Universidade Estadual Paulista "Júlio de Mesquita Filho"
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O papel do estradiol no oviduto bovino: evidências *in vivo* e *in vitro*

Patricia Kubo Fontes
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UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

O PAPEL DO ESTRADIOL NO OVIDUTO BOVINO: EVIDÊNCIAS *IN VIVO* E *IN VITRO*

Patricia Kubo Fontes

Orientador: Anthony César de Souza Castilho

Co-orientador: Mário Binelli

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“*Science and everyday life cannot and should not be separated*”
(A ciência e a vida cotidiana não podem e não devem ser separadas)

— Rosalind Franklin

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RESUMO

O oviduto desempenha diversas funções até a formação do embrião, como maturação e transporte final dos gametas, processo de fertilização, transporte do embrião e desenvolvimento inicial do embrião. No entanto, nenhuma dessas etapas precisa ocorrer obrigatoriamente no oviduto. A capacidade de desenvolver embriões até o estágio de blastocisto sem qualquer contato com o trato reprodutivo materno gerou a hipótese de que o oviduto seria secundário e não essencial no estágio inicial da vida. Hoje, no entanto, é amplamente conhecido que a interação embrião-oviduto, ou interação com produtos de células ovidutais, pode impactar o embrião de diferentes maneiras, especialmente em relação às características epigenéticas, metabolismo e vários marcadores de qualidade. Para reconectar o embrião em desenvolvimento às células ovidutais (OC), muitas abordagens artificiais foram delineadas. Nesta tese, investigamos as CO diante de duas dessas abordagens: os protocolos de superestimulação ovariana (SOV) e a cocultura *in vitro* de embrião-CO. No primeiro estudo, as vacas foram submetidas à: i) protocolo SOV apenas com hormônio folículo-estimulante (FSH), ii) protocolo SOV usando FSH com gonadotrofina coriônica equina (eCG), ou iii) ovulação única por sincronização hormonal. O uso de protocolos SOV permite o desenvolvimento de múltiplos embriões dentro do oviduto, possibilitando a interação dos embriões com as CO. Este primeiro estudo demonstrou que as CO de vacas submetidas à SOV com FSH + eCG apresentam níveis mais elevados de estradiol, que também modulou a expressão gênica de genes relacionados ao processo de fertilização. Em seguida, esse perfil hormonal foi aplicado em um sistema de cultivo *in vitro* de OC. As CO podem ser cultivadas para aplicação em sistema de cocultura com embriões, o que permite que o embrião produzido *in vitro* interaja com as CO. A cultura de OC ainda não possui protocolo padrão ouro. Portanto, para analisar o efeito dos diferentes perfis hormonais obtidos como resultado do primeiro estudo, foi necessário incluir outra variação no segundo estudo: diferentes perfis hormonais x CO coletadas em diferentes fases do ciclo estral. Ao analisar os dados obtidos na análise de RNAseq, após o cultivo *in vitro*, as OC coletadas durante a fase folicular apresentam um perfil de transcriptoma muito diferente do perfil das células coletadas na fase lútea, caso nenhum tratamento hormonal for aplicado. Porém, quando altos níveis de estradiol são aplicados na cultura *in vitro* dessas células, o perfil do transcriptoma das CO torna-se muito semelhante, independentemente do ciclo estral em que as CO foram coletadas. Em conclusão, essas duas abordagens artificiais para possibilitar a interação dos embriões com o oviduto modulam as CO em função dos perfis hormonais. Portanto, o perfil hormonal é um fator importante a ser considerado nas estratégias para estabelecer a interação de embriões e células ovidutais.

ABSTRACT

The oviduct plays many roles in the embryo formation, as gametes final maturation and transport, fertilization process, embryo transport, and embryo early development. However, none of these steps need to occur in the oviduct mandatorily. The ability to develop embryos up to the blastocyst stage without any contact with the maternal reproductive tract hypothesized that the oviduct would be minor and inessential at the early stage of life. Today, however, it is widely known that the embryo-oviduct interaction, or interaction with oviductal cells' products, can impact the embryo in different ways, especially regarding epigenetic characteristics, metabolism, and several quality markers. To reconnect the embryo in development to the oviductal cells (OC), many artificial approaches have been designed. In this thesis, we investigated the OC from two of these approaches: the ovarian superstimulation protocols (OVS) and the embryo-OC *in vitro* coculture. In the first study, cows were submitted to: i) OVS protocol only with follicle-stimulating hormone (FSH), ii) OVS protocol using FSH with equine chorionic gonadotrophin (eCG), or iii) single ovulation by hormone synchronization. The use of OVS protocols allows the development of multiples embryos inside the oviduct, making possible the interaction of the embryos with the OC. This first study demonstrated that OC from cows submitted to OVS using FSH+eCG has higher levels of estradiol, which also modulated gene expression of genes related to the fertilization process. Next, this hormone profile was applied in an *in vitro* culture system of OC. The OC can be cultured for application in a coculture system with embryos, which enable the *in vitro* produced embryo to interact with the OC. The culture of OC has still no gold standard protocol. Therefore, to analyze the effect of different hormone profiles obtained as a result of the first study, it was necessary to include another variation in the second study: different hormone profiles x OC collected in a different phase of the estrous cycle. When analyzing the data obtained from the RNAseq analysis, after *in vitro* culture, OC collected during the follicular phase presents a very different transcriptome profile than cells collected during the luteal phase, if no hormone treatment is applied. However, when high levels of estradiol are applied in the *in vitro* culture, the transcriptome profile of the OC become very similar to each other, independently of the estrous cycle when OC was collected. In conclusion, these two artificial approaches to enable the interaction of embryos with the oviduct modulate the OC depending on the hormone levels presented. Therefore, the hormone profile is an important factor to be considered in strategies to establish the interaction of embryos and oviductal cells.

INTRODUCTION

After reproductive biotechnologies have been developed, exponential growth was observed in embryo production numbers. Currently, the total number of bovine embryos produced by *in vitro* and *in vivo* techniques exceeds 1.5 million per year worldwide. Associated with numerical expansion, many studies investigate how to improve embryo quality and increase the chance of establishing a successful pregnancy after embryo transfer. The oviduct is the organ responsible for establishing a suitable microenvironment for the final oocyte maturation, sperm reserve formation and capacitation, fertilization, gamete/embryo transport, and initial embryo development. Although the interaction with the oviduct does not impede bovine embryo formation and initial development, when the embryo is in natural circumstances with oviduct cells, its quality is unquestionably superior. Thus, the improvement of embryonic quality and consequently increasing pregnancy success may lie in the interaction of the embryo with oviduct cells, or oviductal cells products. To provide this embryo-oviduct contact, several artificial methods have been developed and are constantly evolving. A general selection of these methods is presented in **Chapter 1**. This literature review addresses the main *in vivo* and *in vitro* methods available for providing interaction between the embryo and the oviduct, their limitations, and perspectives.

Superovulation protocol is one of these methods. Although cattle are monovulatory animals, hormonal treatments stimulate multiple follicle growth and ovulation, enabling the formation and development of several embryos in the oviduct lumen. This interaction with the oviduct is very beneficial to these embryos, although their quality is still inferior when compared to embryos produced from single ovulation. The cause of this inferior embryonic quality may be multifactorial, and changes in oviductal cells can be one of them. In **Chapter 2,** oviduct tissues were evaluated after cows had been treated with two ovarian superstimulation protocols and one synchronization protocol for single ovulation. The oviducts were evaluated one day before ovulation, to understand the effects of the protocols without interference from the gametes/embryos presence. Cows submitted to the

ovarian superstimulation protocols, specifically treated with follicle-stimulating hormone associated with equine chorionic gonadotropin, had their oviducts with higher estradiol levels and modulation on transcription levels of genes related to the fertilization process in the oviduct.

In **Chapter 3**, another method that provides interaction between the embryo and the oviduct was studied, in this case, an approach for embryos produced *in vitro*. During the *in vitro* culture of embryos, it is possible to establish a coculture system with oviductal cells. Currently, compartmentalized culture systems are more efficient at this task, as it provides cellular nutrition through the basolateral segment, which mimics blood nutrition in oviductal cells and cell apical region free of culture medium, which mimics the organ lumen. In these compartmentalized systems, it is possible to place embryos in the zygote stage in the apical compartment to develop in contact with oviductal cells. At the same time, it is possible to modulate the culture medium in the basolateral compartment to mimic blood supply. This modulation, especially with hormonal treatments, is not yet fully established. For this reason, in **Chapter 3** the data from the analysis of the culture of bovine oviduct epithelial cells submitted to hormonal treatments in a compartmentalized system is present. It was observed that treatment with high levels of estradiol seems to be the major modulator of transcription in oviductal cells and more importantly, the results suggest that the moment of the estrous cycle when the cells are obtained for later *in vitro* culture is an essential aspect that should be considered for the cultivation of bovine oviduct epithelial cells. Finally, in **Chapter 4,** the final considerations of the findings of these studies are presented.

CAPÍTULO 1

– CHAPTER 1 –

LITERATURE REVIEW

─ LITERATURE REVIEW ─

1. THE OVIDUCT

The oviduct is an organ responsible for providing a suitable microenvironment from gametes' final maturation until initial embryo development. The main events that occur in the oviduct are shown in Figure 1 [1]. To be able to develop all these functions, the oviduct presents a distinct morphology and anatomy. Three distinct segments are identified in the oviduct: infundibulum, ampulla, and isthmus, respectively, the part closest to the ovary, the medial segment, and the part closest to the uterus. In between these segments are the ampulla-isthmic junction and the utero-tubal junction, which are the transitions parts [2]. Dynamic morphological and functional changes are observed in all these segments, mainly orchestrated by steroid hormones estradiol and progesterone (reviewed by [3]). The synchronism of the oviductal events needs to be present to ensure successful embryo formation, followed by transportation in a coordinated relationship with the uterus receptivity (Figure 1, [4]).

The oviduct's most external structure consists of tunica serosa (not shown in the figure 1), followed by the muscular cells organized in longitudinal and circular layers, which are almost inexistent in the infundibulum segment, and become progressively thicker from the ampulla toward the isthmus, where the muscular layer is observed as the thickest (Figure 1, [2]). The internal layer is the mucosa, composed mainly of ciliated and secretory cells (Figure 1) and organized in folds arrangements specific to each oviductal segment [2].

2. ROLE OF THE OVIDUCT IN EARLY EMBRYO DEVELOPMENT

The most-reported way that oviductal cells interact with the embryo is through the oviductal fluid [5]. The oviductal fluid is generated by (i) transudation from plasma into the oviductal lumen together with (ii) the secretion of substances synthesized by the secretory cells [6]. The oviductal cells and oviductal fluid affect the early embryo development in many ways, for instance, the epigenetic regulation [7-9], lipid content [10], transcriptome regulation, and ultrastructure (reviewed by [11]).

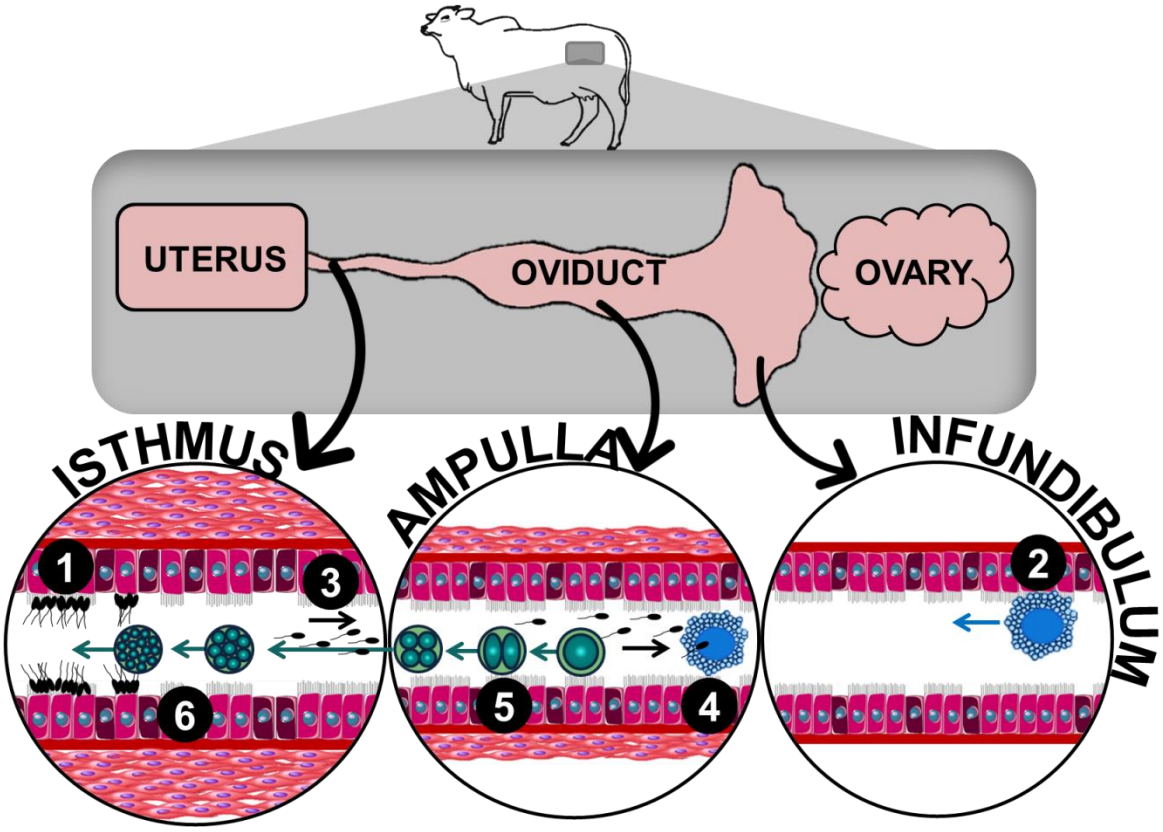

Figure 1: Schematic view of oviduct functions and its segments. In chronological order, the primary roles of the oviduct are: (1) formation of sperm reservoir in the isthmus that prolongs the sperm viability until the ovulation and regulates polyspermy; (2) Cumulus-oocyte complex pick-up by the infundibulum and forward it into the ampulla tubular segment; (3) sperm release from the isthmus and sperm

transportation toward the oocyte, which is also associated to sperm capacitation and hyperactivation; (4) promotion of suitable environment for gametes interaction and fertilization in the ampulla; (5) promotion of suitable environment for early embryo development along with embryo transportation into the uterus; (6) management of embryo migration (delay until the uterus is suitable to accomplish further embryo development). Note that the isthmus is proximal to the uterus, the ampulla is the medial segment, and the infundibulum is proximal to the ovary. The muscular layer (red cells) is almost inexistent in the infundibulum segment and becomes progressively thicker from the ampulla toward the isthmus, while ciliated (pink cells) and secretory (purple cells) cells are present in the mucosa of all three segments.

Because of the oviduct's benefits for early embryo development, many artificial techniques have been developed to assist the embryo with these oviduct goods. Artificial reproductive technologies have been applied to make the oviduct available in *in vivo* conditions for the embryo*. In vitro* techniques have been applied to mimic the embryos' interaction with oviductal epithelial cells or with the oviductal cells products (e.g., oviductal fluid). In the next sections, cattle's primary artificial approaches to connect the embryo to the oviduct are addressed. Additionally, the limitations of these approaches and possibilities for improvement are also described.

3. ARTIFICIAL *IN VIVO* APPROACHES TO SUPPLY EMBRYO WITH OVIDUCT GOODS

Artificial *in vivo* approaches are defined here to develop the embryo inside the oviduct lumen of an alive animal. Two techniques are described below: ovarian superstimulation protocols and transfer of zygote into the oviduct of intra- and inter-species.

3.1. OVARIAN SUPERSTIMULATION PROTOCOLS

Since the first ovarian superstimulation protocol was performed in cattle in 1943 by Casida and collaborators (reviewed by [12]), superstimulation methods have been submitted to a process of improvement mainly by applying different hormones and evaluating different arranges. Due to a greater understanding of follicular wave dynamics and synchronization of the estrus, current ovarian superstimulation protocols can be initiated at any moment of the estrous cycle stage and admit artificial insemination in a fixed time, which makes these protocols independent of animal estrous behavior detection and more comfortable to perform (Reviewed by [13]). In monovulatory species, including cattle, the ovarian superstimulatory protocol is a way to produce many embryos per estrous cycle from a specific animal, named donor. These embryos can be collected by nonsurgical methods and transferred into the uterus of cows named recipients.

The collection/transfer process is usually executed between days 6-8 after estrus, which means that all the events since gametes transport until the early embryo development occur in the lumen of the oviduct of donors' cows. In other words, the period of early embryo development is assisted by the environment produced by the oviduct cells and all the previous steps, for instance, sperm capacitation, final oocyte maturation, and fertilization. Because of this, the embryos produced by ovarian superstimulation protocols are named *in vivo*-derived embryos. Gad and collaborators [14] observed a lower quality in embryos produced by ovarian superstimulation protocols than non-stimulated single-ovulating heifers; Havlicek and collaborators [15], on the other hand, demonstrated a superior quality of superstimulation-derived embryos compared to *in vitro*-

derived embryos. Therefore, embryos' production by ovarian superstimulation protocols is a successful artificial approach to guarantee the benefits of oviductal goods for the embryo (Figure 2A).

3.2. ZYGOTE TRANSFER TO OVIDUCT OF INTRA- AND INTER-SPECIES

The technique applied before establishing *in vitro* embryo production, the transfer of one/two-cell embryos into the oviduct of rabbits [16] or sheep [17], successfully produced the firsts live calf after *in vitro* oocyte maturation followed by *in vitro* fertilization. Nowadays, the procedure of transferring one/two-cells embryos into the oviduct of other species (heterologous) or same species (homologous) guarantees the association of the benefits of *in vitro* maturation and fertilization (almost unlimited source of oocytes from slaughterhouse and necessity of much less semen per batch, respectively) to the benefit of the oviduct environment for early embryo development. In particular, the culture of *in vitro*-derived bovine zygotes in the oviducts of sheep [18-20] or cattle [15,21,22] significantly improves the quality of the resulting blastocysts. Even though this is a laborious technique that requires experienced professionals and animal management, this approach also guarantees the benefits of oviductal goods for the embryo (Figure 2B).

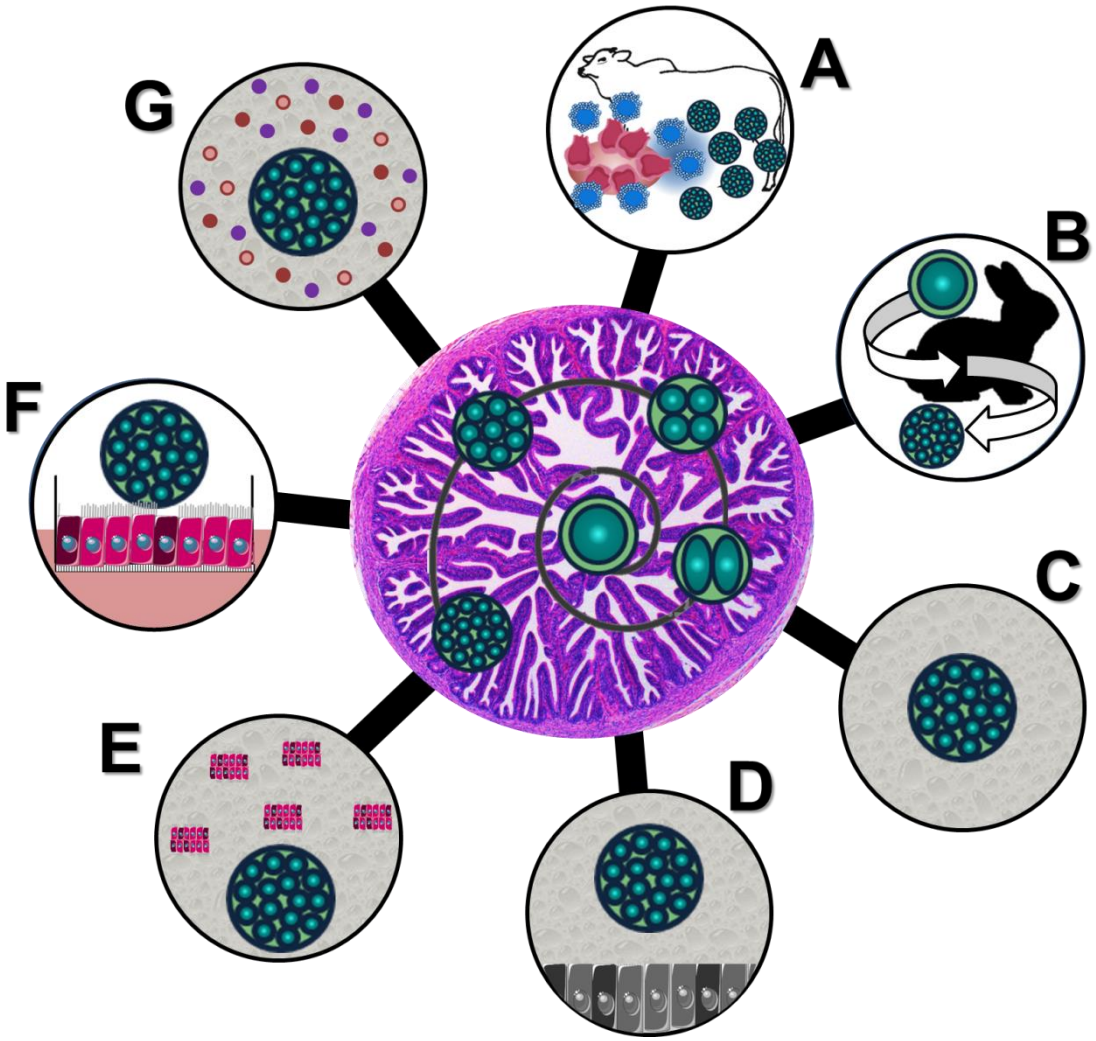

Figure 2: Artificial approaches to establish interaction between embryo and oviduct. (A) ovarian superstimulation protocol; (B) inter-species zygote transfer; (C) embryo developing in synthetic oviduct fluid (SOF); (D) embryo-oviductal cells co-culture in the 2D system, note the dedifferentiation of oviductal cells in this system; (E) embryo-oviductal cells co-culture in suspended vesicle system, note the maintenance of oviductal cells morphology in this system; (F) embryo-oviductal cells co-culture in the 3D system (static), note the maintenance of oviductal cells morphology in this system, associated to the compartmentalized system with basolateral medium feeding the oviductal cells, an embryo in the apical part of the oviductal cells; (G) embryo developing in SOF medium supplemented with oviductal fluid and oviductal fluid products (e.g., extracellular vesicles). In the center of the scheme, the stages (from zygote until 16-cell embryo) of embryo development occur in the oviduct (represented by a histological image from a section of bovine oviduct ampulla stained with hematoxylin & eosin) are indicated.

4. ARTIFICIAL *IN VITRO* APPROACHES TO SUPPLY EMBRYO WITH OVIDUCT GOODS

Bovine embryos can develop until blastocyst stage entirely out of any animal organism, called *in vitro*-derived blastocyst. Even though *in vitro*-derived embryos are not placed in the lumen of the organ, different *in vitro* approaches can also guarantee the assistance of oviduct goods to the embryo, as described in this section.

4.1. SYNTHETIC OVIDUCTAL FLUID

The first calves produced entirely through *in vitro* procedure, including oocyte maturation, fertilization, and culture, were born in 1987 as a result of the work of Lu and collaborators (reviewed by [12]). The biggest challenge to establish the conditions for *in vitro* culture was the barrier to pass the 8-16 cell blockage in bovine embryos. The blockage is *in vitro*-derived bovine embryos was overcome by the development of a synthetic medium formulated based on the compounds of the oviductal fluid [23], named Synthetic Oviductal Fluid (SOF, [24]), which is still the media currently in use for embryo production.

In a way, the SOF medium could be classified as the most “artificial” approach to mimic the oviductal condition since it is cell- and cell product-free and formulated by synthetic biochemical compounds only. Embryo development until the blastocyst stage can be achieved in a wholly defined condition, supplementing SOF medium with polyvinyl alcohol (PVA) to avoid the embryos' stickiness to the petri dish [25,26]. However, the most common conditions for *in vitro* culture of

bovine embryos are semi-defined or non-defined media by adding, respectively, Bovine Serum Albumin (BSA) and Fetal Bovine Serum (FBS) for improvement on rates of development [25,26].

Beyond the presence of BSA and/or FBS in the culture medium, several studies evaluate the effect of adding different compounds into the SOF medium to improve embryo development. Studies have been adding the most expected modulators, for instance, Oviductal Glycoprotein 1 (OVGP1), the specific protein secreted by oviductal cells and present in the oviductal fluid during *in vivo* embryo development [27], until unnatural pharmacological interventions to modulate specific pathways, as the addition of sildenafil to modulate the embryo lipid metabolism [28]. Between them, a huge number and amount of types of investigations are performed aimed to improve bovine embryo development. However, this information is not the scope of this review.

Additionally, an investigation of the SOF biochemical composition *per se* has also been done. Defined in 1972 based on oviductal sheep fluid, this pioneer and still most used SOF medium was compared to a novel SOF formulation based on bovine oviductal fluid and supported by more precise detection methods currently available [29], as a result, the novel SOF proved to be more efficient for bovine embryo development than the sheep-based one [30]. Altogether, the definition of biochemical SOF composition and its supplementation is still ongoing and can represent a good approach to guarantee the benefits of oviductal goods for the embryo (Figure 2C).

4.2. OVIDUCT EPITHELIAL CELL 2D CULTURE SYSTEM

Before the successful development of the SOF medium, embryos were *in vitro* cultured with an oviductal epithelium cell (OEC) monolayer in a co-culture system to be able to pass the 8-16 cell blockage in bovine embryos [31,32]. Over the last few years, the application of co-culture systems with OEC is used to provide a better approximation of the *in vitro*-produced embryo with the maternal environment. On the same line, the monolayer culture of bovine OEC (BOEC) has been characterized [33], and the co-culture system with *in vitro*-derived embryo has improved the blastocyst rate [34] and embryo quality [35]. However, the co-culture system's beneficial effect has been shown to be independent of oviductal cell activity. Embryos co-cultured with cumulus cells [36] or non-reproductive tract cells (Buffalo Rat Liver and Vero Cells, [37]) have showed a positive effect for *in vitro* embryo cultivation.

