DAMIANA CHELLO

Factors that affect the embryo production and fertility of buffalo donors and recipients

São Paulo
2020

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Dissertation submitted to the Postgraduate Program in Animal Reproduction of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Master's degree in Sciences.

Department:

Animal Reproduction

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Prof. Dr. Pietro Sampaio Baruselli

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CERTIFICADO

Certificamos que a proposta intitulada "Fatores que influenciam a produção de embriões de doadoras e a fertilidade de receptoras búbalinas.", protocolada sob o CEUA nº 8091270619 (ID 006944), sob a responsabilidade de **Pietro Sampaio Baruselli** *e equipe; DAMIANA CHELLO* - 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 **aprovada** 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 04/09/2019.

We certify that the proposal "Factors that affect the embryo production and fertility of buffalo donors and recipients.", utilizing 380 Buffalos (380 females), protocol number CEUA 8091270619 (ID 006944), under the responsibility of **Pietro Sampaio Baruselli** *and team; DAMIANA CHELLO* - 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 School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 09/04/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2019 a 12/2019 Área: Reprodução Animal

Origem:	Animais de proprietários						
Espécie:	Bubalinos	sexo:	Fêmeas	idade:	1 a 17 anos	N:	75
Linhagem:	Murrah			Peso:	240 a 650 kg		
Origem:	Animais de proprietários						
Espécie:	Bubalinos	sexo:	Fêmeas	idade:	4 a 17 anos	N:	70
Linhagem:	Murrah-mestiça Murrah x Mediterrane			Peso:	400 a 700 kg		
Origem:	Animais de proprietários						
Espécie:	Bubalinos	sexo:	Fêmeas	idade:	4 a 15 anos	N:	88
Linhagem:	Murrah-mestiça Murrah x Mediterrane			Peso:	400 a 700 kg		
Origem:	Animais de proprietários						
Espécie:	Bubalinos	sexo:	Fêmeas	idade:	4 a 17 anos	N:	97
Linhagem:	Murrah-mestiça Murrah x Mediterrane			Peso:	400 a 700 kg		
Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bubalinos	sexo:	Fêmeas	idade:	4 a 17 anos	N:	50
Linhagem:	Murrah-mestiça Murrah x Mediterrane			Peso:	400 a 700 kg		

Local do experimento: -Mangas e Corrais prefeitura do Campus USP de Pirassununga - Bubalinocultura -Mangas e Corrais da Fazenda Ouro Preto, Registro - SP -Mangas e Corrais da Fazenda Santa Olga, Formosa, Argentina -Mangas e Corrais do Polo Regional Vale do Ribeira, Unidade de Pesquisa e Desenvolvimento, Registro, SP -Mangas e Corrais da Fazenda Pinari do Igaì Os animais serão submetidos à aspiração folicular (OPU) dentro do curral da fazenda e após o tratamento serão liberados nos respectivos piquetes.A Aspiração Folicular Ovariana Guiada por Ultrassom (OPU-Ovum pick-up) compreende a obtenção dos óvulos diretamente do ovário das vacas doadoras com o auxílio de um equipamento de ultrassonografia. Os folículos ovarianos visualizados no aparelho de ultrassom são penetrados com uma agulha ligada a um sistema de vácuo, aspirando o conteúdo líquido e com ele o óvulo. Estes óvulos coletados são rastreados, selecionados e envasados para envio a um laboratório onde serão produzidos os embriões.

São Paulo, 14 de janeiro de 2020

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Dedico

A Gianluca, l'uomo della mia vita senza il quale non sarei qui adesso.

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RESUMO

CHELLO, D. **Fatores que influenciam a produção de embriões de doadoras e a fertilidade de receptoras bubalinas.** 2020. 82 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

Atualmente, a aspiração folicular guiada por ultrassonografia (OPU), combinada com a produção *in vitro* de embriões (PIVE), são tecnologias que apresentam resultados promissores na espécie bubalina para multiplicar indivíduos de alto valor genético. As características reprodutivas peculiares da espécie tornaram a superovulação e a produção *in vivo* de embriões uma tecnologia de baixa eficiência. Para melhor avaliar a resposta à OPU/PIVE, dois estudos foram propostos em doadoras e receptoras de embriões. No Experimento 1, estudou-se o efeito da categoria (novilhas pré-puberes, púberes e vacas) e das concentrações plasmáticas do hormônio Anti-Mulleriano (AMH) das doadoras na eficiência da OPU/PIVE. Foram utilizadas 72 doadoras da raça Murrah pertencentes às três categorias. Os animais foram aspirados no mesmo dia e a PIVE foi realizada com a mesma partida de sêmen de um único touro. No momento da OPU uma amostra de sangue foi colhida para medir os níveis circulantes de AMH. A categoria pré-púbere apresentou menor produção de embriões quando comparada com as vacas. Já os animais adultos (vacas) apresentaram maiores taxas de oócitos viáveis e de embriões produzidos, com maior número de blastocisto/OPU. Verificou-se correlação positiva entre as concentrações plasmáticas de AMH e a quantidade de folículos (AFP), o número de oócitos recuperados e a produção de embriões em todas as categorias. No Experimento 2, avaliou-se o efeito do tratamento com GnRH no momento da TE na formação de CL acessório, no aumento das concentrações plasmáticas de P4 e na taxa de prenhez por transferência de embriões (P/TE). Não houve efeito do tratamento com GnRH no aumento das concentrações de P4 e na P/TE. Verificou-se baixa taxa de ovulação e formação de CL acessório (apenas 28%) após o tratamento com GnRH. Entretanto, foi encontrada uma relação positiva entre a concentração plasmática de P4 e a vascularização do CL, avaliada por ultrassonografia Doppler. Conclui-se que o AMH é um marcador endócrino relacionado a eficiência da OPU/PIVE em bubalinos. Doadoras pré-púberes apresentam menor produção de embriões quando comparadas com vacas. Ainda, o

tratamento com GnRH no momento da TE não aumentou as concentrações plasmáticas de P4 e a P/TE.

Palavras-chave: Búfalo, AMH, GnRH, Corpo Lúteo Acessório, Categoria, TET.

ABSTRACT

CHELLO, D. **Factors that affect the embryo production and fertility of buffalo donors and recipients.** 2020. 82 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

Currently, ultrasound-guided follicular aspiration (OPU) combined with *in vitro* embryo production (IVEP) are technologies that show promising results in buffalo to multiply individuals of high genetic value. The peculiar reproductive characteristics of the buffalo species have made superovulation and *in vivo* embryo production a low efficiency technology. To better evaluate the response to OPU/IVEP, two studies were proposed in buffalo donors and recipients. In Experiment 1, the effect of the donor category (prepubertal and pubertal heifers and cows) and donor anti-Müllerian (AMH) plasma concentrations on the efficiency of OPU/IVEP was studied. Seventy-two Murrah donors belonging to the three categories were used. The animals were aspirated on the same day and IVEP was performed with the same semen batch of a single bull. At the time of OPU a blood sample was taken to measure circulating AMH levels. The prepubertal category showed lower embryo production when compared to cows. On the other hand, adult animals (cows) presented higher viable oocyte and embryo production rates. Positive correlation was found between plasma AMH concentrations and follicle number (AFP), number of oocytes retrieved and embryo production in all categories. In Experiment 2, the effect of GnRH treatment at ET on accessory CL induction, increased plasma P4 concentrations, and embryo transfer pregnancy rate (P/ET) was evaluated. There was no effect of GnRH treatment on increasing P4 concentrations and P/TE. Low ovulation rate and accessory CL formation (only 28%) were found after GnRH treatment. However, a positive relationship was found between plasma P4 concentration and CL vascularization assessed by Color Doppler ultrasonography. It was concluded that AMH is an endocrine marker for OPU/IVEP efficiency in buffalo donors. Pre-pubertal donors have lower embryo production compared to cows. Also, treatment with GnRH at the time of ET did not increase plasma P4 concentrations and P/TE.

Key words: Buffalo, AMH, GnRH, Accessory Corpus Luteum, Category, TET.

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SUMMARY

1 GENERAL INTRODUCTION

Over the past two decades, interest in buffalo breeding has increased throughout the world due to the easy adaptability to different regions (tropical and subtropical) and environment. In fact, both in developing countries as in developed countries, buffalo is considered as an important source of high quality animal protein, both milk and meat (CAMPANILE et al., 2010). FAO data (2017) state that the world buffalo population has increased by 9.2% during the period 2007-2017 and 201 million heads are currently estimated. Nowadays, buffalo herds are responsible for producing almost 120 million tons of milk in 2017 (18% of the world milk production). The Brazilian herd has also grown from 2007 to 2017, from 1.131.986 heads in 2007 to 1.381.395 in 2017, with about 65% of this herd dislocated in the north of the country. Currently Brazil has the largest buffalo population of all Central and South America (FAO, 2017).

As for other species of zootechnical interest, the growth of the buffalo herd must be associated with the control of productivity, which, consequently, depends on the reproductive performance. Furthermore, the associated use of different assisted reproductive technologies can improve the reproductive indexes by creating a selected high-quality genetics for both meat and milk production.

Artificial insemination (AI) is a profitable reproductive biotechnology, which has radically changed animal breeding. AI is worldwide used for dissemination of superior male material to improve genetic progress, control venereal diseases and accelerate improvements in shortest possible time (SINGH; BALHARA, 2016). Nevertheless, AI has the limit of being able to improve genetics only through male lineage, slowing down genetic progress.

On the other hand, the use of embryo transfer (ET) allows the propagation of the best genetics both female and male, granting a rapid progression of genetic gain in many breeds and ensuring a higher volume of offspring to be produced in quantity and quality and greater economic return.

In bovine, donor SOV following by timed embryo transfer (TET) in recipients is one of the most widely used biotechniques to disseminate high value genetic material (PHILLIPS; JAHNKE, 2016). Embryo transfer (ET) may be an indispensable tool for productivity improvement.

In buffalo, studies have been conducted to evaluate the efficiency of superovulation and *in vivo* embryo production (SOV) and embryo transfer (ET; ALVAREZ et al., 1994; BARUSELLI et al., 1994; OBA et al., 1994; CARVALHO et al., 2002; CAMPANILE et al., 2010). However, the results in buffalo are still unsatisfactory. The embryo production technique still requires many in-depth studies on buffaloes in order to seek explanations for the intrinsic species-specific reduced population of primordial and antral follicles, a high variability within animals in follicular recruitment and a lower response of superovulatory treatment in buffaloes compared to cattle (DROST, 2007).

Oogenesis in cow and buffalo gonads begins in the early third of pregnancy, originating the number of primordial follicles (pool), which remains the only source of oocytes during the animal's lifetime. The number of primordial and antral follicles is genetically determined and species-specific and it has been reported by being lower in River type buffalo than in cattle (ERICKSON, 1966; ROCHE; BOLAND, 1991; DRIANCOURT et al., 1993; SAMAD; NASSERI, 1979; DANELL, 1987; KUMAR et al., 1997).

Despite buffalo presents a follicular response to the superstimulation treatments, the recovery of ova/embryo is still unsatisfactory (CHELLO, 2013; GASPARRINI et al., 2014; BOMBONATO; DANGELO; BARUSELLI, 2011; DE CARVALHO et al., 2012; CARVALHO et al., 2002). Currently, studies showed that the most appropriate system to produce buffalo embryos is represented by ultrasound-guided follicular aspiration (Ovum Pick Up - OPU) combined with *in vitro* embryo production (IVEP; BONI et al., 1996; GALLI et al., 2001; NEGLIA et al., 2003). The OPU is a non-invasive technique that allows the recovery of satisfactory numbers of competent oocytes from antral follicles of living animals. It can be performed twice a week for many weeks without side effects on the donor's reproductive career (GALLI et al., 2001). The *in vitro* system has been developed and established in buffalo species by extrapolating information acquired in cattle (NANDI et al., 2002). IVEP technology involves several steps. The first step is the recovery of oocytes through transvaginal aspiration, the selection of viable ones and their *in vitro* maturation (IVM). The next step is in vitro fertilization (IVF), and the final step is *in vitro* co-culture (IVC) of the embryos until the morula or blastocyst stage. The *in vitro*-produced embryos can then be transferred to synchronized recipients or cryopreserved and stored. The acquisition of more insights into buffalo embryo

physiology, metabolism, and *in vitro* oocyte maturation and embryo culture requirements is critical to optimize the efficiency of advanced reproductive strategies in this species.

Nowadays, the major constraint to apply OPU and IVEP technologies in this species is the lesser recovery of viable oocytes (GUPTA et al., 2006; MANJUNATHA et al., 2008, 2009; SÁ FILHO et al., 2009; DI FRANCESCO et al., 2012; NEGLIA et al., 2011), arising from physiological peculiarities, such as the greater incidence of atresia (OCAMPO et al., 1994; PALTA et al., 1998) and lesser number of primordial (SAMAD; NASSERI, 1979; DANELL, 1987) and antral follicles (KUMAR et al., 1997) in buffalo ovaries. A recent study has investigated the effect of superstimulation with FSH previous a session of OPU in three categories of buffaloes: nulliparous, primiparous and multiparous (DE CARVALHO et al., 2019). Oocyte recovery did not increase following superovulation treatment; however the number of oocytes recovered and embryo production / OPU was greater in nulliparous buffaloes than in multiparous (DE CARVALHO et al., 2019).

