the efficiency of IVEP in Holstein donors, especially those that are lactating.

The outcome of IVEP programs has also been associated with the stage of follicular growth at which ovum pick-up (OPU) is performed (PAVLOK; LUCAS-HAHN; NIEMANN, 1992; BLONDIN; SIRARD, 1995; FAIR; HYTTEL; GREVE, 1995; HAGEMANN et al., 1999; HENDRIKSEN et al., 2004). The acquisition of developmental potential of oocytes (e.g., the ability of the oocyte to reach the blastocyst stage) has been associated with follicular growth, i.e., developmental competence continues to be enhanced as follicular diameter increases and approaches the LH surge (LONERGAN et al., 1994; ARLOTTO et al., 1996; SIRARD et al., 2006; CAIXETA et al., 2009; SIRARD, 2011; SIRARD, 2012). It has been shown that during oocyte growth in cattle mRNA and proteins are stored in the oocyte (BREVINI-GANDOLFI; GANDOLFI, 2001) and the composition of RNA is essential to sustain the first few cell cycles of early embryo development (DIELEMAN et al., 2002). Therefore, there may be an ideal range during follicular development where IVEP is optimized; and in that context, the use of protocols for follicular wave synchronization, and superstimulation prior to OPU may be a strategy to improve the efficiency of this technology in dairy cattle.

Superstimulation with porcine FSH (pFSH) prior to the OPU has been used successfully for IVEP programs in non-lactating *Bos taurus* donors, resulting in increased total embryo yields per OPU session (GOODHAND et al., 1999; SENDAG et al., 2008), possibly due to the greater follicular diameters of the aspirated follicles. Another important and positive

aspect regarding the improved efficiency of IVEP following superstimulation is associated with the “coasting” period (i.e., a period of FSH withdrawal/starvation) in Holstein heifers and lactating cows (BLONDIN et al., 2002; NIVET et al., 2012). Similar to the effect of follicular stage, the effect of gonadotropin starvation on the efficiency of IVEP has been reported to be a simulation of the physiological alterations observed immediately prior to ovulation, resulting in improved oocyte competence (BLONDIN et al., 2002). However, the efficacy of “coasting” following superstimulation used in IVEP in lactating donor cows has yet limited data. Considering the concerns related to the oocyte developmental competence (e.g., the oocyte capacity to yield into blastocyst) and donor lactational status, adjustments were studied to ascertain a follicular synchronization protocol for OPU which encompasses the beneficial effects of superstimulation (higher proportion of medium-sized follicles) and gonadotropin withdrawal (coasting), with the overall objective of improving the use of this biotechnology, especially in lactating dairy cattle.

Thus, the challenge is to promote the growth of a homogeneous follicle population and to recover competent oocytes suitable for IVEP procedures. The present study evaluated the effect of superstimulation with pFSH in Holstein oocyte donors (lactating and non-lactating cows) submitted to an OPU-IVEP program. The hypothesis was that superstimulation with pFSH prior to OPU in lactating and non-lactating Holstein donors would alter the proportion of medium-sized follicles available for OPU and enhance the *in vitro* competence of the recovered oocytes, increasing the general efficiency of IVEP programs (number of embryos produced per OPU). We also hypothesized that OPU-IVEP procedures would result in a higher number of blastocysts per OPU session in non-lactating than in lactating donors.

4.2 MATERIALS AND METHODS

4.2.1 Farm and animals

The present experiment was conducted in a commercial dairy farm in southeast Brazil (22°01’27’’S/47°53’19’’W) during January to March, 2013. The herd was composed of 1,500 lactating Holstein cows housed in free stall facilities, milked three times daily and with an

average milk production of 30.1±0.3 kg per day. The non-lactating cows were maintained in dry lot pens.

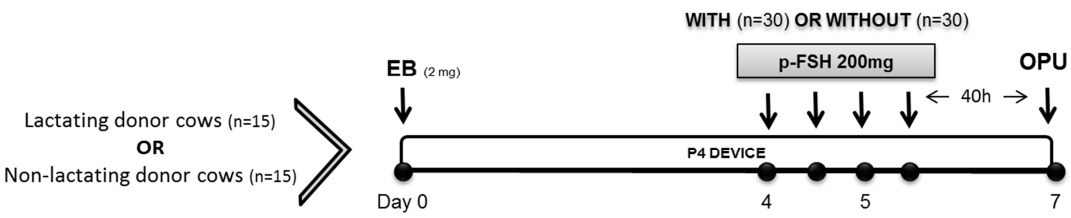
Lactating cows enrolled in the experiment trial were normal cycling cows with 172.9 ± 24.9 DIM (±SEM), had a daily milk production of 30.0 ± 1.3 (±SEM), and were on lactation number 1.7 ± 0.2 (±SEM). Non-lactating cows were normal cycling and on lactation number 2.2 ± 0.4 (±SEM).

All animals were fed a TMR formulated to meet or exceed the minimum nutritional requirements for lactating and non-lactating Holstein cows (NRC, 2001). Briefly, the main ingredients were corn silage and Tifton hay as forage and a corn, soybean, and cottonseed meal-based concentrate.

4.2.2 Experimental design

A total of 30 Holstein donors (15 lactating cows and 15 non-lactating cows) were enrolled in cross-over experimental design, so that all females were submitted to both treatments: Control and pFSH. On random days of the estrous cycle (Day 0; AM) all cows received an intravaginal progesterone device (P4; Primer®, Tecnopec, Brazil) and 2.0 mg intramuscular (IM) of estradiol benzoate (RIC-BE®, Tecnopec, Brazil). The Control group received no further treatments, while the pFSH group received 200 mg NIH-FSH-P1 (Folltropin®, Bioniche Animal Health, Belleville, ON, Canada) divided into four decreasing doses (57, 57, 43 and 43 mg, 12 h apart) on Days 4 and 5. On Day 7 AM (40 h of “coasting” period in the pFSH group), the P4 devices were withdrawal immediately before OPU (Figure 3).

Figure 3 - Superstimulation protocol to evaluate the use of pFSH prior to the ovum pick-up (OPU) in lactating (n = 15) and non-lactating (n = 15) Holstein donors in a cross-over experimental design. EB = 2.0 mg estradiol benzoate; P4 device = intravaginal device containing 1.0 mg of progesterone; pFSH = 200 mg porcine follicle-stimulating hormone (57, 57, 43 and 43 mg; 12 h apart)



Fonte: Vieira, L. M. (2016).

4.2.3 Ultrasonography examinations

Immediately before the OPU session, both ovaries were examined by transrectal ultrasonography using a portable scanner (Aloka SSDV 500; Aloka, Tokyo, Japan) with 5 MHz convex array transducer housed in a plastic vaginal probe. All visible follicles were quantified and classified according to their diameters [small (SF < 6 mm), medium (MF = 6 to 10 mm) and large (LF = > 10 mm) follicles].

4.2.4 Ovum pick-up (OPU)

All donors were subjected to OPU in the morning of Day 7 (40 h after the last treatment in the pFSH group or "coasting" period). For the oocyte collection procedure, cattle were restrained in a chute and epidural anesthesia was administered with lidocaine hydrochloride 2% (Lidovet®, Bravet, Brazil) to facilitate the handling of the ovaries through the rectum. The perineal area was cleaned using water, dried and sprayed with alcohol prior to each session. All follicles ≥ 2 mm were aspirated using the portable scanner with a 5-MHz convex array transducer housed in a plastic vaginal probe with a stainless steel needle guide connected to aspiration equipment and a vacuum system. Follicular aspirates were recovered via a 1.1 mm i.d. by a 120 cm length circuit (Watanabe Tecnologia Aplicada, Cravinhos, SP, Brazil), connected directly to a disposable 20-gauge x 2 inch hypodermic needle (0.9 x 50 mm; Terumo Europe NV – Belgium) and a 50 mL conical tube containing 15 mL of Dulbecco PBS (DPBS; Nutricell Nutrientes Celulares, Campinas, SP, Brazil) supplemented with 1% (vol/vol) fetal calf serum (FCS; Gibco Life Technologies, Grand Island, NY) and 5,000 IU/mL sodium heparin (Parinex, Hipolabor, Belo Horizonte, MG, Brazil) at 35 to 37°C. The vacuum connected to the needle was set at 85 to 90 mm Hg. All retrieval procedures were performed by the same veterinarian. The conical tube containing the follicular aspirate was transported to a field laboratory and cumulus–oocyte complexes (COCs) were recovered using a 75 μm filter (Watanabe Tecnologia Aplicada) and DPBS supplemented with 1% FCS. The COCs were washed once in DPBS supplemented with 1% FCS at 37°C and morphologically evaluated under a stereomicroscope at 8–20X magnification). The COCs were morphologically classified based upon the number of cumulus cell layers as follow:

Grade 1, more than three layers of compact cumulus cells; Grade 2, at least one layer of cumulus cells; Grade 3, denuded; and Grade 4, atretic, with dark cumulus cells and signs of cytoplasmic degeneration (SENEDA et al., 2001). After evaluation, only Grade 4 COCs were considered non-suitable to culture and discarded. The COCs considered suitable to culture were transported to the IVEP laboratory in 1.5 mL cryotubes containing HEPES-buffered tissue culture medium 199 (TCM-199; Gibco Life Technologies, Grand Island, NY), 10% FCS, 49.4 mg/mL sodium pyruvate (Sigma-Aldrich Chemical Co.; St. Louis, MO), and 50 mg/mL gentamycin at 37 to 39°C.

4.2.5 In vitro embryo production (IVEP)

Prior to *in vitro* maturation (IVM), COCs were washed 3 times in HEPES-buffered TCM-199, supplemented with 10% FCS and 50 μg/mL gentamycin, and once in maturation medium, composed of bicarbonate-buffered TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 50 μg/mL LH (APL, Ayerst, Rouses Point, NY), 5 μg/mL FSH (Folltropin-V, Bioniche Animal Health, Canada), 0.1 μg/mL estradiol (Estradiol 17β; Sigma-Aldrich Chemical Co.), 22 μg/mL sodium pyruvate, and 50 μg/mL gentamycin. The COCs of each cow were cultured separately for 24 h in 70 μL drops of maturation medium under mineral oil (D'Altomare, São Paulo, SP, Brazil) at 39°C in an atmosphere of 5% CO_2 in humidified air. After IVM, the COCs were washed and subjected to *in vitro* fertilization (IVF) in 70 μL drops of IVF medium under mineral oil. The IVF medium was Tyrodes albumin lactate pyruvate (TALP; BAVISTER; YANAGIMACHI, 1977) supplemented with 10 μg/mL heparin, 22 μg/mL sodium pyruvate, 50 μg/mL gentamycin), 6 mg/mL fatty acid-free BSA, and PHE solution (2 μM penicillin, 1 μM hypotaurine, and 0.25 μM epinephrine).