When BOEC is cultured in monolayer 2D systems, some limitations need to be considered. First of all, a singular culture medium is used for both somatic and embryonic cells due to the sharing space conditions of the culture system, which most of the time represent suboptimal conditions for the development of OEC, embryo, or both. Additionally, the most critical limitation of the 2D system is the OEC dedifferentiation, which consists of the loss of morphologic characteristics and functional activity, including reduction of cell height, loss of cilia, loss of secretory granules, and loss of bulbous protrusions [37]. Therefore, the co-culture system of embryos with BOECs as 2D monolayer culture might not represent the best maternal environment conditions since it loses the capacity to secrete embryotrophic substances. It also might explain the lack of specialized oviductal

actions of OECs submitted to the co-culture system compared to other cells (e.g., cumulus cells or even non-reproductive tract cells). The cellular dedifferentiation of OEC generates an environment not related to the oviductal conditions anymore, which exposes the embryo to general benefits comparable to any other co-culture system, for instance, reducing harmful free oxygen radicals by lower the oxygen levels [36,37].

Although co-culture embryos with 2D monolayer oviduct cells are not the ideal system, the 2D culture system may be a suitable method for studying specific or individual factors related to embryo-maternal interaction and as a pre-experimental application (Figure 2D).

4.3. SUSPENDED VESICLES OF OVIDUCT EPITHELIAL CELLS

In situations where OEC is submitted to a short-term (24 hours) culture, the cell attachment to the petri dish's bottom can be avoided. These cells flow in the medium aggregate to each other and form structures named ‘vesicles’ or 'explants.' The morphology of the cellular surface of these structures is highly similar to oviduct epithelium *in vivo*, which keeps the intact cilia, secretory activity, and the ultrastructural characteristics of the tissue, including cell organelles, such as basal bodies, secretory vacuoles, mitochondria, and cell polarity during the 24 hours of culture [38]. Even though the culture period of suspended vesicles is relatively short, this system supports the maintenance of 3D cellular morphology and cellular activity similar to *in vivo* conditions, which makes it a useful approach to establish a co-culture system and possibly assist the embryo development with the oviductal goods (Figure 2E).

4.4. OVIDUCT EPITHELIAL CELL 3D CULTURE SYSTEM

Another system to culture OECs as a monolayer has been demonstrated to retain columnar epithelial cell morphology's polarity. Because of that, this system is called the '3D culture system'. This method is composed of a porous membrane that divides the system culture into compartments, or chambers. Within this compartmentalized system, it is possible to apply differential culture media for the somatic cells (OEC) and embryonic cells (from 1-cell embryo until blastocyst). Most importantly, the cells present the apical-basolateral polarity, ciliation, secretory activity, and responsiveness to steroid treatment for long-term cultivation (for at least six weeks, reviewed by [39]). Many studies have characterized BOEC cultured in this system, demonstrating a highly *in vivo* similarity of these *in vitro* cells submitted to the 3D culture system.

Two differential 3D culture systems have been used for BOEC culture. The first one is a transwell insert, where BOECs are cultured under static conditions. In this system, cells are seeded directly onto the porous membrane's apical surface for a monolayer formation. Once a confluent cell layer is reached, the medium from the apical compartment can be removed, leaving the basolateral compartment's medium to nurture the BOECs. At this point, this culture is called the air-liquid interface (ALI-BOEC), referring to the medium-free condition in the apical part and medium-presence in the basolateral part [40,41]. The apical medium-free part and cells receiving their nutrition by the basal surface are crucial for the maintenance of cell polarity and cellular differentiation in this system. The second mechanism concerns a microfluidic system developed by Ferraz and collaborators [7], called ‘oviduct-on-a-chip.' This system uses the same principle as the transwell insert.

However, the basolateral compartment has holes for inlets and outlets that allow a dynamic culture system, with continuous or pulsatile perfusion of culture media in the basolateral part [7].

Both static and microfluidic systems allow manipulating the medium in the basolateral compartment, allowing nutritional blood circulation stimulation and/or the introduction of exogenous factors (*e.g.*, steroid hormones). Another similarity of this system to the *in vivo* condition has been demonstrated by the production of some oviduct fluid surrogate (also called '*in vitro*-derived oviduct fluid') in the apical compartment, mimicking the production of oviductal fluid in the luminal region of the oviduct [40,41].

Finally, the capacity to support embryo development has been demonstrated in both 3D culture systems. In the static system, *in vitro*-derived bovine zygotes have been placed within the apical compartment of transwell inserts and developed until blastocyst stage with no other medium, only in the presence of oviduct fluid surrogate [40]. In the microfluidic system, bovine *in vitro*-matured oocytes have been fertilized and cultured until the 16-cell stage in the apical compartment of the oviduct-on-a-chip [7]. Even though embryos could develop until the blastocyst stage in the static system, the production yield and embryo quality were, respectively, inferior and similar to the standard *in vitro* embryo production [42]. On the other hand, embryos produced in the oviduct-on-a-chip presented a high-quality similarity compared to *in vivo*-derived embryos [7]; however, no bovine blastocyst formation has been demonstrated in this system up until now.

Thus, it is unquestionable that the culture of oviductal epithelial cells in the 3D system is a great approach to mimic the *in vivo* conditions for the early embryo

development (Figure 2F), and more studies will be able to improve and expand the application of these culture systems.

4.5. OVIDUCTAL FLUID AND OVIDUCTAL FLUID FRACTIONS

The oviductal fluid is how oviductal cells reach the embryo during their first days of development in *in vivo* conditions. Because of this, the addition of oviductal fluid into the SOF medium is an excellent approach to positively modulate embryo development [8]. Similarly, extracellular vesicles isolated from the oviductal fluid content have also promoted embryo development improvement [43,44]. Extracellular vesicles are formed by a phospholipid bilayer and secreted by different cell types, including oviduct epithelial cells. They act by modulating intercellular communication and carry bioactive molecules, such as proteins, lipids, mRNA, and microRNA (reviewed by [45]). Interestingly, extracellular vesicles isolated from *in vitro* culture of bovine oviduct epithelial cells also demonstrated to be beneficial for the embryo development when added into the SOF medium [43]. Therefore, the addition of oviductal fluid or oviductal fluid fractions (*i.e.*, extracellular vesicles) *in vivo*- or *in vitro*-derived into the SOF medium is a feasible approach to mimic the oviductal microenvironment in the *in vitro* production of bovine embryos (Figure 2G).

5. IS THERE SPACE TO IMPROVEMENT FOR THESE APPROACHES?

5.1. *IN VITRO* CULTURE SYSTEMS NEED STANDARDIZATION

The cultivation of OEC has a complex methodology, especially concerning its ability to maintain cells in their differentiated state and actively functional. Considering that these cells are modulated by hormone activity, an ideal *in vitro* oviduct model should at least allow the possibility to mimic the hormonal changes that occur during the estrous cycle stages [39].

Independent of the BOEC *in vitro* culture system (2D, 3D, or suspended vesicles), a large methodological variability is present between studies, suggesting that a gold-standard method has still not been established. Moreover, this variability makes the works difficult to compare. The main variables factors between studies about *in vitro* culture of BOEC have been listed in table 1.

Eventually, the 3D systems are more suitable for the cell morphology maintenance compared to the 2D system (see information above). Associated with that, factors listed in table 1 should be considered when developing or refining an *in vitro* oviduct model. Most importantly, the methodology should be very well described in scientific articles to better comprehend the results and better compare different studies. Therefore, the standardization of BOEC *in vitro* culture should be the aim of researches developed in this field to improve the application of BOEC in co-culture systems with embryos in development.

Table 1: Aspects variables between studies about *in vitro* culture of BOEC

Animal source	reference
Synchronized	[38]
Slaughterhouse	[7,34,35,41,42,46-59]
Cell harvesting technique	**reference**
Trypsin	[41-43,46,48,50,53]
Collagenase	[42,50]
Pancreatin	[41,46]
Mechanical	[7,34,35,38,41,43,47-49,51-59]

Oviduct segment of cell harvesting	reference
Ampulla	[38]
Isthmus	[56,57]
Entirely tubular oviduct	[7,35,41-43,46-50,53,55,58,59]
Not specified, probably the entirely oviduct	[34,51,52,54]
Stage of the estrous cycle of cell harvesting	reference
Follicular/Pre-ovulatory	[46,48,49,58]
Post-ovulatory/Metestrus/Early-luteal phase	[35,38,49,54-58]
Mid-luteal phase	[41,43,49]
Late-luteal phase	[49]
Luteal phase (not specified which one)	[42,46,50]
Not specified	[7,34,47,51-53,59]
Oviduct side of cell harvesting	reference
Ipsilateral (closer to CL or pre-ovulatory follicle)	[35,43,46,54,58]
Both (ipsilateral and contralateral)	[38,42,49-52]
Not specified, probably both oviducts	[7,34,41,47,48,53,55-57,59]
Epithelial cell culture purity	reference
Differential adhesion time	[7,41,47,48,51-53]
Epithelial cell marker evaluation	[34,35,38,41-43,48-50,54-59]
Culture system	reference
2D	[34,35,43,46-49,51,53-59]
3D – static	[38,41-43,50-52,54,55]
3D – microfluidic	[7,47]
Suspended vesicles	[38,55]
Cultured of cell from individual animal or pool	reference
Pool	[7,34,35,41,43,46,48,53-59]
Individual Animal	[38,42,47,49-52]
Type of cultured related to passage number	reference
Primary cell culture	[7,34,35,38,41-43,46,47,49-52,54-59]
Passage	[46,48,49,53,59]
Culture media base	reference
DMEM/F12	[7,35,41,42,46,47,49-52,55]
TCM199	[34,38,48,51-54,56,58]
DMEM	[43,50]
Ham’s 10	[59]
SOF	[43,57]
Main supplementation in the culture media	reference
Fetal bovine serum	[7,34,35,41-43,46,48-59]
Bovine serum albumin	[41,47,51,52,55]
Epidermal Growth Factor	[7,42,47,51-53]
Apo-transferrin	[7,42,47,51,52]
Insulin	[7,42,47,50-52]
Gentamicin	[7,34,38,47,49-52,56,58]
Penicillin/Streptomycin	[35,41-43,46,48-50,55]
Amphotericin B	[7,35,41,42,47,49-52,55,56]
Hydrocortisone	[47,51,52]
Epinephrine	[47,51,52]

trans-retinoic acid	[7,42,47-49,51,52]
bovine pituitary extract	[42,47,51,52]
tri-iodothyronine	[47,51,52]
Fungizone	[46]
Glucose	[43,49]
L-Glutamine	[41,43]
Bronchial epithelial cell basal medium	[51,52]
Glutathione	[7,50]
Newborn Calf Serum	[55]
Ascorbic acid	[50]
Pyruvate	[43]
Hormone supplementation	reference
Estradiol	[7,38,41,46,48,50,53,59]
Progesterone	[7,38,41,48,53]
Testosterone	[41]
LH	[48]

5.2. EFFECTS OF OVARIAN SUPERSTIMULATION PROTOCOLS ON OVIDUCT CELLS NEED TO BE BETTER EVALUATE

As mentioned above, the oviductal functions are performed in a refined chronological sequence. In normal conditions, sperm starts the reservoir formation a few minutes after ejaculation and remains in the isthmus segment until ovulation. Around 24 hours after LH-peak, the cumulus-oocyte complex is ovulated and transported into the oviduct's tubular segment. As soon as the oocyte arrives in the ampulla, the sperm become hyperactivated and are released from the oviductal epithelium. Next, the oocyte is penetrated by the spermatozoa minutes up to few hours after ovulation, and an embryo is transported into the uterus within the first cellular cleavages taking place in the oviduct lumen [60].

In cows submitted to ovarian superstimulation protocol, ovulation can occur between 24-33 hours after LH-peak, which results in 4-12 hours of ovulatory process duration [60]. This massive difference between the ovulation of the first and the last cumulus-oocyte complex cause a significant asynchrony between

embryo formation and oviductal functions. The main complication is related to sperm release and hypercapacitation, which occurs in the moment of the first ovulation, impairing the fertilization of later ovulated oocytes [61]. However, this is not the only problem. Rodrıguez-Alonso and collaborators [4] recently demonstrated that the transfer of *in vitro*-produced embryos (Day 1 of development) into asynchrony ahead status oviducts (Day 3 after ovulation) has a negative effect on embryo development compared to synchronized conditions (Day 1 embryo transferred into Day 1 oviduct). Even though the hormonal milieu in this study is not similar to ovarian superstimulation conditions, the presence of embryos in asynchrony circumstances is comparable. Talking about the hormonal milieu, the supra-physiological progesterone levels, associated with multiples C*orpus Luteum* formation after multiple ovulations, have been related to impaired embryo development [14]. All this information makes evident that there is space for improvement on ovarian superstimulation protocol design.

Impressive results were observed by lengthening the superstimulatory treatment from the traditional 4-day protocol to 7 days of follicle-stimulating hormone (FSH) treatment, without increasing the total amount of FSH administered. This long-protocol increased the synchrony of ovulations and the number of transferable embryos produced [62,63]. The increase in ovulation synchrony and higher transferable embryo production can be, respectively, the cause and consequence of oviduct performance: more cumulus-oocyte complex arriving at the same time in the oviduct lumen (cause) would benefit from a suitable oviductal microenvironment during gametes interaction and embryo formation, which increase the production of transferable embryos (consequence).

Another strategy that should be considered to improve the yield and quality of embryos produced by ovarian superstimulation is the evaluation of hormonal milieu induced by each protocol. The post-ovulatory period has been associated to the deleterious effect of supra-physiological levels of progesterone [14]; however, the estrus phase is less investigated. Santos and collaborators [64] reported differing estradiol levels in animals submitted to ovarian superstimulation protocol with FSH-only compared to FSH combined with equine chorionic gonadotropin (eCG). Higher estradiol levels have been positively associated with oviduct fluid secretion [65], which means that this differential hormone milieu between protocols should be more explored to investigate which one is the most beneficial for embryo development.

Altogether, the use of ovarian superstimulation protocol is a practicable technique and an interesting artificial approach to guarantee early embryo development under *in vivo* conditions; however, this method also has limitations on its application. Lastly, it is essential to keep in mind: albeit the quality of superstimulation-derived embryo is inferior to a single-ovulation-derived embryo, the quality of superstimulation-derived embryos is superior compared to *in vitro*-derived embryo [15], demonstrating the benefits of the superstimulatory protocols and motivating for improvement on this field.

6. EFFECT OF STEROID HORMONES IN THE OVIDUCT CELLS: *IN VIVO* AND *IN VITRO*

The effects of steroid hormones, estradiol and progesterone, have been described in muscles and epithelial cells of the oviduct [66]. In cattle, the nuclear receptors of both hormones have been immunolocalized in all these cells during all stages of the estrous cycle [67]. Interestingly, there is a variation of expression of these receptors during the estrous cycle stages, demonstrating the existence of a control system related to steroid hormones activity in the oviduct depending on hormone levels, and this control system is related to the orchestration of the roles performed by the oviduct.

In concern to embryo development, the effect of steroid hormones in the epithelial cells has a greater interesting, mainly the effect of the estradiol. Higher levels of estradiol is present in the peri-ovulatory period, and this higher levels of estradiol is correlated to higher secretion of oviductal fluid, as observed in cows submitted to cannulation procedure [68]. On the other hand, during the luteal phase, moment of higher levels of progesterone, the oviductal fluid secretion was reduced [68]. Even though each cycle stage has different hormone predominance profile, the estradiol and progesterone are not unique in each moment. To ensure the specific effect of estradiol and progesterone, McDaniel and collaborators [69] performed the investigation in ovariectomized cows. In the absence of the ovaries (the main source of estradiol and progesterone production in cyclic animals), the steroid hormones were separately administrated by injections. As result, this study demonstrated that indeed the estradiol is responsible for the presence of largest amount of mucoprotein in the oviduct, while the smallest amounts were observed in progesterone-treated animals [69].

Besides the effect on oviductal fluid secretion, steroid hormones also act on regulating many others parameters in the oviduct under *in vivo* conditions, such us, transcription profile [70-72], proteomic profile [73], and extracellular vesicles content [74]. However, these studies have been performed under *in vivo* conditions; more specifically, ex-vivo condition (which means that the animal needs to be slaughtered for sample collection) has an important bias to be considered: the information obtained from samples collected during the follicular phase of the estrous cycle can be caused by the raise of estradiol levels or the falling (almost absence) of progesterone levels. In accordance with that, a study developed within porcine oviduct cells submitted to *in vitro* culture with different hormone treatment conditions demonstrated that the main alteration in oviduct cells transcription activity during follicular phase is due to the decrease of progesterone levels, and not due to increase levels of estradiol [75]. The modulation of hormone levels and different hormone profiles treatment are easier to be performed in *in vitro* conditions, reinforcing the importance and necessity of *in vitro* culture models for epithelial oviduct cells.

Even though *in vitro* culture models have the advantage of a controlled hormone levels treatment, they also have some general disadvantages related to *in vitro* conditions, such us the cellular senescence, lack of reproducibility, and lack of the organ architecture. Moreover, each *in vitro* culture model has also others specific disadvantages, such us the cell de-differentiation in 2-D culture systems, static systems for culture media in most of the systems, and complexity methodology in microfluidic system. Even with these limitations, bovine oviduct epithelial cells have been cultured in *in vitro* conditions under simulation of estrous

cycle hormone milieu with satisfactory results of cells response to hormone treatment [7], demonstrating that *in vitro* culture models can be used to study the effect of steroid hormone in the oviduct cells.

7. THE NECESSITY OF THESE APPROACHES

The achievement of *in vitro* embryo production technique has been one of the most remarkable events of the Artificial Reproduction Technique (ART). Since then, research has been performed, and notable improvement has already been reached (reviewed by [76,77]). However, there are still unresolved drawbacks that limit a more comprehensive implementation of embryo transfer technology, especially the relatively low pregnancy rate. As we move towards the present, the communication between the embryo and maternal environment has been shown to be an essential target to improve embryo quality, and consequently, its capacity to establish a pregnancy.

Indeed, the animal independence for embryo production accomplished by *in vitro* techniques and the development of multiple offspring at once from the desired animal achieved by ovarian superstimulation protocols made immensurable progress on the reproduction field possible. After this period of detachment from the physiological maternal reproductive tract, it seems that now is the era of finding a way to reconnect. Some approaches have been presented herein, and as observed, there is a place for their improvement and also a place for the development of new approaches. Therefore, to those who are looking for places for the next journey, these are some addresses that you can visit.

8. REFERENCES

[1] W.C. Buhi, Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein., Reproduction 123 (2002) 355-362.
[2] J.L. Yániz, F. Lopez-Gatius, P. Santolaria, K.J. Mullins, Study of the functional anatomy of bovine oviductal mucosa., Anat Rec 260 (2000) 268-278. 10.1002/1097-0185(20001101)260:3<268::AID-AR60>3.0.CO;2-L [pii].
[3] M. Binelli, A.M. Gonella-Diaza, F.S. Mesquita, C.M.B. Membrive, Sex Steroid-Mediated Control of Oviductal Function in Cattle, Biology (Basel) 7 (2018). 10.3390/biology7010015.
[4] B. Rodríguez-Alonso, J.M. Sánchez, M. Hamdi, M. McDonald, V. Havlicek, U. Besenfelder, P. Lonergan, D. Rizos, Asynchrony between the early embryo and the reproductive tract affects subsequent embryo development in cattle, Reprod Fertil Dev 32 (2020) 564-571. 10.1071/RD19360.
[5] M. Saint-Dizier, J. Schoen, S. Chen, C. Banliat, P. Mermillod, Composing the Early Embryonic Microenvironment: Physiology and Regulation of Oviductal Secretions, Int J Mol Sci 21 (2019). 10.3390/ijms21010223.
[6] Y. Menezo, P. Guerin, The mammalian oviduct: biochemistry and physiology, Eur J Obstet Gynecol Reprod Biol 73 (1997) 99-104.
[7] M.A.M.M. Ferraz, H.S. Rho, D. Hemerich, H.H.W. Henning, H.T.A. van Tol, M. Hölker, U. Besenfelder, M. Mokry, P.L.A.M. Vos, T.A.E. Stout, S. Le Gac, B.M. Gadella, An oviduct-on-a-chip provides an enhanced in vitro environment for zygote genome reprogramming, Nat Commun 9 (2018) 4934. 10.1038/s41467-018-07119-8.
[8] R. Lopera-Vasquez, M. Hamdi, V. Maillo, V. Lloreda, P. Coy, A. Gutierrez-Adan, P. Bermejo-Alvarez, D. Rizos, Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro, Reprod Fertil Dev 29 (2017) 621-629. 10.1071/RD15238.
[9] S. Canovas, P.J. Ross, G. Kelsey, P. Coy, DNA Methylation in Embryo Development: Epigenetic Impact of ART (Assisted Reproductive Technologies), Bioessays 39 (2017). 10.1002/bies.201700106.
[10] M.J. Sudano, E.S. Caixeta, D.M. Paschoal, A. Martins, R. Machado, J. Buratini, F.D. Landim-Alvarenga, Cryotolerance and global gene-expression patterns of Bos taurus indicus and Bos taurus taurus in vitro- and in vivo-produced blastocysts, Reprod Fertil Dev (2013). 10.1071/RD13099.
[11] U. Besenfelder, V. Havlicek, G. Brem, Role of the oviduct in early embryo development, Reprod Domest Anim 47 Suppl 4 (2012) 156-163. 10.1111/j.1439-0531.2012.02070.x.
[12] S.G. Moore, J.F. Hasler, A 100-Year Review: Reproductive technologies in dairy science, J Dairy Sci 100 (2017) 10314-10331. 10.3168/jds.2017-13138.
[13] C.M. Barros, R.A. Satrapa, A.C. Castilho, P.K. Fontes, E.M. Razza, R.L. Ereno, M.F. Nogueira, Effect of superstimulatory treatments on the expression of genes related to ovulatory capacity, oocyte competence and embryo development in cattle, Reprod Fertil Dev 25 (2013) 17-25. 10.1071/RD12271.
[14] A. Gad, U. Besenfelder, F. Rings, N. Ghanem, D. Salilew-Wondim, M.M. Hossain, D. Tesfaye, P. Lonergan, A. Becker, U. Cinar, K. Schellander, V. Havlicek, M. Hölker, Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: implications for animal breeding and human assisted reproduction, Hum Reprod 26 (2011) 1693-1707. 10.1093/humrep/der110.

[15] V. Havlicek, F. Wetscher, T. Huber, G. Brem, M. Mueller, U. Besenfelder, In vivo culture of IVM/IVF embryos in bovine oviducts by transvaginal endoscopy, J Vet Med A Physiol Pathol Clin Med 52 (2005) 94-98. 10.1111/j.1439-0442.2005.00694.x.
[16] M.A. Sirard, R.D. Lambert, Birth of calves after in vitro fertilisation using laparoscopy and rabbit oviduct incubation of zygotes, Vet Rec 119 (1986) 167-169. 10.1136/vr.119.8.167.
[17] K.H. Lu, I. Gordon, M. Gallagher, H. McGovern, Pregnancy established in cattle by transfer of embryos derived from in vitro fertilisation of oocytes matured in vitro, Vet Rec 121 (1987) 259-260. 10.1136/vr.121.11.259.
[18] B.P. Enright, P. Lonergan, A. Dinnyes, T. Fair, F.A. Ward, X. Yang, M.P. Boland, Culture of in vitro produced bovine zygotes in vitro vs in vivo: implications for early embryo development and quality, Theriogenology 54 (2000) 659-673. 10.1016/S0093-691X(00)00381-2.
[19] D. Rizos, F. Ward, P. Duffy, M.P. Boland, P. Lonergan, Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality, Mol Reprod Dev 61 (2002) 234-248. 10.1002/mrd.1153.
[20] D. Rizos, P. Lonergan, M.P. Boland, R. Arroyo-García, B. Pintado, J. de la Fuente, A. Gutiérrez-Adán, Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality, Biol Reprod 66 (2002) 589-595. 10.1095/biolreprod66.3.589.
[21] D. Tesfaye, P. Lonergan, M. Hoelker, F. Rings, K. Nganvongpanit, V. Havlicek, U. Besenfelder, D. Jennen, E. Tholen, K. Schellander, Suppression of connexin 43 and E-cadherin transcripts in in vitro derived bovine embryos following culture in vitro or in vivo in the homologous bovine oviduct, Mol Reprod Dev 74 (2007) 978-988. 10.1002/mrd.20678.
[22] F. Wetscher, V. Havlicek, T. Huber, M. Gilles, D. Tesfaye, J. Griese, K. Wimmers, K. Schellander, M. Müller, G. Brem, U. Besenfelder, Intrafallopian transfer of gametes and early stage embryos for in vivo culture in cattle, Theriogenology 64 (2005) 30-40. 10.1016/j.theriogenology.2004.11.018.
[23] B.J. Restall, R.G. Wales, The fallopian tube of the sheep. 3. The chemical composition of the fluid from the fallopian tube, Aust J Biol Sci 19 (1966) 687-698. 10.1071/bi9660687.
[24] H.R. Tervit, D.G. Whittingham, L.E. Rowson, Successful culture in vitro of sheep and cattle ova, J Reprod Fertil 30 (1972) 493-497. 10.1530/jrf.0.0300493.
[25] C. Wrenzycki, D. Herrmann, J.W. Carnwath, H. Niemann, Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA, Mol Reprod Dev 53 (1999) 8-18. 10.1002/(SICI)1098-2795(199905)53:1<8::AID-MRD2>3.0.CO;2-K.
[26] H.M. Kubisch, M.A. Larson, A.D. Ealy, C.N. Murphy, R.M. Roberts, Genetic and environmental determinants of interferon-tau secretion by in vivo- and in vitro-derived bovine blastocysts, Anim Reprod Sci 66 (2001) 1-13. 10.1016/s0378-4320(01)00086-0.
[27] B. Algarra, V. Maillo, M. Avilés, A. Gutiérrez-Adán, D. Rizos, M. Jiménez-Movilla, Effects of recombinant OVGP1 protein on in vitro bovine embryo development, J Reprod Dev 64 (2018) 433-443. 10.1262/jrd.2018-058.
[28] R.C. Botigelli, E.M. Razza, E.M. Pioltine, P.K. Fontes, K.R.L. Schwarz, C.L.V. Leal, M.F.G. Nogueira, Supplementing in vitro embryo production media by NPPC and sildenafil affect the cytoplasmic lipid content and gene expression of bovine cumulus-oocyte complexes and embryos, Reprod Biol 18 (2018) 66-75. 10.1016/j.repbio.2018.01.004.
[29] J. Lamy, J. Gatien, F. Dubuisson, L. Nadal-Desbarats, P. Salvetti, P. Mermillod, M. Saint-Dizier, Metabolomic profiling of bovine oviductal fluid across the oestrous cycle using proton nuclear magnetic resonance spectroscopy, Reprod Fertil Dev (2018). 10.1071/RD17389.
[30] E.C. dos Santos, A.M. Fonseca Junior, C.B. de Lima, J. Ispada, K. Annes, J.V.A. da Silva, M.P. Milazzotto, Embryonic Culture Supplementation (ECS) – a new culture system based on oviduct