In the ongoing process to improve efficiency in the use of biotechnology associated to reproduction, the studies has recently focused on the anti-Müllerian hormone (AMH), a molecular marker of the ovarian follicular pool in women, cattle, goats, and other species (MONNIAUX et al., 2011; FANCHIN et al., 2005). AMH can be considered a useful endocrine marker of ovarian reserve (IRELAND et al., 2007 2008; MONNIAUX et al., 2011), suitable for the selection of the best oocytes donors. In cattle, circulating AMH concentration is already used to predict antral follicular population (AFP) in ovaries (IRELAND et al., 2008; RICO et al., 2009; GUERREIRO et al., 2014; BATISTA et al., 2014), response to SOV treatments (RICO et al., 2009; SOUZA et al., 2015), and also as a marker to predict IVEP performance of *Bos taurus*; (GUERREIRO et al., 2014; GAMARRA et al., 2015; VERNUNFT et al., 2015) and *Bos indicus* breeds (GUERREIRO et al., 2014). A recent study in buffalo of LIANG et al., 2016, confirmed a positive correlation between the concentration of intrafollicular AMH and the AFP, demonstrating that AMH can be an interesting reproductive parameter to select more efficient donors for OPU/IVEP. Comparing Murrah (*Bubalus bubalis*), Holstein (*Bos taurus*) and Gyr (*Bos indicus*) results that Murrah and Holstein heifers presented lower AFP and lower plasma AMH concentration when compared to Gyr (BALDRIGHI et al., 2014). However, despite the differences between genetic groups, a significant positive relationship between

AFP and AMH concentrations was detected within buffalo. More research is needed to identify if AMH can be considered a good marker even among different categories of buffaloes. In fact, as related by BARUSELLI et al., 2015 in cattle, AMH have been correlated with AFP of different categories, showing that AMH plasma concentration (ng/ml) in Holstein and Nelore calves (aging 2 to 4 months) is almost three times greater than in cycling heifers.

The purpose of the following studies was to determine the most effective approach possible to apply assisted reproduction technologies in buffalo, a species that notoriously has lower embryo production efficiency compared to cattle. Additionally, the aim of these experiments was to enhance a more effective selection of donors throughout the use of AMH as an endocrine embryo production marker and subsequently increase the efficiency of buffalo embryo recipients using GnRH at the TET to increase P4 circulation and conception rate.

2 HYPOTHESES

2.1 HYPOTHESES OF CHAPTER 1

There is a positive correlation between AMH plasma concentration, ovarian follicular population and *in vitro* embryo production for pre-pubertal, pubertal and adult buffaloes (*Bubalus bubalis*), being an endocrine marker for IVEP (Figure 1). Furthermore, the pre-pubertal animals present a higher AMH plasma concentration, antral follicle population (AFP), and oocytes recovery, however, a lower viable oocytes and embryo production than cows.

2.2 HYPOTHESES OF CHAPTER 2

The treatment with GnRH at the time of FTET induces the formation of an accessory CL, which produces higher blood progesterone concentrations and increases conception rates of recipients when compared to control group (Figure 2).

Figure 1 - Hypothetical model design of Chapter 1.

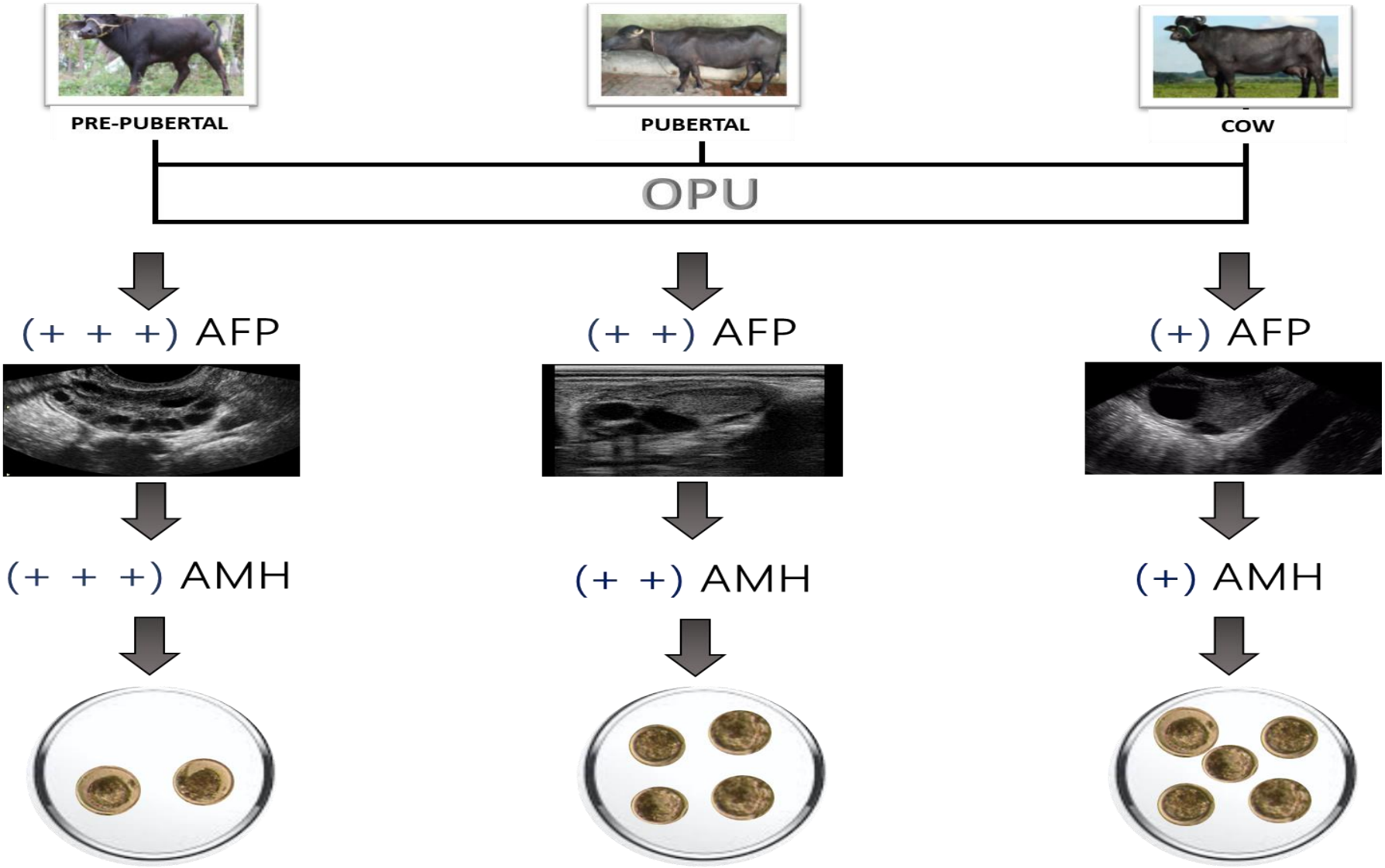
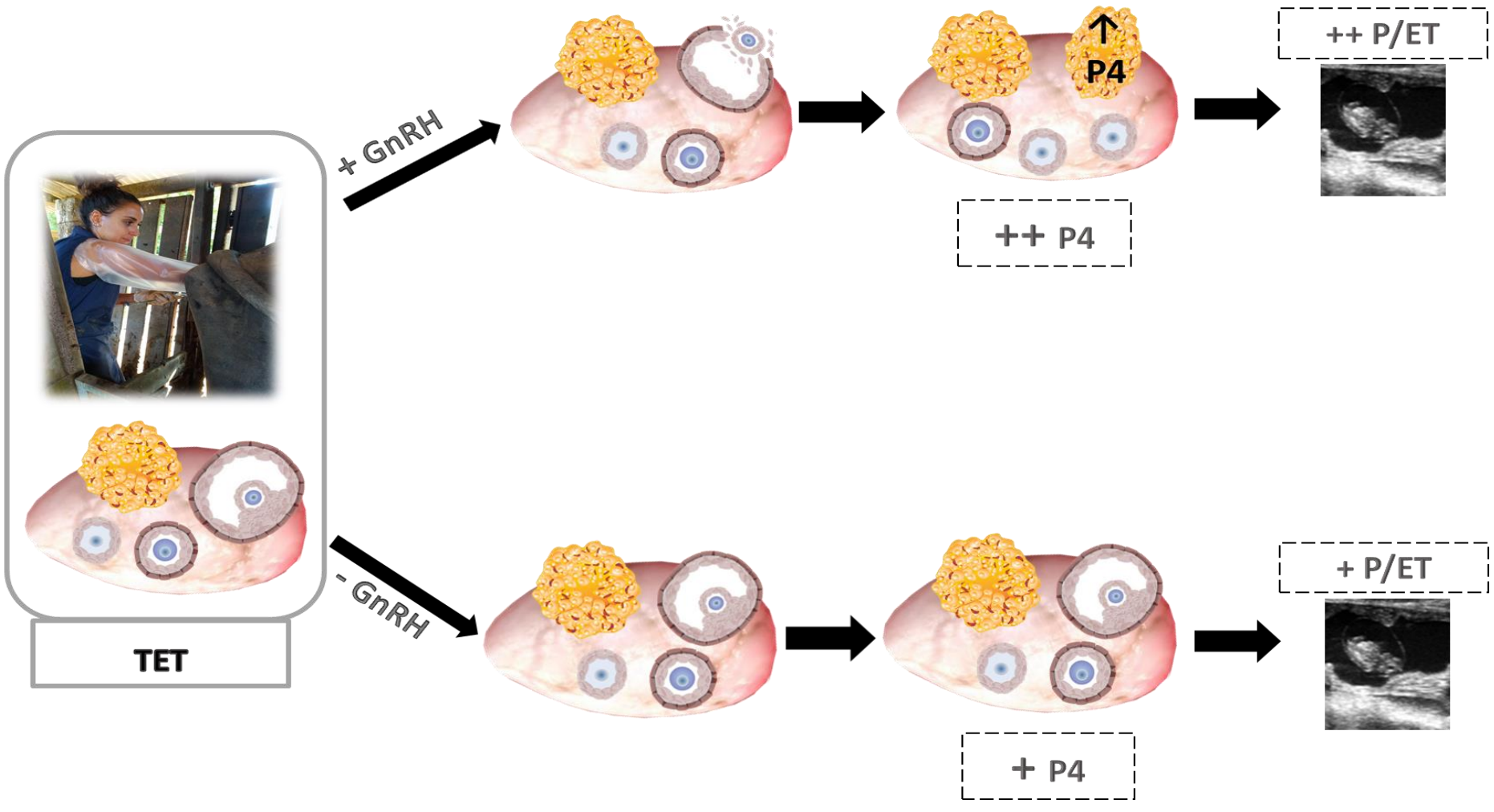


Figure 2 - Hypothetical model design of Chapter 2



3 OBJECTIVES

3.1 OBJECTIVES OF CHAPTHER 1

The aim of the study is to evaluate the association between plasma anti-Müllerian hormone (AMH) concentration and *in vitro* embryo production (IVEP) from prepubertal and pubertal heifers and cow buffalo (*Bubalus bubalis*) donors.

3.2 OBJECTIVES OF CHAPTHER 2

Evaluate efficiency of buffalo recipients treatment with GnRH at the time of TET:

- Ovulation rate and accessory CL formation.
- Progesterone plasma concentration, area, vascularization and diameter of CL.
- Number of pregnant/transferred (P/ET).

4 CHAPTER 1: RELATIONSHIP BETWEEN AMH CONCENTRATION, DONOR CATEGORY AND *IN VITRO* EMBRYO PRODUCTION IN BUFFALOES

ABSTRACT

To guarantee greater IVEP efficiency, it is important to identify donors with greater oocyte recovery-per-OPU potential. The aim of this research is to study the correlation between AMH plasma concentration and the number of ovarian follicles at the moment of OPU, the oocyte recovery during the OPU session and consequent embryo production in different buffalo category. The experiment was conducted at “Fazenda Paineiras da Ingaí”, Sarapuì, SP, Brazil and involved 72 Murrah buffalo donors. The animals were divided into 3 groups, based on age and weigh: pre-pubertal heifers (n=23; PRE-P), 14.0 ± 0.32 months old and 273 ± 5.97 kg; pubertal heifers (n=26; PUB), 26.9 ± 0.77 months old and 456.2 ± 12.1 kg and cows (n=23; COW), 111.8 ± 11.6 months old and 673.1 ± 16.4 kg . The animals were subjected to an OPU session carried out by the same professional during an aleatory day of the estrous cycle. On the day of OPU a blood sample was collected for AMH measurement, and the number of follicles was recorded. Statistical analyses have been performed with the software SAS® 9.4 GLIMMIX procedure. Positive correlation was found between plasma AMH concentrations and follicle number (AFP), number of oocytes recovered and embryo production in all categories. AMH plasma concertation resulted higher in PRE-P than in COW (P=0.04). Donors classified as having high AMH had a greater number of aspirated follicles (P<0.0001), retrieved (P<0.0001) and cultured COCs (P<0.0001) and produced blastocysts (P=0.0063). COW presented higher number of large follicles (LF) than PRE-P and PUB animals (P<0.0001). However, the number of small and medium FL was not different (P>0.05). No statistical difference among the categories has been detected for what concerns the oocytes yielded. Moreover, pre-pubertal donors showed lower embryo production when compared to cows. On the other hand, adult animals (COW) presented higher viable oocyte and embryo production rates. In conclusion, AMH represent a valid endocrine marker for IVEP and a promising tool to enhance the overall efficiency of OPU–IVEP programs in buffalo. Furthermore, PRE-P donors presented a high AMH plasma concentration, however low viable oocyte and blastocyst rates and low number of blastocyst produced per OPU than COW.

Key words: AMH, buffalo, category, OPU, IVEP

4.1 INTRODUCTION

The use of ovum Pick-up (OPU), together with subsequent *in vitro* embryo production (IVEP), is considered today the best way for buffalo to enhance genetic progression through the maternal lineage (BONI et al., 1996; GALLI et al., 2001; NEGLIA et al., 2003). OPU is a very versatile technique that allows a repeated production of embryos from donors of genetic value and it is a serious alternative to *in vivo* superovulation. It represents a valid system to recover oocytes from living donors, in almost any physiological status (prepubertal, pubertal, non-pregnant cow or pregnant up to the third or fourth month) (GALLI et al., 2001; BAYEUX et al., 2017; BARUSELLI et al., 2018). In buffalo it is reported a high individual variability in the number of oocytes retrieved per OPU (from 0 to 30), with a mean of 8.9 ± 5.0 per donor, and hence in the number of embryos produced, making it necessary to distinguish between desirable and undesirable donors (BARUSELLI et al., 2018). In fact, exist a large variability of antral follicular population (AFP) among different females, however the AFP count is highly repeatable within animal (BURNS et al., 2005; IRELAND et al., 2007; BATISTA et al., 2014).