For IVF, semen straws were thawed for 30 s in a 35°C water bath and semen was deposited on a 90 to 45% Percoll gradient prepared with sperm wash medium (modified Tyrode medium) and centrifuged at 320 × g for 30 min to separate the motile sperm and to remove the diluents and seminal plasma. Then, the sperm pellet was evaluated for motility and concentration. Each fertilization droplet received 5 μL of sperm, to achieve a final concentration of 1×10^6 live sperm/mL. Sperm and COCs were incubated at 38.5°C in an atmosphere of 5% CO_2 in humidified air for 18 to 20 h. The same sire was used with each donor during the *cross-over*.

Approximately 18 h after insemination, presumptive zygotes were stripped of cumulus cells by mechanical pipetting in TALP medium. Groups of presumptive zygotes were co-cultured on a monolayer of cumulus cells that had attached to the surface of the plate during IVM. Thus, to maintain the maximum amount of cumulus cells, the IVM medium was gently replaced with 50 μL of CR2aa medium (WATANABE et al., 1999) supplemented with 2% FCS and 30 mg/mL BSA for embryo culture at 39°C in an atmosphere of 5% CO_2 in humidified air for 48 to 72 h, at which time 30 μL of fresh culture medium was added (first feeding). Cleavage rate was recorded after 3 d of embryo culture. The second feeding was done on the sixth day of embryo culture and the blastocyst rate (total number of blastocysts divided by total number of cultured oocytes) was recorded on the seventh day of embryo culture.

4.2.6 Statistical Analysis

Statistical analyses were performed using the GLIMMIX procedure of the Statistical Analysis System for Windows 9.3 (SAS 9.3). The variables evaluated were the number of follicles in each size category at the time of OPU (small, medium and large), total number of follicles aspirated, total number of COCs recovered, recovery rate (total number of COCs recovered per total number of follicles aspirated), number and percentage of cultured COCs (number of COCs cultured per total structures recovered), cleavage rate (number of cleaved zygotes per total number of COCs cultured), blastocyst rate (number of blastocysts produced per total number of COCs cultured) and number of embryos produced per OPU procedure.

For the analysis, a binomial distribution was assumed for the categorical response variables. Continuous data were tested for normality of the residues and homogeneity of variances using the Guided Data Analysis, and transformed when necessary. The fixed effects included in the model were treatment (Control vs. pFSH), lactation status (lactating vs. non-lactating) and their interactions. The individual effect was included as a random effect.

Means (±SE) are used to describe all of the response variables. Differences with $P \leq 0.05$ were considered statistically significant, and $0.05 < P < 0.10$ were designated as a tendency.

4.3 RESULTS

There were no significant interactions between treatment and donor lactational status for any response variable (Table 1). There was no effect ($P = 0.92$) of treatment on the total number of follicles aspirated; however, pFSH-treated donors had a lower proportion of small ($P < 0.001$) and large follicles ($P = 0.03$), and a higher proportion of medium follicles ($P < 0.001$) at the time of OPU (Figure 4). Also, due to a tendency for a reduction in the number of COCs recovered from pFSH- treated donors ($P = 0.10$), the lower recovery rate was significant ($P < 0.001$; Table 1).

Although there was no treatment effect on the number of COCs considered suitable to culture ($P = 0.52$), a higher percentage ($P = 0.05$) of COCs from pFSH-treated donors was considered viable to use in IVEP (Table 1). Although there were no differences in cleavage rates ($P = 0.81$), the pFSH group had a higher blastocyst rate ($P < 0.001$; Table 1), and as a result, superstimulated Holstein donors produced more embryos per OPU session ($P = 0.01$; Table 1).

Regardless of treatment groups, donor lactational status did not affect any of the follicular, COCs or cleavage characteristics (Table 1). However, non-lactating cows produced a higher blastocyst rate ($P = 0.001$) and a higher number of transferable embryos ($P = 0.003$) than lactating Holsteins cows (Table 1).

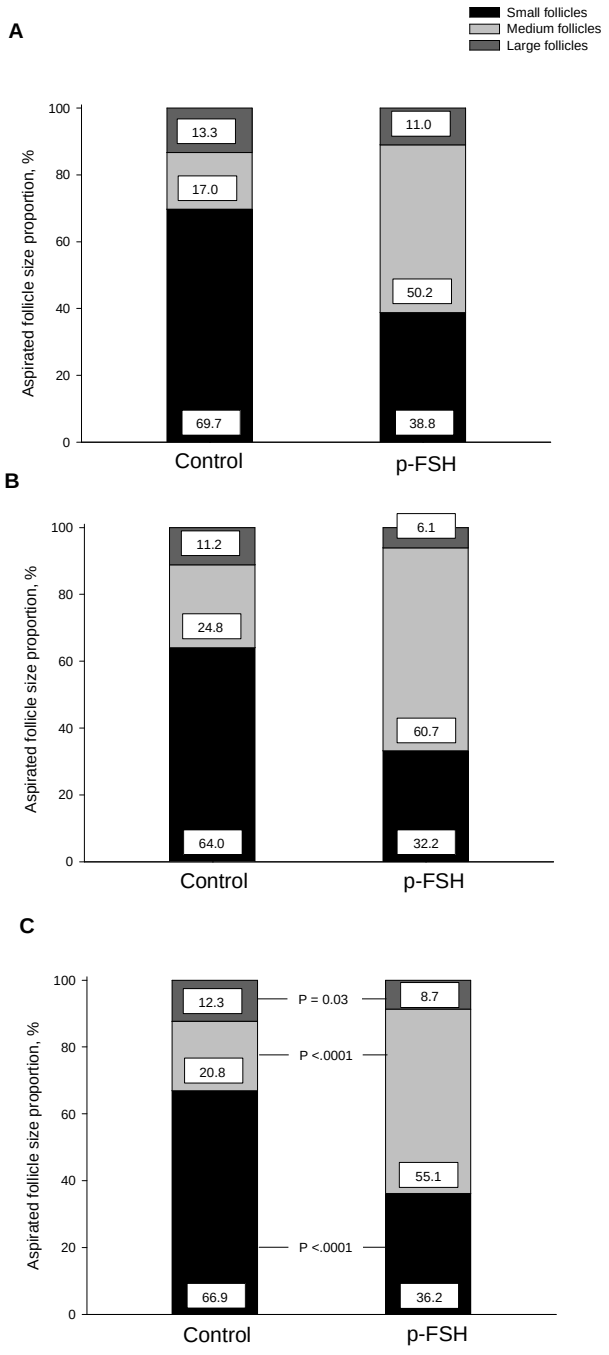
Table 1 - Summary of oocyte and embryo results (mean ±SE) after OPU-IVEP in Control and pFSH-treated donors (lactating and non-lactating Holstein cows)

	Lactating donors		Non-lactating donors		*P-value* [5]		
	Control	pFSH	Control	pFSH	Treatment	Lactation	Treat*Categ
No.	15	15	15	15	.	.	.
Total follicles aspirated	17.6±1.6	18.2±2.1	16.7±1.5	16.3±1.6	0.92	0.52	0.62
Total oocytes retrieved	13.0±1.7	10.7±1.5	10.9±1.6	9.9±1.5	0.10	0.51	0.54
Recovery rate, % [1]	73.9 (195/264)	59.0 (161/273)	65.6 (164/250)	61.1 (149/244)	< 0.001	0.89	0.08
COCs cultured	10.0±1.3	8.9±1.3	8.5±1.4	8.3±1.3	0.52	0.58	0.57
COCs culture rate, % [2]	76.9 (150/195)	82.6 (133/161)	78.0 (128/164)	83.9 (125/149)	0.05	0.77	0.88
Cleavage rate, % [3]	65.3 (98/150)	63.2 (84/133)	72.7 (93/128)	72.8 (91/125)	0.81	0.16	0.69
Blastocyst rate, % [4]	10.8 (15/150)	17.3 (23/133)	31.3 (40/128)	52.8 (66/125)	< 0.001	0.001	0.16
Embryos produced per OPU	1.0±0.4	1.5±0.5	2.7±0.6	4.4±0.8	0.01	0.003	0.17

Fonte: Vieira, L. M. (2016).

[1] No. COCs /no. follicles aspirated; [2] No. COCs cultured /no. total COCs retrieved; [3] No. cleaved zygotes /no. oocytes cultured; [4] No. blastocysts /no. oocytes cultured; [5] Treatment = effect of treatment (Control vs. pFSH); Lactation = effect of donor lactation status (lactating vs. non-lactating); Treat*Categ = interaction between treatment and donor lactation status.

Figure 4 - Proportion of small (< 6 mm), medium (6 to 10 mm) and large follicles (> 10 mm) immediately before OPU (Day 7) in lactating (A; n=30), non-lactating (B; n=30) and both lactating and non-lactating Holstein donors (C; n=60) submitted to OPU with and without pFSH superstimulation. *P values* within follicle size ≤ 0.05 differ significantly. No donor lactation status or interaction between treatment and lactation status was observed (P > 0.05)



Fonte: Vieira, L. M. (2016).

4.4 DISCUSSION

The results of the present study confirm the positive effect of superstimulation of bovine donors with pFSH on the overall efficiency of the OPU-IVEP technology (BLONDIN et al., 2002; MERTON et al., 2003; NIVET et al., 2012). Donors treated with pFSH in the present study had a greater proportion of medium-sized follicles, an increased proportion of COCs suitable to culture and higher developmental rates of recovered COCs (higher blastocyst rate and numbers of embryos per OPU session); however, the lower recovery rate in pFSH treated donors lessened the overall benefit to superstimulation. These data provide support for an alternative method to enhance the IVEP production among lactating and non-lactating Holstein donors.

Previously, it was shown that 70 to 80% of the small follicle (< 5 mm) population viewed at the beginning of the superstimulation treatments responded to exogenously administered (SINGH et al., 2004; DUROCHER; MORIN; BLONDIN, 2006) and in another study, became available for OPU (DUROCHER; MORIN; BLONDIN, 2006). Accordingly, the FSH treatments have been used to superstimulate donors for *in vitro* embryo production (GOODHAND et al., 1999; BLONDIN et al., 2002; MONTEIRO et al., 2009). Therefore, the reasoning for increasing the proportion of medium-sized follicles for aspiration, as reported previously (ALLER et al., 2010; NIVET et al., 2012), and as observed in the present study was based on the observation that oocyte development competence following OPU was influenced by the stage of follicular development (SIRARD, 2012).