and uterus fluid composition improves bovine embryo production and quality 33rd Annual Meeting of the Brazilian Embryo Technology Society, Animal Reproduction, 2019, pp. 674.
[31] F. Gandolfi, R.M. Moor, Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells, J Reprod Fertil 81 (1987) 23-28. 10.1530/jrf.0.0810023.
[32] W.H. Eyestone, N.L. First, Co-culture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium, J Reprod Fertil 85 (1989) 715-720.
[33] N. Ouhibi, Y. Menezo, G. Benet, B. Nicollet, Culture of epithelial cells derived from the oviduct of different species, Hum Reprod 4 (1989) 229-235. 10.1093/oxfordjournals.humrep.a136877.
[34] B. Schmaltz-Panneau, A. Cordova, S. Dhorne-Pollet, C. Hennequet-Antier, S. Uzbekova, E. Martinot, S. Doret, P. Martin, P. Mermillod, Y. Locatelli, Early bovine embryos regulate oviduct epithelial cell gene expression during in vitro co-culture, Anim Reprod Sci 149 (2014) 103-116. 10.1016/j.anireprosci.2014.06.022.
[35] A.K. Talukder, M.B. Rashid, M.S. Yousef, K. Kusama, T. Shimizu, M. Shimada, S.S. Suarez, K. Imakawa, A. Miyamoto, Oviduct epithelium induces interferon-tau in bovine Day-4 embryos, which generates an anti-inflammatory response in immune cells, Sci Rep 8 (2018) 7850. 10.1038/s41598-018-26224-8.
[36] K. Goto, Y. Kajihara, S. Kosaka, M. Koba, Y. Nakanishi, K. Ogawa, Pregnancies after co-culture of cumulus cells with bovine embryos derived from in-vitro fertilization of in-vitro matured follicular oocytes, J Reprod Fertil 83 (1988) 753-758. 10.1530/jrf.0.0830753.
[37] S.E. Ulbrich, K. Zitta, S. Hiendleder, E. Wolf, In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct, Theriogenology 73 (2010) 802-816. 10.1016/j.theriogenology.2009.09.036.
[38] R. Rottmayer, S.E. Ulbrich, S. Kölle, K. Prelle, C. Neumueller, F. Sinowatz, H.H. Meyer, E. Wolf, S. Hiendleder, A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization, Reproduction 132 (2006) 637-648. 10.1530/rep.1.01136.
[39] M.A. Ferraz, H.H. Henning, T.A. Stout, P.L. Vos, B.M. Gadella, Designing 3-Dimensional In Vitro Oviduct Culture Systems to Study Mammalian Fertilization and Embryo Production, Ann Biomed Eng (2016). 10.1007/s10439-016-1760-x.
[40] S. Chen, S.E. Palma-Vera, M. Langhammer, S.P. Galuska, B.C. Braun, E. Krause, A. Lucas-Hahn, J. Schoen, An air-liquid interphase approach for modeling the early embryo-maternal contact zone, Sci Rep 7 (2017) 42298. 10.1038/srep42298.
[41] C.A. Simintiras, T. Fröhlich, T. Sathyapalan, G.J. Arnold, S.E. Ulbrich, H.J. Leese, R.G. Sturmey, Modelling oviduct fluid formation in vitro, Reproduction (2016). 10.1530/REP-15-0508.
[42] V.A. van der Weijden, S. Chen, S. Bauersachs, S.E. Ulbrich, J. Schoen, Gene expression of bovine embryos developing at the air-liquid interface on oviductal epithelial cells (ALI-BOEC), Reprod Biol Endocrinol 15 (2017) 91. 10.1186/s12958-017-0310-1.
[43] R. Lopera-Vásquez, M. Hamdi, B. Fernandez-Fuertes, V. Maillo, P. Beltrán-Breña, A. Calle, A. Redruello, S. López-Martín, A. Gutierrez-Adán, M. Yañez-Mó, M. Ramirez, D. Rizos, Extracellular Vesicles from BOEC in In Vitro Embryo Development and Quality, PLoS One 11 (2016) e0148083. 10.1371/journal.pone.0148083.
[44] C. Simon, D.W. Greening, D. Bolumar, N. Balaguer, L.A. Salamonsen, F. Vilella, Extracellular Vesicles in Human Reproduction in Health and Disease, Endocr Rev 39 (2018) 292-332. 10.1210/er.2017-00229.
[45] A. Bridi, F. Perecin, J.C.D. Silveira, Extracellular Vesicles Mediated Early Embryo-Maternal Interactions, Int J Mol Sci 21 (2020). 10.3390/ijms21031163.
[46] M. Hishinuma, Y. Takahashi, H. Kanagawa, Isolation and monolayer culture of bovine oviduct epithelial cells, Nihon Juigaku Zasshi 51 (1989) 1201-1208. 10.1292/jvms1939.51.1201.

[47] M.A.M.M. Ferraz, H.H.W. Henning, P.F. Costa, J. Malda, F.P. Melchels, R. Wubbolts, T.A.E. Stout, P.L.A.M. Vos, B.M. Gadella, Improved bovine embryo production in an oviduct-on-a-chip system: prevention of poly-spermic fertilization and parthenogenic activation, Lab Chip 17 (2017) 905-916. 10.1039/c6lc01566b.
[48] M.P. Wijayagunawardane, S.P. Kodithuwakku, D. Yamamoto, A. Miyamoto, Vascular endothelial growth factor system in the cow oviduct: a possible involvement in the regulation of oviductal motility and embryo transport, Mol Reprod Dev 72 (2005) 511-520. 10.1002/mrd.20379.
[49] S. Danesh Mesgaran, J. Sharbati, R. Einspanier, C. Gabler, mRNA expression pattern of selected candidate genes differs in bovine oviductal epithelial cells in vitro compared with the in vivo state and during cell culture passages, Reprod Biol Endocrinol 14 (2016) 44. 10.1186/s12958-016-0176-7.
[50] S. Palma-Vera, R. Einspanier, J. Schoen, Bovine oviductal epithelial cells: long term culture characterization and impact of insulin on cell morphology, Reprod Biol 14 (2014) 206-212. 10.1016/j.repbio.2014.04.006.
[51] R. Gualtieri, V. Mollo, S. Braun, V. Barbato, I. Fiorentino, R. Talevi, Long-term viability and differentiation of bovine oviductal monolayers: bidimensional versus three-dimensional culture, Theriogenology 78 (2012) 1456-1464. 10.1016/j.theriogenology.2012.06.010.
[52] R. Gualtieri, V. Mollo, S. Braun, V. Barbato, I. Fiorentino, R. Talevi, Bovine oviductal monolayers cultured under three-dimension conditions secrete factors able to release spermatozoa adhering to the tubal reservoir in vitro, Theriogenology 79 (2013) 429-435. 10.1016/j.theriogenology.2012.10.014.
[53] M.P. Wijayagunawardane, N. Hambruch, J.D. Haeger, C. Pfarrer, Effect of epidermal growth factor (EGF) on the phosphorylation of mitogen-activated protein kinase (MAPK) in the bovine oviduct in vitro: Alteration by heat stress, J Reprod Dev 61 (2015) 383-389. 10.1262/jrd.2014-061.
[54] A. Cordova, C. Perreau, S. Uzbekova, C. Ponsart, Y. Locatelli, P. Mermillod, Development rate and gene expression of IVP bovine embryos cocultured with bovine oviduct epithelial cells at early or late stage of preimplantation development, Theriogenology 81 (2014) 1163-1173. 10.1016/j.theriogenology.2014.01.012.
[55] L. Jordaens, M. Arias-Alvarez, I. Pintelon, S. Thys, S. Valckx, Y. Dezhkam, P.E. Bols, J.L. Leroy, Elevated non-esterified fatty acid concentrations hamper bovine oviductal epithelial cell physiology in three different in vitro culture systems, Theriogenology 84 (2015) 899-910. 10.1016/j.theriogenology.2015.05.030.
[56] M. Hamdi, M.J. Sánchez-Calabuig, B. Rodríguez-Alonso, S. Bagés Arnal, K. Roussi, R. Sturmey, A. Gutiérrez-Adán, P. Lonergan, D. Rizos, Gene expression and metabolic response of bovine oviduct epithelial cells to the early embryo, Reproduction 158 (2019) 85-94. 10.1530/REP-18-0561.
[57] E.V. García, M. Hamdi, A.D. Barrera, M.J. Sánchez-Calabuig, A. Gutiérrez-Adán, D. Rizos, Bovine embryo-oviduct interaction, Reproduction 153 (2017) 631-643. 10.1530/REP-16-0654.
[58] A.V. Carvalho, E. Canon, L. Jouneau, C. Archilla, L. Laffont, M. Moroldo, S. Ruffini, E. Corbin, P. Mermillod, V. Duranthon, Different co-culture systems have the same impact on bovine embryo transcriptome, Reproduction 154 (2017) 695-710. 10.1530/REP-17-0449.
[59] B.P.S. Cometti, R.K. Dubey, B. Imthurn, M. Rosselli, Natural and environmental oestrogens induce TGFB1 synthesis in oviduct cells, Reproduction 155 (2018) 233-244. 10.1530/REP-17-0425.
[60] T. Greve, H. Callesen, Rendez-vous in the oviduct: implications for superovulation and embryo transfer, Reprod Nutr Dev 41 (2001) 451-459.
[61] R. Sartori, M.R. Bastos, M.C. Wiltbank, Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle, Reprod Fertil Dev 22 (2010) 151-158. 10.1071/RD09221.

[62] F.C. Dias, M.I. Khan, M.A. Sirard, G.P. Adams, J. Singh, Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle, Reproduction 146 (2013) 181-191. 10.1530/REP-13-0114.
[63] A. García Guerra, A. Tribulo, J. Yapura, J. Singh, R.J. Mapletoft, Lengthening the superstimulatory treatment protocol increases ovarian response and number of transferable embryos in beef cows, Theriogenology 78 (2012) 353-360. 10.1016/j.theriogenology.2012.02.010.
[64] P.H. Santos, R.A. Satrapa, P.K. Fontes, F.F. Franchi, E.M. Razza, F. Mani, M.F.G. Nogueira, C.M. Barros, A.C.S. Castilho, Effect of superstimulation on the expression of microRNAs and genes involved in steroidogenesis and ovulation in Nelore cows, Theriogenology 110 (2018) 192-200. 10.1016/j.theriogenology.2017.12.045.
[65] S. Ghersevich, E. Massa, C. Zumoffen, Oviductal secretion and gamete interaction, Reproduction 149 (2015) R1-R14. 10.1530/REP-14-0145.
[66] B.E. Barton, G.G. Herrera, P. Anamthathmakula, J.K. Rock, A. Willie, E.A. Harris, K.I. Takemaru, W. Winuthayanon, Roles of steroid hormones in oviductal function, Reproduction 159 (2020) R125-R137.
[67] S.E. Ulbrich, A. Kettler, R. Einspanier, Expression and localization of estrogen receptor alpha, estrogen receptor beta and progesterone receptor in the bovine oviduct in vivo and in vitro., J Steroid Biochem Mol Biol 84 (2003) 279-289. S0960076003000396 [pii].
[68] G.J. Killian, D.A. Chapman, J.F. Kavanaugh, D.R. Deaver, H.B. Wiggin, Changes in phospholipids, cholesterol and protein content of oviduct fluid of cows during the oestrous cycle, J Reprod Fertil 86 (1989) 419-426. 10.1530/jrf.0.0860419.
[69] J.W. McDaniel, H. Scalzi, D.L. Black, Influence of ovarian hormones on histology and histochemistry of the bovine oviduct, J Dairy Sci 51 (1968) 754-761. 10.3168/jds.S0022-0302(68)87067-5.
[70] S. Bauersachs, S. Rehfeld, S.E. Ulbrich, S. Mallok, K. Prelle, H. Wenigerkind, R. Einspanier, H. Blum, E. Wolf, Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle, J Mol Endocrinol 32 (2004) 449-466.
[71] K.L. Cerny, E. Garrett, A.J. Walton, L.H. Anderson, P.J. Bridges, A transcriptomal analysis of bovine oviductal epithelial cells collected during the follicular phase versus the luteal phase of the estrous cycle, Reprod Biol Endocrinol 13 (2015) 84. 10.1186/s12958-015-0077-1.
[72] A.M. Gonella-Diaza, S.C. Andrade, M. Sponchiado, G. Pugliesi, F.S. Mesquita, V. Van Hoeck, R.e.F. Strefezzi, G.R. Gasparin, L.L. Coutinho, M. Binelli, Size of the Ovulatory Follicle Dictates Spatial Differences in the Oviductal Transcriptome in Cattle, PLoS One 10 (2015) e0145321. 10.1371/journal.pone.0145321.
[73] J. Lamy, V. Labas, G. Harichaux, G. Tsikis, P. Mermillod, M. Saint-Dizier, Regulation of the bovine oviductal fluid proteome, Reproduction 152 (2016) 629-644. 10.1530/REP-16-0397.
[74] C. Almiñana, G. Tsikis, V. Labas, R. Uzbekov, J.C. da Silveira, S. Bauersachs, P. Mermillod, Deciphering the oviductal extracellular vesicles content across the estrous cycle: implications for the gametes-oviduct interactions and the environment of the potential embryo, BMC Genomics 19 (2018) 622. 10.1186/s12864-018-4982-5.
[75] S. Chen, S.E. Palma-Vera, B. Kempisty, M. Rucinski, A. Vernunft, J. Schoen, In Vitro Mimicking of Estrous Cycle Stages: Dissecting the Impact of Estradiol and Progesterone on Oviduct Epithelium, Endocrinology 159 (2018) 3421-3432. 10.1210/en.2018-00567.
[76] L.B. Ferré, M.E. Kjelland, L.B. Strøbech, P. Hyttel, P. Mermillod, P.J. Ross, Review: Recent advances in bovine, Animal 14 (2020) 991-1004. 10.1017/S1751731119002775.
[77] M.A. Sirard, 40 years of bovine IVF in the new genomic selection context, Reproduction 156 (2018) R1-R7. 10.1530/REP-18-0008.

CAPÍTULO 2

– CHAPTER 2 –

EQUINE CHORIONIC GONADOTROPIN INCREASES ESTRADIOL LEVELS IN THE BOVINE OVIDUCT AND DRIVES THE TRANSCRIPTION OF GENES RELATED TO FERTILIZATION IN SUPERSTIMULATED COWS

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Equine chorionic gonadotropin increases estradiol levels in the bovine oviduct and drives the transcription of genes related to fertilization in superstimulated cows

Authors: Patricia Kubo Fontes[1]; Eduardo Montanari Razza[1]; Antônio Guilherme Roncada Pupulim[2]; Ciro Moraes Barros[1]; Anthony César de Souza Castilho[3]*

[1] Departament of Pharmacology, Institute of Biosciences, Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil; [2] Centro Universitário Cesumar (UNICESUMAR), Maringá, Paraná, Brazil; [3] Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, São Paulo, Brazil.

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Abstract

In the bovine oviduct, estradiol (E2) stimulates secretion and cell proliferation, whereas progesterone (P4) suppresses them. In this study, we have evaluated the effect of two superstimulatory protocols (follicle-stimulating hormone [FSH] or FSH combined with equine chorionic gonadotropin [eCG]) on the oviductal levels of E2 and P4 and its outcome on oviductal cells. Compared with the control group (a single pre-ovulatory follicle), we have observed that the cows submitted to FSH/eCG treatment showed a higher concentration of E2 in the oviduct tissue, together with a higher abundance of messenger RNA encoding steroid receptors (ESR1 and progesterone receptor), and genes linked to gamete interactions and regulation of polyspermy (oviduct-specific glycoprotein 1, heat-shock protein family A member 5, α-L-fucosidase 1 [FUCA1], and FUCA2) in the infundibulum and ampulla segments of the oviduct. However, we did not observe any modulation of gene expression in the isthmus segment. Even though the FSH protocol upregulated some of the genes analyzed, we may infer that the steady effect of FSH combined with eCG on oviduct regulation might benefit fertilization and may potentially increase pregnancy rates.

Key words: gametes, female reproductive tract, cattle, superovulation, steroids, gene expression

1. Introduction

The oviduct is responsible for transporting gametes to the site of fertilization and embryos to the uterus (Besenfelder, Havlicek, & Brem, 2012; Suarez & Pacey, 2006; Talbot, Geiske, & Knoll, 1999). Additionally, the oviductal fluid plays a major role in the final maturation of gametes, gamete interactions, fertilization, and early embryo development (Besenfelder et al., 2012; Coy, García-Vázquez, Visconti, & Avilés, 2012). All these functions are performed by the segments of the oviduct (infundibulum, ampulla, and isthmus), which undergoes significant morphological, biochemical, and physiological changes during the estrous cycle in cows (Binelli, Gonella-Diaza, Mesquita, & Membrive, 2018).

The steroid hormones, estradiol (E2) and progesterone (P4), are the main factors controlling changes in the oviduct (Binelli et al., 2018). Temporal changes in steroid concentrations during the estrous cycle induce modulation of the oviductal transcriptional profile (Bauersachs et al., 2004; Binelli et al., 2018; Cerny, Garrett, Walton, Anderson, & Bridges, 2015). In general, E2 activity is associated with the stimulation of oviductal secretory cells, and cell proliferation, whereas P4 exhibits a suppressive effect (Binelli et al., 2018; Gonella-Diaza et al., 2017). A detrimental effect of supra-physiological P4 concentrations in the female reproductive tract as a result of ovarian superstimulation (OVS) protocols has been reported in cows (Forde et al., 2012; Gad et al., 2011; Roque et al., 2013). Recently, Santos et al. (2018) reported higher plasma levels of E2 during the pre-ovulatory phase in cows submitted to the OVS protocol using FSH with equine chorionic gonadotropin (eCG; FSH/eCG protocol) as compared to the control cows (single ovulation). Based on the general role of steroids in the oviduct, we hypothesized that the cows submitted to the OVS protocol show higher levels of E2 and that the genes involved in bovine oviductal functions can be stimulated in these cows.

The aim of this study was to evaluate E2 levels in cows submitted to OVS protocols during the pre-ovulatory stage. However, rather than evaluating plasma E2

levels, we measured local oviductal E2 levels as the latter is expected to be higher than the former (Lamy, Liere, et al., 2016; Wijayagunawardane et al., 1998). This suggests that the local oviductal hormone measurement may be a more reliable indicator of the effect of E2 on oviductal cells. Additionally, we have also assessed the transcript abundance of steroid receptors and genes related to gamete interactions and regulation of polyspermy in the oviduct segments.

2. Results

2.1. Direct effects of hormones from the OVS protocols on the oviduct

Thirty Nelore cows were equally divided into three experimental groups: cows submitted to OVS with FSH protocol (n = 10), cows submitted to OVS with FSH associated with eCG (which has a dual effect on FSH and LH receptors, n = 10), and hormonally synchronized cows (control, n = 10). First, we investigated the potential direct effects of the gonadotropins used in the OVS protocols on the oviductal cells. Detection of mRNA was carried out individually for each of the oviduct segment (infundibulum, ampulla, and isthmus). The mRNA abundance of gonadotropin receptors (*FSHR* and *LHCGR*) in all the bovine oviduct samples was highly variable with low concentration levels or undetectable, and, therefore, could not be analyzed. This was true for all the experimental groups, resulting in no difference among treatments.

2.2. Effects of the OVS protocols on sex steroid hormones

Although the gonadotropins have been reported to modulate some processes of oviductal cells cultured *in vitro* (Li et al., 2014; Wijayagunawardane, Kodithuwakku, DE Silva, & Miyamoto, 2009; Wijayagunawardane, Kodithuwakku, Yamamoto, & Miyamoto, 2005; Wijayagunawardane et al., 2001), E2 and P4 are the main regulators of oviductal functions and modifications (Binelli et al., 2018). Therefore, we have

quantified steroid hormone concentrations in the follicular fluid, the major source of E2 and P4. The E2 concentration in the follicular fluid of pre-ovulatory follicles was higher in cows submitted to the FSH/eCG protocol (n = 10 follicles, one follicle per cow) than in the control group (n = 9 follicles, one follicle per cow; Figure 1A). P4 concentration did not vary among the groups (Figure 1C).

To further investigate the effect of sex steroid hormones on oviductal cells, we measured the hormonal levels in oviduct cell homogenates. Higher E2 concentrations were observed in the oviducts of cows submitted to the FSH/eCG protocol (n = 5) when compared with the control group (n = 5; Figure 1B), as was the case with the follicular fluid. Again, no differences in P4 concentration were observed among the groups (Figure 1C). To evaluate the capacity of oviductal cells to produce E2, we analyzed the transcript levels of aromatase (*CYP19A1*), which codes for the enzyme that catalyzes the last step in E2 biosynthesis. *CYP19A1* mRNA was not detected in any of the oviductal segments, confirming that E2 is not produced by oviductal cells.

To further understand the key roles of steroids in the bovine oviduct, we investigated the expression levels of mRNA encoding the steroid receptors *ESR1, ESR2*, and *PGR.* In summary, we found higher levels of *ESR1* in the ampulla of cows submitted to the FSH/eCG protocol (n = 4), relative to the control group (n = 5; Figure 1D, Table S1), as well as higher levels of *PGR* in the infundibulum and ampulla of cows submitted to both the OVS treatments relative to the control group (Figure 1D, Table S1).

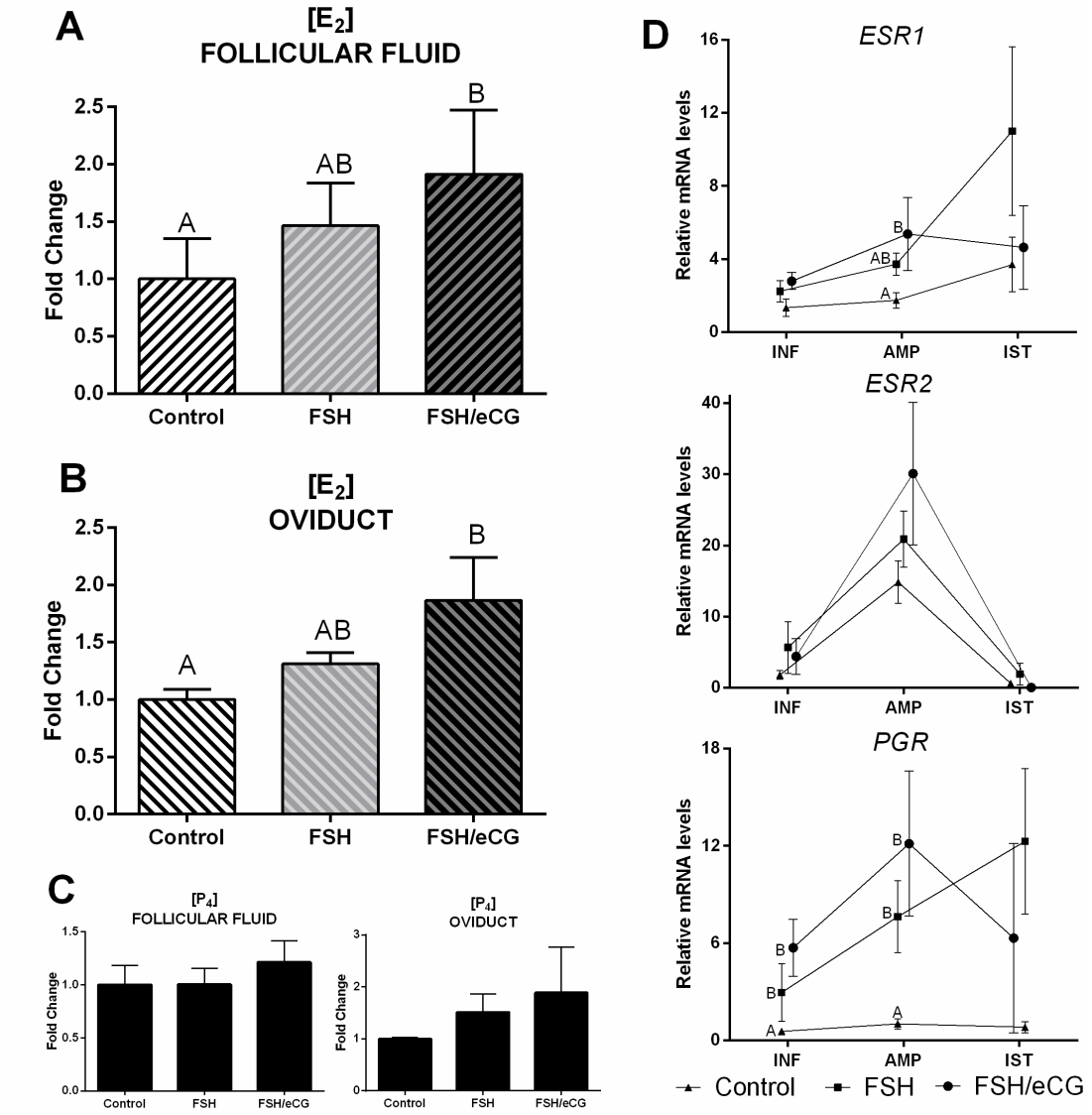

Figure 1. Sex steroid parameters in the superstimulated groups (FSH and FSH/eCG) and the control group (single pre-ovulatory follicle). A) E2 levels in the follicular fluid (n = 9 follicles in the control group; n = 10 follicles in each OVS group). B) E2 levels in the oviduct (n = 5 oviduct/group). C) P4 levels in the follicular fluid and oviduct samples. All hormonal measurements are presented as fold change compared to the control group ± standard error of the mean. D) Sex steroid receptor transcripts abundance (*ESR1, ESR2,* and *PGR*) in the infundibulum, ampulla, and isthmus from control (n = 5), FSH (n = 5), and FSH/eCG (n = 4) groups. Transcript abundance data are presented as mean of the relative mRNA levels (target gene/geometric mean of reference genes [*18S* and *H2A*]). Different letters (A, B) indicate significant differences ($p < 0.05$) between the groups. [E2]: estradiol concentration; [P4]: progesterone concentration; INF: infundibulum; AMP: ampulla; IST: isthmus.

2.3. Effects of the OVS protocols on the abundance of genes related to fertilization

Finally, we have investigated whether the increase in E2 levels in the cows submitted to FSH/eCG protocol was involved in oviductal functionality by quantifying the abundance of genes related to fertilization. The composition of the oviductal fluid is complex (Pillai, Weber, Phinney, & Selvaraj, 2017) and can be differently regulated during the estrous cycle stages (Lamy, Labas, et al., 2016). Some of the factors, such as Oviduct-specific glycoprotein 1 (OVGP1), heat-shock protein family A member 5 (HSPA5), and alpha-L-fucosidase (FUCA) have been reported to become highly abundant as the time of ovulation approaches (Bauersachs et al., 2004; Buhi, 2002a; Carrasco, Romar, Aviles, Gadea, & Coy, 2008; Lamy, Labas, et al., 2016).