To implement the use of IVEP, it is necessary to understand in advance that final results may be affected by primarily physiological characteristics, such as AFP and oocyte competence (TANEJA et al., 2000). Buffaloes presented 20% less antral follicles than those present in cow ovaries (VAN TY et al.,1989) and a greater incidence of follicular atresia can be found in buffalo (66.7%) compared to cattle (50%) (CRUZ, 2006; OCAMPO et al., 1994; PALTA. et al., 1998). Studies evaluated the effect of bovine somatotropin (bST) administration to increase the AFP and oocyte competence in buffalo donors submitted OPU/IVEP. The bST treatment improved the follicular population, however no effect was found on *in vitro* blastocyst outcomes (Sà FILHO et al., 2009; FERRAZ et al., 2015). These are important characteristics to take into consideration when choosing an appropriate reproductive strategy for this species.

In order to optimize the IVEP procedure, it is critical to identify reliable markers that permit a selection of embryo donors to enroll buffaloes in embryo production programs. Recently, especially in human medicine, the focus has been on anti-Müllerian hormone (AMH); a glycoprotein expressed in females by granulosa cells of ovarian follicles (VIGIER et al., 1984) .One of the few studies carried out on Murrah

heifers reported a correlation between plasma AMH concentration and antral follicular population (AFP), suggesting that AMH is a potential marker of follicular population in this species (BALDRIGHI et al., 2014; LIANG et al., 2016) and proved the commercial potential of these techniques in buffalo.

It has already been demonstrated in different mammalian females: mice (JONES; KROHN, 1961), cattle (TAKEO et al., 2017) and women (FANCHIN et al., 2005) that fertility declines with increasing age in association with a gradual decrease in AMH circulating. Generally, in beef cows, a decreased pregnancy rate, poor oocyte quality, decreased superovulatory response, impaired CL function, and increased ovulation failure is linked to a low follicle number (CUSHMAN et al., 2010). The AFP rapidly decreases with age (ERICKSON, 1966; BARUSELLI et al., 2015). Therefore, the use of calves and prepubertal heifer, with more follicles in their ovaries (DESJARDINS and HAFS, 1969), as donors in embryo transfer programs could increase the efficiency of the techniques and offers considerable potential for accelerated genetic gain through a reduction in generation interval (LOHUIS, 1995; BATISTA et al., 2016).

Endocrine AMH marker together with ultrasound techniques can be used to estimate the ovarian reserve. While direct follicle count with ultrasound may vary during the estrus cycle, AMH levels fluctuate minimally during this period (RICO et al., 2009; IRELAND et al., 2010; SOUZA et al., 2015) and, consequently, blood samples can be taken at any time to evaluate circulating AMH, indicating that it may be more accurate to determine the AFP.

Thus, the present study was designed to evaluate the effective correlation between AFP, AMH plasma concentration, IVEP in buffalo donor from different category (pre-pubertal, pubertal and cow). The results of this research will be helpful in the selection process of the best subjects to introduce in IVEP programs.

4.2 MATERIALS AND METHODS

This study was approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science, University of São Paulo (protocol #8091270619).

4.2.1 Farm and animals

The experiment was conducted in a commercial milk buffalo farm in Sarapuí (Sao Paulo, Brazil, located at latitude 23° 34' 57.5" S and longitude 47°49' 06.4" W) during the beginning of breeding season (autumn and winter). Murrah buffaloes (*Bubalis bubalis*) females (n=72) of three categories [Prepubertal heifers (n=23) (PRE-P); pubertal heifers (n=26) (PUB) and cows (n=23) (COW)] were used.

All the animals presented a body condition score (BCS) ≥ 2.5 (scale 1 – 5, where 1 = very thin and 5 = very fat). Pre-pubertal heifers were non-cycling animals, 14.0 ± 0.32 months old. Pubertal heifers were normal cycling, 26.9 ± 0.77 months old and Cows were cycling animals, 111.8 ± 11.6.

The animals were kipped on *Cynodon* and *Panicum maximum* pasture with free access to water and supplemented with mineralized salt.

4.2.2 Ultrasonography examinations

Immediately before the OPU procedure, ovaries were scanned by ultrasonography, using a 7.5-MHz linear-array transrectal transducer (Mindray® DP-2200Vet; Shenzhen, Guangdong, China). The same technician counted and measured all the visible antral follicles (> 2 mm in diameter, AFP) present on both ovaries and reported the presence and the size of the CL. Follicles were classified in small [SF < 5 mm], medium [MF = 5-8 mm], and large [LF>8 mm] follicles.

4.2.3 AMH plasma concentration

Before each OPU session, all donors of the different categories were subjected to a blood sample in vacuum tubes containing EDTA (Vacutainer®, Becton Dickinson and Company, USA) by jugular venipuncture. All the samples were immediately placed on ice and later centrifuged at 3,000g for 15 min. Plasma samples were frozen at -25°C until analysis. The Bovine AMH enzyme-linked immunosorbent assay AL-114 kit (Ansh Labs, Webster, TX, USA) was used to determine plasma AMH concentrations. The AMH analysis was performed at the Animal Endocrinology Laboratory of the São Paulo State University "Júlio de Mesquita Filho", Faculty of Veterinary Medicine, Campus of Araçatuba; and yielded a sensitivity of 0.02 ng/mL and an intra-assay variation coefficient of less than 6%. (low intra-assay variation coefficient = 5% and high intra-assay variation coefficient = 1,61%).

4.2.4 Ovum pick-up (OPU)

All the donors were subjected to an OPU session on an aleatory day of the estrous cycle without previous synchronization of the follicular wave or hormonal stimulation. The same technical performed all the oocyte aspiration procedures. Before oocytes aspiration, the animals were restrained in a chute and epidural anesthesia was administered using 4 mL of 2% lidocaine hydrochloride (Lidovet, Bravet, Brazil) to facilitate the manipulation of the ovaries through rectal palpation. Prior to each session, the vulva and perineal area were cleaned and disinfected with alcohol. All follicles ≥ 2mm were aspirated using a portable scanner with a 5-MHz convex array transducer (Mindray DP 2200Vet; Shenzhen, Guangdong, China) housed in a plastic vaginal probe with a stainless steel needle guide (20G; 0.9 x 50mm; Terumo Europe NV, Belgium) connected to a vacuum pump system (85–90 mm Hg of negative pressure; V-MAR 5000, Cook Australia, Queensland, Australia). Follicular contents were then recovered using a via a 1.1-mm inner diameter by a 120-cm length circuit (Watanabe Tecnologia Aplicada, WTA Ltda, Cravinhos, São

Paulo, Brazil) that was directly connected to a 50-mL conical tube containing 15mL of Dulbecco phosphate-buffered saline (DPBS; Nutricell Nutrientes Celulares, Campinas, São Paulo, Brazil) and 5,000 IU/mL sodium heparin (Parinex, Hipolabor, Belo Horizonte, Minas Gerais, Brazil) at a temperature of 37°C. . The conical tube containing the follicular aspirate was directly transported to a field laboratory and cumulus-oocyte complexes (COCs) were recovered using a 75-µ filter (Watanabe Tecnologia Aplicada) and Dulbecco modifed phosphate-buffered saline (DMPBS; IMV technologies, Vila São Jorge, Campinas, São Paulo, Brazil). The COCs were washed once in DMPBS at 37°C and morphologically evaluated under a stereomicroscope at x8 to x20 magnification. The COCs were morphologically classified on the basis of 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 cytoplasm degeneration (SENEDA et al., 2001). After evaluation, only grade 4 COCs were considered non-viable for culture and were 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), 10% fetal bovine serum (FBS), 49.4 mg/mL sodium pyruvate (Sigmae-Aldrich Chemical Co., St. Louis, MO, USA), and 50 mg/mL gentamycin. They were kept at a temperature between 37 and 39 °C.

4.2.5 COCs processing and *in vitro* embryo production (IVEP)

After OPU the viable COCs of each buffalo were cultured for 24 h in 400-µl of maturation medium (IVB® Technologies, ABS® Global) covered under 300-µl of mineral oil (Sigma-Aldrich Chemical Co) in an atmosphere with 5% CO^2, at 38.5°C of temperature and saturated humidity.

After *in vitro* maturation, the COCs were washed in TL-SEMEN medium and *in vitro* fertilization (IVF) medium (IVB® Technologies, ABS® Global) and placed in culture plates containing 50-µL drops of IVF medium covered with mineral oil. For IVF, semen straws were thawed for 30 s in a 37°C water bath and semen was

deposited on a 90% to 45% Percoll gradient prepared with sperm wash medium (modified Tyrode's medium) and centrifuged at 900g for 5 min to separate the motile sperm and remove the diluents and seminal plasma. The sperm pellet was then analyzed to determine motility and concentration. Each fertilization droplet received 10 µL of sperm. Sperm and COCs were incubated at 38.5°C in humidified air with 5% CO_2 for 18 to 20 h. Semen from a single bull was used for all replicates and donor categories. Approximately 18 h after insemination, the cumulus cells were removed by pipetting from the presumptive zygotes, which were then transferred to 100-µL drops of embryo culture medium (IVB® Technologies, ABS® Global) and incubated in an atmosphere with 5% CO^2, 5% O^2, 90% N^2, at 38.5°C of temperature. After 4 days of culture, 50% of the culture medium was replaced with fresh medium (feeding) from a stock of the same medium used at the beginning of the culture. The cleavage and blastocyst rates were recorded at day 4 and day 7 of culture, respectively.

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 plasmatic AMH concentration, total number of follicles aspirated, total number of COCs retrieved, 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), blastocyst rate (number of blastocysts produced per total number of COCs cultured), and number of blastocysts produced per OPU procedure. 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 AMH categories (low and high) and animals within each animal category (pre-pubertal, pubertal and cow) were included as a random effect in the statistical model. Therefore, aware of the limitation related to the analyses of the category effect (always maintained as random effect), results and discussion will only be presented as AMH concentration (continuous or categorized in high or low) within each category. For the correlation studies, significance was ascertained by Bravais–Pearson r critical values, as performed in

PROC CORR and PROC REG of SAS 9.3 to obtain the regression functions. Means (± standard error of the mean) are used to describe all the response variables. And for all analysis, differences with $P \leq 0.05$ were considered statistically significant and P between 0.05 and 0.10 was considered a trend.

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4.3 RESULTS

There was no interaction between animal category and AMH plasma concentration for all analyzed variables (Table 1).

As supposed in the hypotheses of the present study, the pre-pubertal heifers presented the greatest plasma AMH concentrations (0.19 ± 0.02 ng/mL) compared with those in pubertal heifers (0.15 ± 0.02 ng/mL) and cows (0.12 ± 0.02 ng/mL, P = 0.04; Table 2). No difference was found in the total average of follicles visualized during the OPU in the three categories (P = 0.88), but a greater presence of large follicles (>8mm) was observed in cows compared to the other two categories (P <0.0001, Graphic 1).

However, no category effect was observed for recovered COCs (P = 0.92), COCs recovery rate (P = 0.91) and number of Viable COCs (P = 0.36). Furthermore, the viable oocytes rate (number of COCs cultured over the number of follicles aspirated) was greater in cows than in pre-pubertal and pubertal heifers (P = 0.02, Table 2). The number of blastocysts produced per OPU (P = 0.09) and the blastocyst rate (P = 0.05) was higher in cows compared to pre-pubertal heifers and similar to the results obtained in pubertal heifers (Table 2).

Table 1 - Plasma AMH concentrations and cumulus oocytes complexes (COCs) and embryo results (mean ± SEM) after OPU in pre-pubertal, pubertal and cow donors classified into 2 AMH (high and low) groups.