The acquisition of oocyte developmental potential has been shown to be associated with follicular growth (LONERGAN et al., 1994; ARLOTTO et al., 1996; CAIXETA et al., 2009). A sequence of molecular and transcriptomal alterations during follicular (and oocyte) growth has been related to final oocyte development potential (BREVINI-GANDOLFI; GANDOLFI, 2001; SIRARD, 2012; LABRECQUE; SIRARD, 2013), indicating that oocyte development competence is acquired gradually during follicle growth. Mourot et al. (2006) have reported differences in oocyte gene expression profiles according to the size of the follicle from which the oocyte was retrieved e.g., higher mRNA levels for PSMB2, SKIIP, CDC5L, RGS16, and PRDX1 in oocytes of follicles more than 8 mm. In addition, Chu, Dufort and Sirard (2012) demonstrated increased expression of PTTG1, BTG4, PAPOLA and LEO1 genes in oocytes retrieved from superstimulated donors compared to untreated donors. Considering that these genes are related to transcription and cell cycle regulation, the

authors suggested that stimulation with exogenous FSH could allow for the accumulation of more messenger which would result in the preservation of oocyte quality. Collectively, these observations suggest that potential factors affecting oocyte developmental competence might be altered by superstimulation with exogenous FSH prior to the OPU.

The oocyte quality concept involves the competence to yield a viable blastocyst within an *in vitro* production system (MERTON et al., 2003). In this context, donors superstimulated with pFSH in the present study had increased blastocyst production rates compared to the untreated donors. Similar results were reported by Blondin et al. (2002) in a study performed in *Bos taurus* beef cattle, also treated with exogenous FSH. However, recent report has shown that the oocyte quality gained after an optimal coasting period has limited lifespan, possibly due to an intensification of the transcript degradation mechanism (LABRECQUE et al., 2013). Therefore, the present data suggest that the increased *in vitro* embryo production of donors superstimulated with pFSH prior to OPU was related to the gonadotropin stimulus effect. However, it is important to highlight that this positive effect can be a response to an associative influence of the pFSH treatment added to the fact that all pFSH-treated donors had a 40 h coasting period.

Although oocyte quality has been shown to improve with follicle growth (FAIR; HYTTEL; GREVE, 1995), follicle size was negatively correlated with the recovery rate in *Bos taurus* cows (SENEDA et al., 2001) and heifers (GOODHAND et al., 1999). Similar results were observed in the present study with Holstein donor cows. Seneda et al. (2001) suggested that the reduced volume and viscosity of follicular fluid and the lower intra-follicular pressure of small follicles might favor the OPU procedure (i.e., increased COCs recovery rates following intra-follicular needle insertion) compared to the large follicles. Therefore, as a matter of necessity, the mechanism to retrieve the maximum proportion of COCs with optimal development potential and without adversely affecting recovery rate needs to be studied and established to enhance IVEP systems.

Despite the improvement in the *in vitro* embryo production in lactating donors following pFSH treatment in the present study, the efficiency of the *in vitro* system was even greater in non-lactating Holstein donors. Superovulation and embryo production has also been reported to be higher in non-lactating cows or heifers compared to lactating Holstein cows (SARTORI et al., 2002; SARTORI; BASTOS; WILTBANK, 2010; VIEIRA et al., 2014b). Dairy cows present a peculiar metabolic system, linked to nutrition and disruption of endocrine profiles. Lactating dairy cows metabolic profile are commonly characterized by the lower concentrations of progesterone and estradiol (WILTBANK et al., 2006) and increased

concentrations of NEFA (nonesterified fatty acids) and BHBA [β-hydroxybutyrate (LEROY, 2005)]; and this peculiar metabolism has been associated with a suboptimal follicle microenvironment, compromising oocyte quality and resulting in a failure to conceive (SARTORI; ROSA; WILTBANK, 2002; SARTORI et al., 2004; WILTBANK et al., 2006; LEROY et al., 2008a,b; WALSH; WILLIAMS; EVANS, 2011). Therefore, the greater challenge of lactating cows to maintain an optimal reproductive efficiency might be part of the explanation to the lower results observed in the *in vitro* embryo production. Although *in vitro* embryo production was improved following treatment with pFSH in lactating donors, lactation results in such highly modified metabolism that it will be difficult to completely overcome treatment strategies. Accordingly, non-lactating donors may be considered the preferred donor to be enrolled in OPU-IVEP programs, due to the higher yield of embryos per OPU session.

In conclusion, superstimulation with pFSH increased the proportion of medium-sized follicles available for the OPU procedure. Consequently, the treatment also enhanced the proportion of COCs suitable for culture and resulted in greater blastocyst rates and embryo yield per OPU-IVEP session. Regardless of gonadotropin treatment, non-lactating donors had higher *in vitro* oocyte competence and produced more embryos per OPU session, resulting in an overall higher OPU-IVEP efficiency compared to lactating donors. It can be concluded that pFSH superstimulation treatment can effectively improve embryo yield of *in vitro* production systems in lactating or non-lactating Holstein donors. Importantly, non-lactating donors should be the elective lactation status to be used in OPU-IVEP programs, regardless superstimulation treatments.

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5 EFFICACY OF A SINGLE INTRAMUSCULAR INJECTION OF PORCINE FSH IN HYALURONAN PRIOR TO OVUM PICK-UP IN HOLSTEIN CATTLE

ABSTRACT

Plasma FSH profiles, in vitro embryo production (IVP) following ovum pick-up (OPU) and establishment of pregnancy with IVP embryos were compared in untreated Holstein oocyte donors and those superstimulated with multiple injections or a single intramuscular (IM) injection of porcine FSH (pFSH) in hyaluronan (HA). Plasma FSH profiles were determined in 23 heifers randomly allocated into 1 of 4 groups. Controls received no treatment, while the F200 group received 200 mg of pFSH in 4 doses, 12 h apart. The F200HA and F300HA groups received 200 or 300 mg pFSH in 5 mL or 7.5 mL, respectively of a 0.5% HA solution by a single IM injection. Plasma FSH levels were determined before the first pFSH treatment and every 6 h over 96 h. All data were analyzed by orthogonal contrasts. Circulating FSH area under curve (AUC) in pFSH-treated animals was greater than in the Control group ($P = 0.02$). Although the AUC did not differ among FSH-treated groups ($P = 0.56$), the total period with elevated plasma FSH was greater in the F200 group than in the HA groups ($P < 0.0001$). However, the F300HA group had a greater AUC than the F200HA group ($P = 0.006$), with a similar total period with elevated plasma FSH ($P = 0.17$). The IVP was performed in 90 non-lactating Holstein cows randomly allocated to 1 of the 4 treatment groups as in the first experiment. A greater proportion of medium sized (6-10 mm) follicles was observed in cows receiving pFSH, regardless of treatment group ($P < 0.0001$). Also, numbers of follicles ($P = 0.01$), cumulus oocyte complexes (COCs) retrieved ($P = 0.01$) and matured ($P = 0.02$), cleavage rates ($P = 0.002$) and blastocysts produced per OPU session ($P = 0.06$) were greater in cows receiving pFSH, regardless of treatment group. Cows in the F200HA group had a greater recovery rate ($P = 0.009$), number of COCs cultured ($P = 0.04$) and blastocysts produced per OPU session ($P = 0.06$) than cows in the F300HA group. Similar pregnancy rates were observed 50 to 60 d after transferring IVP embryos from donors in the different treatment groups ($P > 0.05$). In conclusion, a single IM injection of pFSH combined in 0.5% HA resulted in similar plasma FSH profiles as twice-daily pFSH treatments. Treatment of non-lactating donors with pFSH, with or without HA, resulted in increased IVP over untreated controls. A single dose of 200 mg of pFSH in 0.5% HA resulted in greater IVP than 300 mg pFSH in HA. Finally, pregnancy rates with IVP embryos were similar, regardless donor treatment.

Keywords: Bovine. *Bos Taurus.* FSH half-life. *In vitro* embryo production. Oocyte competence.

5.1 INTRODUCTION

Genetic improvement has led the effort to enhance dairy cattle productivity. Among breeding alternatives, *in vitro* produced embryo transfer is a robust tool used to enhance genetic progress through both the female and male lineage. However, large scale efficient use of this reproductive strategy in dairy cattle has been a challenge. The main factors affecting the efficacy of ovum pick-up (OPU) and *in vitro* embryo production (IVP) include: 1) reduced oocyte quality (LEROY et al., 2008a; SARTORI; BASTOS; WILTBANK, 2010; BARUSELLI et al., 2012; LEROY et al., 2012), mainly in lactating Holstein cows compared to Holstein heifers (FERREIRA et al., 2011) and beef cattle (RATTO et al., 2011); 2) low antral follicle populations, especially in *Bos taurus* breeds (BATISTA et al., 2014); and 3) variation between females (MERTON et al., 2003; PONTES et al., 2009). Therefore, various strategies have been designed to improve the results from IVP programs with Holstein donors.

Among the strategies used, ovarian antral follicle counts in the donors, evaluated directly by ultrasonography (BURNS, 2005; IRELAND et al., 2007; IRELAND et al., 2008; SILVA-SANTOS et al., 2014a,b) or indirectly by the concentrations of anti-Mullerian hormone (RICO et al., 2009; MONNIAUX et al., 2011; BALDRIGHI et al., 2014; BATISTA et al., 2014; GUERREIRO et al., 2014) are beginning to be applied in the field. Another alternative is the use of follicular wave synchronization protocols associated with superstimulation treatments prior to the OPU (GOODHAND et al., 1999; SENDAG et al., 2008; VIEIRA et al., 2014a). Earlier studies reported associations between IVP outcomes and the stage of follicular growth at which OPU is performed (PAVLOK; LUCAS-HAHN; NIEMANN, 1992; BLONDIN; SIRARD, 1995; FAIR; HYTTEL; GREVE, 1995; HAGEMANN et al., 1999; HENDRIKSEN et al., 2004). During follicular and oocyte growth, the oocyte acquires its developmental potential progressively (LONERGAN et al., 1994; ARLOTTO et al., 1996; SIRARD et al., 2006; CAIXETA et al., 2009; SIRARD, 2011; SIRARD, 2012) due to the constant increase in the cytoplasmic storage of mRNA and proteins (BREVINI-GANDOLFI; GANDOLFI, 2001), essential for early embryo development (DIELEMAN et al., 2002). Superstimulation treatments with porcine FSH (pFSH) prior to the OPU has improved IVP in *Bos taurus* donors, resulting in increased total embryo yields per OPU session (GOODHAND et al., 1999; DE ROOVER et al., 2008; SENDAG et al., 2008; VIEIRA et al., 2014a), possibly due to healthier and more competent oocytes recovered from the greater proportion of medium-sized follicles.