We have found that all the four genes – *OVGP1*, *HSPA5*, *FUCA1*, and *FUCA2* –were up-regulated in the infundibulum and ampulla of cows submitted to the FSH/eCG protocol (n = 4) relative to the control group (n = 5; Figure 2, Table S1). In the cows submitted to the FSH treatment (n = 5), *OVGP1* mRNA levels in the infundibulum and ampulla and *HSPA5* mRNA levels in the ampulla were higher relative to the control group (Figure 2, Table S1). The treatments did not have any effects on the transcript levels in the isthmus.

3. Discussion

In the present study, we investigated the effect of two OVS protocols on bovine oviductal cells. The use of eCG in combination with FSH during the OVS protocol resulted in a considerable increase in local E2 concentration, and the effect appeared to be segment-specific in the bovine oviduct, with an increase in the mRNA abundance of the steroid receptors (*ESR1* and *PGR*) and genes related to gamete interaction and fertilization (*OVGP1*, *HSPA5*, *FUCA1*, and *FUCA2*). In contrast, P4 concentration was similar among the different groups, as demonstrated in other studies that measured the

P4 levels during the pre-ovulatory stage (Dias, Khan, Sirard, Adams, & Singh, 2013; Lamy, Liere, et al., 2016; Wijayagunawardane et al., 1998).

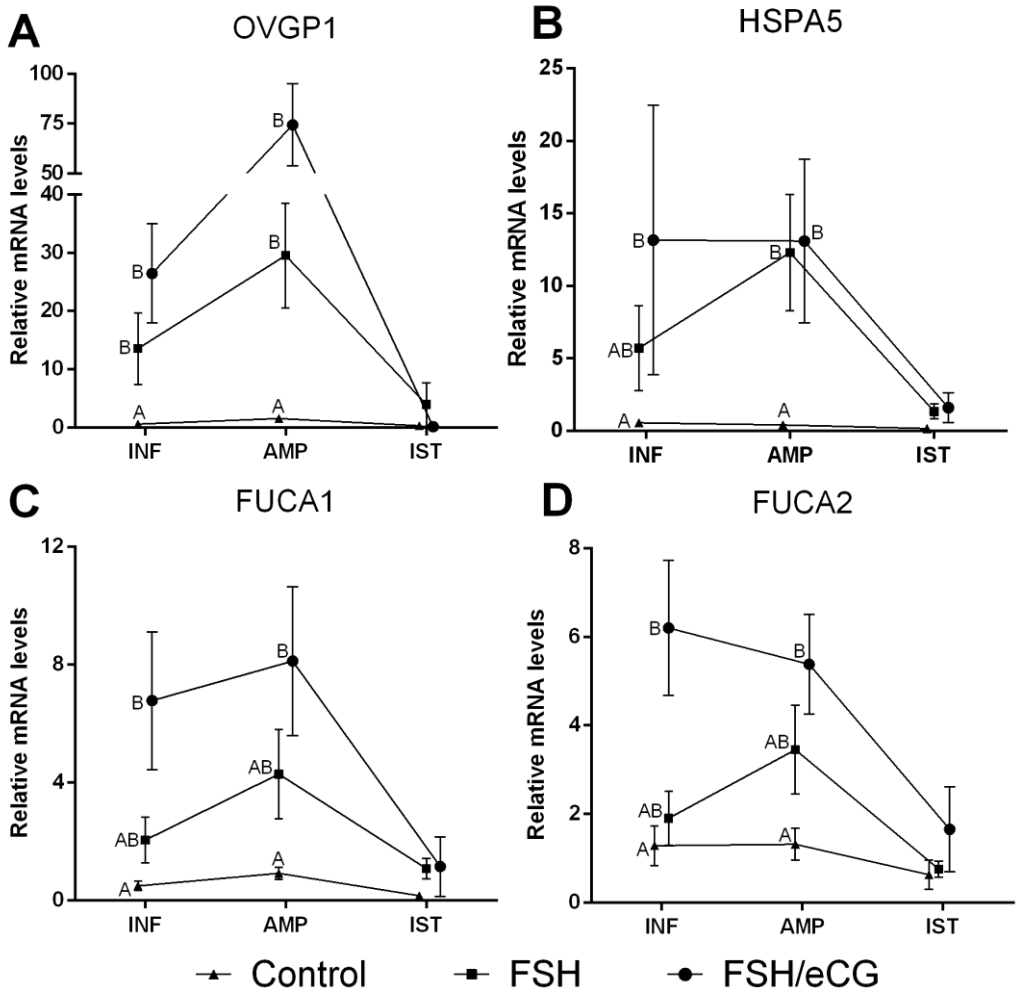

Figure 2. Transcript abundance of the genes related to gamete interactions and the fertilization process. Transcript abundance of A) *OVGP1*, B) *HSPA5*, C) *FUCA1*, and D) *FUCA2* in the infundibulum, ampulla, and isthmus segments from all the three treatments. Data are presented as mean of the relative mRNA levels (target gene/geometric mean of reference genes [*18S* and *H2A*]). Different letters (A, B) indicate a significant difference ($p < 0.05$) between groups: control (n = 5, circle), FSH (n = 5, square), and FSH/eCG (n = 4, triangle). INF: infundibulum; AMP: ampulla; IST: isthmus.

Follicle development is correlated to an FSH surge (Adams, Matteri, Kastelic, Ko, & Ginther, 1992; Fortune, 1994). During follicular deviation in cattle, the decreasing FSH levels and the inhibitory factors released from the dominant follicle inhibit acquisition of ovulatory capacity in the next follicle (Wiltbank, Fricke, Sangsritavong,

Sartori, & Ginther, 2000). Based on this, treatment with FSH and/or eCG (which stimulates both the LH and FSH receptors (Murphy & Martinuk, 1991)) has been adopted in several OVS protocols because of their capacity to stimulate follicle development until the follicles acquire ovulatory capacity. To assess the possible direct effect of the gonadotropins used for the induction of multiple ovulations, we evaluated the transcript abundance of gonadotropin receptors (*FSHR* and *LHCGR*) in the oviduct of cows submitted or not submitted to the FSH or FSH/eCG protocols. In the present study, both the receptors showed variable or undetectable expression in many samples, suggesting that there is no potential direct contribution of FSH and eCG to bovine oviductal cells during the pre-ovulatory period in cattle.

Several studies have shown a possible role for LH in the bovine oviduct related to its stimulatory effect on oviductal contraction mediated by the vascular endothelial growth factor (Wijayagunawardane et al., 2005), angiotensin (Wijayagunawardane et al., 2009), and prostaglandins pathways (Wijayagunawardane, Choi, et al., 1999; Wijayagunawardane, Miyamoto, & Sato, 1999; Wijayagunawardane et al., 2001) as well as the secretion of oviductal glycoprotein (Sun, Lei, & Rao, 1997). Moreover, there are reports of detection of *LHCGR* and *FSHR* transcripts and their modulation by growth factors in the bovine oviduct (Li et al., 2014). However, all the information is based on *in vitro* epithelial cell culture samples. In contrast, LHR and FSHR proteins are well described in mice (Zhang, Shi, Segaloff, Van Voorhis, & Zheng, 2001), pigs (Gawronska, Paukku, Huhtaniemi, Wasowicz, & Ziecik, 1999; Małysz-Cymborska & Andronowska, 2016), sheep (Wang, Zhang, Gao, & Wang, 2012), and humans (Zheng, Magid, Kramer, & Chen, 1996). Therefore, to understand their function, the bovine LHR and FSHR transcripts and proteins should be further characterized *in situ* in oviductal cells, including splice variants.

Several studies have investigated the role of steroid hormones as major regulators of oviductal functions (Bauersachs et al., 2004; Binelli et al., 2018; Lamy, Labas, et al., 2016). Santos et al. (2018) recently reported higher plasma levels of E2

during the pre-ovulatory phase in cows submitted to the FSH/eCG protocol relative to control animals (single ovulation). Together, these data encouraged us to investigate the effect of OVS protocols in the oviductal cells. However, instead of evaluating E2 plasma levels, we measured its local oviductal concentrations, as measuring the local oviductal hormone levels may be a more reliable way to evaluate the effect of E2 on oviductal cells. The local concentrations of steroid hormones in the bovine oviduct can be ten to fifty-folds higher than the concentrations found in the circulating blood (Lamy, Liere, et al., 2016; Wijayagunawardane et al., 1998) due to local countercurrent transfer of ovarian products (including the transfer of steroids to the oviduct) (Einer-Jensen & Hunter, 2005; Zervomanolakis et al., 2009), resulting in distinction between ipsilateral and contralateral oviducts in monovulatory animals (Lamy, Liere, et al., 2016; Wijayagunawardane et al., 1998). In our study, the oviductal levels of E2 were higher in the cows submitted to the FSH/eCG protocol relative to the control group. Corroborating previous hypothesis that oviductal hormone levels may be influenced by the local countercurrent system (Einer-Jensen & Hunter, 2005; Zervomanolakis et al., 2009), in this study, E2 levels in the follicular fluid were also found to be higher in cows submitted to the FSH/eCG protocol relative to the animals from the control group.

Besides countercurrent system, local synthesis of steroids by the oviductal tissue is another mechanism postulated to control the levels of E2 in the oviduct. In agreement with this, the presence of steroidogenic enzymes, including aromatase (that irreversibly converts androgens into estrogens (Grumbach & Auchus, 1999)), has been detected in the oviduct of frog (Kobayashi, Zimniski, & Smalley, 1996), mare (Nelis et al., 2016), and pig (Martyniak, Franczak, & Kotwica, 2018). However, to date, the presence of aromatase in the bovine oviduct has not been reported. Similar to the reports of Ulbrich *et al.* (2003), aromatase transcripts were not detected in the oviductal samples from any experimental group in our study, suggesting that there is no local E2 synthesis in cattle.

Steroid hormones exert their effects through their intracellular receptors via a classical mechanism involving members of the nuclear receptor superfamily, namely estrogen receptor α and β (ERα and ERβ) and the progesterone receptor (PR), which are transcribed from three genes: *ESR1*, *ESR2*, and *PGR*, respectively (Binelli et al., 2018). E2 stimulation up-regulated only *ESR1* and *PGR* but not *ESR2* (Kowalik, Martyniak, Rekawiecki, & Kotwica, 2016; Lee & Gorski, 1996, 1998; Ulbrich et al., 2003). In agreement with this, variation in *ESR2* levels was not observed among the different groups, whereas higher levels of *ESR1* and *PGR* were detected in the oviducts of cows submitted to the FSH/eCG protocol than to the control group, potentially due to increased oviductal levels of E2. This effect was shown in the infundibulum (*PGR*) and ampulla (*ESR1* and *PGR*), but not in the isthmus, demonstrating the specificity of each segment of the oviduct.

The oviduct plays specific roles in gamete maturation, fertilization, and early embryo development, where the oviductal fluid has a pivotal function. The oviductal fluid has a complex composition (Lamy, Labas, et al., 2016; Pillai et al., 2017) consisting mainly of plasma transudate and derivatives from the secretory epithelium (Leese, 1988). Some proteins produced by the oviductal cells have been reported to regulate gamete interactions and fertilization. The stimulatory effect of E2 on oviduct fluid secretion (Ghersevich, Massa, & Zumoffen, 2015) results in the production of the greatest volume of fluid by the oviduct during estrus (Killian, 2011). Proteins like OVGP1, HSPA5 (also known as GRP78), FUCA1, and FUCA2 have been associated with spermatozoa and/or the cumulus-oocyte complex (Boilard et al., 2004; Coy et al., 2008; Phopin, Nimlamool, Bartlett, & Bean, 2012; Phopin et al., 2013), acting on polyspermy control by hardening the *zona pellucida* before fertilization (Coy et al., 2008; Miller, Gong, Decker, & Shur, 1993; Mondéjar, Martínez-Martínez, Avilés, & Coy, 2013), and modulating sperm-*zona pellucida* interactions, indicating an active role in the oviduct during the fertilization process (Coy et al., 2008; Marín-Briggiler et al., 2010; Romero-Aguirregomezcorta, Matás, & Coy, 2015; Venditti, Swann, & Bean, 2010). In

this study, owing to the increase in local E2 levels in cows submitted to the FSH/eCG superstimulation protocol, we analyzed the transcript levels of genes involved in fertilization and gamete interactions.

Several authors (Bauersachs et al., 2004; Buhi, 2002b; Buhi, Alvarez, & Kouba, 2000; Carrasco, Coy, Avilés, Gadea, & Romar, 2008; Fontes et al., 2018; Lamy, Labas, et al., 2016) have reported that *OVGP1, HSPA5, FUCA1,* and *FUCA2* levels are stimulated by E2 during the periovulatory period. In our study, the increased levels of E2 resulted in the up-regulation of *OVGP1*, *HSPA5*, *FUCA1*, and *FUCA2* in the infundibulum and ampulla of the cows submitted to the FSH/eCG protocol relative to the control animals, and no effect of the treatments was observed in the isthmus. This latter result agrees with the findings of other studies showing that each oviductal region responded differently to E2 stimulation, and that transcription in the isthmus does not change under estrogen treatment (Buhi et al., 2000).

Two OVS protocols were the targets of our study. As we have demonstrated, the FSH/eCG protocol altered all the parameters evaluated in the oviduct samples in comparison with the control group (single pre-ovulatory follicle), while the regulation by the FSH protocol was inconsistent, either with positive or null effects on transcript abundance in the bovine oviduct. Although a subtle effect of FSH protocol on bovine oviduct regulation cannot be discarded, the stable effect of FSH/eCG protocol on the oviduct reinforces the benefits of replacing the last two doses of FSH by eCG to the ovarian superstimulation technology in Nelore cows. In other studies, the FSH/eCG protocol, in comparison with the FSH protocol, increases the total number of oocytes/embryo recovery (Barros et al., 2007). Moreover, the number and percentage of transferable embryos were greater for the FSH/eCG females than for the FSH females (Mattos et al., 2011). In other words, the embryo quality was improved with FSH/eCG treatment and it could be associated with the modifications in the proteins produced by the oviduct.

Considering the information presented in this study regarding the effect of steroid hormones on oviduct function, we strongly suggest measuring hormones levels in the oviductal cells rather than in the plasma. We have demonstrated that the OVS protocol with FSH/eCG results in increased E2 levels in the follicular fluid from the pre-ovulatory stage and we suggest that increased E2 levels may reflect a higher concentration of E2 in the oviduct due to the countercurrent system. Finally, we propose a mechanism whereby higher levels of E2 regulate oviductal gene expression to guarantee the successful fertilization of multiple oocytes in superstimulated cows (Figure 3).

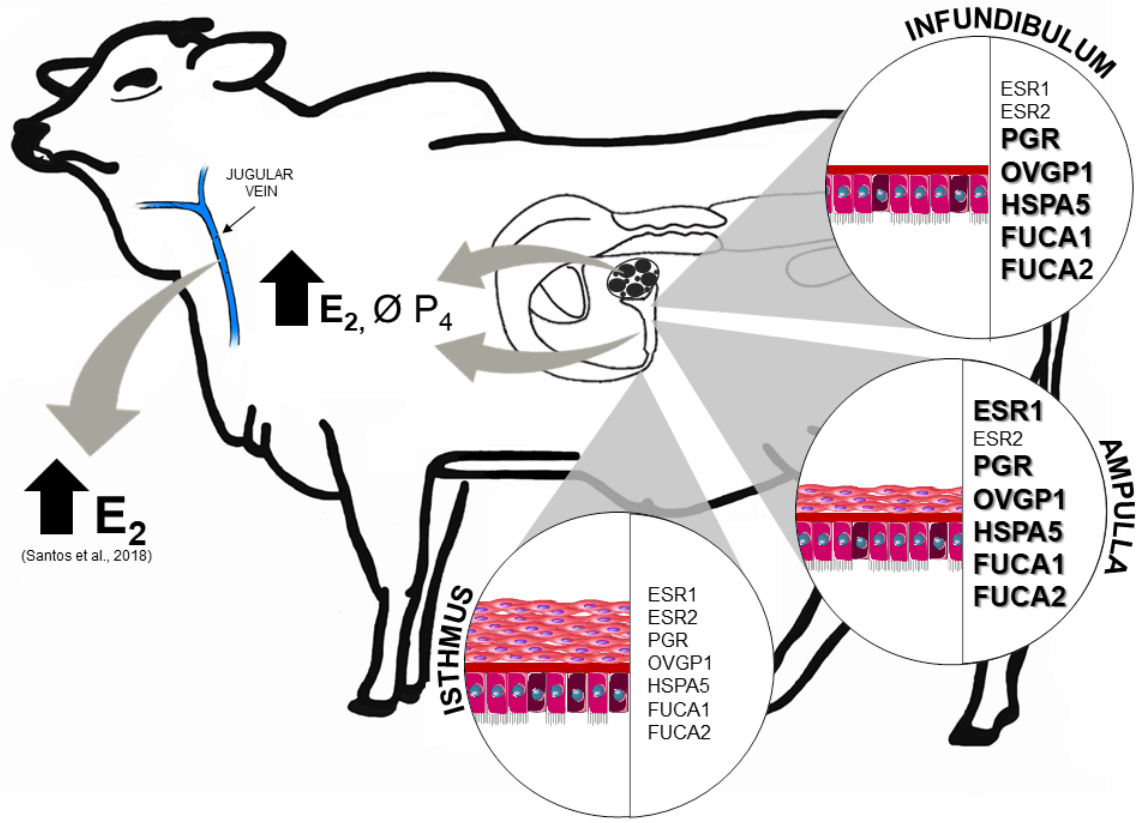

Figure 3. General considerations of the cows submitted to the FSH/eCG protocol compared to the control group. The use of eCG in the OVS protocols has been shown to increase the plasma levels of E2 relative to non-superstimulated cows during the pre-ovulatory period (Santos et al., 2018). We further analyzed the sex steroid hormone levels in the follicular fluid and oviduct and found higher levels of E2 but similar levels of P4 in stimulated animals as compared to non-stimulated ones. We suggest that the source of E2 present in the oviduct is from the countercurrent system. This higher level of E2 in the oviductal cells may stimulate the transcription of the estradiol receptor (*ESR1*) in the ampulla and the progesterone receptor (*PGR*) in the infundibulum and ampulla, and the genes associated with the fertilization process (*OVGP1, HSPA5, FUCA1,* and *FUCA2*) in the infundibulum and ampulla. However, differences in the transcript levels between the FSH/eCG and control groups were not

observed in the isthmus segment. E2: estradiol; P4: progesterone; bold font (*ESR1, PGR, OVGP1, HSPA5, FUCA1,* and *FUCA2*) indicates higher levels in the FSH/eCG than control group; regular font indicates no difference between groups.

Based on all the findings, we concluded that ovarian superstimulation using FSH combined with eCG up-regulates E2 levels in oviductal cells and positively drives the transcription profile of steroid receptors (*ESR1* and *PGR*) and the genes associated with fertilization (*OVGP1, HSPA5, FUCA1,* and *FUCA2*) in the infundibulum and ampulla segments of oviducts of Nelore cows. We may infer that such effects triggered by superstimulation with FSH combined with eCG might benefit fertilization and potentially increase pregnancy rates.

4. Material and methods

All experimental procedures involving animals were approved by the Ethics Committee on the Use of Animals of the Biosciences Institute of the São Paulo State University (IB/UNESP-CEUA, number 379). The protocol was in accordance with the ethical principles in animal research.

4.1. Experimental groups

Thirty Nelore (*Bos taurus indicus*) non-lactating multiparous cows ranging from 5 to 7 years of age with body condition scores ranging from 2.0 to 3.5 were randomly distributed in three experimental groups (n = 10 animals/group): ovarian superstimulation with FSH (FSH group), ovarian superstimulation with FSH and eCG (FSH/eCG group), and control group (hormonally synchronized, single pre-ovulatory follicle). These protocols are used commercially for *Bos taurus indicus*. This study was conducted on a farm located in Santa Cruz do Rio Pardo (São Paulo, Brazil; 22º 53′ 56″ S, 49º 37′ 57″ W; elevation 467 m). The cows were maintained on pasture (*Brachiaria brizantha*) with *ad libitum* access to water and a mineral supplement. The hormonal and protocol details are shown in Figure 4.

Briefly, at a random stage of the estrous cycle designated as Day 0 of the protocols, all cows received a progesterone-releasing vaginal device (1.0 g, PRIMER®, Tecnopec, São Paulo, Brazil) and IM administration of estradiol benzoate (EB, 2.5 mg, Estrogin®, Farmavet, São Paulo, Brazil). Cows from the FSH group received pFSH (a total of 200 mg, Folltropin®-V, Bioniche Animal Health, Belleville, ON, Canada) twice daily for four consecutive days (40%, 30%, 20%, and 10% of the total amount was administered from Day 5 to Day 8, respectively). For cows from the FSH/eCG group, the last two doses of FSH were replaced with two doses of eCG (a total of 400 IU, IM, Novormon®, Syntex, Buenos Aires, Argentina). All the cows received IM administration of a PGF2α analogue (d-cloprostenol, 150 mg, Prolise®, Tecnopec, São Paulo, SP, Brazil) on Day 7 (superstimulated groups) or Day 8 (control group) and the progesterone-releasing vaginal inserts were removed at 7 p.m. on Day 8.

All the cows were slaughtered in a commercial abattoir at 7 a.m. on Day 9 of the protocol, i.e., at the supposed time of exogenous LH administration if continuing with the protocol. Three or four animals, from each experimental group, were slaughtered in each of the three independent sessions. Due to a possibility of an endogenous LH surge, blood samples were collected from the jugular vein on Day 8 (7 p.m.) and Day 9 (7 a.m.) to quantify the plasma concentration of LH and verify an endogenous LH surge. None of the cows were excluded from the experiment as the animals did not present an endogenous LH surge.

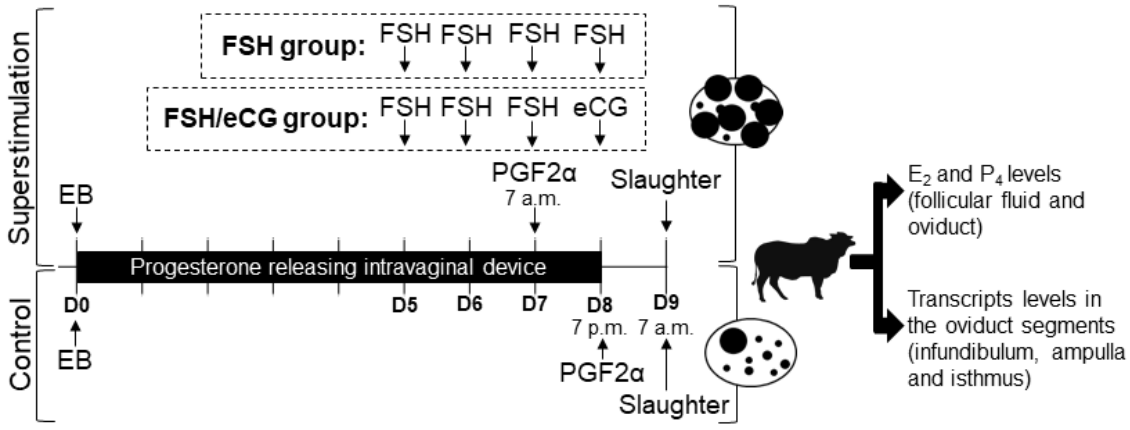

Figure 4. Experimental design. At a random stage of the estrous cycle, all 30 animals received a progesterone releasing intravaginal device (PRIMER) with administration of EB (Day 0). Ovarian superstimulation was performed with two derivation protocols: the FSH group (received FSH twice daily from Day 5 to Day 8) and the FSH/eCG group (the last two doses of FSH were replaced with two doses of eCG). A PGF2α agonist was administered to the cows submitted to superstimulation protocols on Day 7 (7 a.m.) and in cows from the control group (ovulation synchronized) on Day 8 (7 p.m.). All animals had the PRIMER removed on Day 8 (7 p.m.) and were slaughtered on Day 9 (7 a.m.). Cows submitted to ovarian superstimulation protocols presented multiple pre-ovulatory follicles and cows submitted to ovarian synchronization presented one pre-ovulatory follicle. Hormonal levels (E2 and P4) were measured in the follicular fluid and local oviduct and the transcript abundance was evaluated in a tissue section from each oviduct segments (infundibulum, ampulla, and isthmus). EB: estradiol benzoate; PGF2α: Prostaglandin F2 alpha; D: Day.

4.2. Sample collection

After the animals were slaughtered, the reproductive tracts of all the cows were transported to the laboratory on ice within 2 h in a bag containing saline. The samples were processed for follicular fluid hormonal analysis, oviductal supernatant hormonal analysis, and quantification of the abundance of oviductal transcripts (detailed description below, Figure 4).

4.2.1. Follicular fluid collection

The pre-ovulatory follicles were dissected from isolated ovaries. Using a caliper, two perpendicular measurements gave the average diameter of each follicle. The follicle diameter for all the cows ranged from 11 to 14 mm. Approximately 20 follicles were present in each superstimulated cow and the number did not differ between the FSH and FSH/eCG groups. Follicular fluid was collected using a needle and syringe. The samples were centrifuged at 7000 ×*g* for 10 min to remove cellular debris and the supernatant was stored in -80 ºC. The follicular fluid from the dominant follicle was collected from the cows belonging to the control group (n = 9; one follicle was lost during the dissection process) and from one of the three largest follicles from the FSH and FSH/eCG groups (n = 10 follicles/group).

4.2.2. Oviductal tissue collection for measurement of hormonal levels

The oviducts (n = 5/group) ipsilateral to the pre-ovulatory follicle from the control animals and one of the oviducts from the superstimulated cows (n = 5/group), were isolated from the reproductive tract. The surrounding connective tissues and infundibulum were trimmed with only the tubal portion of the oviduct remaining. The samples were processed according to Wijayagunawardane *et al.* (1998) with some modifications. Each sample was weighed, minced, and homogenized separately in CK28-R tubes (2 mL, with ceramic beads) with a Precellys® homogenizer (Bertin Technologies, Montigny le Bretonneux, France) for three cycles of 30 s at 6800 rpm with 15 s intervals. The samples were then centrifuged for 10 min at 570 ×*g* at 4 ºC and the supernatant was stored at -20 ºC.