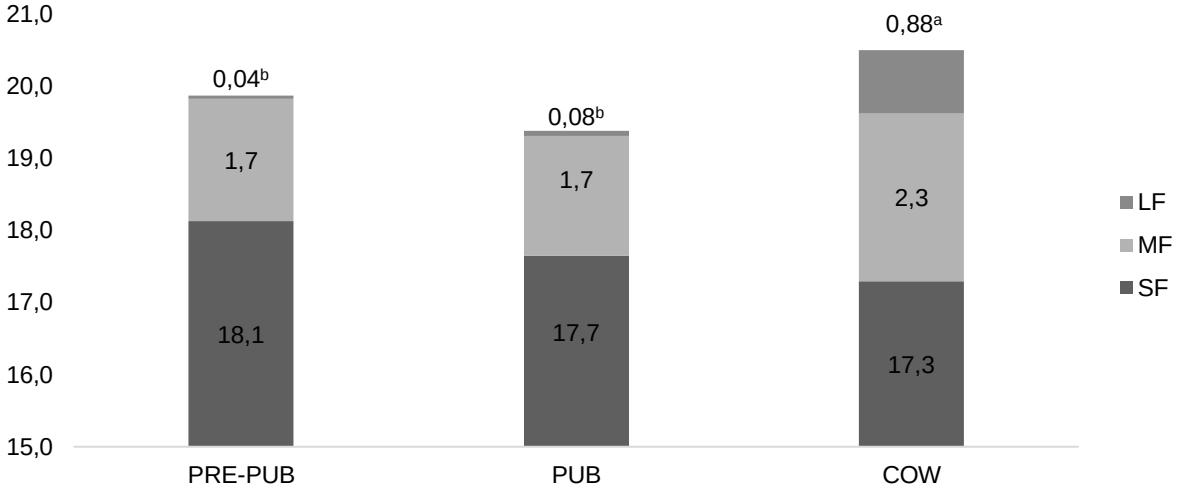
	Pre-pubertal		Pubertal		Cow		*P* Category	*P* AMH	*P* Cat*AMH
	High AMH	Low AMH	High AMH	Low AMH	High AMH	Low AMH			
Number of donors	12	11	13	13	11	12			
Plasma AMH (ng/mL)	0.26 ± 0.02^{x}	0.10 ± 0.01	0.22 ± 0.02^{x-y}	0.09 ± 0.01	0.17 ± 0.02^{y}	0.07 ± 0.007	0.0015	<0.0001	0.3074
Number of aspirated follicles	22.5 ± 2.47	17.0 ± 1.91	25.9 ± 1.55	12.8 ± 1.64	23.9 ± 2.43	17.5 ± 1.88	0.7919	<0.0001	0.1144
Number of recovered COCs	17.6 ± 2.46	10.2 ± 1.33	18.6 ± 1.68	7.84 ± 1.13	17.4 ± 2.24	10.2 ± 1.37	0.8954	<0.0001	0.5048
COCs recovery rate (%)	77.9 ± 5.36	59.8 ± 8.08	72.2 ± 5.30	64.0 ± 8.42	73.6 ± 5.40	59.8 ± 5.24	0.9098	0.0156	0.7403
Number of viable COCs	11. 3 ± 1.85	5.72 ± 0.83	10.6 ± 0.78	4.23 ± 0.76	12.7 ± 1.94	6.75 ± 1.15	0.2011	<0.0001	0.9545
Viable COCs rate (%)	63.5 ± 5.01^{b}	51.0 ± 5.87	60.0 ± 4.26	48.2 ± 5.58^{ab}	71.3 ± 3.93	63.3 ± 5.91^{a}	0.0229	0.0191	0.7915
Number of blastocysts produced per OPU	1.33 ± 0.66 ^{b}	0.90 ± 0.43	2.84 ± 0.57^{ab}	0.69 ± 0.32	3.81 ± 1.20 ^{a}	1.66 ± 0.51	0.0593	0.004	0.3307
Blastocyst rate* (%)	7.62 ± 3.61	6.92 ± 3.17	15.1 ± 2.54	7.44 ± 3.36	18.7 ± 4.57	13.4 ±3.26	0.0531	0.0992	0.5860

Table 2 - Plasma AMH concentration and the number of aspirated follicles, oocytes (COCs) and blastocysts (mean ± SEM) after OPU-IVEP in buffalo pre-pubertal, pubertal and cow donors.

	Pre-pubertal	Pubertal	Cow	P value
Number of donors	23	26	23	-
Age in months	14.0 ± 0.32^{c}	27.9 ± 0.77^{b}	111.8 ± 11.6^{a}	<0.0001
Plasma AMH (ng/mL)	0.19 ± 0.02^{a}	0.15 ± 0.017^{ab}	0.12 ± 0.015^{b}	0.04
Number of aspirated follicles	19.9 ± 1.65	19.3 ± 1.7	20.5 ± 1.6	0.88
Number of recovered COCs	14.08 ± 7.7	13.2 ± 1.4	13.7 ± 1.47	0.92
COCs recovery rate (%)	69.2 ± 5.04	68.1 ± 4.9	66.4 ± 3.9	0.91
Number of viable COCs	8.6 ± 1.18	7.4 ± 0.8	9.6 ± 1.25	0.36
Viable COCs rate (%)	57.5 ± 3.9^{b}	54.1 ± 3.6^{b}	68.2 ± 3.6^{a}	0.02
Number of blastocysts produced per OPU	1.13 ± 0.4^{b}	1.8 ± 0.4^{ab}	2.7 ± 0.6^{a}	0.09
Blastocysts rate* (%)	7.3 ± 2.3^{b}	11.3 ± 2.2^{ab}	15.9 ± 2.7^{a}	0.05

***** Blastocysts rate: Number of blastocyst over total recovered oocytes

Graphic 1 - Proportion of small (<5 mm; SF; P=0.95), medium (5-8 mm; MF; P=0.49) and large follicles (>8 mm; LF; *P* <0.0001), immediately before the OPU, in pre-pubertal (n = 23), pubertal (n = 26) and cow (n = 23) donors. Data presented on average.



In the three categories, donors were classified into low and high AMH groups, according to the average AMH concentration (divided according to median). The average AMH concentrations in the low and high categories were 0.08 ± 0.005 ng/mL versus 0.22 ± 0.01 ng/mL (*P* <0.0001; Table 3). Moreover donors classified as having high AMH had a greater number of aspirated follicles (*P* <0.0001), retrieved (*P* <0.0001) and cultured COCs (P<0.0001) and produced blastocysts (*P* = 0.0063; Table 3). Furthermore, COCs recovery rate (*P* = 0.0128) and viable COCs rate (*P* = 0.0273) were greater in high AHM groups, while blastocyst rate did not show differences between groups (*P* = 0.1264).

Table 3 - Plasma AMH concentrations and cumulus oocytes complexes (COCs) and embryo results (mean ± SEM) after OPU in pre-pubertal, pubertal and cow donors classified into 2 AMH categories.

	AMH category		*P* - Values
	Low AMH	High AMH	
N of donors	36	36	-
Plasma AMH (ng/mL)	0.08 ± 0.005[b]	0.22 ± 0.01[a]	<0.0001
Number of aspirated follicles	15.6 ± 1.07[b]	24.1 ± 1.2[a]	<0.0001
Number of recovered COCs	9.3 ± 0.7[b]	17.9 ± 1.2[a]	<0.0001
COCs recovery rate (%)	61.3 ± 4.1[b]	74.5 ± 3.03[a]	0.0128
Number of viable COCs	5.5 ± 0.5[b]	11.5 ± 0.9[a]	<0.0001
Viable COCs rate (%)	54.8 ± 3.4[b]	64.6 ± 2.6[a]	0.0273
Number of blastocysts produced per OPU	1.08 ± 0.25[b]	2.6 ± 0.5[a]	0.0063
Blastocyst rate* (%)	9.2 ± 1.9	13.7 ± 2.1	0.1264

***** Blastocysts rate: Number of blastocysts over total recovered oocytes

A high positive correlation was observed between the plasma AMH concentration and the number of follicles aspirated (*P* <0.0001; R= 0.58), number of retrieved COCs (*P* <0.0001; R=0.62), number of cultured COCs (*P* <0.0001; R=0.55) and produced blastocysts (*P* = 0.0064; R=0.32) in all the categories (Figure 3). However, a lower correlation was detected between the plasma AMH concentration and COCs recovery rate (R= 0.26; *P* = 0.0251), viable COCs rate (R=0.13; *P* = 0.2943) and blastocysts rate (R=0.19; *P* = 0.0979; Figure 4).

Figure 3 - Correlations between plasma anti-Müllerian (AMH) concentrations (ng/mL) and the number of follicles visualized at the moment of OPU (A), the cumulus-oocyte complexes (COCs) retrieved (B), COCs cultured (C) and the blastocysts produced (D) in buffalo donors. Blood samples for the plasma AMH measurements were collected by jugular venipuncture immediately before the OPU session.

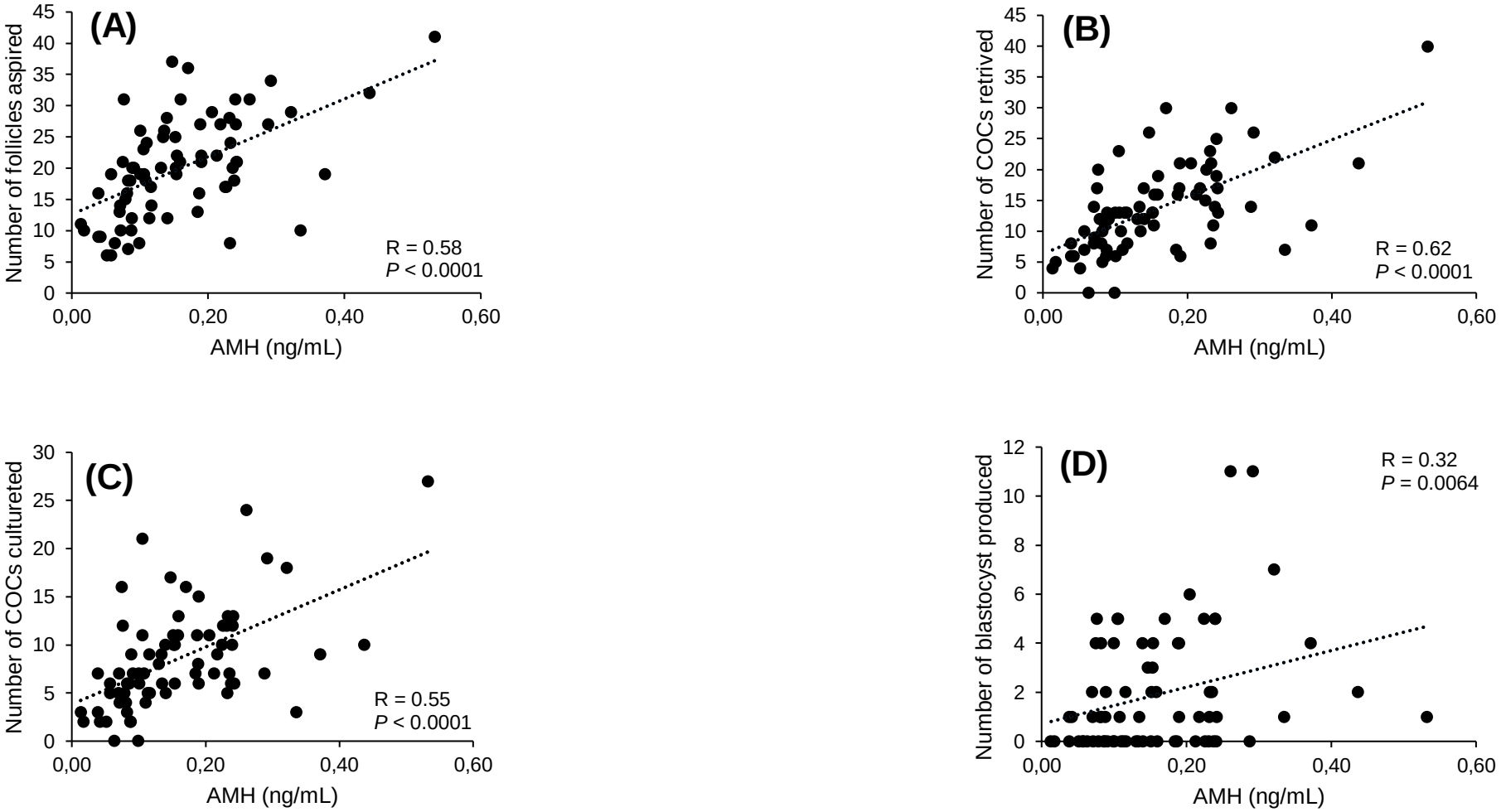
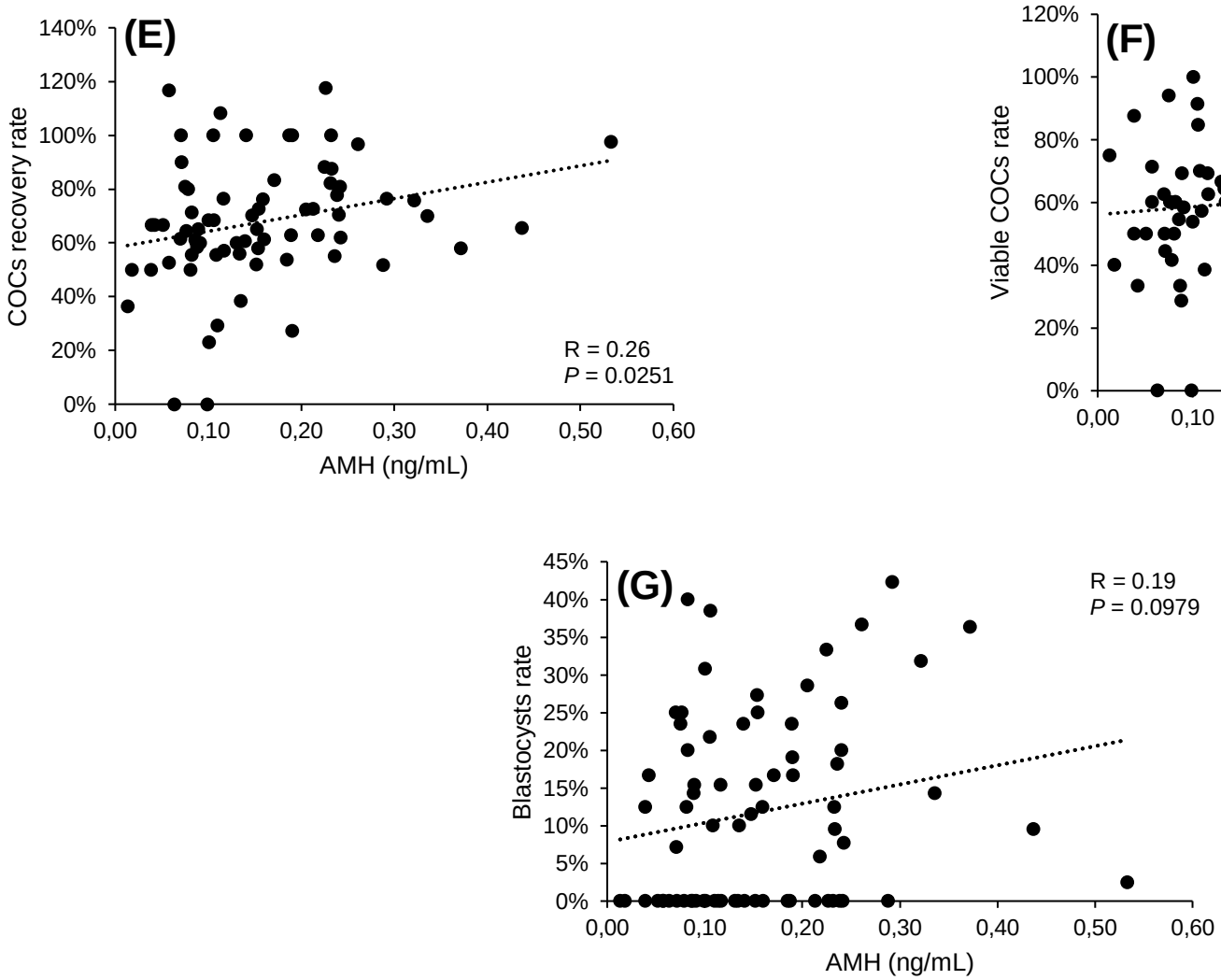


Figure 4 - Correlations between plasma anti-Müllerian (AMH) concentrations (ng/mL) and COCs recovery rate (over the total follicles aspirated; E), viable COCs rate (over the total of COCs retrieved; F) and blastocysts rate (over total recovered oocytes; G) in buffalo donors. Blood samples for the plasma AMH measurements were collected by jugular venipuncture immediately before the OPU session.