Traditional superstimulatory treatments consist of twice daily IM injections of pFSH for *in vivo* (MAPLETOFT; BÓ, 2012) or *in vitro* (ROOVER et al., 2005; VIEIRA et al., 2014a) embryo production. The need of frequent applications to induce ovarian superstimulation (DEMOUSTIER et al., 1988; MALHI et al., 2008) is due to the short half-life of FSH (5 h) in cattle (LASTER, 1972; DEMOUSTIER et al., 1988). Explicitly, superstimulation protocols require precision and attention to minimize mishandling during the treatments. Therefore, the application of traditional superstimulatory protocols in large scale programs can lead to failures and poor results, suggesting the need for simplified protocols that can be implemented efficiently and easily in the field by reducing handling and consequently, the incidence of potential errors.

Considering the need to develop a simplified superstimulation protocol, studies have focused on alternative methods to maintain FSH release during a prolonged period of time. An alternative that has been studied for *in vivo* embryo production is the use of a single injection of pFSH in a hyaluronan (HA) solution (TRÍBULO et al., 2011). The HA is a biodegradable polymer that apparently results in a sustainable slow release of hormones. Previous studies reported a similar number of transferable embryos when a single (in 2% HA) (TRÍBULO et al., 2011) or two (in 0.5 or 1% HA) (TRÍBULO et al., 2012) IM injections of pFSH was administered compared to the traditional twice-daily IM injection protocol. The same authors emphasized that the need for the second injection is related to the concentration of HA; the lower concentrations are less viscous and easier to mix with pFSH, but are not as efficacious in sustaining circulating FSH levels (TRÍBULO et al., 2011, 2012). Therefore, considering the shorter period of FSH support required for superstimulation prior to OPU (BLONDIN et al., 2002; VIEIRA et al., 2014a) compared to traditional multiple ovulation and *in vivo* embryo procedures [reviewed by Mapletoft and Bó (2012)], the use of a single injection of pFSH in 0.5% HA (5 mg/mL) might be an alternative to simplify the superstimulatory protocol for OPU-IVP programs. In a preliminary study performed in beef donor cows, the administration of 160 mg of pFSH diluted in 0.5% HA resulted in a comparable number of COCs recovered and blastocysts produced as the administration of 160 mg pFSH in twice daily IM injections (ONGARATTO et al., 2011).

Thus, the objectives of the present study were: 1) to compare plasma FSH profiles when two different doses of pFSH were combined with 0.5% HA with twice daily injections of pFSH in saline; 2) to evaluate the FSH support needed to obtain growth of a homogeneous population of medium-sized follicles for OPU; 3) to compare OPU-IVP outcomes after

superstimulation with two different doses of pFSH administrated as a single IM injection in 0.5% HA with twice daily injections of pFSH in saline, and; 4) to determine the viability of IVP embryos following superstimulation by transfer to synchronous beef heifer recipients.

5.2 MATERIALS AND METHODS

5.2.1 Experiment 1: plasma FSH profiles

5.2.1.1 Farm and animals

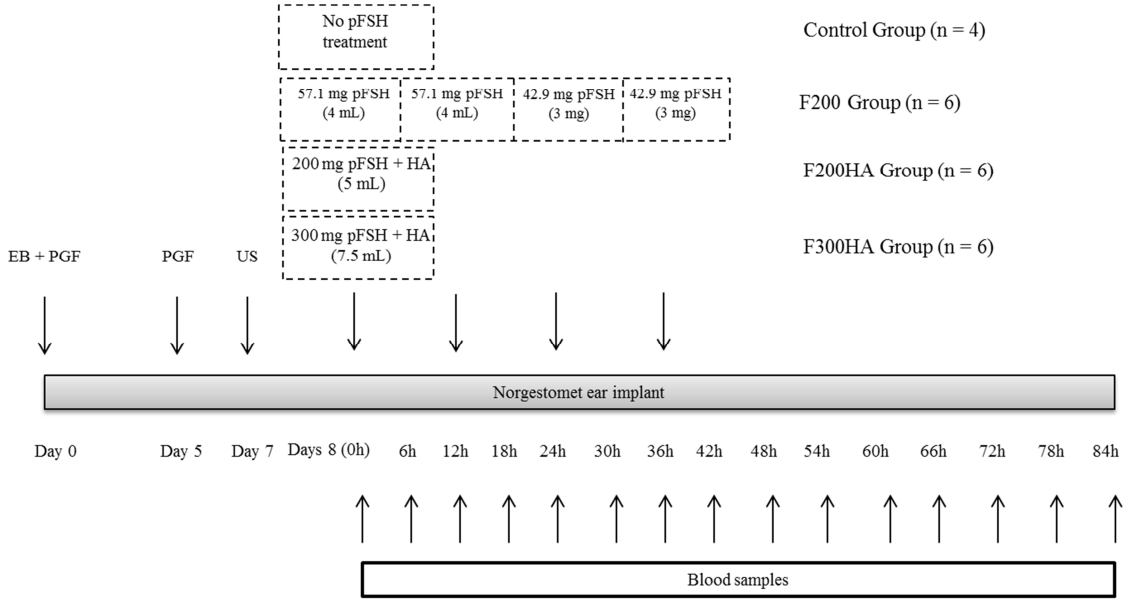
The experiment was conducted during July and August of 2014, on a state research farm (Institute of Animal Science) located in Nova Odessa, São Paulo, Brazil. The heifers enrolled in the experiment were maintained in dry-lot pens and fed with a TMR formulated to meet or exceed the minimum nutritional requirements for Holstein heifers (NRC, 2001). All heifers had free access to water and mineralized salt. On the first Day of the trial, animals were on average of 330.0 ± 52.9 Kg (± SD) in weight, 13.5 ± 2.2 months (± SD) of age and a body condition score of 2.7 ± 0.3 (BCS; 1 – 5 scale (AYRES et al., 2009).

5.2.1.2 Experimental design

A total of 23 Holstein heifers were randomly allocated to one of four groups (Control, F200, F200HA or F300HA groups). On a random days of the estrous cycle (Day 0), all heifers received a Norgestomet ear implant (Crestar®, MSD Animal Health, The Netherlands), 2.0 mg IM of estradiol benzoate (RIC-BE®, Tecnopec, Brazil) and 0.150 mg of cloprostenol (ESTRON®, Tecnopec, Brazil). On Day 5, all heifers received another 0.150 mg of cloprostenol and on Day 7 were submitted to an ultrasound evaluation to obtain the diameter of the largest visible follicle present in the ovaries (average diameter of 10 ± 0.7 mm). Control heifers received no further treatments. On Days 8 and 9, the F200 group received a total dosage of 200 mg of pFSH (14.3 mg/mL in saline diluent; Folltropin®,

Bioniche Animal Health, Belleville, ON, Canada) administrated IM in four doses (57.1, 57.1, 42.9 and 42.9 mg), 12 h apart. The HA treated heifers received a single dose of pFSH (IM) on Day 8 AM in 5.0 mL (F200HA) or 7.5 mL (F300HA) of 0.5% hyaluronan (40 mg/mL of FSH; MAP-5®, Bioniche Animal Health, Belleville, ON, Canada) On Day 12 AM, the Norgestomet ear implant was removed after the last blood sample (Figure 5).

Figure 5 - Experiment 1 design. Heifers were assigned to one of four groups to compare plasma FSH profiles. Holstein heifers were not treated (Control, n = 4), treated with 200 mg of pFSH divided into twice daily injections (F200, n = 6) or 200 or 300 mg of pFSH in 0.5% hyaluronan as a single injection (F200HA, n = 6 and F300HA, n = 6). EB = 2.0 mg of estradiol benzoate; PGF = 0.150 mg of cloprostenol; US = ultrasound evaluation to obtain the largest follicle diameter; pFSH = porcine follicle-stimulating hormone; HA = Hyaluronan



Fonte: Vieira, L. M. (2016).

The methodology used to suppress endogenous FSH release was based on reproductive physiology and knowledge of endocrinology. The synchronization of follicular wave emergence permitted the initiation of FSH treatments 4 days after follicular wave emergence, when a growing dominant follicle should have been selected and when endogenous FSH concentrations should be at baseline [reviewed by Baruselli et al. (2012)]. As reviewed by Mapletoft, Bó and Baruselli (2009), endogenous FSH is suppressed by estradiol and inhibin, hormones produced by the growing follicles, and especially the dominant follicle. Additionally, two doses of cloprostenol were administered to ensure low progesterone levels and, thus, support the dominant follicle growth. Lastly, recommended

doses of Norgestomet ear implant are associated with greater LH support of the development of the dominant follicle (BÓ et al., 2002).

5.2.1.3 Blood sample collection and plasma FSH quantification

Starting on Day 8 (immediately before the first pFSH treatment) blood samples were collected from the jugular vein at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, 90 and 96 h (n = 23) for plasma pFSH concentrations. Approximately, 8 mL were collected into heparinized tubes and immediately placed in ice. Plasma was separated by centrifugation (2,000 × g for 15 min at room temperature), and stored at −20 ◦C until assayed. Quantification of FSH was performed by radioimmunoassay (RIA) validated for bovine FSH using USDA-bFSH for iodination and reference standards, and NIDDKanti-oFSH antiserum (BOLT; ROLLINS, 1983). Two assays were performed on each sample; sensitivity was 0.03 ng/mL and intra- and inter-assay coefficients of variation were 20.3% and 10.5% for high concentrations and 9.2% and 18.4% for low concentrations. The analyses were performed in the Laboratory of Animal Endocrinology at the University of the State of São Paulo (UNESP), Araçatuba, São Paulo.

5.2.2 Experiment 2: ovum pick-up, in vitro embryo production and establishment of pregnancy

5.2.2.1 Farm and animals

This experiment was conducted on two commercial dairy farms in southeast Brazil (22°01’27’’S/47°53’19’’W) during October of 2013 and February of 2014. The herds were composed of approximately 1,500 lactating Holstein cows housed in free stall facilities, milked three times daily and with an average milk production of 30.1±0.3 kg per day.