4.2.3. Oviductal tissue collection to RT-qPCR

The ipsilateral oviducts from control animals (n = 5) and one randomly selected oviduct from each cow superstimulated either with FSH (n = 5) or FSH/eCG (n = 4, one cow was excluded from analysis due to low quality of the RNA sample) were isolated from the reproductive tract and the surrounding connective tissues were trimmed. A section (whole tissue) of each of segments, infundibulum, ampulla, and isthmus were collected separately from each oviduct and the transition regions were discarded (as previously described (Gonella-Diaza et al., 2015). The samples were placed in TRIzol® (Invitrogen, São Paulo, SP, Brazil) and homogenized with a Polytron® homogenizer (Ultraturrax, Luzern, Switzerland). Total RNA was extracted according to the manufacturer's protocol and stored at -80 °C. RNA concentrations were quantified using a spectrophotometer (Nanodrop; Thermo Fisher Scientific, Waltham, MA, USA).

4.3. Hormonal assays

The E2 and P4 concentrations in the follicular fluid and oviduct samples were measured by chemiluminescence, a solid-phase competitive immunoassay (Immulite®

1000; Siemens Flanders, New Jersey, USA) as reported previously (Santos et al., 2018). All follicular fluid samples were diluted ten-fold for the E2 assay. For the E2 assay, the concentration ranged from 20 to 2000 pg/mL and sensitivity was 15 pg/mL; for the P4 assay, the concentration ranged from 0.2 to 40 ng/mL and sensitivity was 0.2 ng/mL.

4.4. Quantitative PCR

Total RNA (1 µg) from each sample was incubated with DNAse I (Invitrogen, São Paulo, SP, Brazil) and then reverse transcribed with SuperScript® III (Invitrogen, São Paulo, SP, Brazil) using Oligo-d(T) primers, following the manufacturer's instructions. The cDNA obtained was diluted eight-fold and stored at -20 °C to be used for gene expression assays by qPCR.

Real time PCR analysis was performed using StepOne Plus® (Life Technologies, Carlsbad, CA) with the Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). Primers were designed using bovine-specific sequences (Table S2). The reactions were optimized by primer dilution and an annealing temperature gradient to achieve maximum amplification efficiency for each gene (90-110%). The reaction volume was 25 µL with 1 µL of each sample and variable primer concentrations (Table S2). PCR cycling conditions were 95° C for 10 min, followed by 40 cycles of 95° C for 10 s, and annealing and extension for 1 min at specific temperature to each gene (Table S2). Each sample was analyzed in duplicates and the specificity of each PCR product was determined by melting curve analysis and amplicon size determination on agarose gels. Negative template control (NTC) was run with every plate.

To select reference genes, the GeNorm and Normfinder software were used to evaluate gene variability. An ANOVA was then performed with Cq (quantification cycle) values to evaluate the inter-group variability. Seven reference genes candidates were tested (Table S2): *18S, ACTB, GAPDH, H2A, PPIA, RPL15*, and *RPL30*. The relative

expression of target genes was calculated from the geometric mean of the most stable reference genes (*18S* and *H2A*) using the ΔΔCt method (Livak & Schmittgen, 2001). Ten target genes were analyzed (Table S2): hormone receptors (*LHCGR, FSH, ESR1, ESR2*, and *PGR*), a steroidogenic enzyme (*CYP19A1*), and genes related to the fertilization process (*OVGP1, HSPA5, FUCA1*, and *FUCA2*).

4.5. Statistical analysis

The effect of ovarian superstimulation on E2 and P4 concentrations (follicular fluid and oviduct) and on transcript abundance in the infundibulum, ampulla, and isthmus were tested by ANOVA and comparisons of means were performed with the Tukey–Kramer HSD test. The hormone concentrations are presented as fold change compared to the control group. The data are presented as the mean ± standard error of the mean (S.E.M.). The analyses were performed using JMP software (SAS Institute, Cary, NC, USA). Differences were considered significant when $p < 0.05$.

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References

Adams, G. P., Matteri, R. L., Kastelic, J. P., Ko, J. C., & Ginther, O. J. (1992). Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *J Reprod Fertil, 94*(1), 177-188.

Barros, C. M., Barcelos, A. C. Z., Gouvêa, L. M., Meneghel, M., Barcelos, D. S., Barcelos, L. N., & Trinca, L. A. (2007). Improvement of a superstimulatory protocol in Nelore cows: replacing the last two doses of pFSH by eCG. *Reproduction, Fertility and Development, 20*(1), 152-152. Retrieved from doi:doi.org/10.1071/RDv20n1Ab143

Bauersachs, S., Rehfeld, S., Ulbrich, S. E., Mallok, S., Prelle, K., Wenigerkind, H., . . . Wolf, E. (2004). Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. *J Mol Endocrinol, 32*(2), 449-466.

Besenfelder, U., Havlicek, V., & Brem, G. (2012). Role of the oviduct in early embryo development. *Reprod Domest Anim, 47 Suppl 4*, 156-163. doi:10.1111/j.1439-0531.2012.02070.x

Binelli, M., Gonella-Diaza, A. M., Mesquita, F. S., & Membrive, C. M. B. (2018). Sex Steroid-Mediated Control of Oviductal Function in Cattle. *Biology (Basel), 7*(1). doi:10.3390/biology7010015

Boilard, M., Reyes-Moreno, C., Lachance, C., Massicotte, L., Bailey, J. L., Sirard, M. A., & Leclerc, P. (2004). Localization of the chaperone proteins GRP78 and HSP60 on the luminal surface of bovine oviduct epithelial cells and their association with spermatozoa. *Biol Reprod, 71*(6), 1879-1889. doi:10.1095/biolreprod.103.026849

Buhi, W. C. (2002a). Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction, 123*(3), 355-362.

Buhi, W. C. (2002b). Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction, 123*(3), 355-362.

Buhi, W. C., Alvarez, I. M., & Kouba, A. J. (2000). Secreted proteins of the oviduct. *Cells Tissues Organs, 166*(2), 165-179. doi:10.1159/000016731

Carrasco, L. C., Coy, P., Avilés, M., Gadea, J., & Romar, R. (2008). Glycosidase determination in bovine oviducal fluid at the follicular and luteal phases of the oestrous cycle. *Reprod Fertil Dev, 20*(7), 808-817.

Carrasco, L. C., Romar, R., Aviles, M., Gadea, J., & Coy, P. (2008). Determination of glycosidase activity in porcine oviductal fluid at the different phases of the estrous cycle. *Reproduction, 136*(6), 833-842. doi:10.1530/REP-08-0221

Cerny, K. L., Garrett, E., Walton, A. J., Anderson, L. H., & Bridges, P. J. (2015). A transcriptomal analysis of bovine oviductal epithelial cells collected during the follicular phase versus the luteal phase of the estrous cycle. *Reprod Biol Endocrinol, 13*, 84. doi:10.1186/s12958-015-0077-1

Coy, P., Cánovas, S., Mondéjar, I., Saavedra, M. D., Romar, R., Grullón, L., . . . Avilés, M. (2008). Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proc Natl Acad Sci U S A, 105*(41), 15809-15814. doi:10.1073/pnas.0804422105

Coy, P., García-Vázquez, F. A., Visconti, P. E., & Avilés, M. (2012). Roles of the oviduct in mammalian fertilization. *Reproduction, 144*(6), 649-660. doi:10.1530/REP-12-0279

Dias, F. C., Khan, M. I., Sirard, M. A., Adams, G. P., & Singh, J. (2013). Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle. *Reproduction, 146*(2), 181-191. doi:10.1530/REP-13-0114

Einer-Jensen, N., & Hunter, R. (2005). Counter-current transfer in reproductive biology. *Reproduction, 129*(1), 9-18. doi:10.1530/rep.1.00278

Fontes, P. K., Ereno, R. L., Peixoto, A. R., Carvalho, R. F., Scarano, W. R., Trinca, L. A., . . . Castilho, A. C. S. (2018). Can the antral follicular count modulate the gene expression of bovine oviducts in Aberdeen Angus and Nelore heifers? *PLoS One, 13*(8), e0202017. doi:10.1371/journal.pone.0202017

Forde, N., Carter, F., di Francesco, S., Mehta, J. P., Garcia-Herreros, M., Gad, A., . . . Lonergan, P. (2012). Endometrial response of beef heifers on day 7 following insemination to supraphysiological concentrations of progesterone associated with superovulation. *Physiol Genomics, 44*(22), 1107-1115. doi:10.1152/physiolgenomics.00092.2012

Fortune, J. E. (1994). Ovarian follicular growth and development in mammals. *Biol Reprod, 50*(2), 225-232.

Gad, A., Besenfelder, U., Rings, F., Ghanem, N., Salilew-Wondim, D., Hossain, M. M., . . . Hölker, M. (2011). Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: implications for animal breeding and

human assisted reproduction. *Hum Reprod, 26*(7), 1693-1707. doi:10.1093/humrep/der110

Gawronska, B., Paukku, T., Huhtaniemi, I., Wasowicz, G., & Ziecik, A. J. (1999). Oestrogen-dependent expression of LH/hCG receptors in pig Fallopian tube and their role in relaxation of the oviduct. *J Reprod Fertil, 115*(2), 293-301.

Ghersevich, S., Massa, E., & Zumoffen, C. (2015). Oviductal secretion and gamete interaction. *Reproduction, 149*(1), R1-R14. doi:10.1530/REP-14-0145

Gonella-Diaza, A. M., Andrade, S. C., Sponchiado, M., Pugliesi, G., Mesquita, F. S., Van Hoeck, V., . . . Binelli, M. (2015). Size of the Ovulatory Follicle Dictates Spatial Differences in the Oviductal Transcriptome in Cattle. *PLoS One, 10*(12), e0145321. doi:10.1371/journal.pone.0145321

Gonella-Diaza, A. M., da Silva Andrade, S. C., Sponchiado, M., Pugliesi, G., Mesquita, F. S., Van Hoeck, V., . . . Binelli, M. (2017). Oviductal transcriptional profiling of a bovine fertility model by next-generation sequencing. *Genom Data, 13*, 27-29. doi:10.1016/j.gdata.2017.06.004

Grumbach, M. M., & Auchus, R. J. (1999). Estrogen: consequences and implications of human mutations in synthesis and action. *J Clin Endocrinol Metab, 84*(12), 4677-4694. doi:10.1210/jcem.84.12.6290

Killian, G. (2011). Physiology and endocrinology symposium: evidence that oviduct secretions influence sperm function: a retrospective view for livestock. *J Anim Sci, 89*(5), 1315-1322. doi:10.2527/jas.2010-3349

Kobayashi, F., Zimniski, S. J., & Smalley, K. N. (1996). Characterization of oviductal aromatase in the northern leopard frog, Rana pipiens. *Comp Biochem Physiol B Biochem Mol Biol, 113*(3), 653-657.

Kowalik, M. K., Martyniak, M., Rekawiecki, R., & Kotwica, J. (2016). Expression and immunolocalization of membrane progesterone receptors in the bovine oviduct. *Domest Anim Endocrinol, 55*, 83-96. doi:10.1016/j.domaniend.2015.12.001

Lamy, J., Labas, V., Harichaux, G., Tsikis, G., Mermillod, P., & Saint-Dizier, M. (2016). Regulation of the bovine oviductal fluid proteome. *Reproduction, 152*(6), 629-644. doi:10.1530/REP-16-0397

Lamy, J., Liere, P., Pianos, A., Aprahamian, F., Mermillod, P., & Saint-Dizier, M. (2016). Steroid hormones in bovine oviductal fluid during the estrous cycle. *Theriogenology, 86*(6), 1409-1420. doi:10.1016/j.theriogenology.2016.04.086

Lee, Y. J., & Gorski, J. (1996). Estrogen-induced transcription of the progesterone receptor gene does not parallel estrogen receptor occupancy. *Proc Natl Acad Sci U S A, 93*(26), 15180-15184.

Lee, Y. J., & Gorski, J. (1998). Estrogen receptor down-regulation is regulated noncooperatively by estrogen at the transcription level. *Mol Cell Endocrinol, 137*(1), 85-92.

Leese, H. J. (1988). The formation and function of oviduct fluid. *J Reprod Fertil, 82*(2), 843-856.

Li, C., Ma, Y., Yi, K., Wang, C., Li, W., Liu, Z., . . . Zhou, X. (2014). The interactions between nerve growth factor and gonadotrophins in bovine oviduct. *Anim Reprod Sci, 149*(3-4), 117-123. doi:10.1016/j.anireprosci.2014.06.030

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*(4), 402-408. doi:10.1006/meth.2001.1262

Małysz-Cymborska, I., & Andronowska, A. (2016). Downregulation of LH and FSH receptors after hCG and eCG treatments in the porcine oviduct. *Domest Anim Endocrinol, 57*, 48-54. doi:10.1016/j.domaniend.2016.06.003

Marín-Briggiler, C. I., González-Echeverría, M. F., Munuce, M. J., Ghersevich, S., Caille, A. M., Hellman, U., . . . Vazquez-Levin, M. H. (2010). Glucose-regulated protein 78 (Grp78/BiP) is secreted by human oviduct epithelial cells and the recombinant protein modulates sperm-zona pellucida binding. *Fertil Steril, 93*(5), 1574-1584. doi:10.1016/j.fertnstert.2008.12.132

Martyniak, M., Franczak, A., & Kotwica, G. (2018). Synthesis of steroid hormones in the porcine oviduct during early pregnancy. *Reprod Biol*. doi:10.1016/j.repbio.2018.02.002

Mattos, M. C., Bastos, M. R., Guardieiro, M. M., Carvalho, J. O., Franco, M. M., Mourao, G. B., . . . Sartori, R. (2011). Improvement of embryo production by the replacement of the last two doses of porcine follicle-stimulating hormone with equine chorionic gonadotropin in Sindhi donors. *Anim Reprod Sci, 125*(1-4), 119-123. doi:10.1016/j.anireprosci.2011.02.028

Miller, D. J., Gong, X., Decker, G., & Shur, B. D. (1993). Egg cortical granule N-acetylglucosaminidase is required for the mouse zona block to polyspermy. *J Cell Biol, 123*(6 Pt 1), 1431-1440.

Mondéjar, I., Martínez-Martínez, I., Avilés, M., & Coy, P. (2013). Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals. *Biol Reprod, 89*(3), 67. doi:10.1095/biolreprod.113.111385

Murphy, B. D., & Martinuk, S. D. (1991). Equine chorionic gonadotropin. *Endocr Rev, 12*(1), 27-44.

Nelis, H., Wojciechowicz, B., Franczak, A., Leemans, B., D'Herde, K., Goossens, K., . . . Smits, K. (2016). Steroids affect gene expression, ciliary activity, glucose uptake, progesterone receptor expression and immunoreactive steroidogenic protein expression in equine oviduct explants in vitro. *Reprod Fertil Dev, 28*(12), 1926-1944. doi:10.1071/RD15044

Phopin, K., Nimlamool, W., Bartlett, M. J., & Bean, B. S. (2012). Distribution, crypticity, stability, and localization of α-L-fucosidase of mouse cauda epididymal sperm. *Mol Reprod Dev, 79*(3), 208-217. doi:10.1002/mrd.22016

Phopin, K., Nimlamool, W., Lowe-Krentz, L. J., Douglass, E. W., Taroni, J. N., & Bean, B. S. (2013). Roles of mouse sperm-associated alpha-L-fucosidases in fertilization. *Mol Reprod Dev, 80*(4), 273-285. doi:10.1002/mrd.22164

Pillai, V. V., Weber, D. M., Phinney, B. S., & Selvaraj, V. (2017). Profiling of proteins secreted in the bovine oviduct reveals diverse functions of this luminal microenvironment. *PLoS One, 12*(11), e0188105. doi:10.1371/journal.pone.0188105

Romero-Aguirregomezcorta, J., Matás, C., & Coy, P. (2015). α-L-fucosidase enhances capacitation-associated events in porcine spermatozoa. *Vet J, 203*(1), 109-114. doi:10.1016/j.tvjl.2014.11.006

Roque, M., Lattes, K., Serra, S., Solà, I., Geber, S., Carreras, R., & Checa, M. A. (2013). Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. *Fertil Steril, 99*(1), 156-162. doi:10.1016/j.fertnstert.2012.09.003

Santos, P. H., Satrapa, R. A., Fontes, P. K., Franchi, F. F., Razza, E. M., Mani, F., . . . Castilho, A. C. S. (2018). Effect of superstimulation on the expression of microRNAs and genes involved in steroidogenesis and ovulation in Nelore cows. *Theriogenology, 110*, 192-200. doi:10.1016/j.theriogenology.2017.12.045

Suarez, S. S., & Pacey, A. A. (2006). Sperm transport in the female reproductive tract. *Hum Reprod Update, 12*(1), 23-37. doi:10.1093/humupd/dmi047

Sun, T., Lei, Z. M., & Rao, C. V. (1997). A novel regulation of the oviductal glycoprotein gene expression by luteinizing hormone in bovine tubal epithelial cells. *Mol Cell Endocrinol, 131*(1), 97-108.

Talbot, P., Geiske, C., & Knoll, M. (1999). Oocyte pickup by the mammalian oviduct. *Mol Biol Cell, 10*(1), 5-8.

Ulbrich, S. E., Kettler, A., & Einspanier, R. (2003). Expression and localization of estrogen receptor alpha, estrogen receptor beta and progesterone receptor in the bovine oviduct in vivo and in vitro. *J Steroid Biochem Mol Biol, 84*(2-3), 279-289.

Venditti, J. J., Swann, J. M., & Bean, B. S. (2010). Hamster sperm-associated alpha-L-fucosidase functions during fertilization. *Biol Reprod, 82*(3), 572-579. doi:10.1095/biolreprod.109.076695

Wang, L. H., Zhang, W., Gao, Q. X., & Wang, F. (2012). Expression of the luteinizing hormone receptor (LHR) gene in ovine non-gonadal tissues during estrous cycle. *Genet Mol Res, 11*(4), 3766-3780. doi:10.4238/2012.October.15.8

Wijayagunawardane, M. P., Choi, Y. H., Miyamoto, A., Kamishita, H., Fujimoto, S., Takagi, M., & Sato, K. (1999). Effect of ovarian steroids and oxytocin on the production of prostaglandin E2, prostaglandin F2alpha and endothelin-1 from cow oviductal epithelial cell monolayers in vitro. *Anim Reprod Sci, 56*(1), 11-17.

Wijayagunawardane, M. P., Kodithuwakku, S. P., DE Silva, N. T., & Miyamoto, A. (2009). Angiotensin II secretion by the bovine oviduct is stimulated by luteinizing hormone and ovarian steroids. *J Reprod Dev, 55*(5), 570-575.

Wijayagunawardane, M. P., Kodithuwakku, S. P., Yamamoto, D., & Miyamoto, A. (2005). Vascular endothelial growth factor system in the cow oviduct: a possible involvement in the regulation of oviductal motility and embryo transport. *Mol Reprod Dev, 72*(4), 511-520. doi:10.1002/mrd.20379

Wijayagunawardane, M. P., Miyamoto, A., Cerbito, W. A., Acosta, T. J., Takagi, M., & Sato, K. (1998). Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. *Theriogenology, 49*(3), 607-618.

Wijayagunawardane, M. P., Miyamoto, A., & Sato, K. (1999). Prostaglandin E2, prostaglandin F2 alpha and endothelin-1 production by cow oviductal epithelial cell monolayers: effect of progesterone, estradiol 17 beta, oxytocin and luteinizing hormone. *Theriogenology, 52*(5), 791-801. doi:10.1016/S0093-691X(99)00172-7

Wijayagunawardane, M. P., Miyamoto, A., Taquahashi, Y., Gabler, C., Acosta, T. J., Nishimura, M., . . . Sato, K. (2001). In vitro regulation of local secretion and contraction of the bovine oviduct: stimulation by luteinizing hormone, endothelin-1 and prostaglandins, and inhibition by oxytocin. *J Endocrinol, 168*(1), 117-130.

Wiltbank, M. C., Fricke, P. M., Sangsritavong, S., Sartori, R., & Ginther, O. J. (2000). Mechanisms that prevent and produce double ovulations in dairy cattle. *J Dairy Sci, 83*(12), 2998-3007. doi:10.3168/jds.S0022-0302(00)75201-5

Zervomanolakis, I., Ott, H. W., Müller, J., Seeber, B. E., Friess, S. C., Mattle, V., . . . Wildt, L. (2009). Uterine mechanisms of ipsilateral directed spermatozoa transport: Evidence for a contribution of the utero-ovarian countercurrent system. *Eur J Obstet Gynecol Reprod Biol, 144 Suppl 1*, S45-49. doi:10.1016/j.ejogrb.2009.02.012

Zhang, M., Shi, H., Segaloff, D. L., Van Voorhis, B. J., & Zheng, M. (2001). Expression and localization of luteinizing hormone receptor in the female mouse reproductive tract. *Biol Reprod, 64*(1), 179-187.

Zheng, W., Magid, M. S., Kramer, E. E., & Chen, Y. T. (1996). Follicle-stimulating hormone receptor is expressed in human ovarian surface epithelium and fallopian tube. *Am J Pathol, 148*(1), 47-53.

Supplementary Information

Table S1: Relative mRNA abundance (mean ± standand error mean) and *p*-values

		Groups			***p*-value**
		CTRL	FSH	FSH/eCG	Comparisons between groups
Gene	Segment	MEAN ± STANDARD ERROR MEAN			
ESR1	INF	1.34 ± 0.48	2.24 ± 0.58	2.79 ± 0.46	0.192
	AMP	1.73 ± 0.43**a**	3.72 ± 0.61**ab**	5.37 ± 2.00**b**	**0.021**
	IST	3.70 ± 1.50	11.00 ± 4.61	4.63 ± 2.29	0.351
ESR2	INF	1.80 ± 0.59	5.67 ± 3.65	4.40 ± 2.54	0.979
	AMP	14.85 ± 2.95	20.89 ± 3.96	30.08 ± 9.99	0.278
	IST	0.63 ±0.23	1.93 ± 1.54	0.03 ± 0	0.165
PGR	INF	0.57 ± 0.17**a**	2.95 ± 1.77**b**	5.72 ±1.75**b**	**0.012**
	AMP	1.03 ± 0.31**a**	7.63 ± 2.22**b**	12.14 ±4.47**b**	**0.002**
	IST	0.82 ± 0.34	12.28 ± 4.48	6.31 ± 5.83	0.148
OVGP1	INF	0.56 ± 0.21**a**	13.54 ± 6.13**b**	26.45 ± 8.53**b**	**0.009**
	AMP	1.54 ± 0.66**a**	29.54 ± 9.01**b**	74.40 ± 20.72**b**	**<0.001**
	IST	0.32 ± 0.19	3.95 ± 3.76	0.13 ±0.10	0.607
HSPA5	INF	0.55 ±0.15**a**	5.70 ± 2.93**ab**	13.15 ± 9.29 **b**	**0.034**
	AMP	0.40 ± 0.08**a**	12.29 ± 4.00**b**	13.09 ± 5.65 **b**	**<0.001**
	IST	0.15 ± 0.08	1.33 ± 0.51	1.59 ± 1.02	0.106
FUCA1	INF	0.50 ± 0.16**a**	2.05 ± 0.78**ab**	6.78 ± 2.34**b**	**0.027**
	AMP	0.92 ±0.20**a**	4.29 ± 1.52**ab**	8.12 ± 2.53**b**	**0.012**
	IST	0.16 ± 0.11	1.08 ± 0.34	1.15 ± 1.01	0.105
FUCA2	INF	1.28 ± 0.45**a**	1.90 ± 0.61**ab**	6.21 ± 1.52**b**	**0.042**
	AMP	1.32 ± 0.36**a**	3.45 ± 1.00**ab**	5.38 ± 1.13**b**	**0.024**
	IST	0.63 ± 0.33	0.76 ± 0.18	1.65 ± 0.96	0.662

Data are presented as mean ± standard error mean of relative mRNA abundance (ΔΔCt method). Different letters (a, b) means differences ($p < 0.05$) between groups (Control, FSH, and FSH/eCG). INF: infundibulum, AMP: ampulla, IST: isthmus.

Table S2. Bovine specific oligonucleotide forward and reverse primer sequences (5'-3')

Gene Symbol	Gene Name	Primer sequence (5'-3')	NCBI Reference	[mM]†	ºC‡
Hormone receptor					
ESR1	Estradiol Receptor 1	F: CAGGCACATGAGCAACAAAG R: TCCAGCAGCAGGTCGTAGAG	NM_001001443.1	300	60
ESR2	Estradiol Receptor 2	F: GTAGAGAGCCGCCATGAATAC R: CAATGGATGGCTAAAGGAGAGA	NM_174051.3	300	60
FSHR	Follicle Stimulating Hormone Receptor	F: AGCCCCTTGTCACAACTCTATGTC R: GTTCCTCACCGTGAGGTAGATGT	NM_174061.1	300	60
LHCGR	Luteinizing Hormone/Choriogonadotropin Receptor	F: GCATCCACAAGCTTCCAGATGTTACGA R: GGGAAATCAGCGTTGTCCCATTGA	NM_174381.1	300	60
PGR	Progesterone Receptor	F: ACTACCTGAGGCCGGATT R: CCCTTCCATTGCCCTCTTAAA	NM_001205356.1	300	60
Steroidogenic enzyme					
CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1 (Aromatase)	F: CTGAAGCAACAGGAGTCCTAAATGTACA R: AATGAGGGGCCCAATTCCCAGA	XM_019968827.1	400	62
Genes related to fertilization process					
FUCA1	Alpha-L-Fucosidase 1	F: TGGTTCCATCCTCTCTACCTAC R: CCACTCTCCATCAGACCAAATC	NM_001046035.2	300	60
FUCA2	Alpha-L-Fucosidase 2	F: CTGGAATCGCTGACTATCTCAC R: CCATCTTGTGTGGGTCCAATA	NM_001205818.1	300	60
HSPA5	Heat Shock Protein Family A Member 5	F: GTTCTTGTTGGTGGCTCTACT R: ACAGCCTCATCTGGGTTTATG	NM_001075148.1	300	60
OVGP1	Oviductal Glycoprotein 1	F: GTCGTCCAGAAAGCGTATGA R: CAGAGAGAACAGAGGGCTATTG	NM_001080216.1	300	60
Reference					
18S	RNA, 18S Ribosomal	F: AGAAACGGCTACCACATCCAA R: CCTGTATTGTTATTTTTCGTCACTACCT	NR_036642.1	200	60
ACTB	Actin Beta	F: GCGTGGCTACAGCTTCACC R: TTGATGTCACGGACGATTTC	NM_173979.3	300	60
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAG	NM_001034034.2	200	60
H2A	H2A Histone type 2-A	F: GAGGAGCTGAACAAGCTGTTG R: TTGTGGTGGCTCTCAGTCTTC	XM_019956604.1	300	60
PPIA	Peptidylprolyl Isomerase A	F: GCCATGGAGCGCTTTGG R: CCACAGTCAGCAATGGTGATCT	NM_178320.2	300	60
RPL15	Ribosomal Protein L15	F: CTCATCGTTGGTGCCAATGCAAGT R: TCACATCCACCCTGGGAAACAGAA	NM_001077866.1	300	60
RPL30	Ribosomal Protein L30	F: TGGTGTCCATCACTACAGTGGCAA R: ACCAGTCTGTTCTGGCATGCTTCT	NM_001034434.2	300	60

† Primer concentration per PCR reaction (mM); ‡ Temperature of annealing and extension (ºC)

CAPÍTULO 3

– CHAPTER 3 –

SHOULD THE PHASE OF THE ESTROUS CYCLE BE CONSIDERED WHEN HARVESTING BOVINE OVIDUCT EPITHELIAL CELLS FOR APPLICATION IN *IN VITRO* COCULTURE SYSTEMS?