4.4 DISCUSSION

For our knowledge, this is the first study that describes the correlation between plasma AMH concentration and IVEP in three different categories of buffalo donors (*Bubalus bubalis*). These data provide new insights on both the reproductive physiology and the biotechnology responses of this species. Measurement of plasma AMH concentration, combined with genetic information on a specific individual, can prove useful method for donor selection, even among young animals, to predict results of *in vitro* embryo production programs.

Previous studies in cattle showed that AMH concentrations can reliably predict the number of healthy follicles and ovarian reserve of heifers and cow (IRELAND et al. 2008; BATISTA et al. 2014). In the present study, plasma AMH concentrations were higher in pre-pubertal buffaloes than in pubertal heifers or cows. In fact, early trials suggest that the age of the animal influences the antral follicle count in cattle (BURNS et al. 2005; CUSHMAN et al. 2009). The depletion of the follicular ovarian reserve is linked to reproductive senescence in mammalian females, and there is a positive association between the follicular ovarian reserve and the number of antral follicles on the surface of the ovary (ERICKSON, B.H. 1966). Our study also showed that the category of cows had a greater presence of large follicles at the time of the OPU and lower levels of anti-Müllerian hormone compared to pre-pubertal category. These results are in line with the findings of other studies which demonstrated that AMH expression is maximum in granulosa cells of preantral and small antral follicles and progressively diminishes at later stages of follicle development (DURLINGER et al. 2002; VISSER et al. 2006).

In the present study the viable oocyte and blastocyst rate and the number of blastocyst produced per OPU were higher in adult than in young buffalo. Previous reports established that oocytes recovered from young animals are less competent in developing to blastocyst stage compared with oocytes retrieved from cows (ARMSTRONG, D.T., 2001; CAMARGO et al., 2005; BARUSELLI et al., 2016). The quality of oocytes is significantly affected by the sexual maturity status of the donor (WARZICH et al., 2017). Prepubertal calves exhibited poorer cleavage and blastocyst development than adult cattle (REVEL et al., 1995; PERSICCE et al., 1997). WARZICH et al., 2017 found that oocytes of prepubertal heifers have a lower quality, resulting in lower number of lipid droplets, reduced levels of glucose and fatty

acids content in follicular fluid and also decreased transcript level of the SCD gene (one of seven genes involved in energy metabolism) in the granulosa cells. Additionally, in several other studies, follicular diameter was clearly associated with developmental competence. Some authors reported a relationship between follicle size and oocyte competence in calves (GANDOLFI et al., 1998; KAUFFOLD et al., 2005), whereas others noted that low-quality ooplasm as well as altered patterns of protein expression produced a direct effect on developmental competence in heifers' oocytes (LEVESQUE, J.T; SIRARD M.A., 1994; SALAMONE et al., 2001). Oocytes derived from medium and large follicles reached the morula stage and had greater developmental competence than the ones derived from small follicles (BLONDIN, P.; SIRARD, M.A., 1995). A recent study provided evidence that superstimulation with FSH prior to OPU session increased the proportion of large and medium-sized follicles available for aspiration, enhancing the proportion of viable oocytes and resulting in greater blastocyst rates and embryo yield per OPU-IVEP production in nulliparous, primiparous and multiparous buffalo donors (DE CARVALHO et al., 2019). Basing on the results of our study, the lower number of large follicles in younger donors could explain in part the lower number of embryos produced.

The results pointed out also a high positive correlation between four quantitative parameters (total follicles aspirated, total COCs retrieved, total COCs viable for culture and number of embryos produced per OPU) and the plasma AMH concentration. Previous studies have established that the antral follicular population (AFP) is directly and positively correlated to AMH plasma concentrations in buffalo (BALDRIGHI et al., 2014), like in cattle of different genetic groups (BALDRIGHI et al., 2014; BATISTA et al., 2014, GUERREIRO et al., 2014) and categories (BATISTA et al., 2016). Other studies on *Bos indicus* and *Bos taurus* have confirmed the positive correlation between plasma AMH concentrations and total follicles aspirated, total cumulus oocytes complexes (COCs) retrieved, number of COCs cultured, and number of blastocyst produced per OPU session (GUERREIRO et al., 2014; BATISTA et al., 2016; VERNUNFT et al., 2015; GAMARRA et al., 2014). This is the first report that confirm these positive correlations also in *Bubalus bubalis*. Moreover, the lower correlation obtained between AMH plasma concentration and the variables associated with *in vitro* developmental competences, such as viable COCs rate and blastocyst rate, confirmed that AMH concentration can not be related to oocytes quality, as found in other similar reports (BARUSELLI et al., 2015; SENEDA et al.,

2019). However, more studies are required to confirm these findings, since others authors found a positive association between AFP and AMH on fertility (IRELAND et al., 2007; SILVA-SANTOS et al., 2014).

In the present trial, all the donors from each category were divided based on the low and high AMH concentration. It was observed that buffalo with a higher plasma average of AMH concentration present superior IVEP efficiency. These animals presented a greater number of visualized follicles at the time of the OPU, a greater number of oocytes recovered/cultivated and a greater production of embryos, confirm previous studies in cattle (MOSSA et al., 2017) and sheep (LAHOZ et al., 2014; PINTO et al., 2018). These data support the use of AMH like useful tool for improving embryo production in buffalo.

The results presented suggest that AMH concentrations could be used as an endocrine marker and possible predictor of *in vitro* embryo production in buffalo, proving the importance of identifying donors who have a greater potential for retrieval of oocytes / OPUs in order to have higher success in IVEP. These data are relevant especially for buffaloes, that present a lower production of oocytes per OPU than cattle (BARUSELLI et al., 2018).

4.5 CONCLUSION

The results obtained show that the measurement of plasma AMH can be a valid tool to identify best oocyte-donors within IVEP programs, confirm the initial hypothesis of the present study. Furthermore, positive correlation was found between AMH plasma concentration, AFP and IVEP in buffalo donors. Donors classified as having high AMH had a greater number of aspirated follicles, retrieved and cultured COCs, produced blastocysts. The pre-pubertal heifers proved to be less effective for the implementation of IVEP results than cows. Pubertal heifers present similar results compared to adult animals and pre-pubertal ones.

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5 CHAPTER 2: EFFECT OF TREATMENT WITH GNRH AT THE TIMED EMBRYO TRANSFER ON THE FOLLICULAR DYNAMICS AND CONCEPTION RATE OF BUFFALO RECIPIENTS

ABSTRACT

The incorporation of techniques designed to control follicular wave dynamics and ovulation reduces the problem of estrous detection in buffalo and provides possibilities for the application of efficient timed embryo transfer (TET) programs. The objective of the study is to evaluate the effect of GnRH treatment at the time of ET on the pregnant per transferred embryo (P/ET) in buffalo recipients. The hypothesis is that treatment with GnRH induces the formation of an accessory corpus luteum (CL), increases the plasma concentrations of progesterone (P4), and increase P/ET. This study involved 256 Murrah and crossbreeding Murrah x Mediterranea buffaloes, aged from 2 to 15 years, with BCS> 2.5 and belonging to 4 different farms: F1 (n=70), F2 (n=97), F3 (n=63) and F4 (n=22). On D0 the recipients received an intravaginal P4 device, associated with i.m. 2.0 mg of estradiol benzoate and 0.53 mg of sodium cloprostenol (PGF2α). On D9, the P4 device was removed and 0.53 mg PGF, 400 IU equine chorionic gonadotrophin and 1.0 mg estradiol cypionate were administrated i.m. On D18 the buffaloes that had a CL> 10 mm received an embryo and the recipients were divided into two groups: Group GnRH (G-GnRH; n=133) which received 25 µg of lecirelin acetate i.m.; and the Control Group (G-CONT; n=123), which did not receive any treatment. A subset of 34 recipients was subjected to Doppler ultrasound examination and blood collection in the following days: D18, D22, D25, D28 to evaluate the ovulation rate, the formation of an accessory CL, the vascularization of the CLs and the P4 plasma concentration. After 30 and 60 days of TET, ultrasound examination was performed to evaluate P/ET. No difference was observed in P/ET at 60 days (G-GnRH: 31%, G-CONT: 29%, P = 0.74) and in pregnancy loss between 30 and 60 days (P=0.49) between groups. After GnRH treatment, 28% of showed ovulation and subsequent CL accessories establishment. However, no increase of plasma P4 concentration was observed. Treatment with GnRH at the time of TET did not increase plasma P4 concentration and P/ET in buffaloes.

Key words: GnRH, Accessory Corpus Luteum, FTET, buffalo recipients

5.1 INTRODUCTION

The profitability of buffalo breeding highly depends on the efficiency of reproduction and genetic improvements and hence on the utilization of reproductive biotechnologies. In recent years, several therapies have been proposed for the manipulation of the ovarian cycle and ovulation in buffalo, regardless of buffalo reproductive seasonality (BARUSELLI et al., 2007b; (CAMPANILE et al., 2010; DE CARVALHO; SOARES; BARUSELLI, 2016). Considering that *in vivo* embryo production in buffaloes (CARVALHO et al., 2002; MISRA; TYAGI, 2007; NEGLIA et al., 2010) generates, on average, less viable embryos than bovine (BOLAND; GOULDING; ROCHE, 1991); *in vitro* embryo production (IVEP) is the most effective procedure to increase the maternal contribution to genetic improvement for this species. In fact, the ovum pick-up (OPU) technique combined with IVEP allows a greater production of embryos on a long-term period (GALLI et al., 2001; NEGLIA et al., 2003).

In this context, the pregnancy after ET in buffaloes still has low rates when compared to cattle (MISRA et al., 1999). Previous studies have indicated that bovine females with elevated P4 plasma concentrations generated embryos with greater ability to synthesize and secrete Bovine Interferon-Tau (bIFN-τ) positively affecting maternal recognition of pregnancy (MAN; LAMMING, 1995; MANN; LAMMING, 1999; BAZER; SPENCER; OTT, 1997) and, consequently, had higher conception rates (NISHIGAI et al., 1998; BARUSELLI et al., 2010).

bIFN- τ is a glycoprotein secreted by the trophoblast of the conceptus (BAZER; JOHNSON, 1991; JOHNSON et al., 1994) that performs a suppressive function of the transcription of genes for estrogen and oxytocin receptors on uterine epithelium blocking the release of prostaglandin F2α (PGF2α). This prevents luteolysis and ensures P4 secretion which leads to the establishment of pregnancy (SPENCER AND BAZER 1996; FUENTES; DE LA FUENTES, 1997; HANSEN et al., 1999; MANN et al., 1999; BARUSELLI et al., 2000; BINELLI et al., 2001; SANTOS et al., 2001; SPENCER et al., 2007; HANSEN et al., 2010; BAZER, 2013).

These studies have suggested that the increase in P4 concentrations, due to a more functional CL during early diestrus, may have a positive effect on embryonic development through improved maternal-fetal recognition, favoring embryonic secretion of bIFN-τ (GEISERT et al., 1992; MANN; LAMMING, 1995; MANN;

LAMMING, 2001; MANN et al., 2003; CARTER et al., 2008). Following this path, several researches have been carried out with the objective of defining the best strategies to increase P4 concentrations during this critical period (15 to 19 days after ovulation; BINELLI et al., 2001; THATCHER et al., 2002). Such strategies include exogenous progesterone supplementation (ROBINSON et al., 1989; MACMILLAN; THATCHER, 1991; SCHMITT et al., 1996a,1996b; TRIBULO et al., 1997) and induction of formation of an accessory corpus luteum (SANTOS et al., 2001; MARQUES et al., 2012; NASCIMENTO et al., 2013). It is possible to promote the formation of an accessory CL by injecting of GnRH, LH or hCG during the early luteal phase to produce ovulation of the first-wave dominant follicle (RAJAMAHENDRAN; SIANANGAMA, 1992; SCHMITT et al., 1996a; AMBROSE et al., 1998; DIAZ et al., 1998; SIANANGAMA; RAJAMAHENDRAN, 1996; SANTOS et al., 2001).

The gonadotrophin releasing hormone (GnRH) is a decapeptide synthesized by the hypothalamus, which acts on the pituitary and stimulates to the release of the hormones LH (luteinizing hormone) and FSH (follicle stimulating hormone). The administration of GnRH and GnRH agonists induces an acute release of gonadotrophins in cattle (MARTINEZ et al., 2003) and buffaloes (BARUSELLI et al., 2003; RASTEGARNIA et al., 2004; JACOMINI et al., 2014) and produces ovulation in follicles that are at the appropriate maturation stage and have the relevant LH receptors (XU et al., 1995). In buffaloes, treatment with GnRH has proved to be effective in inducing ovulation, accompanied by the appearance of luteal tissue in the ovary within 14 h of ovulation, an increase in circulating concentrations of progesterone within 96 hours (RASTEGARNIA et al., 2004) and the formation of a CL accessory (CAMPANILE et al., 2007a, 2008; CARVALHO et al., 2007).