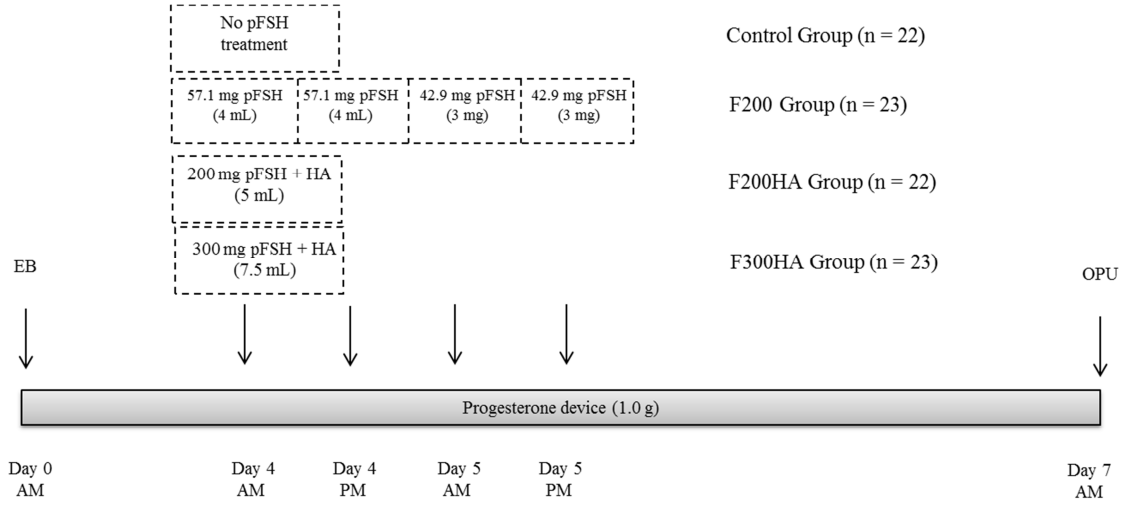
The donors were non-lactating Holstein cows selected by genetic merit, 5 years of age with two to three previous lactations, maintained in dry-lot pens and fed a TMR formulated to

meet the minimum nutritional requirements for non-lactating Holstein cows (NRC, 2001). Briefly, the main ingredients were corn silage and Tifton hay as forage, and corn, soybean and cottonseed meal-based concentrate. The embryo recipients were crossbred (*Bos indicus x Bos taurus*) beef heifers maintained on *Brachiaria decumbens* pastures. All animals had free access to water and mineralized salt.

5.2.2.2 Experimental design

A total of 90 non-lactating Holstein donors were enrolled at random in one of the four groups: Control (n=22); F200 (n=23); F200HA (n=22) and F300HA (n=23). On random days of the estrous cycle (Day 0), all cows received an intravaginal progesterone device (P4; Primer®, Tecnopec) and 2.0 mg of estradiol benzoate (BE, RIC-BE®, Tecnopec) IM. The Control group received no superstimulatory treatment. On Days 4 and 5, the F200 group received total dosage of 200 mg of pFSH (Folltropin®) in saline divided in four doses (57.1, 57.1, 42.9 and 42.9 mg), administered 12 h apart. The groups containing pFSH in hyaluronan (40 mg/mL; MAP-5®, received a single dose (IM) on Day 4 AM, 5.0 mL (F200HA) and 7.5 mL (F300HA). Immediately before OPU (Day 7 AM) the P4 devices were withdrawn (Figure 6).

Figure 6 - Experiment 2 design. Non-lactating Holstein cows were allocated to one of four groups for in vitro embryo production following OPU. Cows were not treated (Control, n = 22), treated with 200 mg of pFSH (F200, n = 23) in twice daily injections or 200 or 300 mg of pFSH combined with 0.5% hyaluronan solution (F200HA, n = 22 and F300HA, n = 23) as a single intramuscular injection. EB = 2.0 mg of estradiol benzoate; pFSH = porcine follicle-stimulating hormone; HA = 0.5% hyaluronan; OPU: ovum pick-up



Fonte: Vieira, L. M. (2016).

5.2.2.3 Ultrasonography examinations

Immediately before the OPU session, both ovaries were examined by transrectal ultrasonography using a portable scanner (Aloka SSDV 500; Aloka, Tokyo, Japan) with 5 MHz convex array transducer housed in a plastic vaginal guidance device. All follicles suitable to be punctured (diameter ≥ 2 mm) were quantified and classified according to their diameters [small (SF < 6 mm), medium (MF = 6 to 10 mm) and large (LF = > 10 mm) follicles].

5.2.2.4 Ovum pick-up (OPU)

For the cumulus–oocyte complexes (COCs) collection, cows were restrained in a chute and epidural anesthesia was administered with 2% lidocaine hydrochloride (Lidovet®, Bravet, Brazil) to facilitate the handling of the ovaries through the rectum. The perineal area was cleaned using water, dried and sprayed with 70% alcohol prior to each session. All

follicles ≥ 2 mm were aspirated using the portable scanner with a 5-MHz convex array transducer housed in a plastic vaginal guidance device with a stainless steel needle guide connected to aspiration equipment and a vacuum system. Follicular aspirates were recovered via a 1.1 mm i.d. by a 120 cm length circuit (Watanabe Tecnologia Aplicada, Cravinhos, SP, Brazil), connected directly to a disposable 18-gauge x 2 inch hypodermic needle (0.9 x 50 mm; Terumo Europe NV – Belgium) and a 50 mL conical tube containing 15 mL of Dulbecco PBS (DPBS; Nutricell Nutrientes Celulares, Campinas, SP, Brazil) supplemented with 1% (vol/vol) fetal calf serum (FCS; Gibco Life Technologies, Grand Island, NY) and 5,000 IU/mL sodium heparin (Parinex, Hipolabor, Belo Horizonte, MG, Brazil) at 35 to 37°C. The vacuum connected to the needle was set at 100 mm Hg. To avoid reduced recovery rate in superstimulated donors (VIEIRA et al., 2014a), a greater needle diameter (18-ga) and vacuum pressure (100 mmHg) was used based on Bols et al. (1996). All retrieval procedures were performed by two veterinarians. The conical tube containing the follicular aspirate was transported to a field laboratory and COCs were recovered using a 75 μm filter (Watanabe Tecnologia Aplicada) and DPBS supplemented with 1% FCS. The COCs were washed once in DPBS supplemented with 1% FCS at 37°C and evaluated under a stereomicroscope at 8–20X magnification). The COCs were morphologically classified based upon the number of cumulus cell layers as follows: Grade 1, more than three layers of compact cumulus cells; Grade 2, at least one layer of cumulus cells; Grade 3, denuded; and Grade 4, atretic, with dark cumulus cells and signs of cytoplasmic degeneration (SENEDA et al., 2001). After evaluation, only Grade 4 COCs were considered unsuitable and discarded. The remaining COCs were considered suitable for culture, maintained in maturation medium and transported to the commercial IVP laboratory (Bioembryo Biotecnologia da Reprodução Animal, Bauru, SP or Neogen Reprodução Assistida, Tabiratiba,SP) at 37 to 39°C.

5.2.2.5 In vitro embryo production (IVEP)

The *in vitro* maturation (IVM) medium was composed of bicarbonate-buffered TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 50 μg/mL LH (APL, Ayerst, Rouses Point, NY), 5 μg/mL pFSH (Folltropin-V), 0.1 μg/mL estradiol (Estradiol 17β; Sigma-Aldrich Chemical Co.), 22 μg/mL sodium pyruvate, and 50 μg/mL gentamycin. The COCs of each cow were cultured separately for 24 h (considering the transport period to the

lab) in 100 µL drops of maturation medium under mineral oil (D'Altomare, São Paulo, SP, Brazil) at 39°C in an atmosphere of 5% CO_2 in humidified air. After 24 h of IVM, the COCs were washed and subjected to *in vitro* fertilization (IVF) in 100 µL drops of IVF medium under mineral oil. The IVF medium was Tyrodes albumin lactate pyruvate (TALP) supplemented with 10 µg/mL heparin, 22 µg/mL sodium pyruvate, 50 µg/mL gentamycin), 6 mg/mL fatty acid-free BSA, and PHE solution (2 µM penicillin, 1 µM hypotaurine, and 0.25 µM epinephrine; (BAVISTER; YANAGIMACHI, 1977).

For IVF, semen straws of three sires, homogeneously distributed among experimental groups, were thawed for 30 s in a 35°C water bath and semen was deposited on a 90 to 45% Percoll gradient prepared with sperm wash medium (modified Tyrode medium) and centrifuged at 320 × g for 30 min to separate the motile sperm and to remove the diluents and seminal plasma. Then, the sperm pellet was evaluated for concentration and motility by the addition of IVF medium. Each fertilization droplet received 5 µL of sperm, to achieve a final concentration of 1×10^6 live sperm/mL. Sperm and COCs were incubated at 38.5°C in an atmosphere of 5% CO_2 in humidified air for 18 to 20 h (starting 24 h after OPU procedure).

Approximately 18 h after IVF, presumptive zygotes were stripped of cumulus cells by mechanical pipetting in TALP medium. Groups of presumptive zygotes were co-cultured on a monolayer of cumulus cells that had attached to the surface of the plate during IVM. Thus, to maintain the maximum amount of cumulus cells, the IVM medium was gently replaced with CR2aa medium (WATANABE et al., 1999) supplemented with 2% FCS and 30 mg/mL BSA for embryo culture in 100 µL drops at 39°C in an atmosphere of 5% CO_2 in humidified air for 48 to 72 h. During the first (Day 3 of culture) and second feeding (Day 5 of culture), half of the drop volume (50 µL) was replaced by fresh medium during all embryo culture procedures. Cleavage rate was recorded after 3 d of embryo culture (number of cleaved zygotes divided by number of cultured COC) and blastocyst rate after 7 d of embryo culture (number of blastocysts divided by number of cultured COC).

5.2.2.6 Embryo transfer and pregnancy diagnosis

A subset of the fresh *in vitro* produced embryos (Control: n = 18, F200: n = 23, F200HA: n = 11 and F300HA: n = 20) were transferred non-surgically into the uterine horn ipsilateral to the CL of recipients based on detection of estrus synchronous with the stage of

development of the embryos, as previously described in detail by Rodrigues et al. (2010). On the day of embryo transfer, recipients were assessed by rectal palpation for the presence of corpus luteum (CL). Pregnancy diagnosis was performed by transrectal palpation 50 to 60 days after embryo transfer. The detection of asymmetry of the uterine horns and amniotic vesicle were used as indicator of pregnancy.

5.2.2.7 Statistical Analysis

Statistical analyses were performed using Statistical Analysis System for Windows (SAS 9.3). In Experiment 1, area under the curve (AUC) of plasma FSH was calculated by the trapezoid method. Total period with elevated plasma FSH concentration was determined as the time interval from the onset of the increase of FSH (baseline concentration before the increase in FSH concentration induced by the first pFSH treatment) to its last return to baseline concentrations after the last pFSH treatment. The increase in FSH was defined as twice the standard deviation above the overall within-cow mean of FSH concentrations. In the control group, one heifer had an increased FSH concentration at 72 h, therefore, it was excluded from the analysis. In Experiment 2, the variables evaluated were: number of follicles suitable to be punctured in each size category at the time of OPU (small, medium and large), number of follicles suitable to be punctured, number of COCs recovered, recovery rate (number of COCs recovered per number of follicles suitable to be punctured), number and percentage of cultured COCs (number of COCs cultured per structures recovered), cleavage rate (number of cleaved zygotes per number of COCs cultured), blastocyst rate (number of blastocysts produced per number of COCs cultured), number of embryos produced per OPU procedure and pregnancy rate after embryo transfer.