SHOULD THE PHASE OF THE ESTROUS CYCLE BE CONSIDERED WHEN HARVESTING BOVINE OVIDUCT EPITHELIAL CELLS FOR APPLICATION IN *IN VITRO* COCULTURE SYSTEMS?

The experimental part of this work was developed in collaboration with the group of Bart M. Gadella from Utrecht University, Utrecht, Netherlands. This is part of the work developed by Patricia K. Fontes during her international internship at Utrecht University during her Ph.D.

ABSTRACT

The improvement of *in vitro* culture of oviductal epithelial (OEC) cells has been the focus of many studies due to its capacity to mimic the maternal environment for *in vitro* embryo production. This coculture system with OECs and embryos has resulted in more *in vivo*-like embryos. However, the *in vitro* culture of OEC still does not have a gold-standard system. One fact that can be related to variation in cell culture is the phase of the estrous cycle when OECs are harvested. Using bovine species as an experimental model, we demonstrated that indeed cells harvested during follicular or luteal phases from the estrous cycle keep a transcriptome profile difference after long-term culture if no hormone treatment is applied. Cultured under four conditions related to estradiol and progesterone treatments, these cells demonstrated to have a distingue profile when not stimulated by hormone treatments, but hormone treatments were able to similarly modulate the cells' transcription activity. Therefore, when culturing BOECs for application in *in vitro* coculture system with *in vitro* produced embryos, the phase of the estrous cycle should be considered if no hormone treatment will be performed. In the case of BOECs cultured *in vitro* be submitted to a simulation of the estrous cycle phases before the coculture system, BOECs can be harvested at any time of the estrous cycle.

1. INTRODUCTION

The *in vitro* embryo production (IVEP) is the most explored artificial reproduction technique (ART) in many different species and for various proposes, e.g. rescue of endangered animals, increment in beef production, helping couples with fertility problems/single parent/same-sex couples to conceive a baby, and biomedicine research. The development of the IVEP technique has continuously been improved since the first mammal birth in 1959 (1), mainly to achieve a better embryo quality and to improve the microenvironment conditions that this embryo is exposed to. Aiming that, some bridges are being built to connect embryos produced *in vitro* to their natural environment, the oviduct.

Even though the oviduct plays a pivotal function in *in vivo* embryo formation and early development, its roles could be bypass during many years of *in vitro* production of embryos. However, the importance of the oviduct had been considered underestimated and new approaches to establish contact between embryo and oviduct conditions have been developed. Lately, the benefits of oviductal fluid for the development of *in vitro* produced embryos have been assessed by using *in vivo*-derived oviductal fluid in the embryo culture media, as well as, embryo-oviductal cells coculture systems (2-4). The latest most notary benefit about the interaction between embryo and oviduct is the embryonic epigenetic modulation, which has been listed to be a key factor for a more *in vivo*-like produced embryo (2,4-6).

The oviduct is the organ that connects the ovary to the uterus, meaning it has an intra-abdominal location (7). Besides its difficult access location, the oviduct has highly tortuous anatomy, which means that in the practical situation of using oviduct for ART, the necessity of surgery for its access and its cannulation process is not easily performed for fluid and cells collection, for instance. Therefore, in some situations, the animal is

slaughtered for oviduct cells/fluid collection, or, more frequently, the material is collected from animals slaughtered for beef production.

The collection of oviductal fluid for the isolation of extracellular vesicles has been successfully demonstrated to benefit IVEP (4,8). However, the volume of oviductal fluid is very limited, and the extracellular vesicles profile is variable depending on the estrous cycle stages (9). Hence, the culture of oviductal cells has been an advantageous alternative for the establishment of embryo-oviductal cell coculture systems, or even for *in vitro*-derived oviductal fluid collection (10). Even though the *in vitro* culture of oviductal cells is a more gainful strategy to establish contact with embryos, up until now, no culture system has been proved to be completely feasible on mimicking the *in vivo* conditions to blastocyst production (11). The absence of a gold-standard protocol to culture oviductal cells is proof that there is room for improvement. A variation on cell collection method (mechanically *vs.* enzymatically), sub-culture passages number, culture media compositions, and presence/concentrations of estradiol and progesterone hormones are the main examples of variations between protocols (12,13).

Besides the examples listed above, another factor highly variable when performing *in vitro* culture of oviductal cells is at which phase from estrous cycle cells are harvested. And this fact is even more evident in studies that use material from slaughterhouses. In some cases, a postmortem evaluation/estimation of the animal's estrous cycle phase is performed based on their ovaries morphology (14). Adopting these criteria, some studies are performed using oviductal cells collected during the luteal phase (early, mid, and/or late (3,12,15,16) or follicular phase (17-19). However many other studies do not specify and/or do not evaluate the estrous cycle phase when the oviductal cells had been harvested (2,11,20,21). This variety can have a great impact on the outcome of *in vitro* cell culture, whereas oviductal cells have distinct known profiles across the estrous cycle phases (22-27). Therefore, we decided to perform an extensive evaluation of the

transcriptome profile of oviductal cells submitted to *in vitro* culture harvested in two phases of the estrous cycle: mid-luteal and follicular. To do that, we use the bovine species as an experimental model, and the bovine oviduct epithelial cells (BOECs) were submitted to *in vitro* culture with estradiol (E2) and progesterone (P4) treatments simulating the estrous cycle phase. The estrous cycle phases were simulated because in *in vivo* conditions the presence of gametes and embryos in the oviduct occurs during the periovulatory period. In this way, the knowledge developed in this study can have great applicability in embryo-oviduct coculture systems. Considering this information, the hypothesis is that the origin of BOEC influences the behavior of cells submitted to *in vitro* culture. Therefore, we aimed to determine whether the moment of the estrous cycle when cells were harvested (origin of BOEC: follicular *vs.* luteal) is an important factor to be considered during oviductal cells *in vitro* culture. Since E2 and P4 are known to act modulating transcription activity, we elected the transcriptome analysis by RNA-seq as the main tool to reach our objective to evaluate the behavior of the cells.

2. MATERIALS AND METHODS

2.1. Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) of the highest available purity.

2.2. Experimental design

Bovine oviduct epithelial cells were collected and *in vitro* cultured following the experimental design in Figure 1.

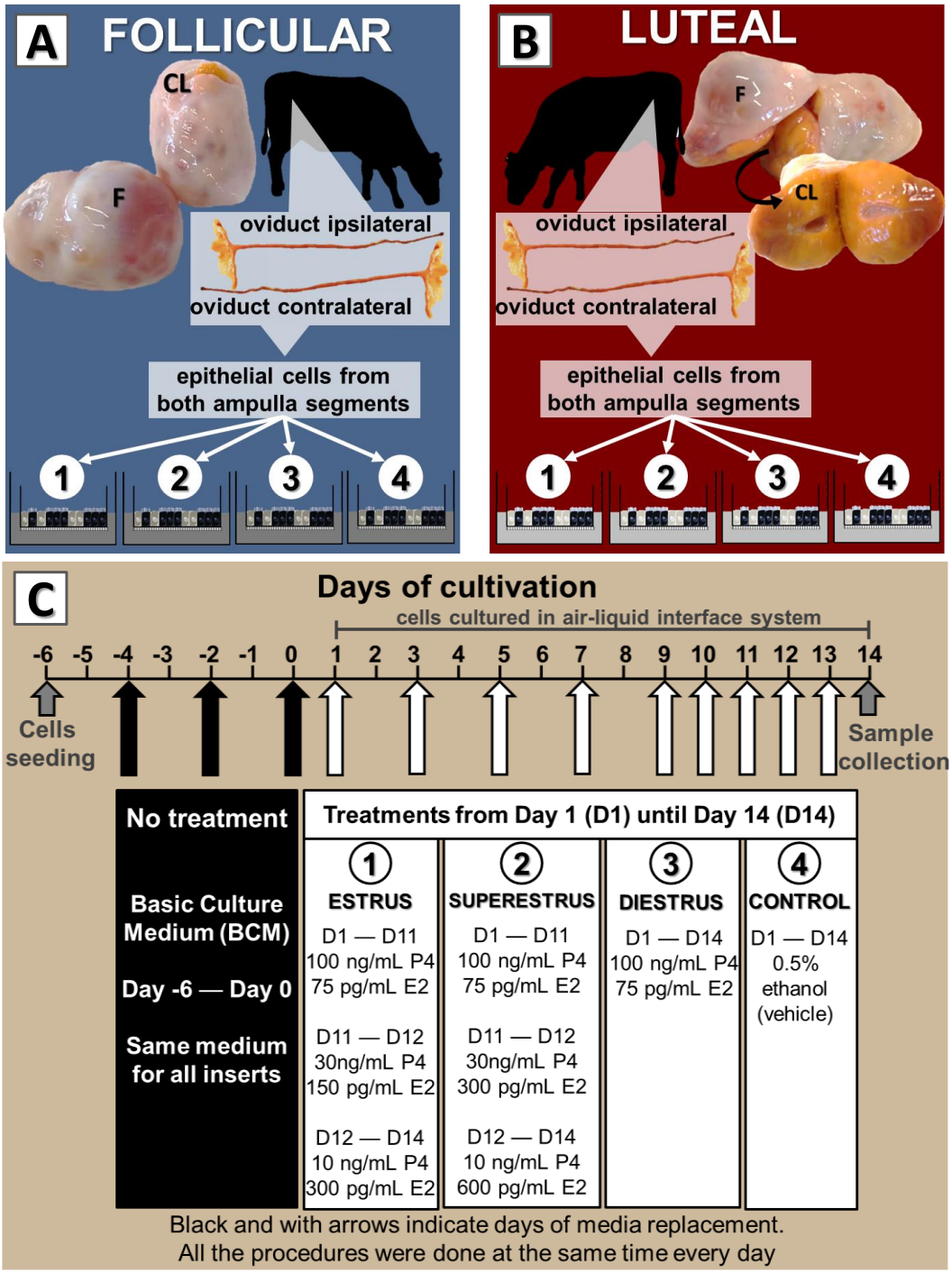

Figure 1: Experimental design. **(A)** Based on ovarian morphology evaluation (14), cows were classified as at follicular phase when a pre-ovulatory follicle and a Corpus Luteum in the regression phase were identified (n = 3 animals). BOECs were collected from the ampulla segment from both oviducts (ipsi- and contralateral) from each animal and seeded in four transwell inserts per animal. **(B)** Similarly, cows were classified as at luteal phase when a dominant follicle and a Corpus Luteum bigger than 1.6 centimeters in diameter, red/brown color in the apex only, and orange color in the remaining portion were identified in the ovaries (n = 3 animals). BOECs from ampulla segments (both oviducts) from each animal were seeded

in four transwell inserts per animal. **(A, B)** Three independent replicates were performed using one animal from each estrous cycle stage (follicular and luteal) per replicate. **(C)** The day of cell seeding was day -6 of the protocol. BOEC were cultured until confluence for 7 days. During this period, half of the media from the apical compartment and total media from the basolateral compartment were renewed every 48 h (black arrows) with no treatment. On day 1, the medium from the apical compartment was removed to establish the air-liquid interface. And BOECs were submitted to four different protocols of hormone treatments added in the media from the basolateral compartment: 1) Estrus; 2) SuperEstrus; 3) Diestrus; 4) Control. The white arrows indicate the days of media replacement from day 1 until day 14, during this period the full media from the basolateral compartment were renewed. The white boxes indicate the specifics hormone treatment for each one of the four treatments. On day 14, cells were collected for transcriptome analysis. BOECs: bovine oviduct epithelial cells; CL: corpus Luteum; F: dominant or pre-ovulatory follicle; D: day, P4: progesterone; E2: estradiol.

2.3. Oviduct cells isolation and pre-culture

Reproductive tracts from cows were collected at a local slaughterhouse. The tissues were collected immediately after slaughter (~15 min) and were transported to the laboratory on ice within one hour. Based on ovarian morphology (14), the estrous cycle stages were classified post-mortem in:

1) Follicular (follicular phase: estimated to be between 18-20 days of the estrous cycle), when a Corpus Luteum with less than 1 centimeter (cm) in diameter, light yellow/white external color, and the yellow internal color was present, in addition to the presence of a preovulatory follicle more than 10 mm in diameter (Figure 1A);

1) Luteal (mid-luteal phase: estimated to be between 5-10 days of the estrous cycle), when a Corpus Luteum with more than 1.6 cm in diameter, red/brown color in the apex only and orange color in the remaining portion was present, in addition to the presence of a dominant follicle more than 10 mm in diameter (Figure 1B).

A total of six animals were used in this study (three of each estrous cycle stage), divided into three independent replicates. Two animals (one animal from each estrous stage) were processed per replicate. Both oviducts of each animal (ipsilateral and contralateral) were dissected free of surrounding tissue. The tubular segment of oviducts (from isthmus until ampulla) was quickly washed in 70% ethanol, followed by three washes in PBS supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL

gentamycin, and 2.5 µg/mL amphotericin B (room temperature). The length of the total tubular segment of the oviducts was measured; only oviducts within a minimal 25 cm were used. No difference between phases of the estrous cycle was observed (Follicular: 29±3.6 cm; Luteal: 28.4±2.7 cm).

Only BOECs from the ampulla segment were collected for culture. Both oviducts of the same cow (ipsilateral and contralateral) were mechanically squeezing with tweezers, cells were pooled in warm (37ºC) HEPES buffered Medium 199 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamycin, and 2.5 µg/mL amphotericin B (Wash Medium - WM), and centrifuged at 200 x g for 5 min at 25 ºC. The supernatant medium was discarded and the cells were resuspended in 3 mL of warm (37 ºC) 1% Collagenase A (Roche Diagnostics, Indianapolis, IN) diluted in PBS supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamycin, and 2.5 µg/mL amphotericin B. The enzyme digestion was performed for 10 min at a warm plate (37 ºC) associated with pipetting up and down every 2 min to separate the cells. Next, the enzyme activity was inactivated by adding 7 mL of WM supplemented with 10% fetal bovine serum (FBS), followed by two rounds of centrifugation at 100 x g for 5 min at 25 ºC with 7 mL WM supplemented with 10% FBS. Last, the cells were placed in six-well plates (one plate per cow) and cultured for 18-20 h in WM supplemented with 10% FBS for fibroblast exclusion by time adhesion to the plate (defined as pre-culture day).

2.4. BOECs culture in transwell inserts

Basic Culture Medium (BCM) was prepared as followed: DMEM/Ham's F12 (DMEM/F-12, Gibco BRL, Paisley, UK), no phenol red due to hormonal treatment (28), supplemented with 5% FBS, 5 µg/mL insulin, 5 µg/mL transferrin, 25 ng/mL epidermal growth factor, 50 µg/mL gentamycin, 0.25 µg/mL amphotericin B, 100 U/mL penicillin, and 100 µg/mL streptomycin. Before cells seeding, the transwell inserts (Nunc™

Polycarbonate, 0.4 µm pores, 24-well plate; ThermoFisher Scientific, Rolkilde, Denmark) were coated with the BCM in the basolateral compartment (800 µL) and the apical compartment (100 µL) of the inserts for one hour in the incubator (37 ºC, 5% CO_2, in humidified air). Eight inserts were prepared at the same time, four inserts per cow.

After the pre-culture day, the cells floating in the medium were collected, centrifuged at 100 x g for 5 min at 25 ºC, and resuspended in BCM. Cell viability was analyzed with double stain to total (Hoechst 33342) and dead (propidium iodide) cells identification. At least 200 cells were analyzed per cow, within a proportion of live cells higher than 75% in all the samples. No difference between the phases of the estrous cycle was observed (Follicular: 83.3±7.0%; Luteal: 83.3±4.5%). Cell count was performed with a Bürker Türker Counting Chamber. BOECs were diluted to a concentration of 3 x 10^6 cells/mL, 140 µL of this solution was added into the apical compartment of the inserts (pre-coated with BCM) resulting in 0.9 x 10^6 cells/cm^2. The inserts were kept in a liquid-liquid interface (liquid in both compartments: basolateral and apical) for seven days in the incubator (37 ºC, 5% CO_2, in humidified air) to allow cell attachment and to reach confluence. During these first seven days, the BCM was replaced every other day in the basolateral compartment (100%) and the apical compartment (50% of total media volume) (Figure 1C). Next, the medium from the apical compartment was completely removed to establish the air-liquid interface (ALI), and the hormonal stimulation was performed in the media from the basolateral compartment for 14 days. This long-term culture (21 days) was performed in the incubator (37 ºC, 5% CO_2, in humidified air).

2.5. Hormone treatment and quantification

Since the first day of ALI, E2 and P4 were added to the BCM in the basolateral compartment. Each treatment was performed in one insert per animal (Figure 1C):

(1) Estrus (EST): diestrus phase was simulated from day 1 – 11 with 100 ng/mL P4 and 75 pg/mL E2, followed by one day transition of 30 ng/mL P4 and 150 pg/mL E2, and simulation of estrus milieu for two days with 10 ng/mL P4 and 300 pg/mL E2;

(2) Super Estrus (SUP): diestrus phase was simulated from day 1 – 11 with 100 ng/mL P4 and 75 pg/mL E2, followed by one day transition of 30 ng/mL P4 and 300 pg/mL E2, and simulation of superestrus milieu for two days with 10 ng/mL P4 and 600 pg/mL E2;

(3) Diestrus (DIE): diestrus phase simulation from day 1 – 14 with 100 ng/mL P4 and 75 pg/mL E2;

(4) Control (Ctrl): 0.5% ethanol for 14 days of culture (vehicle).

The medium in the basolateral compartment of all inserts was completed replaced every other day during the first nine days. Then, from day 10 until day 13, the medium was completed replaced every day. The cells were collected on day 14, approximately 24h after the last medium replacement (Figure 1C).

Hormone treatments were implemented using values similar to the ones described locally in the oviducts, which has been demonstrated to be much higher than the plasmatic levels (26). The P4 and E2 concentrations of EST and DIE treatments were based on ex-vivo oviductal fluid concentrations reported in cows under physiological conditions (26). The E2 concentration of SUP treatment (E2: 600 pg/mL) is based on ex-vivo oviductal cells analysis in cows submitted to ovarian superstimulation protocols (29), the higher levels of E2 has been suggested to improve oviductal cells functions.

2.6. Samples collection for RNA extraction

On day 14, the culture medium was completely removed and 250 µL of kit Lysis-buffer were added in the apical compartment of the inserts, mixed by pipetting, recovered in a 1.5 mL tube, and stored in -80 ºC.

2.7. RNA extraction and reverse transcription

Total RNA was extracted using the Micro Kit RNeasy (Qiagen, Hilden, Germany), including the RNAse-free DNAse treatment, following the manufacturer's instructions. Total RNA concentration was analyzed using a spectrophotometer (Nanodrop 2000™, ThermoFisher Scientific, Wilmington, DE), which also enables the evaluation of contamination of proteins and organic solvents by the 260/280 ratio values. The RNA quality was evaluated using the 2100 Bioanalyzer system with RNA Nano chips (Agilent Technologies, Waldbronn, Germany). All the samples presented an RNA integrity number (RIN) > 9.2. Part of the sample was stored as RNA for RNA-seq analysis, and the other part (200 ng of total RNA/sample) was converted into cDNA using the High Capacity cDNA kit (Applied Biosystems™, Carlsbad, CA), according to the manufacturer's instructions. Individual sample information is presented in Supplementary Table 1.

2.8. Library preparation and RNA-seq data analysis

Approximately 500 ng of total RNA were used for each RNA-seq library preparation using the Illumina TruSeq Stranded mRNA sample Prep LT Protocol (San Diego, CA). Libraries were pair-end sequenced in a 2 x 101 bp protocol using the HiSeq SBS v4 kit (San Diego, CA) and the HiSeq 2500 Illumina platform. All the 24 samples (four treatments/cow; three cows from the follicular phase and three cows from the luteal phase) were sequenced in the same lane of the Flow Cell. All the sequences were 101 bp lengths and 100% similarity in the sequence number between firsts reads (R1) and seconds reads (R2, pair-end). The quality of the sequences was checked using the FastQC program (version 0.11.9) (30). Phred of 32 was the minimum mean quality score of each base position in all the samples. Adaptors content were removed using the Cutadapt software (version 2.10) (31). Reads were mapped using HiSat2 (version 2.2.0) (32) to the bovine reference genome (Bos_taurus.ARS-UCD1.2.dna.toplevel.fa, ENSEMBL,

http://m.ensembl.org/info/data/ftp/index.html, accessed in May 2020), with the option --rna-strandness RF (according to Illumina strand library preparation, which the R1 and R2 are the reverse and forward strands, respectively). The mapping file was sorted using SAMTools (version 0.1.18) (33) with the option -N (name). Read counts were obtained using the script from HTSeq-count (version 0.12.4) (34) with the option strand specificity - reverse (according to Illumina strand library preparation, which the R1 and R2 are the reverse and forward strands, respectively) and the bovine genome assembly (Bos_taurus.ARS-UCD1.2.dna.toplevel.fa, ENSEMBL, http://m.ensembl.org/info/data/ftp/index.html, accessed in May 2020). All the parameters related to RNA-seq bioinformatics are summarized in Supplementary Table 1.

The differential expression analyses were performed with package DESeq2 (version 1.28.1) (35), from R/Bioconductor (36). The fitType was set to 'local'. Analyses of different treatments (CTRL *vs.* DIE, CTRL *vs.* EST, and CTRL *vs.* SUP) for the same cow were performed as a paired test using the option 'design: ~ animal + treatment'; all the others parameters were set as default. The P-value was determined by Wald statistics. An adjusted P-value (P-adj) to correct for multiple testing was calculated using the Benjamini–Hochberg method. Differentially expressed genes (DEGs) were filtered by a false discovery rate (FDR) <10% (P-adj value < 0.1).

Functional enrichment analyses were performed using the plugin ClueGO/CluePedia (version 2.5.7) (37) in the Cytoscape (version 3.8.0). Gene ontology (GO) terms of 'Biological Process' (EBI-UniProt-GOA-ACAP-ARAP_24.07.2020) and Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) pathways (11.01.2018) was evaluated using the Bos taurus genome as reference (version 2018-01-11). Specifications of ClueGo parameters of each analysis are indicated in the results section. All RNA-Seq data will be deposited in the NCBI Gene Expression Omnibus (GEO) database.

3. RESULTS AND DISCUSSION

Successful gestations have been established after the transfer of *in vitro* produced embryos into the uterus of an animal/woman. Usually transferred at morulae or blastocyst stage, embryos will have their first contact with the maternal environment around the 3rd-8th day of their development, depending on the species [1]. Consequently, the role of the oviduct for the achievement of pregnancy has been neglected for a long period. It is a fact that the oviduct is not mandatory for the initiation of a new life; however, it is known nowadays that the interaction of oviduct-embryo facilitates the maternal recognition of pregnancy [2] and improves the embryo quality in many aspects, such as epigenetics pattern, gene expression profile, morphology, and cryotolerance [3].

The coculture system of oviduct cells with embryos is a feasible way to establish the interaction of them in an *in vitro* condition. However, the *in vitro* culture of oviductal cell is still not optimal. The major characteristic of the *in situ* oviduct cells is that epithelial cell undergoes cyclic events of cell proliferation, differentiation, and cell desquamation/nuclei extrusion, which is regulated by the cyclic variation of ovarian steroids [4,5]. Therefore, many studies have been trying to exposure oviductal cells to a timeframe simulation of the estrous cycle for maximal hormone responsiveness and oviductal cells as similar as possible to the *in vivo* cells [6-8]. Because the estrus cycle is defined by consecutive hormonal stages, we hypothesized that the stage of oviductal cells harvesting should be considered in cell culture to maintain the cells in their natural circuit. Therefore, in this study, we harvested BOECs during the follicular and luteal phase and subsequently submitted these cells to a simulation of estrous cycle phases *in vitro*. In this way, we aimed to determine whether BOECs collected in different estrous cycle phases and after being

submitted to *in vitro* culture system would have different outcomes, and possibly indicate which phase is more appropriate for the BOECs collection.

Even though the presence of gametes and embryos takes place in the bovine oviduct during the periovulatory period in natural *in vivo* conditions, in many studies the *in vitro* coculture system is performed in oviduct cells with no hormone stimulation [2,6,9]. Therefore, to have a wide view of the coculture scenario, in this study we evaluated BOECs collected in different estrous cycle phase and submitted to four culture conditions: 1) under no hormone treatment (CTRL), due to many studies that perform coculture of embryos and oviduct cells with no hormone treatment; 2) treatment simulating the diestrus phase (DIE), which is the period of the estrous cycle not receptive for embryo development; 3) treatment simulating the estrus phase (EST), which is the period of the estrous cycle appropriate for embryo development; 4) treatment simulating the estrus phase in cows submitted to ovarian superstimulation protocol (SUP) [10], which is the period of the estrous cycle appropriate for the development of multiple embryos.

Previously study has shown that bovine oviductal cells when cultured *in vitro* with no hormonal stimulation have a lower cell height, decreased cell ciliation, and lower expression of secretory markers (*i.e.* oviductal glycoprotein 1 – OVGP1) compared to cells hormonally stimulated, suggesting loss in morphological and functional differentiation in cells cultured in absence of hormone stimulation [6]. Similarly in porcine, *in vitro* estrus cycle simulation revealed the effects of E2 and P4 on differentiation, gene expression, and cellular function in oviduct epithelial cells [7]. Consequently, it was expected that BOEC submitted to CTRL treatment loses their differentiation after been submitted to a long-term culture without hormone stimulation. Moreover, this dedifferentiation would result in very similar or even identical transcriptome profiles between BOECs from different origins (follicular and luteal). However an unexpected result was observed in the present study: cells submitted to CTRL treatment presented 1134 DEGs when comparing cells from

follicular phase (F_CTRL) *vs.* luteal phase (L_CTRL), of those, 425 and 709 genes were upregulated in F_CTRL and L_CTRL, respectively (Figure 2, Supplementary Table 2).