Furthermore, the secretion of P4 in buffalo is closely related to the vascularization of CL (CAMPANILE et al., 2010). It's therefore interesting to evaluate the characterization of the blood flow of the CL at the time of TET, using Color Doppler Ultrasonography (CDUS). CDUS is a noninvasive diagnostic method used to characterize and measure blood flow and can be also used indirectly to evaluate the functionality of the ovaries and his structures (VIANA et al., 2013).

Based on these premises, the objective of the following study is to analyze the effects of application of GnRH at the time of TET to create an accessory CL that increases the P4 concentration and, consequently, the P/ET.

5.2 MATERIALS AND METHODS

This study was approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science, University of São Paulo (protocol #8091270619).

5.2.1 Farms and animals

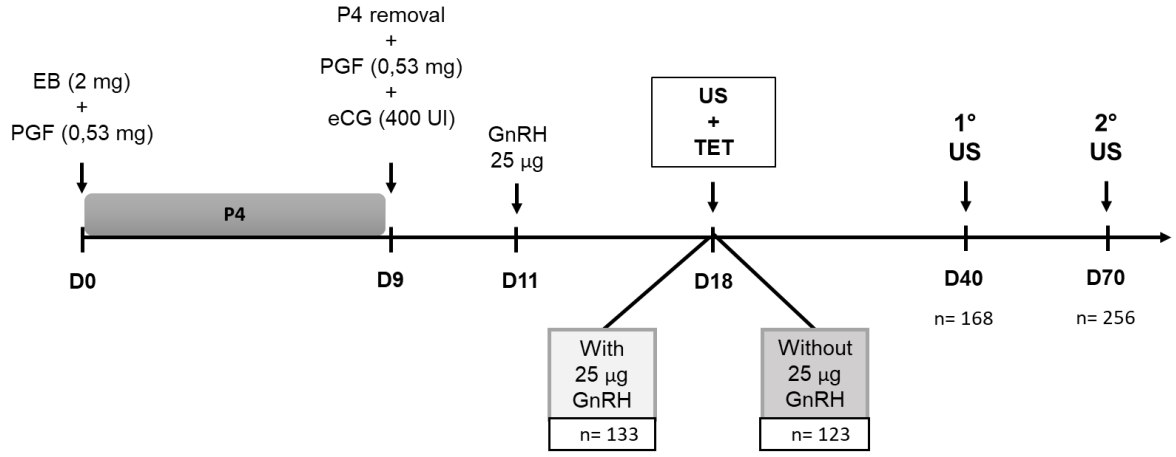
This study involved 256 Murrah and crossbreeding Murrah x Mediterranea buffaloes, aged from 2 to 15 years, without any fertility problems and with a good body condition score (BCS≥ 2.5). The experiment was carried out in 4 different farms: Estância Santa Olga (Road 81, Formosa, Argentina), F1 (n=70); Ribeira Valley Regional Pole (Unit of Research and Development, Registro, São Paulo, Brazil), F2 (n=52); Fazenda Ouro Preto (Registro, São Paulo, Brazil), F3 (n=88) and Campus Fernando Costa – USP/Pirassununga, São Paulo, Brazil, F4 (n=46). The animals were maintained on pasture with free access to water and supplemented with mineralized salt.

5.2.2 Experimental design

The recipients were synchronized with the following protocol: on day 0 (D0) the buffaloes received an intravaginal P4 device (Prociclar® 750 mg; Ceva, Brazil) associated with intramuscular (i.m.) administration of 2 mg of estradiol benzoate (Sincrodiol®, Ourofino, Brazil) and 500 µg of sodium cloprostenol (PGF2α; Sincrocio®, Ourofino, Brazil). On day 9 (D9), the P4 device was removed and 500 µg PGF2α, 400 IU equine chorionic gonadotrophin (eCG; Sincro eCG®, Ourofino, Brazil) were administrated i.m. Forty-eight hours later 25 µg of gonadotropin-releasing hormone (GnRH; Gestran-Plus®, Tecnopec, Brazil) was administered to induce ovulation. On day 18 (D18), an ultrasound evaluation (DP 2200®, Mindray, China) was performed. Only the buffaloes that had a CL> 10 mm received an *in vitro* produced embryo, cryopreserved through the direct transfer technique (DT), vitrified or fresh. At TET (D18), recipients were divided into two groups: Group GnRH (G-GnRH; n=133) which received 25 µg of lecirelin i.m. (Gestran Plus, Tecnopec) and the Control Group (G-CONT; n=123), which did not receive any treatment (Figure 5).

For the balanced formation of the groups, the diameter of the LF and CL was considered. Pregnancy diagnose was performed at Day 60 in all recipients (n= 256), while a subset of 168 animals were also evaluated at Day 30 and Day 60 to check pregnancy loss.

Figure 5 - Schematic diagram of the embryo receptors synchronization protocol.



5.2.3 Doppler Ultrasonography examinations and P4 plasma concentration

A subgroup of 34 buffaloes belonging to Campus Fernando Costa – USP/Pirassununga, São Paulo, Brazil, were synchronized to receive ET with the same protocol described above and were subjected to Doppler ultrasound exam and blood sampling to study the effect of GnRH treatment on P4 blood concentration and accessory CL formation (Figure 6).

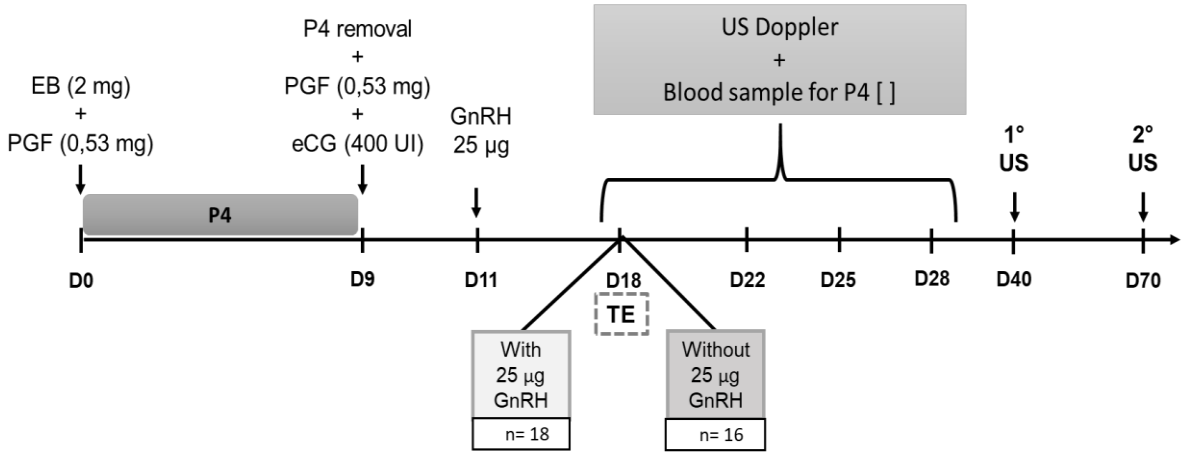
Doppler ultrasonography of ovaries (My Lab™DeltaVET, Esaote, Italia) were performed on Days 18 (day of TET), 22, 25 and 28. All the exams were completed by the same technician. During each exam the dimensions (area and diameter), peripheral and total blood flow of the CL and the formation of an accessory CL were evaluated. Measurement of blood flow intensity of the CLs (i.e., the number of blood cells moving in the vessel per unit of time) was taken by analyzing the images that showed different color intensity according to the concentration of the flow in each point of the evaluated structure (GINTHER, 2007). Colored representations of blood perfusion on the screen were estimated by the proportion of tissue with colored signals (GINTHER, 2007; PUGLIESI et al., 2014). To enable the characterization of blood perfusion and estimate the functionality of the luteal structure, the colored

stimuli of the internal and external portion, showed during the exam have been quantified by assigning different percentages.

- ≤ 25%: structure with few colored signals (CL with impaired functionality);
- >25%: organ with good vascularization and a good functionality.

With the objective of evaluating P4 circulating concentrations, blood samples were collected on Days 18, 22, 25 and 28, from the jugular vein with 4 ml vacuum tubes containing EDTA (Vacutainer®, Becton Dickinson and Company, USA). The blood samples were immediately placed in a box with chopped ice and then centrifugated (3000 g for 15 min). The plasma was decanted and stored in microtubes of 2.0 mL at -20 °C until the assay was performed. The P4 assay was performed at the Animal Endocrinology Laboratory of the São Paulo State University "Júlio de Mesquita Filho", Faculty of Veterinary Medicine, Campus Araçatuba, with a solid-phase radioimmunoassay kit ImmuChem™ Coated Tube Progesterone (MP™ Biomedicals). The limit of detection of the assay was 0.534 and the limit of quantitation was 0.351. The coefficient of variation for high control was 0.64% and for low control for 5.68%.

Figure 6 - Schematic diagram of the embryo receptors synchronization protocol, doppler ultrasound examinations and blood samples.



5.2.4 Statistical analysis

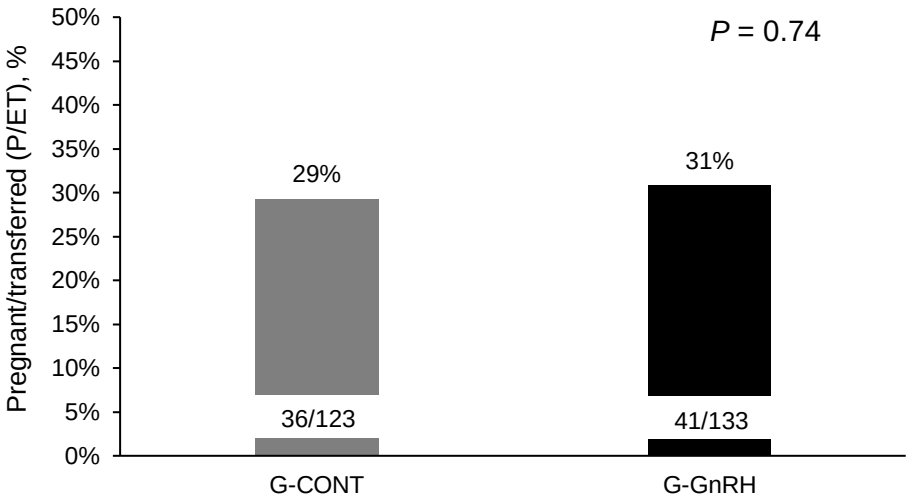
Information from each animal was compiled in a spreadsheet for statistical analysis. All analyzes were performed using Statistical Analysis System for Windows SAS® (SAS Institute Inc., Cary, NC, USA v9.4) using the Enterprise Guide package (v 7.1). Data obtained were analyzed using completely randomized experimental design include treatment (Control Group vs GnRH Group) and four replicates. Recipients were considered the experimental unit. Generalized Linear Mixed Model (GLIMMIX) was used to the statistical inferences. Continuous data were analyzed using the GLIMMIX procedure by adjusting a central trend distribution. Data were tested for residue normality using the UNIVARIATE procedure according to the Shapiro-Wilk test, the undistributed data were normally be transformed prior to analysis if the improvement in residual distribution. Outliers were removed when necessary. The model included treatment, replication, techniques, and significant bidirectional interactions as fixed effects. In addition, it included the option, DDFM = Satterthwaite, to the model declaration adjusted to the degrees of freedom for unequal deviations. Interaction between Parity and Farm was including as random effect. The categorical data were analyzed using the GLIMMIX procedure adjusting a binomial distribution. When means were significantly different, Tukey's post hoc multiple comparison tests were conducted to differentiate between them. Values were presented as means ± SEM and percentages. Statistical significance was declared at $P<0.05$. The P4 concentrations and the CL area were analyzed by repeated measures using the GLIMMIX and PLM procedures. For correlation analysis, a significance verified by the critical values of rho (Speraman) and its significant level of significance (P value), as performed using the correlation procedure (CORR).

5.3 RESULTS

The G-CONT and the G-GnRH presented the same (P > 0.05) CL diameter (G-CONT= 16.1 ± 0.25 vs G-GnRH= 16.2 ± 0.30) and the LF diameter (G-CONT= 9.79 ± 0.41 vs G-GnRH= 9.80 ± 0.45) at the moment of ET.

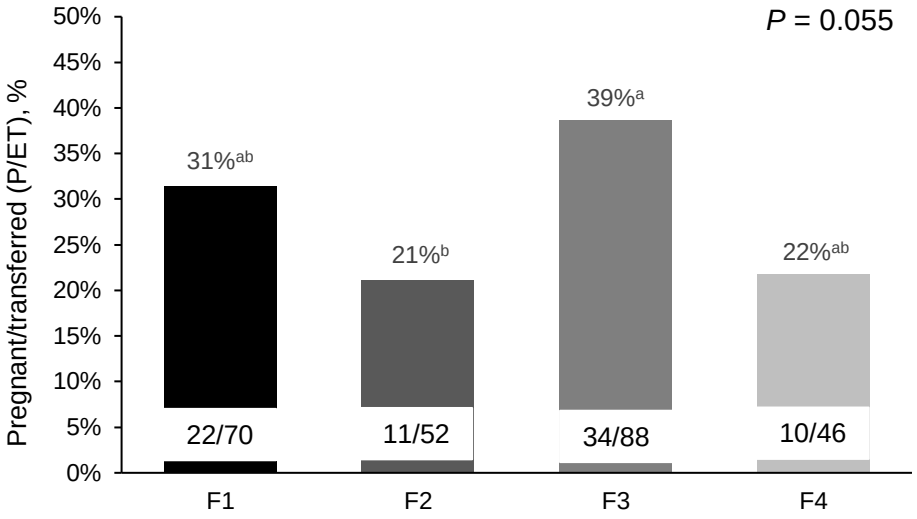
No significant difference was observed in the pregnant/transferred (P/ET) at 60 days (G-CONT = 29% vs. G-GnRH = 31%; P = 0.74; Graphic 2) in recipients treated or not with GnRH at the moment of ET. Also, in a subset of recipients (n=168) the pregnancy diagnose was performed at Day 30 and Day 60 to check pregnancy loss. The pregnancy loss was similar between groups [G-CONT = 13.0% (3/23) vs. G-GnRH = 4.17% (1/24); P = 0.488].