For the analysis, a binomial distribution was assumed for the categorical response variables. Continuous data were tested for normality of the residues and homogeneity of variances using the Guided Data Analysis, and transformed when necessary. The fixed effect included in the model was treatment. The OPU session effect was included as a random effect. The data were analyzed by orthogonal contrasts. The contrasts established were: C1 (Superstimulation effect): Control vs (F200+F200HA+F300HA); C2 (HA effect): F200 vs

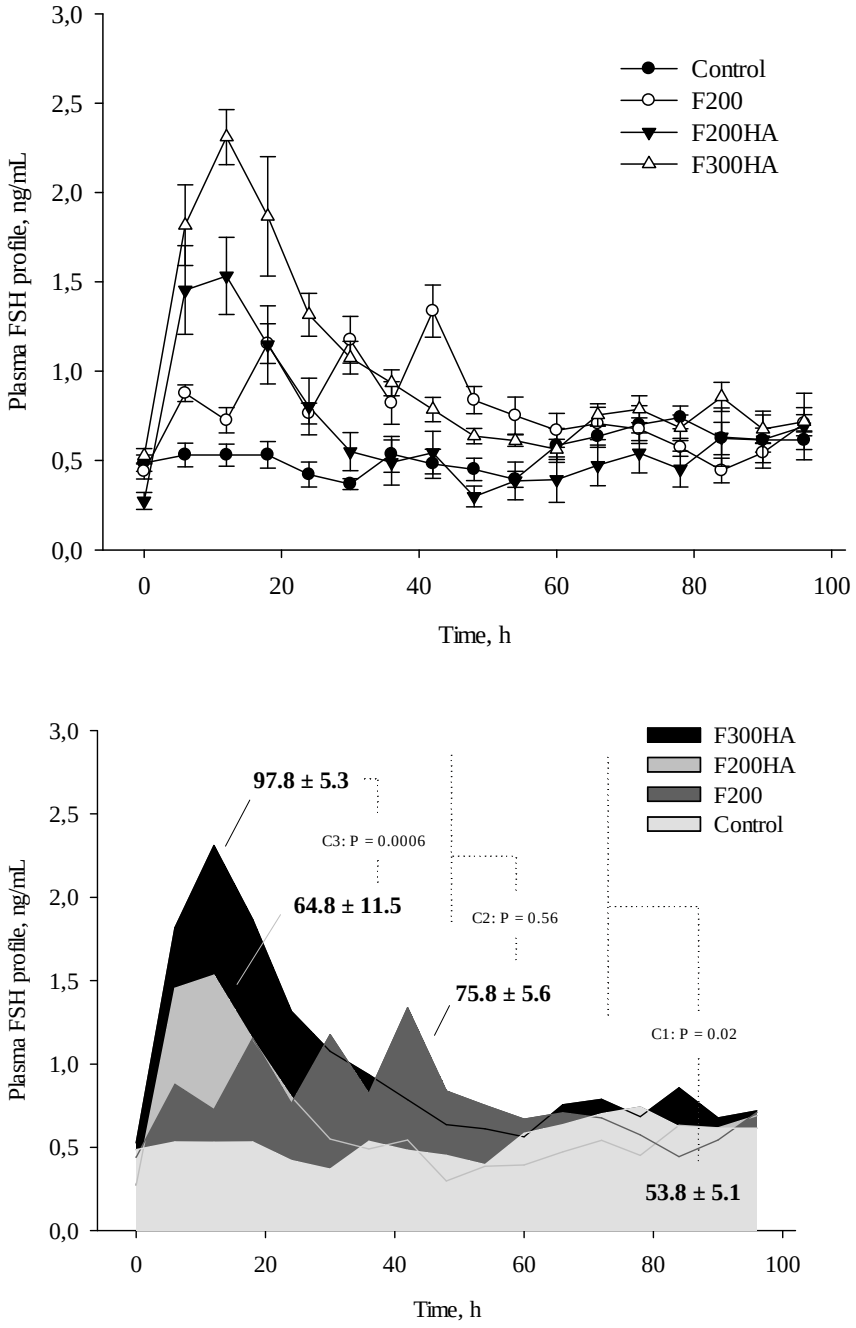
(F200HA+F300HA); and C3 (Dose effect): F200HA vs. F300HA. Means (±SE) or percentages were used to describe the response variables.

5.3 RESULTS

5.3.1 Experiment 1: plasma FSH profiles

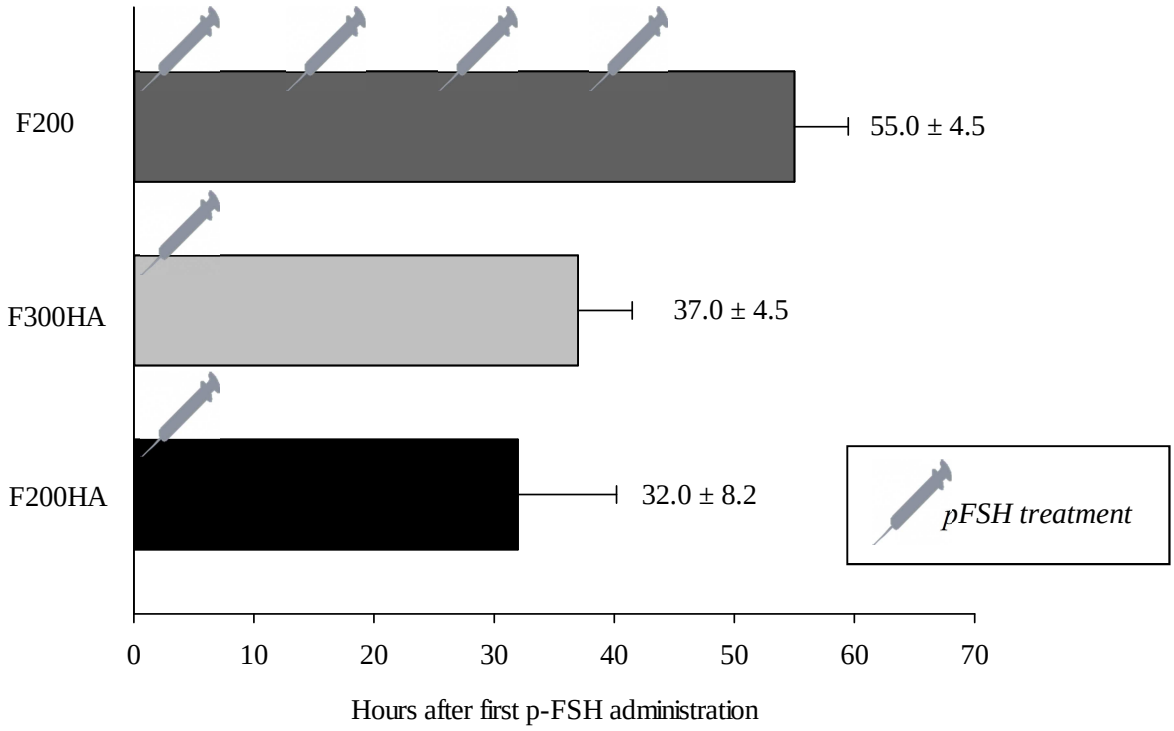
The plasma FSH concentration profiles differed among treatment groups (Figure 7). Heifers receiving pFSH treatment had greater (P = 0.002; C1) area under the curve (AUC) of plasma FSH; however, although F200 group was not different from HA groups (P = 0.56; C2), the F300HA group had greater AUC compared to the F200HA group (P = 0.006, Figure 3; C3). Heifers receiving the F200 treatment had an extended period of greater plasma concentration of FSH compared to the groups that received a single dose of pFSH in HA (P < 0.0001; C2), while both HA groups were similar (P = 0.17, C3; Figure 8).

Figure 7 - Mean ± SEM (upper graph) and area under curve (AUC, ng*h/mL; lower graph) in Holstein heifers following no superstimulation treatment (Control) or treatment with pFSH in twice daily injections (F200) or combined with 0.5% hyaluronan as a single intramuscular injection (F200HA and F300HA). Circulating FSH concentrations were determined by RIA at 6 hour intervals for 96 hours after the first treatment. For statistical analysis, orthogonal contrasts were applied: C1 (FSH Effect): Control x (F200+F200HA+F300HA), P = 0.02; C2 (HA Effect): F200 X (F200HA+F300HA), P = 0.56; and C3 (Dose Effect): F200HA x F300HA), P = 0.0006



Fonte: Vieira, L. M. (2016).

Figure 8 - Total period (in hours) that plasma FSH concentrations were elevated in Holstein heifers treated with four doses of pFSH (F200) 12 h apart or two different doses (200 and 300 mg) of pFSH as a single injection in 0.5% hyaluronan (HA; F200HA and F300HA). The total period with elevated plasma FSH concentration was determined as the time interval from the onset of the FSH increase (baseline concentration before the increase in FSH concentration induced by treatment) to its return to baseline concentrations after the last pFSH treatment. Data are presented as mean ± standard error of the mean. For the duration of pFSH treatment the orthogonal contrasts were used: C1 (HA Effect): F200 X (F200HA+F300HA), P <0.0001and C2 (Dose Effect): F200HA x F300HA, P = 0.17



Fonte: Vieira, L. M. (2016).

5.3.2 Experiment 2: ovum pick-up, in vitro embryo production and establishment of pregnancy

The numbers of follicles suitable for puncture and oocytes retrieved were greater in superstimulated groups (P = 0.01 and P = 0.01, C1, respectively), but were similar among groups that received four doses of pFSH or a single dose of pFSH in HA (P = 0.97 and P = 0.78, C2, respectively; Table 1). The number of COCs retrieved in the F300HA group tended to be lower than in the F200HA group (P = 0.09, C3; Table 1). In addition, pFSH-treated

donors had a lower proportion of small follicles ($P < 0.001$, C1) and a higher proportion of medium follicles ($P < 0.001$, C1) at the time of OPU than the Control group (Figure 9). Cows that received four doses of pFSH had a greater proportion of small follicles compared to the cows that received a single dose of pFSH in HA (C2; $P = 0.08$, Figure 9). Proportions of medium ($P = 0.32$) and large follicles ($P = 0.42$; C2) did not differ between cows that received four doses of pFSH or a single dose of pFSH in HA (Figure 9).

Additionally, the F300HA group had a greater proportion of large follicles compared to the F200HA group ($P = 0.05$, C3). Lastly, no difference was observed in the recovery rate between cows in the control group and superstimulated donors ($P = 0.80$, C1), and no difference was observed in the recovery rate between F200 and the F200HA and F300HA groups ($P = 0.67$, C2). However, cows in the F300HA group had a lower recovery rate ($P = 0.009$, C3) than those in the F200HA group.