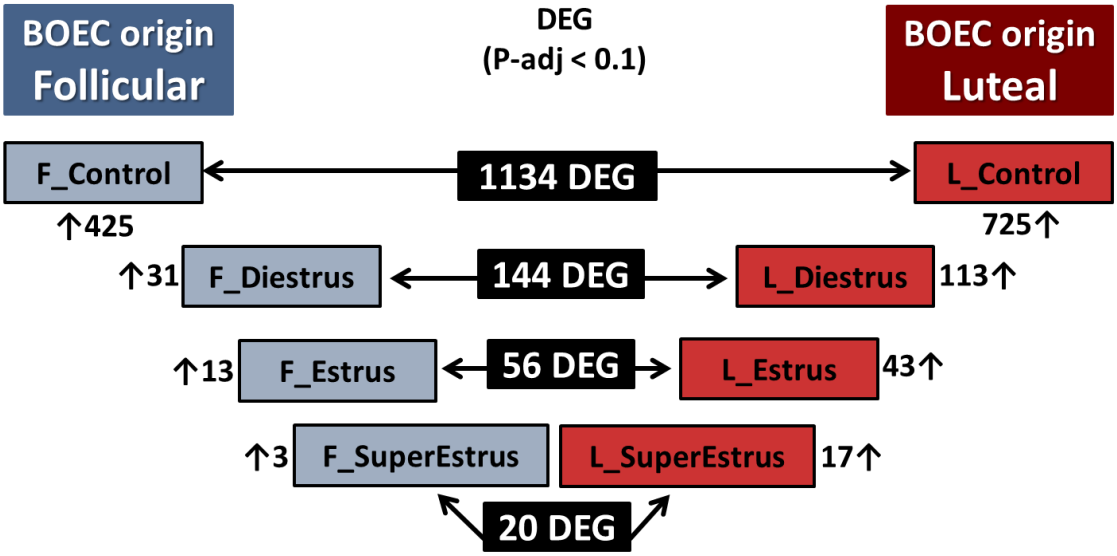

Figure 2: Total number of differentially expressed genes (DEGs, P-adj < 0.1) identified between different origins of BOECs (follicular *vs.* luteal) in all four treatments: SuperEstrus, Estrus, Diestrus, and Control. Numbers inside the black boxes indicate the total DEG in each comparison. Numbers on the left side indicate genes that were upregulated in cells from the follicular origin; numbers on the right side indicate genes that were upregulated in cells from the luteal origin.

Many studies have demonstrated that the state of oviduct tissue analyzed *in vivo* is unique and distinct in the follicular phase compared to the luteal phase [11-16]. Particularities of each phase have been observed concerning the epithelial cell transcriptome profile [13,14,16] and oviductal fluid proteomics [12,17], metabolomics [11], and extracellular vesicles content [18,19], for instance. With this information in mind and the outcome of transcriptome profile observed in the present study, the first assumption was that the BOECs from different origins (follicular and luteal) would, therefore, be capable to keep their characteristics of their *in vivo* state when not hormonally stimulated; even after long-term culture. To evaluate the effectiveness of this theory, functional enrichment analysis was performed using the Cytoscape ClueGO analysis [20], all the 1134 DEGs from the comparison F_Ctrl *vs.* L_Ctrl were used for this analysis. The

analysis was performed using the function ‘cluster’ from ClueGO, which permits the simultaneous visualization of genes up- and downregulated from this comparison. As shown in Figure 3 and Supplementary Table 3, most of the GO/KEGG terms were exclusively enriched in the BOECs originally from the luteal phase (red nodes), some terms were common between follicular and luteal (gray nodes), and only three terms were enriched in the BOECs originally from follicular phase (blue nodes, Figure 3A, Supplementary Table 3). About the terms enriched in BOECs originally from the luteal phase, they were mainly related to cell cycle, cell division, and mitosis, whereas in BOECs from the follicular origin, the terms included regulation of localization, endocytosis, and protein stabilization (Figure 3A, Supplementary Table 3). Next, some genes were selected based on a literature review because of their known differential expression between follicular and luteal phases [7,12,13]. Analyzing these genes in BOECs submitted to F_CTRL and L_CTRL treatment, none of the genes presented the expected expression pattern (Figure 3B). Most of them were not differentially expressed between F_CTRL and L_CTRL (white bars, Figure 3B), and some of them had their expression levels in the opposite direction as expected (*CDC20, CKS2, PNP,* and *TFE3,* Figure 3B). In summary, the GO/KEGG terms from functional enrichment analysis along with the analysis of selected genes drove us to the conclusion that the cells are not connected to their original state, *i.e.* BOECs collected at follicular and luteal phases do not keep their original characteristics after long-term culture under no hormone stimulation; however, these cells present a distinct transcriptome profile between one another.

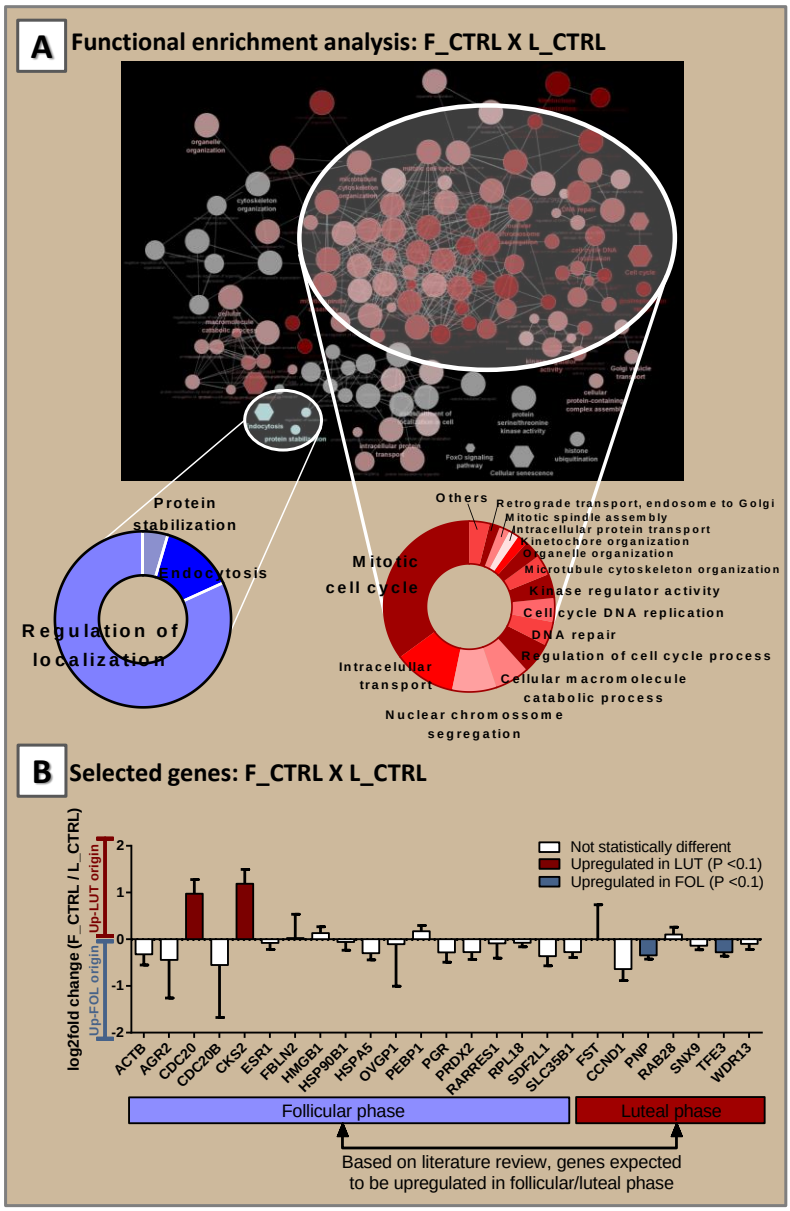

Figure 3: Specific evaluation of cells from the control treatment (F_Ctrl x L_Ctrl). **(A)** Functional enrichment analysis of gene ontology (GO) terms related to biological processes (circles) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (hexagon). The analysis was performed using the ClueGo Cytoscape plug-in. The function 'cluster' allows simultaneous visualization of genes up- and downregulated: gray-shaded nodes represent unspecific biological phenomena, blue and red-shaded nodes represent, respectively, upregulated biological processes in cells from F_Ctrl and L_Ctrl treatments. GO/KEGG terms are presented as nodes and clustered together based on the similarity of genes present in each term or pathway. Node size is also proportional to the relevance of each biological phenomenon. The only three terms enriched in cells from F_Ctrl are highlighted in the left blue circle, and the most expressive terms enriched in cells from L_Ctrl are highlighted in the right red circle. ClueGo parameters were set as indicated: only display pathways with p values < 0.1; Bonferroni step down as correction method; kappa score of 0.4, GO tree interval 3 – 8; GO term minimum 3 genes/4% of genes per pathway for F_Ctrl and 3 genes/6% of genes per pathway for L_Ctrl. The distingue percentage values were set up to balance the different number of DEG between F_Ctrl and L_Ctrl. **(B)** Representation of log2 fold changes (F_Ctrl X L_Ctrl) results of RNA-seq data of some specific genes known to be upregulated during follicular (horizontal blue bar) and luteal (horizontal red bar) phases (23,24,39).

When analyzing the BOECs submitted to *in vitro* culture under hormone treatment, a substantial decrease of DEG was detected when comparing the moment of BOECs collection (Figure 2, Supplementary Table 2). A total of 144 genes were differently expressed between F_DIE *vs.* L_DIE, of which 113 genes were upregulated in L_DIE and 31 genes were upregulated in F_DIE (Figure 2, Supplementary Table 2). The functional enrichment analysis revealed four enriched terms in the F_DIE and 17 enriched terms in the L_DIE treatment (Supplementary Table 3). About the treatment simulating the estrus phase, the evaluation of transcriptome profile resulted in 56 DEGs comparing F_EST *vs.* L_EST, while in cells submitted to SUP treatment, 20 DEGs were identified (Figure 2, Supplementary Table 2). Functional enrichment analysis across all DEGs was performed using the Cytoscape ClueGO analysis (37), and no functional GO/KEGG term was identified comparing F_EST *vs.* L_EST and F_SUP *vs.* L_SUP, probably because of the small number of DEGs.

Considering the transcriptome profile of all these BOECs, the origin of the cells (follicular *vs.* luteal) presented a huge difference in the expression of transcripts after long-term culture under no hormone stimulation. This difference was greatly reduced when cells were submitted to the DIE milieu simulation and almost nonexistent when cells were submitted to the SUP milieu simulation (Figure 2). Therefore, in an attempted to understand how the origin of BOEC could affect the transcriptome profile of cells submitted to long-term culture under no hormone treatment, but turn the cells very similar under hormone treatment, new bioinformatics analyses on RNA-seq data were performed to compare the same cell population under different conditions, in other words, comparisons were executed between 'CTRL *vs.* DIE', 'CTRL *vs.* EST', and 'CTRL *vs.* SUP' separately in BOECs from follicular and luteal origins. As result, there were 331, 794, and 427 DEGs for CTRL *vs.* DIE, CTRL *vs.* EST, and CTRL *vs.* SUP comparisons, respectively, when

analyzing cells from follicular phase origin (Figure 4). In BOECs originally from the luteal phase, there were 949, 1225, and 833 DEGs for CTRL *vs.* DIE, CTRL *vs.* EST, and CTRL *vs.* SUP comparisons, respectively (Figure 4). From this point of view, apparently, the treatments with E2 and P4 modulate greatly the transcription activity of the cells. Indeed the ovarian steroid hormones are transcription controllers well described by many studies, especially by their classical mechanism of action (42). Also known as the genomic mechanism, in the classical signaling pathway E2 and P4 bind to their respective nuclear receptors. Nuclear receptors with their ligand can interact with the DNA sequence in regions known as Hormone Responsive Element (HRE), which are regions most of the time related to gene promoter region (43). This interaction results in the recruitment of transcriptional coregulators, which can induce or repress the expression of the target gene (43). The E2 mechanism of action on its nuclear receptor ERα has been the most studied, acting on its HRE (known as estrogen-responsive element – ERE) and modulating the cellular transcriptional machinery (50). The interaction of ERα-E2-EREs can increase open chromatin, remodeling the DNA by co-modulator recruitment, resulting in an increase in transcriptional activity (50). Based on this, steroid hormones have been described as the main controllers of oviductal gene expression (42), with gene expression patterns during the luteal phase being associated with increased levels of P4 and gene expression pattern during the follicular phase being associated with increased levels of E2. Therefore, in the scenery of the present study, when hormone treatments are applied on BOECs from different origins the modulation of transcriptome regulation might lead the cells to turn very similar to one another (Figure 2).

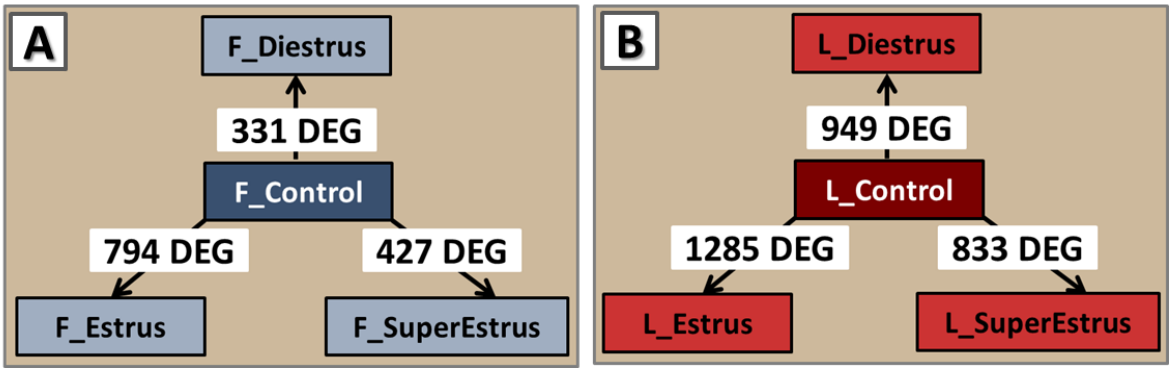

Figure 4: Total number of differentially expressed genes (DEGs, P-adj < 0.1) identified between controls and hormone-treated cells (Diestrus, Estrus, and SuperEstrus) in cells from **(A)** follicular origin and **(B)** luteal origin. F: follicular, L: luteal.

After all, it is also important to consider some limitations of our study. The results of this present study are based on a static culture system, in which the culture media was replaced every other day or every day, depending on the day of the protocol, which is not comparable to the dynamics of *in vivo* conditions. Moreover, the hormone levels were chosen based on data from local oviductal concentrations, to approximate to *in vivo* conditions, when in fact it might be necessary to adjust such levels to a static culture system. Lastly, the *in vitro* culture was performed in isolated epithelial cells, losing the possible interaction of these cells with the multicellular tissue formation. Even though these limitations are important to be considered, the results obtained in this study do not change our conclusion, since all the cells were under this same condition.

In conclusion, the data herein revealed, for the first time, that BOECs collected at follicular or luteal phases resulted in different transcriptome profiles after the cells were submitted to the same *in vitro* culture conditions with no hormone treatment. Moreover, when comparing the hormonally treated cells, the transcriptome profile presents a very similar pattern, independently of BOECs origin. In other words, when culturing BOECs for application in *in vitro* coculture system with *in vitro* produced embryos, the phase of the estrous cycle should be considered if no hormone treatment will be performed. In the case

of BOECs cultured *in vitro* be submitted to a simulation of the estrous cycle phases before the coculture system, BOECs can be harvested at any time of the estrous cycle.

REFERENCES

1. CHANG MC. Fertilization of rabbit ova in vitro. *Nature*. 1959;184(Suppl 7):466-467.
2. Ferraz MAMM, Rho HS, Hemerich D, Henning HHW, van Tol HTA, Hölker M, Besenfelder U, Mokry M, Vos PLAM, Stout TAE, Le Gac S, Gadella BM. An oviduct-on-a-chip provides an enhanced in vitro environment for zygote genome reprogramming. *Nat Commun*. 2018;9(1):4934.
3. Talukder AK, Rashid MB, Yousef MS, Kusama K, Shimizu T, Shimada M, Suarez SS, Imakawa K, Miyamoto A. Oviduct epithelium induces interferon-tau in bovine Day-4 embryos, which generates an anti-inflammatory response in immune cells. *Sci Rep*. 2018;8(1):7850.
4. Lopera-Vasquez R, Hamdi M, Maillo V, Lloreda V, Coy P, Gutierrez-Adan A, Bermejo-Alvarez P, Rizos D. Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro. *Reprod Fertil Dev*. 2017;29(3):621-629.
5. Canovas S, Ross PJ, Kelsey G, Coy P. DNA Methylation in Embryo Development: Epigenetic Impact of ART (Assisted Reproductive Technologies). *Bioessays*. 2017;39(11).
6. Cordova A, Perreau C, Uzbekova S, Ponsart C, Locatelli Y, Mermillod P. Development rate and gene expression of IVP bovine embryos cocultured with bovine oviduct epithelial cells at early or late stage of preimplantation development. *Theriogenology*. 2014;81(9):1163-1173.
7. Hafez B, Hafez ESE. Reprodução Animal. 7 ed. São Paulo.
8. Almiñana C, Corbin E, Tsikis G, Alcântara-Neto AS, Labas V, Reynaud K, Galio L, Uzbekov R, Garanina AS, Druart X, Mermillod P. Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. *Reproduction*. 2017;154(3):153-168.
9. Almiñana C, Tsikis G, Labas V, Uzbekov R, da Silveira JC, Bauersachs S, Mermillod P. Deciphering the oviductal extracellular vesicles content across the estrous cycle: implications for the gametes-oviduct interactions and the environment of the potential embryo. *BMC Genomics*. 2018;19(1):622.
10. Lopera-Vásquez R, Hamdi M, Fernandez-Fuertes B, Maillo V, Beltrán-Breña P, Calle A, Redruello A, López-Martín S, Gutierrez-Adán A, Yañez-Mó M, Ramirez M, Rizos D. Extracellular Vesicles from BOEC in In Vitro Embryo Development and Quality. *PLoS One*. 2016;11(2):e0148083.
11. van der Weijden VA, Chen S, Bauersachs S, Ulbrich SE, Schoen J. Gene expression of bovine embryos developing at the air-liquid interface on oviductal epithelial cells (ALI-BOEC). *Reprod Biol Endocrinol*. 2017;15(1):91.
12. Palma-Vera S, Einspanier R, Schoen J. Bovine oviductal epithelial cells: long term culture characterization and impact of insulin on cell morphology. *Reprod Biol*. 2014;14(3):206-212.

13. Ferraz MA, Henning HH, Stout TA, Vos PL, Gadella BM. Designing 3-Dimensional In Vitro Oviduct Culture Systems to Study Mammalian Fertilization and Embryo Production. *Ann Biomed Eng*. 2016.
14. Ireland JJ, Murphee RL, Coulson PB. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *J Dairy Sci*. 1980;63(1):155-160.
15. Simintiras CA, Fröhlich T, Sathyapalan T, Arnold GJ, Ulbrich SE, Leese HJ, Sturmey RG. Modelling oviduct fluid formation in vitro. *Reproduction*. 2016.
16. Jordaens L, Arias-Alvarez M, Pintelon I, Thys S, Valckx S, Dezhkam Y, Bols PE, Leroy JL. Elevated non-esterified fatty acid concentrations hamper bovine oviductal epithelial cell physiology in three different in vitro culture systems. *Theriogenology*. 2015;84(6):899-910.
17. Wijayagunawardane MP, Kodithuwakku SP, Yamamoto D, Miyamoto A. Vascular endothelial growth factor system in the cow oviduct: a possible involvement in the regulation of oviductal motility and embryo transport. *Mol Reprod Dev*. 2005;72(4):511-520.
18. Danesh Mesgaran S, Sharbati J, Einspanier R, Gabler C. mRNA expression pattern of selected candidate genes differs in bovine oviductal epithelial cells in vitro compared with the in vivo state and during cell culture passages. *Reprod Biol Endocrinol*. 2016;14(1):44.
19. Carvalho AV, Canon E, Jouneau L, Archilla C, Laffont L, Moroldo M, Ruffini S, Corbin E, Mermillod P, Duranthon V. Different co-culture systems have the same impact on bovine embryo transcriptome. *Reproduction*. 2017;154(5):695-710.
20. Cometti BPS, Dubey RK, Imthurn B, Rosselli M. Natural and environmental oestrogens induce TGFB1 synthesis in oviduct cells. *Reproduction*. 2018;155(3):233-244.
21. Chen S, Palma-Vera SE, Langhammer M, Galuska SP, Braun BC, Krause E, Lucas-Hahn A, Schoen J. An air-liquid interphase approach for modeling the early embryo-maternal contact zone. *Sci Rep*. 2017;7:42298.
22. Lamy J, Gatien J, Dubuisson F, Nadal-Desbarats L, Salvetti P, Mermillod P, Saint-Dizier M. Metabolomic profiling of bovine oviductal fluid across the oestrous cycle using proton nuclear magnetic resonance spectroscopy. *Reprod Fertil Dev*. 2018.
23. Lamy J, Labas V, Harichaux G, Tsikis G, Mermillod P, Saint-Dizier M. Regulation of the bovine oviductal fluid proteome. *Reproduction*. 2016;152(6):629-644.
24. Bauersachs S, Rehfeld S, Ulbrich SE, Mallok S, Prelle K, Wenigerkind H, Einspanier R, Blum H, Wolf E. Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. *J Mol Endocrinol*. 2004;32(2):449-466.
25. Cerny KL, Garrett E, Walton AJ, Anderson LH, Bridges PJ. A transcriptomal analysis of bovine oviductal epithelial cells collected during the follicular phase versus the luteal phase of the estrous cycle. *Reprod Biol Endocrinol*. 2015;13:84.
26. Lamy J, Liere P, Pianos A, Aprahamian F, Mermillod P, Saint-Dizier M. Steroid hormones in bovine oviductal fluid during the estrous cycle. *Theriogenology*. 2016;86(6):1409-1420.
27. Acuña OS, Avilés M, López-Úbeda R, Guillén-Martínez A, Soriano-Úbeda C, Torrecillas A, Coy P, Izquierdo-Rico MJ. Differential gene expression in porcine oviduct during the oestrous cycle. *Reprod Fertil Dev*. 2017;29(12):2387-2399.
28. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci U S A*. 1986;83(8):2496-2500.

29. Fontes PK, Razza EM, Pupulim AGR, Barros CM, de Souza Castilho AC. Equine chorionic gonadotropin increases estradiol levels in the bovine oviduct and drives the transcription of genes related to fertilization in superstimulated cows. *Mol Reprod Dev*. 2019;86(11):1582-1591.
30. Andrews S. FASTQC. A quality control tool for high throughput sequence data. 2010.
31. MARTIN M. Cutadapt removes adapter sequences from high-throughput sequencing reads.: **EMBnet journal**; 2011:10-12.
32. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods*. 2015;12(4):357-360.
33. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Subgroup GPDP. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-2079.
34. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics*. 2015;31(2):166-169.
35. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.
36. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 2004;5(10):R80.
37. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman WH, Pagès F, Trajanoski Z, Galon J. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics*. 2009;25(8):1091-1093.
38. Chen S, Einspanier R, Schoen J. Transepithelial electrical resistance (TEER): a functional parameter to monitor the quality of oviduct epithelial cells cultured on filter supports. *Histochem Cell Biol*. 2015;144(5):509-515.
39. Chen S, Einspanier R, Schoen J. In vitro mimicking of estrous cycle stages in porcine oviduct epithelium cells: estradiol and progesterone regulate differentiation, gene expression, and cellular function. *Biol Reprod*. 2013;89(3):54.
40. Soleilhavoup C, Riou C, Tsikis G, Labas V, Harichaux G, Kohnke P, Reynaud K, de Graaf SP, Gerard N, Druart X. Proteomes of the Female Genital Tract During the Oestrous Cycle. *Mol Cell Proteomics*. 2016;15(1):93-108.
41. Gatien J, Mermillod P, Tsikis G, Bernardi O, Janati Idrissi S, Uzbekov R, Le Bourhis D, Salvetti P, Almiñana C, Saint-Dizier M. Metabolomic Profile of Oviductal Extracellular Vesicles across the Estrous Cycle in Cattle. *Int J Mol Sci*. 2019;20(24).
42. Binelli M, Gonella-Diaza AM, Mesquita FS, Membrive CMB. Sex Steroid-Mediated Control of Oviductal Function in Cattle. *Biology (Basel)*. 2018;7(1).
43. Perrotti LI. Estrogen and Progesterone Receptor Signaling and Action. In book: Gene Regulation, Epigenetics and Hormone Signaling2017:329-354.
44. Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C. jvenn: an interactive Venn diagram viewer. *BMC Bioinformatics*. 2014;15:293.
45. Holliday R. Epigenetics: a historical overview. *Epigenetics*. 2006;1(2):76-80.
46. MP M, CB L, AM FJ, EC S, J I. Erasing gametes to write blastocysts: metabolism as the new player in epigenetic reprogramming. Vol 17: Animal Reproduction; 2020.
47. Kölle S, Hughes B, Steele H. Early embryo-maternal communication in the oviduct: A review. *Mol Reprod Dev*. 2020;87(6):650-662.

48. Barton BE, Herrera GG, Anamthathmakula P, Rock JK, Willie A, Harris EA, Takemaru KI, Winuthayanon W. Roles of steroid hormones in oviductal function. *Reproduction*. 2020;159(3):R125-R137.
49. Chen S, Palma-Vera SE, Kempisty B, Rucinski M, Vernunft A, Schoen J. In Vitro Mimicking of Estrous Cycle Stages: Dissecting the Impact of Estradiol and Progesterone on Oviduct Epithelium. *Endocrinology*. 2018;159(9):3421-3432.
50. Houshdaran S, Oke AB, Fung JC, Vo KC, Nezhat C, Giudice LC. Steroid hormones regulate genome-wide epigenetic programming and gene transcription in human endometrial cells with marked aberrancies in endometriosis. *PLoS Genet*. 2020;16(6):e1008601.
51. Magnani L, Lupien M. Chromatin and epigenetic determinants of estrogen receptor alpha (ESR1) signaling. *Mol Cell Endocrinol*. 2014;382(1):633-641.
52. Houshdaran S, Zelenko Z, Irwin JC, Giudice LC. Human endometrial DNA methylome is cycle-dependent and is associated with gene expression regulation. *Mol Endocrinol*. 2014;28(7):1118-1135.
53. Savio JD, Boland MP, Hynes N, Mattiacci MR, Roche JF. Will the first dominant follicle of the estrous cycle of heifers ovulate following luteolysis on day 7? *Theriogenology*. 1990;33(3):677-687.
54. Bisinotto RS, Chebel RC, Santos JE. Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows. *J Dairy Sci*. 2010;93(8):3578-3587.