Graphic 2 - Pregnant/transferred embryo at 60 days in recipients treated or not with GnRH (25 µg of Lecirelin) at ET: Control group (G-CONT) and GnRH group (G-GnRH).



In addition, there was no interaction (P = 0.78) treatment*quality of transferred embryos [blastocyst (BL), expanded blastocyst (EXBL), hatched blastocyst (HBL)]. P/TE at 60 days differed across farms [Farm 1 = 31% (22/70); Farm 2 = 21% (11/52); Farm 3 = 39% (35/88); Farm 4= 22% (10/46); P = 0.0548; Graphic 3].

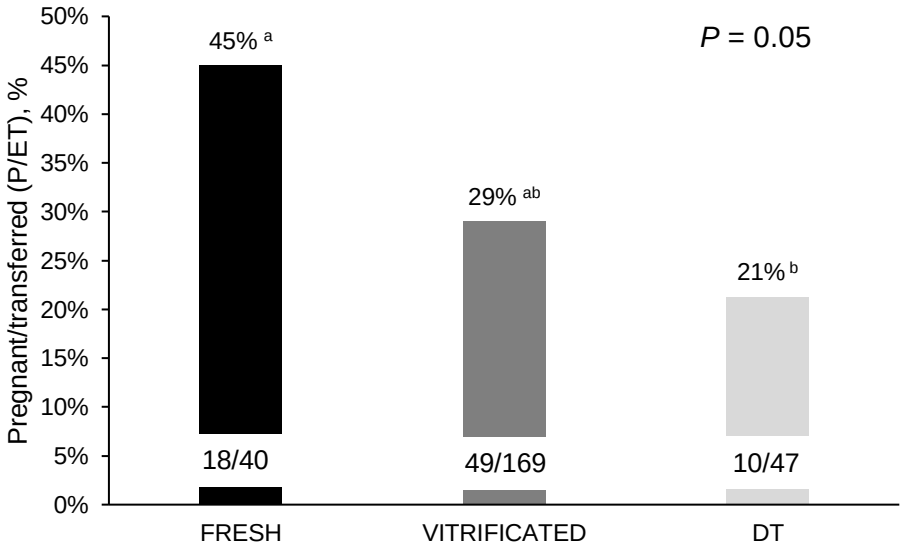
Graphic 3 - Pregnant/transferred embryos at 60 days according to the farms.



Recipient category influenced 60 days P/ET outcomes. Heifers (presented higher P/ET than cow [Heifer = 45.5% (10/22) vs. Cow = 28.6% (67/234); *P* = 0.0465].

Comparing the different techniques of embryo maintenance, it was found that the transfer of fresh embryos resulted in a higher P/ET compared to direct transfer (DT). However, similar results was observed when compared to vitrified embryos (*P* = 0.05; Graphic 4).

Graphic 4 - Pregnant/transferred embryos at 60 days for different techniques of embryo maintenance.



A subset group of recipients was analyses using Doppler ultrasound exams (n=34). To ensure homogeneity, the groups were balanced, before the eventual treatment, depending on the diameter of the CL and the size of the major follicle at the time of transfer (LF; Table 4). No differences were observed for the CL diameter D18 (P=0.89), CL vascularization D18 (P=0.10), CL area D18 (P=0.87) and largest follicle diameter D18 (P=0.61).

It was observed that 28% (n = 5/18) of treated animals showed an ovulation following GnRH treatment, with a subsequent formation of an accessory CL. On the control group, no accessory CL formation was observed (n = 0/16; *P* = 0.026).

Recipients that ovulated after GnRH treatment presented a LF diameter (14.4 ± 0.85 mm; n=5) at the moment of the injection compared to recipients that did not ovulated (11.3 ± 0.51 mm; n=13; *P* = 0.034).

Table 4 - Mean with standard errors of CL diameter, CL vascularization, CL area and diameter of LF on Day 18 for the two experimental groups.

	G-Control	G-GnRH	*P* value
Number of buffaloes	16	18	-
CL diameter D18 (mm)	15.3 ± 1	15.4 ± 0.4	0.89
CL vascularization D18 (%)	50% ± 0.019	56% ± 0.02	0.107
CL Area D18 (cm^3)	2.17 ± 0.168	2.2 ± 0.083	0.87
LF diametrer D18 (mm)	12 ± 0.8	11.3 ± 1.04	0.61
P4 plasma concentration (ng/mL)	1.07 ± 0.16	1.18 ± 0.14	0.60
Ovulation rate %	0%	28%	0.026

A positive correlation between P4 plasma concentration and percentage of CL vascularization (Figure 7) and area of CL (Figure 8) were found in all 4 days of analysis (D18, D22, D25, D28;).

Figure 7 - Correlation between P4 plasma concentration (ng/mL) and vascularization of CL on Day 18 (A), Day 22 (B), Day 25 (C) and Day 28 (D) of protocol.

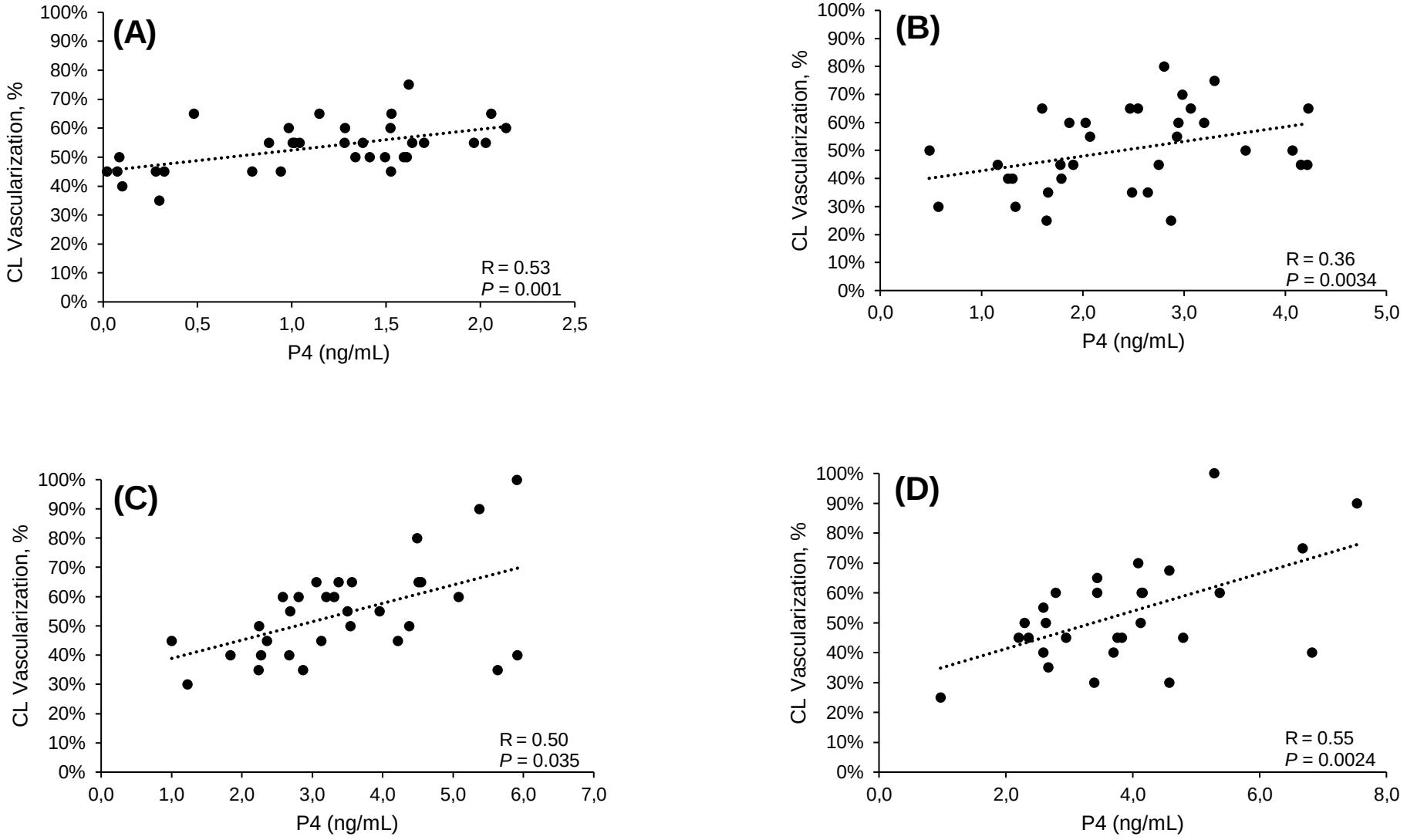
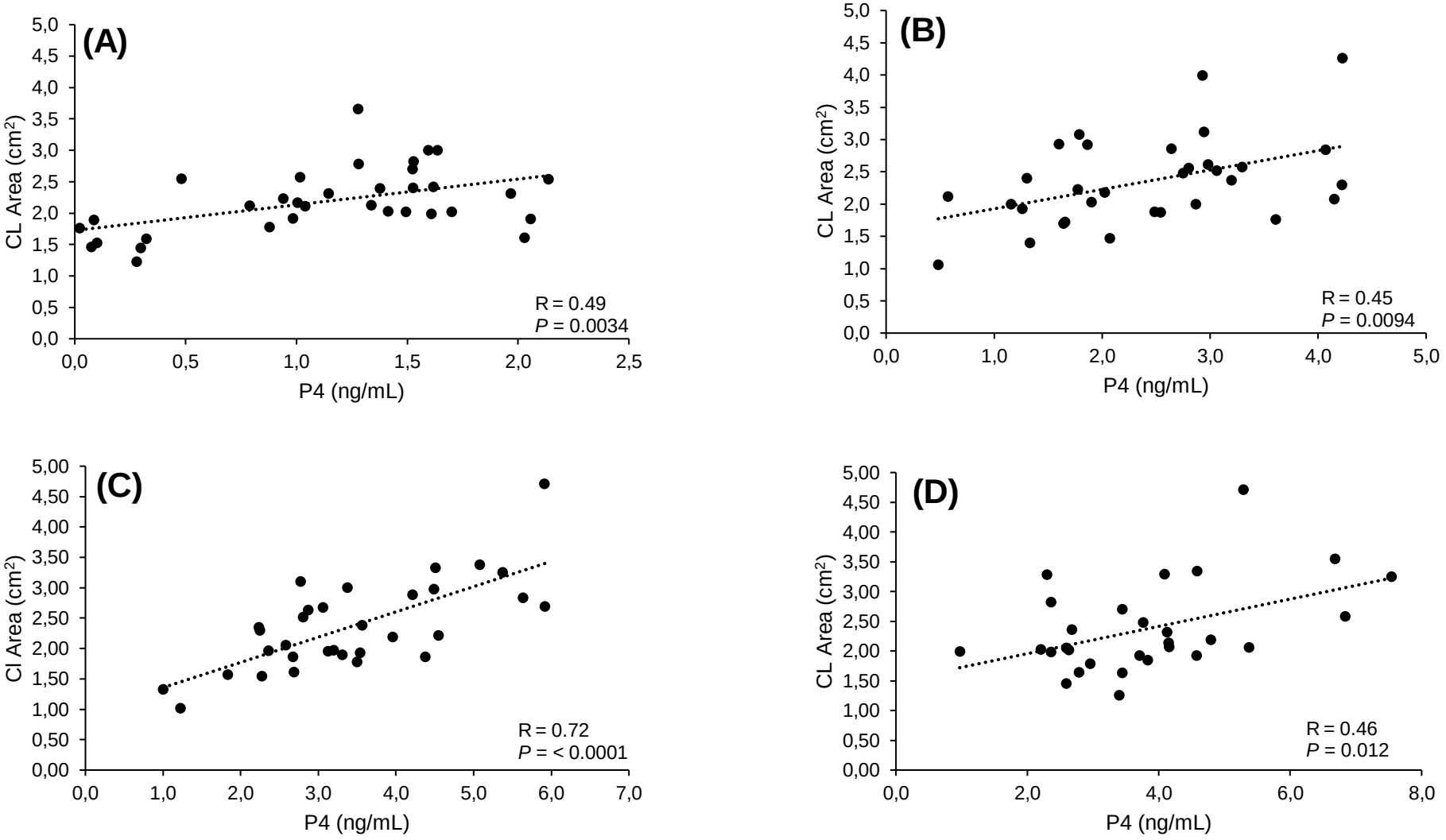