A greater number of COCs were considered suitable for culture in pFSH-treated donors ($P = 0.02$, C1) that in the Control group, but there was no difference among pFSH treatment groups ($P = 0.56$, C2). Within single injection with HA groups, the number of COCs suitable to culture was lower in the F300HA group than in the F200HA group ($P = 0.04$, C3; Table 1). Similar (C1: $P = 0.34$, C2: $P = 0.46$ and C3: $P = 0.30$) percentages of COCs were considered suitable to use in IVP among pFSH-treated groups (Table 1).

A greater cleavage rate ($P = 0.002$, C1) was obtained in COCs collected from donors treated with pFSH, regardless of treatment group and those from cows in the Control group (Table 1) and there was no effect of pFSH treatment group on cleavage rate (C2: $P = 0.99$ and C3: $P = 0.97$). Although, there was no difference in blastocyst production rate among groups ($P = 0.42$, C1; $P = 0.80$, C2; and $P = 0.16$, C3), superstimulated donors produced more embryos per OPU session ($P = 0.06$, C1) than the Control group. Among the superstimulated cows, there was no difference between twice daily injection and single injection groups ($P = 0.61$, C2), but the number of blastocysts produced per OPU session was lower in the F300HA than in the F200HA group ($P = 0.06$, C3; Table 1). Finally, similar pregnancy rates were observed among groups after transferring the subset of the fresh *in vitro* produced embryos (C1: $P = 0.42$, C2: $P = 0.82$ and C3: $P = 0.82$; Table 1).

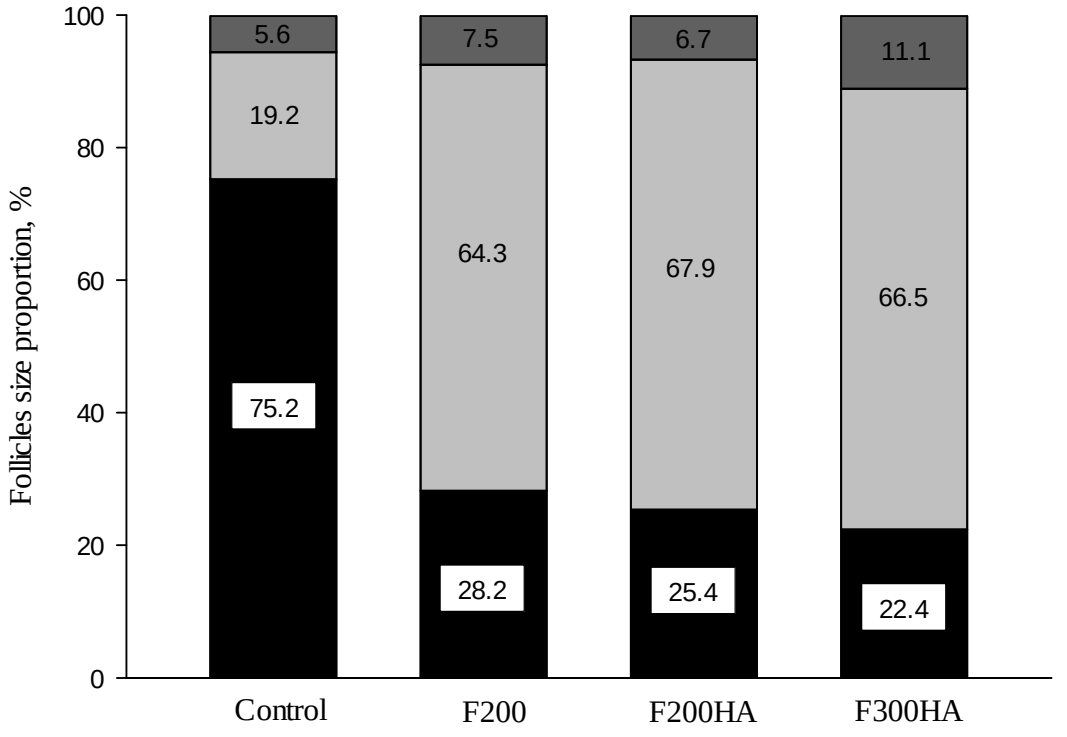
Table 2 - Summary of cumulus-oocyte complex (COC) yield and in vitro embryo production (mean + SE or percentage) in non-lactating Holstein cows that were not treated (Control), treated with 200 mg of pFSH (F200) in twice daily injections or 200 or 300 mg of pFSH as a single injection in 0.5% hyaluronan (F200HA and F300HA)

	Treatments				*P-value* [5]		
	Control	F200	F200HA	F300HA	C1	C2	C3
No.	22	23	22	23	.	.	.
Total follicles suitable to be punctured	16.1 ± 1.1	20.4 ± 1.4	23.2 ± 2.3	19.6 ± 1.6	0.01	0.97	0.24
Total oocytes retrieved	13.1 ± 1.0	16.5 ± 1.2	19.5 ± 2.1	15.4 ± 1.4	0.01	0.78	0.09
Recovery rate, % [1]	80.8 (287/355)	81.0 (379/468)	84.0 (429/511)	78.7 (355/451)	0.80	0.67	0.009
COCs cultured	9.3 ± 0.7	12.2 ± 1.2	15.6 ± 1.7	11.4 ± 1.2	0.02	0.56	0.04
COCs culture rate, % [2]	71.4 (205/287)	74.1 (281/379)	80.0 (343/429)	74.1 (263/355)	0.34	0.46	0.30
Cleavage rate, % [3]	75.6 (155/205)	85.1 (239/281)	79.6 (273/343)	79.4 (210/263)	0.002	0.99	0.97
Blastocyst rate, % [4]	25.9 (53/205)	30.3 (85/281)	30.3 (104/343)	27.0 (71/263)	0.42	0.80	0.16
Embryos produced per OPU	2.4 ± 0.5	3.7 ± 0.7	4.7 ± 0.7	3.1 ± 0.6	0.06	0.61	0.06
Pregnancy per ET, %	33.3 (6/18)	39.1 (9/23)	54.6 (6/11)	60.0 (12/20)	0.42	0.82	0.82

Fonte: Vieira, L. M. (2016).

1 No. COCs /no. follicles suitable to be punctured; 2 No. COCs cultured /no. total COCs retrieved; 3 No. cleaved zygotes /no. COC cultured; 4 No. blastocysts /no. COC cultured; 5 Orthogonal contrasts: C1 (FSH Effect): Control x (F200+F200HA+F300HA); C2 (HA Effect): F200 X (F200HA+F300HA); and C3 (Dose Effect): F200HA x F300HA).

Figure 9 - Proportion of small (< 6 mm), medium (6 to 10 mm) and large (> 10 mm) follicles suitable to be punctured immediately before OPU (Day 7) in non-lactating cows (n=90) submitted to OPU without previous superstimulation treatment (Control), after treatment with pFSH (F200) or pFSH in Hyaluronan (F200HA and F300HA). Orthogonal contrasts: C1 (FSH Effect): Control x (F200+F200HA+F300HA); C2 (HA Effect): F200 X (F200HA+F300HA); and C3 (Dose Effect): F200HA x F300HA



	P-value		
	C1: FSH effect	C2: HA effect	C3: Dose effect
Small follicles	< 0.0001	0.08	0.24
Medium follicles	< 0.0001	0.32	0.94
Large follicles	0.08	0.42	0.05

Fonte: Vieira, L. M. (2016).

5.4 DISCUSSION

The present study confirmed that pFSH treatment results in greater plasma FSH concentrations compared to the non-pFSH treated Controls. In particular, the administration of a single dose of pFSH in HA provided comparable plasma FSH concentrations (as measured by AUC) to the twice-daily pFSH treatment. The maintenance of elevated plasma

FSH concentrations differed among treatment groups; FSH concentration in HA groups reached baseline in approximately 32-37 hours after treatment which was before the twice-daily pFSH treatment over 2 days. Moreover, in Experiment 2, pFSH treatment of cows (with or without HA) increased the efficiency OPU-IVP compared to the untreated Control donors. Although plasma FSH concentrations in HA groups reached baseline before the traditional twice-daily treatment, FSH support seemed to have resulted in similar overall superstimulation and efficiency of the OPU-IVP technology, suggesting plasma FSH concentration does not need to be elevated for more than 36 h to superstimulate donors for OPU-IVP.

Additionally, it is important to highlight that the higher dose of pFSH in HA (300 mg) resulted in greater proportion of large follicles, but reduced COCs recovery rate and the number of embryos produced per OPU session compared to the group that received only 200 mg of pFSH combined with HA. Regardless, these data reinforce the benefits of the superstimulation treatment in the Holstein breed to enhance the IVP production and that only one injection of 200 mg pFSH diluted in 0.5% HA is required, reducing animal handling and the potential noncompliance during the hormonal protocol.

The traditional superstimulatory protocol utilizes saline as a diluent and due to the short half-life of the FSH (LASTER, 1972; DEMOUSTIER et al., 1988) twice daily IM injections are required for *in vivo* (MAPLETOFT; BÓ, 2012) or *in vitro* (ROOVER et al., 2005; VIEIRA et al., 2014a) embryo production. Based on the rapid clearance of FSH (~5 h; DEMOUSTIER et al., 1988), regardless of dose of administered (HIRAIZUMI et al., 2015), an alternative could be to modify hormone absorption. Modifications examined have included changing the route administration (subcutaneous injection - BO et al., 1994; KELLY et al., 1997; HIRAIZUMI et al., 2015) or mixing the pFSH with polymers, such as polyvinylpyrrolidone (CHASOMBAT et al., 2013), aluminum hydroxide gel (HASHIMOTO et al., 2007) or hyaluronan (TRÍBULO, A. et al., 2012) in order to reduce the rate of absorption. Tríbulo et al. (2012), reported satisfactory superovulatory results after treating donors with a single dose of pFSH combined with 2% HA. However, 2% HA was viscous and difficult to mix with pFSH, especially in the field. A 0.5% solution of HA was much less viscous and easy to work with and resulted in a similar number of transferable embryos as twice-daily treatments when divided into two administration 48 h apart. Although a two injection protocol seemed to be required to superovulate donor cows for *in vivo* embryo production, the results of the present study support the notion that only one injection of pFSH diluted in 0.5% HA is required for *in vitro* embryo production following OPU in non-lactating

Holstein donors. These results are also in agreement with those obtained after a single injection of pFSH diluted in 0.5% HA in beef cows (ONGARATTO et al., 2011). The present data verify a shortened, but extended period of elevated plasma concentrations of FSH in HA groups. The extended period of elevated plasma FSH in females treated with the traditional twice-daily treatment protocol is no doubt related to the continued administration of pFSH.