SUPPLEMENTARY MATERIAL

Supplementary table 1: Information of samples (RNA quality and RNA-seq bioinformatics)

To access this table, click in the link below or copy and paste in your internet browser:

https://drive.google.com/file/d/1FzRNoFDwih2tXWrriglgdyF0mtcWpNg6/view?usp=sharing

Supplementary table 2: List of all DEGs in all comparisons.

To access this table, click in the link below or copy and paste in your internet browser:

https://drive.google.com/file/d/1OznX-SmqnXoW-zZYB3tMV5WZZe6YTOR7/view?usp=sharing

Supplementary table 3: List of all GO/KEGG in all comparisons.

To access this table, click in the link below or copy and paste in your internet browser:

https://drive.google.com/file/d/1fg5lu59yrtRGLxkgpZHU90wHL3kOBut1/view?usp=sharing

CONSIDERÇAÇÕES FINAIS

— FINAL REMARKS —

In this thesis, we evaluated the bovine oviduct cells under two conditions: *in vivo* (post-mortem) and *in vitro* culture system. When performing studies with oviduct cells under *in vivo* conditions, it is necessary to keep in mind the intra-abdominal location and difficulty access to the oviduct, which requires surgery or animal slaughter for sample collection. Due to this limitation to evaluate the oviduct under *in vivo* conditions, in the second part of this thesis, we performed a study with oviduct cells submitted to an *in vitro* culture system. However, the experience of using *in vitro* culture systems for oviductal cells had been associated with negative results in the distant past. Due to all these reasons, the study of the oviduct organ has been low performed, and information is still scarce, especially compared to other subjects in the field of reproduction.

As a change in this scenario, in the last decade, it has increased the focus on studying the oviduct organ, mostly using *in vitro* culture systems. An important change was caused by systems that allow 3D cell morphology. These systems have been demonstrated to support a more *in vivo*-like tissue structure and functionality, which enlarge the possibility to better understand this organ. Moreover, the same reason that caused the neglect of the oviduct for a long time is the reason why the oviduct is back in the spotlight, the *in vitro* embryo production. When bovine embryos could be produced until the blastocyst stage without any cell feeder layer, the oviduct cells were considered unnecessary for this artificial reproductive technique (ART). However, currently, information has been made clear on the benefits of the oviducts cells for the improvement of embryo quality and increased chances to establish a pregnancy.

Taking all this information into account, we planned in this thesis to obtain a broad scenario of the oviduct by investigating it under an *in vivo* and an *in vitro* condition. To do that, we chose scenarios where the oviduct can be possible use to improve embryo production: oviduct from cows submitted to ovarian superstimulation protocols, which

produces *in vivo*-derived embryos that can be transferred to recipient cows; and an *in vitro* 3D culture system that can be used for coculture system with *in vitro* produced embryos.

In **Chapter 2**, two ovarian superstimulation protocols were applied in cows and compared to synchronized cows. The first interesting result was related to the evaluation of hormone levels in the oviduct cells, in which we demonstrated that estradiol levels during the preovulatory phase are not similar between the two ovarian superstimulatory protocols and also differ from synchronized cows. These distinct estradiol profiles seem to be related to alterations of transcript activity in the oviduct cells, in which higher levels of transcripts in the oviduct cells related to genes of fertilization process control was present in cows with high estradiol levels. These alterations in the oviduct of cows submitted to ovarian superstimulation protocol could improve the development of multiples embryos present in these oviducts.

To better understand the participation of the estradiol in the oviduct cells, we forward to another investigation of oviduct cells but now in an *in vitro* model. This strategy was chosen due to the difficult access of the oviduct in an *in vivo* condition, which would require the slaughter of the animals. And also due to the easiness of working with more experimental groups when using an *in vitro* model.

In **Chapter 3**, we were interested in better understand the effect of the high levels of estradiol in the oviductal cells found in the results from chapter 2. However, the oviductal cell culture is a technique still with no gold standard, which made us ask which phase of the estrous cycle we should collect these cells to start the simulation of the estrous cycle *in vitro*. This information is very scarce in the literature, which made us go one step back in our plan and add this investigation in our experimental design. Therefore, we analyzed the effect of high levels of estradiol in oviductal cells collected at the follicular and luteal phase of the estrous cycle. To make possible the interpretation of the data, the two sources of oviductal cells were also submitted to other three experimental groups: normal estradiol

levels, high progesterone levels, and no hormone treatment. After all, we observed that the phase of the estrous cycle when the oviductal cells were collected has a huge influence on the outcome transcriptome of cells submitted to *in vitro* culture with no hormone treatment, moreover, the treatment with high estradiol levels could change the transcriptional activity of these cells resulting in a very similar profile after *in vitro* culture.

Both investigations of oviductal cells in this thesis, the ovarian superstimulation protocol and the *in vitro* culture of oviductal cells, are artificial approaches to enable the interaction between embryo and oviductal cells. Based on our results, it is clear that each approach can be very different depending on the hormone levels of each condition. Different ovarian superstimulation protocol has different estradiol levels in the oviductal cells during the preovulatory phase. And oviductal cell *in vitro* culture has different transcriptome profiles depending on which phase the cells were collected and which hormone treatment was applied during the *in vitro* culture.

When synchronization and ovarian superstimulation protocols are designed, rarely the physiology of the female reproductive tract in general (oviduct, uterus, and vagina) is taking into consideration. Related to this absence of importance drive into the oviduct organ during application of protocols for estrus synchronization, studies performed *in vivo* should be interpreted and discussed keeping in mind that the conditions are not natural. Most studies performed *in vivo* use hormone treatment for estrus synchronization, which means that the knowledge produced from these studies might not represent 100% of oviduct physiology.

The oviduct has been neglected for a long period due to the possibility of *in vitro* embryo production. However, its participation has been reactivated after the demonstration of the involvement of oviductal cells on essential epigenetics regulation of *in vitro* produced embryos, higher pregnancy rates of embryos that contacted the oviductal cells, and improvement of embryo quality. Therefore, it has been pivotal the production of a more *in*

vivo-like embryo. For that, more studies involving the understand of oviduct cells should be continuously performed to identify the main factor involved in their function, as well as factors produced by the oviductal cells that improve the embryo production. In parallel, studies performed *in vivo* should start to consider the importance of the full reproductive tract for embryo formation and pregnancy success, such as the oviduct, uterus, and vagina. And maybe, some studies should be performed in an animal under the natural estrous cycle, not hormonally synchronized.

CURRICULUM VITAE

Patricia Kubo Fontes was born on Abril 21st 1990 in Fernandópolis, São Paulo, Brazil. She finished her secondary education in 2007 in the Colégio Coopere in Fernandópolis, São Paulo, Brazil. In 2008 she started to study Biomedicine at São Paulo State University (UNESP) in Botucatu, São Paulo, Brazil, where she got her University degree in 2011. In 2012 she started a master in Pharmacology at UNESP, Botucatu, São Paulo, Brazil. During her master program, she did four months internship at the Faculty of Veterinary Medicine, University of Montreal, Canada. After her graduation in June 2014, she worked as Professor at "Universidade Paulista (UNIP)", Bauru, São Paulo, Brazil and became the researcher responsible for Gene Expression Analysis in High Throughput System at the Laboratory of Phytomedicine, Pharmacology and Technology, UNESP, Botucatu, São Paulo, Brazil. In March 2016 she started her PhD study in the Pharmacology and Biotechnology Program, UNESP, Botucatu, São Paulo, Brazil. During her PhD program, she did an one year internship at the Department of Farm Animal Health of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands. The achievements during her PhD study are described in this thesis.

RESEARCH EXPERIENCE

Laboratory of Molecular Biology and Cell Culture | São Paulo State University, Botucatu, Brazil (Mar/2016 – currently)
Supervision: Dr. Anthony César de Souza Castilho | Co-supervision: Dr. Mário Binelli

Farm Animal Health | Utrecht University, Utrecht, The Netherlands (Jan/2018 – Jan /2019)
Supervision: Dr. Bart M Gadella | Co-supervision: Dr. Tom A E Stout and Dr. Heiko H H W Henning

Laboratory of Phytomedicines, Pharmacology and Biotechnology | São Paulo State University, Botucatu, Brazil (Ago/2014 – Dez/2015)
Supervision: Dr. Marcelo Fábio Gouveia Nogueira and Dr. Anthony César de Souza Castilho

Centre de Recherche en Reproduction Animale | Université de Montréal, Saint-Hyacinthe, Canada (Ago/2013 – Dez/2013)
Supervision: Dr. Christopher Price

Laboratory of Pharmacology of Animal Reproduction | São Paulo State University, Botucatu, Brazil (Jul/2010 – Jun/2014)
Supervision: Dr. Ciro Mores Barros | Co-supervision: Dr. Anthony César de Souza Castilho

MAIN LABORATORY SKILLS

- Molecular Biology: Sample preparation, RNA extraction (Trizol, Column-based kits), RNA quality test, cDNA synthesis, RT-qPCR (96-well plate and High-throughput system/Microfluidics);
- Microscopy: Confocal microscope, Fluorescence microscope, Light microscope (Immunohistochemistry, Immunocytochemistry)
- Cell culture: primary oviduct epithelial cell culture (monolayer, tri-dimensional system);
- Gametes manipulation: ovarian follicular aspiration, oocyte selection and classification, frozen semen preparation;
- Bovine *in vitro* production of embryos: gametes *in vitro* maturation, *in vitro* fertilization, zygote denudation, embryo culture, embryo morphological classification;
- Protein quantification: Sample preparation, Protein extraction, Western blotting, Chemiluminescence;
- Data Analysis: GraphPad Prism, JMP (SAS), RNA-Seq analysis (basic level), DAVID, ClueGO (basic level).

LIST OF PUBLICATIONS

1. MA Lima, F Morotti, BM Bayeux, RG Rezende, RC Botigelli, THC De Bem, **PK Fontes**, MFG Nogueira, FV Meirelles, PS Baruselli, JC Silveira, F Perecin, MM Seneda. Ovarian follicular dynamics, progesterone concentrations, pregnancy rates and transcriptional patterns in Bos indicus females with a high or low antral follicle count. Scientific Reports, **2020**. Accepted.
2. J Ispada, AM Fonseca, CB Lima, EC Santos**, PK Fontes**, MFG Nogueira, VL Silva, FN Almeida, SC Leite, JL Chitwood, PJ Ross, MP Milazzotto. Tricarboxylic Acid Cycle Metabolites as Mediators of DNA Methylation Reprogramming in Bovine Preimplantation Embryos. International Journal of Molecular Sciences, **2020**. Accepted.
3. CB Lima, EC Santos, J Ispada, **PK Fontes**, MFG Nogueira, CR Ferreira, RG Cooks, CMD Santos, MP Milazzotto. The dynamics between in vitro culture and metabolism: embryonic adaptation to environmental changes. Scientific Reproduction, **2020**. Accepted.
4. EM Pioltine, MF Machado, JC Silveira, **PK Fontes**, RC Botigelli, AEV Quaglio, CB Costa, MFG Nogueira. Can extracellular vesicles from bovine ovarian follicular fluid modulate the in-vitro oocyte meiosis progression similarly to the CNP-NPR2 system? Theriogenology, **2020**. *doi.org/10.1016/j.theriogenology.2020.06.031*
5. LB Latorraca, WB Feitosa, C Mariano, MT Moura, **PK Fontes**, MFG Nogueira, FF Paula-Lopes. Autophagy is a pro-survival adaptive response to heat shock in bovine cumulus-oocyte complexes.Scientific Reports,**2020***.doi.org/10.1038/s41598-020-69939-3*
6. **PK Fontes**, ACS Castilho, EM Razza, MFG Nogueira. Bona fide gene expression analysis of samples from the bovine reproductive system by microfluidic platform. Analytical Biochemistry, **2020.** doi.org/10.1016/j.ab.2020.113641
7. CB Costa, PA Lunardelli, **PK Fontes**, MJ Sudano, MFG Nogueira, AA Alfieri, CR Ferreira, CB Lima, LSR Marinho, MM Seneda. Influence of cAMP modulator supplementation of in vitro culture médium on Bos taurus indicus embryos. Theriogenology, **2020**. *doi.org/10.1016/j.theriogenology.2019.09.007*
8. AB Giroto, **PK Fontes**, FF Franchi, PH Santos, EM Razza, MFG Nogueira, MA Maioli, GP Nogueira, GB Nunes, GZ Mingoti, EA Mareco, ACS Castilho. Use of pregnancy-associated plasma protein-A during oocyte in vitro maturation increases IGF-1

and affects the transcriptional profile of cumulus cells and embryos from Nelore cows. Molecular Reproduction and Development, **2019**. doi.org/10.1002/mrd.23259

9. RS Valente, TG Almeida, MF Alves, J Camargo, AC Basso, KRA Belaz, MN Eberlin, FC Landim-Alvarenga, **PK Fontes**, MFG Nogueira, MJ Sudano. Modulation of long-chain Acyl-CoA synthetase on the development, lipid deposit and cryosurvival of in vitro produced bovine embryos. PlosOne, **2019**. doi.org/10.1371/journal.pone.0220731
10. FF Franchi, RA Satrapa, **PK Fontes**, PH Santos, EM Razza, IP Emanuelli, RL Ereno, EA Mareco, MFG Nogueira, CM Barros, ACS Castilho. Equine chorionic gonadotropin drives the transcriptional profile of immature cumulus-oocyte complexes and in vitro-produced blastocysts of superstimulated Nelore cows. Molecular Reprodution and Development, **2019**. doi.org/10.1002/mrd.23251
11. **PK Fontes**, EM Razza, AGR Pupulim, CM Barros, ACS Castilho. Equine chorionic gonadotropin increases estradiol levels in the bovine oviduct and drives the transcription of genes related to fertilization in superstimulated cows. Molecular Reproduction and Development, **2019**. doi.org/10.1002/mrd.23243
12. FM Dalanezi, HDM Garcia, RA Ferrazza, FF Franchi, **PK Fontes**, ACS Castilho, MFG Nogueira, EMS Schmidt, R Sartori, JCP Ferreira. Extracellular vesicles of follicular fluid from heat-stressed cows modify the gene expression of in vitro-matured oocytes. Animal Reproduction Science, **2019**. *doi.org/10.1016/j.anireprosci.2019.04.008*
13. PC Dall'Acqua, GB Nunes, CR Silva, **PK Fontes**, MFG Nogueira, FL Lopes, M Marinho, GZ Mingoti. Differences in embryonic gene expression and quality indicate the benefit of epidermal growth factor receptor inhibitor during prematuration to improve competence in bovine oocytes. Reproduction in Domestic Animals, **2019**. *doi: 10.1111/rda.13405*
14. EM Razza, HS Pedersen, L Stroebech, **PK Fontes**, HN Kadarmideen, H Callesen, M Pihl, MFG Nogueira, P Hyttel. Simulated physiological oocyte maturation has side effects on bovine oocytes and embryos. Journal of Assisted Reproduction and Genetics, **2019**. *doi.org/10.1007/s10815-018-1365-4*
15. GP Alves, FB Cordeiro, CB Lima, K Annes, EC Santos, J Ispada, **PK Fontes**, MFG Nogueira, M Nichi, MP Milazzotto. Follicular environment as a predictive tool for embryo development and kinetics in cattle. Reproduction, Fertility and Development, **2019**. *doi: 10.1071/RD18143*
16. EC Santos, R Varchetta, CB Lima, J Ispada, HS Martinho, **PK Fontes**, MFG Nogueira, B Gasparrini, MP Milazzotto. The effects of crocetin supplementation on the blastocyst outcome, transcriptomic and metabolic profile of in vitro produced bovine embryos. Theriogenology, **2019**. *doi.org/10.1016/j.theriogenology.2018.08.010*
17. **PK Fontes**, RL Ereno, AR Peixoto, RF Carvalho, WR Scarano, LA Trinca, CM Barros, ACSouza Castilho. Can the antral follicular count modulate the gene expression of bovine oviducts in Aberdeen Angus and Nelore heifers? PlosOne, **2018**. *doi.org/10.1371/journal.pone.0202017*
18. EM Razza, MJ Sudano, **PK Fontes**, FF Franchi, KRA Belaz, PH Santos, ACS Castilho, DFO Rocha, MN Eberlin, MF Machado, MFG Nogueira. Treatment with cyclic adenosine monophosphate modulators prior to in vitro maturation alters the lipid composition and transcript profile of bovine cumulus–oocyte complexes and blastocysts. Reproduction, Fertility and Development, **2018**. *doi.org/10.1071/RD17335*
19. RC Botigelli, EM Razza, EM Pioltine, **PK Fontes**, KRL Schmarz, CLV Leal, MFG Nogueira. Supplementing in vitro embryo production media by NPPC and sildenafil affect the cytoplasmic lipid content and gene expression of bovine cumulusoocyte complexes and embryos. Reproductive Biology, **2018**. *doi.org/10.1016/j.repbio.2018.01.004*
20. PH Santos, RA Satrapa, **PK Fontes**, FF Franchi, EM Razza, F Mani, MFG Nogueira, CM Barros, ACS Castilho. Effect of superstimulation on the expression of

microRNAs and genes involved in steroidogenesis and ovulation in Nelore cows. Theriogenology, **2018**. *doi.org/10.1016/j.theriogenology.2017.12.045*

21. J Ispada, CB Lima, MA Sirard, **PK Fontes**, MFG Nogueira, K Annes, MP Milazzotto. Genome-wide screening of DNA methylation in bovine blastocysts with different kinetics of development. Epigenetics & Chromatin, **2018**. *doi.org/10.1186/s13072-017-0171-z*
22. MM Bomfim, GM Andrade, M Collado, JR Sangalli, **PK Fontes**, MFG Nogueira, FV Meirelles, JC Silveira, F Perecin. Antioxidant responses and deregulation of epigenetic writers and erasers link oxidative stress and DNA methylation in bovine blastocysts. Molecular Reproduction and Development, **2017**. doi*: 10.1002/mrd.22929*
23. RF Leite, K Annes, J Ispada, CB Lima, EC Santos, **PK Fontes**, MFG Nogueira, MP Milazzotto. Oxidative Stress Alters the Profile of Transcription Factors Related to Early Development on In Vitro Produced Embryos. Oxidative Medicine and Cellular Longevity, **2017**. *doi.org/10.1155/2017/1502489*
24. M Sponchiado, NS Gomes, **PK Fontes**, T Martins, M Collado, AA Pastore, G Pugliesi, MFG Nogueira, M Binelli. Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. PlosOne, **2017**. *doi.org/10.1371/journal.pone.0175954*
25. PH Santos, **PK Fontes**, FF Franchi, MFG Nogueira, KRA Belaz, A Tata, MN Eberlin, MJ Sudano, CM Barros, ACS Castilho. Lipid profiles of follicular fluid from cows submitted to ovarian superstimulation. Theriogenology, **2017**. *dx.doi.org/10.1016/j.theriogenology.2017.02.002*
26. JS Ticianelli, IP Emanuelli, RA Satrapa, ACS Castilho, B Loureiro, MJ Sudano, **PK Fontes**, RFP Pinto, EM Razza, RS Surjus, R Sartori, MEOA Assumpção, JA Visintin, CM Barros, FF Paula-Lopes. Gene expression profile in heat-shocked Holstein and Nelore oocytes and cumulus cells. Reproduction, Fertility and Development, **2017**. *doi.org/10.1071/RD16154*
27. RFP Pinto, **PK Fontes**, B Loureiro, ACS Castilho, JS Ticianelli, EM Razza, RA Satrapa,J Buratini, CM Barros. Effects of FGF10 on Bovine Oocyte Meiosis Progression, Apoptosis, Embryo Development and Relative Abundance of Developmentally Important Genes In Vitro. Reproduction in Domestic Animals, **2015**. *doi: 10.1111/rda.12452*
28. **PK Fontes**, ACS Castilho, EM Razza, RL Ereno, RA Satrapa, CM Barros. Prostaglandin receptors (EP2 and EP4) and angiotensinreceptor (AGTR2) mRNA expression increases in the oviductsof Nelore cows submitted to ovarian superstimulation. Animal Reproduction Science, **2014**. *dx.doi.org/10.1016/j.anireprosci.2014.10.012*
29. ACS Castilho, MFG Nogueira, **PK Fontes**, MF Machado, RA Satrapa, EM Razza, CM Barros. Ovarian superstimulation using FSH combined with equine chorionic gonadotropin (eCG) upregulates mRNA-encoding proteins involved with LH receptor intracellular signaling in granulosa cells from Nelore cows. Theriogenology, **2014**. *dx.doi.org/10.1016/j.theriogenology.2014.06.011*
30. CM Barros, RA Satrapa, ACS Castilho, **PK Fontes**, EM Razza, RL Ereno, MFG Nogueira. Effect of superstimulatory treatments on the expression of genes related to ovulatory capacity, oocyte competence and embryo development in cattle. Reproduction, Fertility and Development, **2013**. *dx.doi.org/10.1071/RD12271*

SCHOLARSHIPS & AWARDS

2018: PhD scholarship abroad program awarded by São Paulo Research Foundation – FAPESP
2017-2020: PhD scholarship awarded by São Paulo Research Foundation – FAPESP
2017: 3rd Place – Best Poster – XIX Congress Pharmaceutical of São Paulo and XI International Seminar of Pharmaceutical Sciences (São Paulo, SP, Brazil).
2016: PhD scholarship awarded by Brazilian federal government agency – CAPES
2016: 1st Place – Best Work – VI Symposium of Research and Post-Graduation from PPGRA (Pirassununga, SP, Brazil)
2016: 2nd Place – Best Work – XXX Annual meeting of Brazilian Embryo Technology Society (Foz do Iguaçu, PR, Brazil)
2015: 2nd Place – Best Work – XXIX Annual meeting of Brazilian Embryo Technology Society (Gramado, RS, Brazil)
2013: Master scholarship abroad program awarded by São Paulo Research Foundation – FAPESP
2012-2014: Master scholarship awarded by São Paulo Research Foundation – FAPESP
2011: Scientific Initiation scholarship (Bachelor) awarded by São Paulo Research Foundation – FAPESP

ORAL PRESENTATIONS

- Annual meeting of Brazilian Embryo Technology Society (SBTE), Bahia, Brazil (2019)

PK Fontes, BM Gadella, HHW Henning, HTA van Tol, TAE Stout, M Binelli, ACS Castilho. Influences of in vitro mimicking of estrous cycle phases on gene expression profiles of bovine oviduct epithelial cells obtained from pre-ovulatory or mid-luteal phase. *Student Competition Finalist.*

- Annual meeting of Association of Embryo Technology in Europe (AETE), Nantes, France (2018)

PK Fontes, HHW Henning, HTA van Tol, PLAM Vos, TAE Stout, BM Gadella. Detection of adult stem cell marker leucine-rich repeat-containing G-protein-coupled receptor-5 (LGR5) transcripts in bovine epithelial cells. *Student Competition Finalist.*

- International Ruminant Reproduction Symposium (IRRS), Foz do Iguaçu, Brazil (2018)

CB De Lima,EC Dos Santos, J Ispada, **PK Fontes**, MFG Nogueira, CMD Dos Santos, MP Milazzotto. Comprehensive evaluation of metabolic behavior in preimplantation embryos. Society for the Study of

- Reproduction Annual Meeting (SSR), New Orleans, EUA (2018)

CB De Lima EC Dos Santos, J Ispada, **PK Fontes**, MFG Nogueira, CMD Dos Santos, MP Milazzotto. Mapping of metabolic behaviors in embryos as a model for the improvement of cell culture systems in vitro

- Annual meeting of Brazilian Embryo Technology Society (SBTE), Foz do Iguaçu, Brazil (2016)

M Sponchiado, NS Gomes, G Pugliesi, T Martins, M Del Collado, RS Ramos, MR França, ML Oliveira, AM Gonella-Diazza, K Ribeiro, DC Cuadros**, PK Fontes**, AA Patrone, MFG Nogueira, M Binelli M. The bovine embryo modulates endometrial function 7 days after estrus in vivo

- International Congress of Animal Reproduction (ICAR), Tours, France (2016)

RF Leite, K Annes, J Ispada, **PK Fontes**, MFG Nogueira, MP Milazzotto. Influence of oxygen tension in the transcription pattern and cell differentiation of bovine embryos produced in vitro.

SUPERVISING AND MENTORING

- 05/03/2018-04/03/2020: BSc. Talita Raquel Cavichioli Sebastião – Master Student

Supervisor: Anthony Cesar de Souza Castilho, Co-supervisor: **Patricia Kubo Fontes**
Project: Effect of α-L-fucosidase on zona pellucida hardening before fertilization and in vitro embryo production

- 05/06/2019-04/06/2020: Thainá Sallum Bacco Manssur – Bachelor Student

Supervisor: Anthony Cesar de Souza Castilho, Co-supervisor: **Patricia Kubo Fontes**
Project: Effect of α-L-fucosidase during the in vitro fertilization of bovine embryos
Scientific Initiation scholarship awarded by São Paulo Research Foundation – FAPESP

- 05/08/2016-04/08/2017: Natalia Luca Pereira – Bachelor Student

Supervisor: **Patricia Kubo Fontes**
Project: Does GRP78 added to the in vitro fertilization step improve bovine in vitro embryo production?

- 05/09/2015-04/09/2016: Raquiel Bueno Rodrigues – Bachelor Student

Supervisor: Anthony Cesar de Souza Castilho, Co-supervisor: **Patricia Kubo Fontes**
Project: Cellular and molecular modulation of PAPPA protein added to in vitro maturation of bovine oocytes
Scientific Initiation scholarship awarded by São Paulo Research Foundation – FAPESP

- 05/08/2015-04/08/2016: Rayane Isabelle Turkocio – Bachelor Student

Supervisor: **Patricia Kubo Fontes**
Project: Embryo quality marker in bovine blastocyst: evaluation of α-L-fucosidase effect during fertilization

- 05/08/2015-04/08/2016: Andressa Carvalho – Bachelor Student

Supervisor: **Patricia Kubo Fontes**
Project: Bioavailability modulation of IGF-1during in vitro maturation of bovine oocytes

- 05/08/2015-04/08/2016: Carla de Cássia Malavasi – Bachelor Student

Supervisor: **Patricia Kubo Fontes**
Project: Does α-L-fucosidase modulates the in vitro fertilization process of bovine embryos?

LINKS

- ORCID: https://orcid.org/0000-0002-4280-9575
- Research ID: https://publons.com/researcher/R-8362-2017/
- MyCitations: scholar.google.com/citations?hl=en&user=KZPdk3cAAAAJ&view_op=list_works