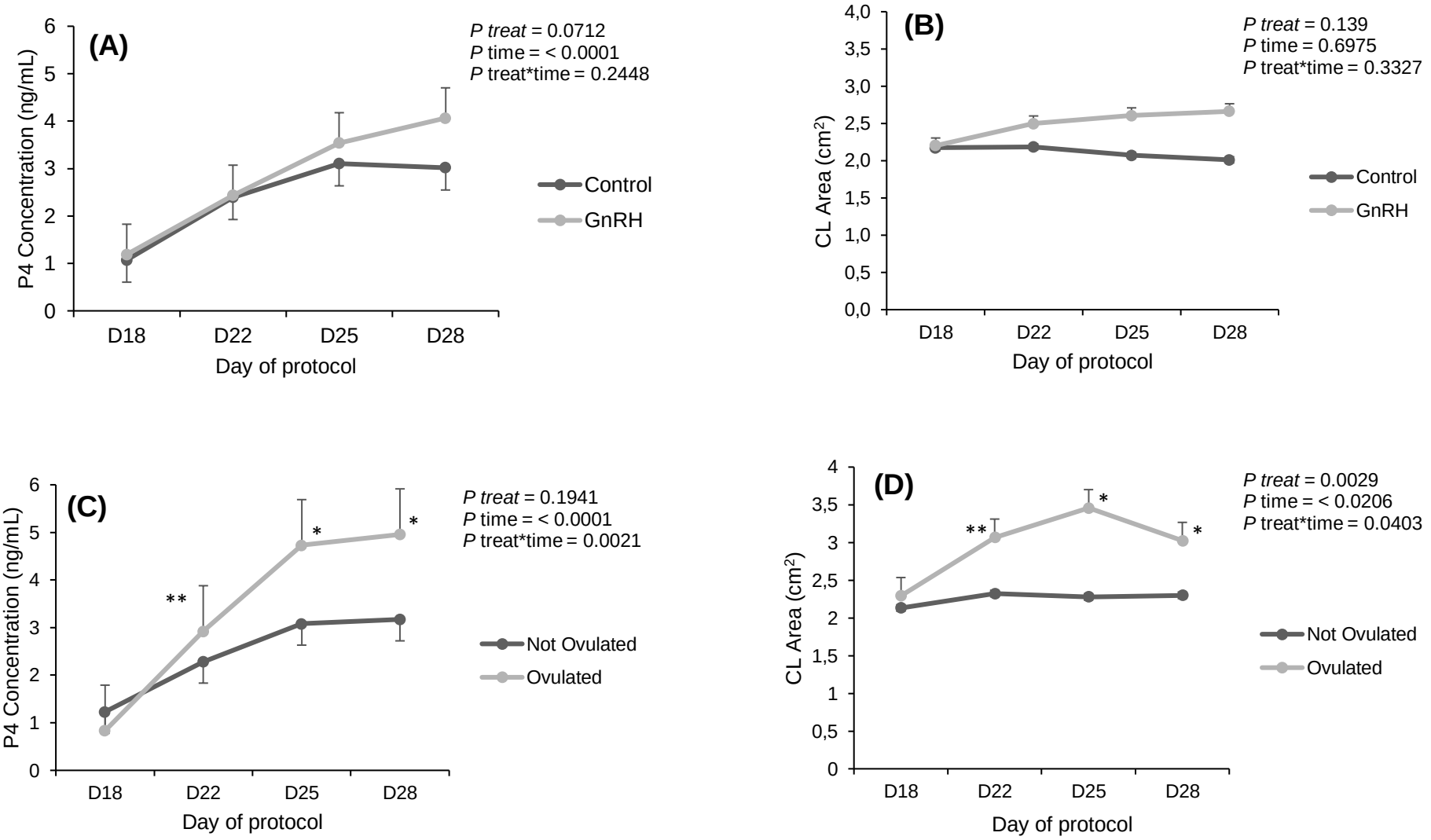
Figure 8 - Correlation between P4 plasma concentration (ng/mL) and CL area on Day 18 (A), Day 22 (B), Day 25 (C) and Day 28 (D) of protocol.



Moreover, on Day 25 and Day 28 a positive correlation was identified between P4 plasma concentration and the diameter of CL (R= 0.54, *P*= 0.0014 and R= 0.42, *P*= 0.02; respectively).

Analyzing the evolution of the plasma P4 concentration values during the days D18, D22, D25 and D28, a positive trend was found for the treated group compared to the control group (*P*= 0.0712; Figure 9), while for CL area values no interaction was observed (*P*= 0.139). Analyzing only the treated animals, those that presented an accessory CL showed an interaction time*treatment for plasma concentration of P4 (*P*= 0.0021) and area of CL (*P*= 0.0403; Figure 9).

Figure 9 - Plasma progesterone concetration (ng/mL) and CL area (cm^2) according to the days of the experimental protocol (D18 = day of the treatment with the GnRH and the ET). A and B are relative to all the recipients (Control, n=16 and GnRH, n=18). C and D are relative to GnRH group according to induced ovulation (Ovulated, n=5 and Not ovulated, n=13). *P < 0.05; ** P = ≥ 0.05 and ≤ 0.10.



5.4 DISCUSSION

The treatment with GnRH did not increase plasma P4 concertation after ET and subsequently the P/ET, rejecting the initial hypothesis. CAMPANILE et al., 2007b noted that the establishment of buffalo pregnancy requires a progressive increase in P4 secretion during the first 2-3 weeks after mating. In this regard, recent studies have corroborated the formation of an accessory CL, a higher conception rate and a higher plasma P4 concentration in Murrah buffaloes treated with 20 µg of buserelin acetate (BA) or 3000 UI of hCG on day 5 post ovulation (PANDEY et al., 2015) or 25 µg of lecirelin 6 days after AI in Ovsynch protocol (CARVALHO et al., 2007). Additionally, in Mediterranean cows it was also observed induction of accessory CL and increase in P4 concentration after application of 12 µg of GnRH or 1500 UI of hCG on Day 5 following AI (CAMPANILE et al., 2007a; 2007b,). In the present study, the treatment with GnRH at the time of ET increased marginally (P = 0.0712; Figure 9) the plasma P4 concentration, probably due to the low ovulation rate obtained (28%). In fact, when the plasma P4 concentration was analyzed only in treated animals, the ovulated recipients presented greater P4 concentration and CL area, confirm the efficiency of induced accessory CL. For this reason, the low response to GnRH treatment could explain the lacking increase in the P/ET in the present study.

In buffalo, almost 60% of the cows ovulated after the GnRH treatment at random stage of estrous cycle (BARUSELLI et al., 2003; NEGLIA et al., 2016). The responses of GnRH could depend on the diameter of the largest follicle at the moment of the treatment (BERBER et al., 2002; BARUSELLI et al., 2003; CAMPANILE et al., 2008). Buffaloes that ovulated after GnRH application presented a larger follicle than animals that did not ovulate (9.5 ± 1.7 vs. 6.7 ± 2.4 mm; BARUSELLI et al., 2003). In Murrah×Mediterranean buffalo heifers, it has been reported that follicles acquired the capacity to ovulate in response to exogenous LH when they reach a diameter of 8.5–10.0mm (GIMENES et al., 2007). At the day of the divergence, the FD and the largest subordinate follicle (FS) have an average diameter of 7.2 ± 0.3 and 6.4 ± 0.3 respectively (GIMENES et al., 2007). During the proestrus, the dominant follicles reach a size of 13–15mm in buffalo (BARUSELLI et al., 1997; NEGLIA et al., 2007) and secrete sufficient estradiol for a positive feedback action to the insurgence of the pre-ovulatory LH surge to induce ovulation. In the present study, the low ovulation rate observed after GnRH treatment suggests that

size of the largest follicle could be determinant for the efficiency of the hormonal strategy. In fact all the cows that presented an accessory CL showed a LF diameter of 14 ± 0.85 mm at the time of GnRH treatment, comparing to the recipients that did not ovulated (11.3 ± 0.51 mm). This data reinforces the importance of the presence of a responsive LF to guarantee an effective action of gonadotropin.

In our research, to ease the handling of the animals, the GnRH treatment was carried out on day 7 of the cycle (day 18 of the protocol). This one/two-day delay in relation to above mentioned studies (CAMPANILE et al., 2007a, 2007b; CARVALHO et al., 2007; PANDEY et al., 2015) may have increased the probability of having LF already undergoing atresia. The end of growth phase of dominant follicle of the first follicular wave is recorded that occurs at 8.55 ± 2.33 and 6.60 ± 1.42 days of the estrous cycle, respectively for buffaloes with 2 and 3-wave cycles (BARUSELLI et al., 1997). This data could explain the low ovulation rate obtained in the present study.

During the estrous cycles, buffalo CL secretes progesterone for around 15 days and the circulating concentrations of P4 increase during the luteal phase (BARUSELLI et al., 1997). Circulating P4 concentrations have a great impact on the exogenous GnRH-induced LH surge of both *Bos indicus* and *Bos taurus* heifers and cows, reducing significantly the ovulation rate of the LF in animals having high circulating P4 concentrations compared to those with low P4 concentration (STEVENSON et al., 2008; GALVÃO; SANTOS, 2010; GIORDANO et al., 2012a; BATISTA et al., 2017). It is possible that the high levels of circulating P4 present at the TET (Day 7 of the estrous cycle) could affect the magnitude of a GnRH-induced LH surge, reducing the ovulation rate and the accessory CL formation. In lactating dairy cows, the use of a higher dose of GnRH can dramatically increase the magnitude of the LH surge, either in the presence or absence of circulating P4 (SOUZA et al., 2009; GIORDANO et al., 2012b). Considering that the effect of GnRH is dose-dependent, it has been observed in beef heifers and in lactating dairy cows that doubling the GnRH dose (200 µg of gonadorelin instead of 100 µg) caused a concomitant doubling in LH release in either high-P4 or low-P4 environment, (DIAS et al., 2010; GIORDANO et al., 2013). Also in buffalo, an increase in the ovulation rate was obtained following a higher GnRH dose (100µg vs 50µg) during a synchronization protocol using a norgestomet implant in association with two injections of PGF2α (RASTEGARNIA et al., 2004). Therefore, increasing the GnRH dose could be a viable alternative to enhance LH secretion, potentially resulting in

enhanced ovulation and increased the efficiency of the treatment at TET, overcoming the suppressive effect of circulating P4.

This study also provided interesting and new information about buffalo CL. In fact, the area and the vascularization of the CL measure by Color Doppler Ultrasonography (CDUS) were positively correlated with circulating P4 concentrations. Thus, the CL blood perfusion evaluation can be very useful because higher P4 concentrations at the beginning of the diestrus are related to a greater development of the conceptus (MANN; LAMMING, 2001) and higher probability of pregnancy. Recognition and maintenance of pregnancy are related to various signals transmitted from the embryo to the mother. These signals are in charge of inhibiting corpus luteum (CL) lysis, and thus enable the maintenance of progesterone (P4) production (MACHADO et al., 2006).

Luteal steroids are delivered to the general circulation due to CL vascularization, which provides also the circulating substrate used by the luteal cells for P4 biosynthesis (CARR; MACDONALD; SIMPSON, 1982). As shown in previous studies, plasma P4 concentrations throughout the estrous cycle are closely associated with the blood flow area and/or blood flow velocity, thus proving that CDUS is a very useful tool to evaluate the CL function (MATSUI; MIYAMOTO, 2009; HERZOG et al., 2010) Previous researches have been performed to determine the functional status of the CL throughout luteal blood flow measurement (ACOSTA et al., 2002; GINTHER et al., 2009; PUGLIESI et al, 2017, 2018). Luteal blood flow level is strongly correlated with P4 production. Studies that have been conducted to evaluate blood flow to the bovine CL using CDUS have reported that an increased vascularity of CL is gradually accompanied by an increase in functionality of the CL and consequently P4 production (ACOSTA et al., 2002; HERZOG et al., 2010; LÜTTGENAU; BOLLWEIN, 2014; KAYA et al., 2017). There are not many studies available on blood flow to the ovarian follicle and CL in buffaloes. A study on Surti buffaloes showed that augmented plasma P4 concentrations from Day 5 of estrus until Day 13 were positive correlated to CL diameter and vascularity (GAUR; PUROHIT, 2019), supporting the results of the present study.

There was showed that farm affected the OPU/IVEP efficiency, showing that animal management has an important influence on the application of reproductive technologies (BARUSELLI et al., 2018).

A study was undertaken to evaluate the efficiency of a TET protocol in three different categories of recipients. The results showed similar efficiency for TET when different categories of recipients (nulliparous, primiparous and multiparous) were used (SOARES et al., 2015). In our trial, no effect was observed when heifers and cows were used as embryo recipients. These data support the use of these categories in an ET program with similar efficiency. Furthermore, other studies evaluated the use of direct transfer (DT) in order to facilitate the ET program (SILVA et al., 2019). No differences were observed when DT or vitrified embryos were transferred (DT = 33.0%; 31/94 vs. Vitrified = 34.9%; 38/109). Also, the pregnancy loss between 30 and 60 days of gestation did not differ for DT and vitrified embryos [9.7% (3/31) vs. 2.6% (1/38), respectively. The results of this experiment showed no difference between DT and Vitrified embryos. However, the fresh embryo produced more P/ET than DT embryos. More studies are needed to improve the efficiency of cryopreservation of the buffalo embryos.

The results obtained in the present study have confirmed the existence of a positive correlation between P4 concentration and the area, diameter and vascularization of CL in buffalo. The study also demonstrates that the use of the CDUS represents a valid tool for the evaluation of luteal function also in this species and being able to facilitate and improve the use of reproductive technologies in the future, especially for the selection of embryo recipients.

5.5 CONCLUSION

The data collected shows that treatment with GnRH does not increase the P/ET and the plasma P4 concentration, rejecting the initial hypothesis of the present study. On the other hand, the positive correlation between plasma P4 concentration, vascularization and CL area is of most interest and deserves to be deepened through further studies. In fact, this correlation could help with the choice of embryo recipients that could present high P/ET. Further experiments are needed to standardize the results, especially on buffalo, which has less academic research compared to cattle.

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6 GENERAL CONCLUSIONS

- A positive correlation between AMH plasma concentration, ovarian follicular population and in vitro embryo production were found in buffalo donors, confirming the initial hypothesis.
- Pre-pubertal donors presented a high AMH plasma concentration, however low viable oocyte and blastocyst rates and low number of blastocyst produced per OPU than cows, confirming the hypothesis of the present study.
- AMH represent a valid endocrine marker for IVEP and a promising tool to enhance the overall efficiency of OPU–IVP programs in in buffalo as a selective criterion for high embryo producing donors.
- The treatment with GnRH at the time of TET was not effective to induce an accessory CL, to improve circulating P4 concentrations and to increase P/ET in buffalo recipients, rejecting the initial hypothesis.
- A positive correlation between plasma P4 concentrations and CL vascularization and CL area were found, supporting for future studies of CL functionality and P/TE in buffalo.

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