As previously reported (ALLER et al., 2010; NIVET et al., 2012; VIEIRA et al., 2014a) and reinforced in the present study, treatment with pFSH increases the proportion of medium-sized follicles for OPU. The reasoning for increasing the proportion of medium-sized follicles for OPU has been based on the observation that oocyte developmental competence was influenced by the stage of follicular development (LONERGAN et al., 1994; ARLOTTO et al., 1996; CAIXETA et al., 2009; SIRARD, 2012). During follicle growth, several morphological, molecular, metabolic and epigenetic changes occur in the COCs (KRISHER, 2014). These changes, including molecular and transcriptional alterations, have been correlated with final oocyte development competence, (BREVINI-GANDOLFI; GANDOLFI, 2001; MOUROT et al., 2006; SIRARD, 2012; LABRECQUE; SIRARD, 2013). In addition, others have shown positive differential gene expression [genes related to transcription and cell cycle regulations (CHU; DUFORT; SIRARD, 2012)] of COCs retrieved from superstimulated donors compared to untreated females. However, negative differential gene expression (genes related to matrix remodeling, disturbance of angiogenesis, apoptosis, and oxidative stress response (DIAS et al., 2013) has also been reported in COCs retrieved from superstimulated donors. Although the present study did not reveal differences in pregnancy rates after transfer of the in vitro-produced embryos, additional trials are required to clarify and confirm the beneficial effects of superstimulation treatment on the overall outcomes.

Although oocyte quality has been shown to improve with follicle growth (FAIR; HYTTEL; GREVE, 1995), follicle size has been reported to adversely affect COC recovery rate in *Bos taurus* cows (SENEDA et al., 2001; VIEIRA et al., 2014a) and heifers (GOODHAND et al., 1999). Seneda et al. (2001), suggested that the increased volume and viscosity of follicular fluid and the greater intra-follicular pressure of larger follicles following superstimulation may hamper COC recovery. In the present study, similar recovery rates were observed among superstimulated and Control groups. However, it is important to note that a greater needle diameter (18-ga) and vacuum pressure (100 mmHg) was used in the present trial, which may have resulted in a greater recovery rate. In another earlier study a greater recovery rate was observed when OPU was performed with larger needles but a similar vacuum pressure (BOLS et al., 1996). Therefore, more detailed studies are necessary

to establish an ideal approach to retrieve the maximum proportion of COCs with optimal development potential and without adverse effects after superstimulation of donors for OPU.

The oocyte development potential can be evaluated by the capacity of the COC to become a viable blastocyst within an IVP system (MERTON et al., 2003). Although the present study reports similar blastocyst rates among treatment groups, donors treated with pFSH, regardless of diluent type (with or without HA), had increased cleavage rates compared to the control (untreated) donors. Other studies have also reported increased blastocyst production rates in *Bos taurus* beef (BLONDIN et al., 2002; DE ROOVER et al., 2008) and dairy cattle (VIEIRA et al., 2014a) following superstimulation with pFSH. Although considerable variation has been reported, an increased oocyte developmental competence (cleavage and/or blastocyst rate) was apparent in all studies, reinforcing the beneficial effects of the superstimulation treatment during OPU-IVP programs.

Although different gene expression and a possible decrease in the quality of embryos produced after ovarian stimulation has been reported in mice (FAUQUE et al., 2007), humans (SATO et al., 2007) and cattle (MUNDIM et al., 2009), the present study indicates that pregnancy rates after the transfer of fresh *in vitro*-produced embryos are likely to be similar, regardless of donor treatment. However, future studies with larger numbers of embryos and recipients are required to confirm these results.

Finally, a greater efficiency of OPU-IVP was obtained in donors treated with a single injection of 200 mg of pFSH in 0.5% HA as compared to those treated with 300 mg of pFSH in 0.5% HA. As reported previously (ROOVER et al., 2005), the larger dose of pFSH resulted in an increased follicular diameter but no increase in the number of visible follicles. As expected a greater proportion of large-sized follicles also resulted in reduced COC recovery rates and consequently, fewer COCs retrieved and cultured and reduced blastocyst production per OPU. Furthermore, the higher pFSH dosage seems to result in more rapid growth rates which may result in altered gene expression in granulosa cells (GARCÍA GUERRA et al., 2015) and compromise the efficiency of OPU-IVP. Therefore, to optimize OPU-IVP improved approaches must be designed to more closely refine the pFSH dosage used as a single injection with HA in potential donors.

In conclusion, the administration of a single dose of pFSH in 0.5% HA provided a comparable plasma FSH concentration (as measured by AUC of FSH) to the twice-daily pFSH treatment. Regardless of diluent type and administration schedule, superstimulation with pFSH enhanced the overall efficiency of the IVP program in non-lactating Holstein cows. The pFSH diluted in HA appears to be an alternative to extend plasma FSH

concentrations, maintaining the beneficial effects of the superstimulation treatment with a simplified protocol and reduced labor. Under the condition of the current trial, 200 mg of pFSH seems to be a more appropriate dosage than 300 mg when combined with HA in OPU-IVP programs of non-lactating Holstein donors. Lastly, pregnancy establishment with *in vitro*-produced embryos was similar, regardless donor treatment prior to OPU.

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6 GENERAL CONCLUSIONS

6.1 The superstimulation with four (twice-daily) pFSH injections in synchronized donors (lactating and non-lactating cows) increased medium sized-follicles and *in vitro* embryo production. ***Initial hypothesis was confirmed.***

6.2 Regardless treatment group, synchronized non-lactating donors had greater *in vitro* embryo production compared to lactating Holstein cows. ***Initial hypothesis was confirmed.***

6.3 A single injection of 200 or 300 mg of pFSH combined with 0.5% hyaluronan presented similar AUC of plasma FSH compared to the traditional treatment (four decreasing doses, 12 h apart). However, the total period with elevated FSH concentration was lower among donors treated with pFSH in Hyaluronan compared to the traditional treatment. ***Initial hypothesis was not confirmed.***

6.4 A single injection of pFSH in 0.5% hyaluronan in synchronized donors (non-lactating cows) increased medium sized-follicles and *in vitro* embryo production; being comparable to the traditional treatment (four decreasing doses, 12 h apart) and greater than the non-superstimulated group. However, a single injection of 200 mg of pFSH in 0.5% hyaluronan in synchronized donors (non-lactating cows) resulted in greater *in vitro* embryo production compared to the dose of 300 mg of pFSH. ***Initial hypothesis was not confirmed.***

7 PRACTICAL IMPLICATIONS

Considering increased IVEP outcome observed among Holstein donors submitted to superstimulation prior to OPU, the treatment can be an alternative strategy to enhance the technology efficiency and viability in dairy farms. Additionally, an increased embryo per OPU session was observed in non-lactating donors, suggesting that *in vitro* embryo production might be even greater when opting for superstimulated non-lactating Holstein donors. However, it is important to highlight that in the literature there are many discrepancy result regarding the benefits of superstimulation prior to OPU (Table 3), therefore more studies are required to specify in which situation and protocol the superstimulation treatment could be recommended.

Still, although the possible positive effect of the superstimulation treatment, the increased animal handling due to the need of consecutive pFSH administrations (12 h apart) hamper the treatment use in large scale in the field. Therefore, the use of pFSH in a slow carrier diluent (i.e. hyaluronan) as a superstimulation treatment enable an effective follicular response and IVEP with half of the managements (six to three handling) required with the traditional twice-daily injections. Therefore, the superstimulation protocol has been adjusted aiming to maintain the possible superstimulation positive results associated to a practicality field application.

Lastly, if the results presented herein are maintained, the superstimulation treatment could be considered profitable to be applied in the field. Considering the present study results and actual market scenario: 1. Increased number of blastocysts per OPU session in superstimulated donors (2.7 and 4.4); 2. Similar pregnancy establishment (45.8%) after transferring *in vitro* produced embryo; 3. R$ 160.00 per pFSH (200mg) treated donor; and 4. R$400.00 per pregnancy. Simulating 100 OPU session, it would be observed 440 instead of 270 produced blastocysts and 202 instead of 124 pregnancies established. Therefore, considering total return with established pregnancy (R$80,800.00 *versus* R$49,600.00), even including the total pFSH cost (R$16,000.00), *in vitro* lab would still have greater return (R$64,800.00) compared to the one obtained with no superstimulated donors (R$49,600.00)[3].

[3] Commercial product prices obtained in Brazil during September 2016 with current USDBRL exchange rate of 3.27BRL.

Table 3 - Effect of superstimulation treatment prior to OPU

Reference	Treatment prior to OPU	Breed/Category	Number of OPU	Donors treatment		Result relative to control group
				Superstimulated	Control	
GOODHAND et al. (1999)	6 decreasing doses of FSH (9.0mg)	Simental heifers	8	2.1 transferable embryos	1.0 transferable embryo	+
GOODHAND et al. (2000)	6 decreasing doses of FSH (9.0mg)	Beef x Friesian cows	64	2.5 transferable embryos	2.0 transferable embryos	=
MERTON et al. (2003)	4 equal doses of FSH (9.0mg)	-	30	3.3 embryos	1.5 embryos	+
DE ROOVER et al. (2008)	4 equal doses of FSH (9.0mg)	Belgian Blue Breed	640	3.4 embryos	0.7 embryos	+
ALLER et al. (2012)	Single dose of eCG (1,600IU)	Pregnant Angus cows	30	2.2 COC/donor/session	1.0 COC/donor/session	+
GERHARDT et al. (2013)	1 dose of FSH (40mg)	Non-lactating Girolando cows	68	1.8 embryos	1.3 embryos	=
MARTINS et al. (2013)	4 decreasing doses of FSH (200mg)	Gir cows	12	3.5 embryos	2.8 embryos	=
LIMA MARTINS et al. (2013)	3 decreasing doses of FSH (200mg), 24h apart	Sindhi cows	6	31.2% of blastocyst rate	27.1% of blastocyst rate	=
SILVA et al. (2014)	4 or 6 decreasing doses of FSH (200mg)	Non-lactating Holstein cows	36	3.4 embryos	3.8 embryos	=
GUERREIRO et al. unpublished data (2015)	4 decreasing doses of FSH (180mg)	Holstein Heifers	6	3.5 embryos	1.3 embryos	+
OLIVEIRA et al. (2016 in press)[3]	6 equal doses of FSH (240mg)	Non-lactating Holstein cows	35	3.0 embryos	2.6 embryos	=

[3] OLIVEIRA, L. H.; SANCHES, C. P.; SEDDON, A. S.; VERAS, M. B.; LIMA, F. A.; MONTEIRO, P. L. J., JR.; WILTBANK, M. C.; SARTORI, R. Short communication: follicle superstimulation before ovum pick-up for in vitro embryo production in Holstein cows. **Journal of Dairy Science**, 2016. In press.

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