PRISCILA HELENA DOS SANTOS

EFEITOS DA PROTEÍNA OVIDUTAL HSPA5 NA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

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Tese apresentada ao programa de Pós-graduação do Instituto de Biociências de Botucatu, Universidade Estadual Paulista – UNESP, para a obtenção do título de Doutor em Farmacologia e Biotecnologia.

Orientador: Prof. Doutor Anthony César de Souza Castilho

Botucatu – SP

2022

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Santos, Priscila Helena dos.
Efeitos da proteína ovidutal HSPA5 na produção in vitro de embriões bovinos / Priscila Helena dos Santos. - Botucatu, 2022

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Anthony César de Souza Castilho
Capes: 21005001

1. Bovinos - Reprodução. 2. Proteínas de choque térmico. 3. Homeostase celular. 4. Fertilização in vitro. 5. Transcrição genética.

Palavras-chave: BiP; GRP78; Heat shock protein; MIV; PIVE.

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Data da defesa: 11 de abril de 2022.

"O que prevemos raramente ocorre; o que menos esperamos geralmente acontece. "

(Benjamin Disraeli)

AGRADECIMENTOS

Aos meus pais, meus maiores apoiadores desta jornada, que mesmo não entendendo o percurso, confiaram, acreditaram e me incentivaram do início ao fim. Ao meu marido, Guilherme, que além de estar pacientemente ao meu lado, me deu suporte, incentivo e deixou a vida mais leve. Às minhas avós pelas constantes orações e amor incondicional. Aos meus avôs, que levo no coração, pelo carinho que será sempre lembrado. Aos meus irmãos e cunhados por serem inspiração. Ao meu orientador, por acreditar em mim desde o início, pela oportunidade de fazer parte de um time de excelência e pela amizade além da pós-graduação. À minha amiga, madrinha, companheira de todas as horas, que mesmo na distância se faz presente, Fernanda (Tico). Obrigada por ouvir, respeitar e estar comigo. Às amigas que além do suporte científico, estiveram comigo nas alegrias e tristezas fora da pós-graduação, Sarah e Patrícia. Botucatu não seria a mesma sem vocês. Durante os cinco anos de estudo, tive a oportunidade (e sorte) de conviver com muitas pessoas que deixaram marcas e aprendizados. Agradeço a cada colega e amigo do LaMem, FitoFarmaTec e Biotera que compartilhou conhecimento, risadas e cafés filosóficos comigo. Talita, Ketlyn, Eduardo, Carol, Elisa, Erika, Vitória, Ana Elise e Alan. À Raquel, pelo companheirismo, por ser prestativa e salvar nossas vidas nas intercorrências. Ao professor Di Stasi pela confiança e acolhimento em seu laboratório. À Janete, que sempre cuidou tão bem dos alunos da Farmacologia e aos demais funcionários. À seção-técnica de pós-graduação do Instituto de Biociências de Botucatu, em especial ao Davi. Aos professores do Departamento de Farmacologia do Instituto de Biociências de Botucatu pelas disciplinas. Aos professores João Ferreira, Mateus Sudano, Isabele Picada e a Raquel Puelker, por atenderem prontamente meu convite para compor a banca de doutorado. À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo financiamento à pesquisa (# 2018/06674-7;

2013/11480-3; 2015/04505-5) e à Coordenação de aperfeiçoamento de Pessoal de Nível Superior – CAPES (Financiamento Cód 001) pelo tempo de bolsa concedida.

RESUMO

A proteína HSPA5 (do inglês, *heat shock 70 kDa protein 5*), também conhecida como GRP78 (do inglês, 78 kDa glucose-regulated protein) e BiP (do inglês, immunoglobulin-binding protein), é uma chaperona do retículo endoplasmático que desenvolve um papel fundamental na manutenção da homeostase celular. Relativamente recente, essa proteína foi descrita como um dos componentes do fluido ovidutal com capacidade de se ligar aos espermatozoides e aumentar a concentração de cálcio intracelular. Considerando que o ambiente ovidutal fornece substratos e fatores importantes para a fusão dos gametas e desenvolvimento inicial embrionário *in vivo* e que a proteína HSPA5está em alta concentração no fluido ovidutal, nós acreditamos que essa proteína pode exercer efeitos benéficos na produção de embriões *in vitro*. Hipotetizando que a HSPA5 pode interagir com o complexo *cumulus*-oócito (COC) durante a maturação *in vivo* (MIV) e também com o embrião em desenvolvimento durante o cultivo *in vitro* (CIV), a temática primordial desta tese foi testar a adição da HSPA5 recombinante humana na produção *in vitro* de embriões (PIVE). Em um primeiro momento, nós analisamos o efeito da HSPA5 nas últimas 4 horas da MIV sobre os níveis de espécies reativas de oxigênio (ROS) e potencial de membrana mitocondrial (MMP) dos oócitos e subsequentes embriões, os níveis de glutationa (GSH) nos COCs e meio de cultivo, a solubilidade da zona pelúcida e penetração espermática, sobre a taxa de embriões produzidos e o perfil de transcritos destes embriões. Na segunda abordagem nós testamos a adição da rHSPA5 nos oito dias de CIV e comparamos com o tratamento na MIV e a associação dos dois tratamentos. Foi avaliado a taxa de embriões produzidos e a transcrição gênica destes embriões. Em suma, os resultados indicaram que a rHSPA5 é capaz de interagir com o COC durante a MIV e com os embriões em desenvolvimento no CIV. A proteína foi capaz de aumentar taxa de embriões produzidos quando adicionada nas últimas 4 horas de MIV, manter os níveis de ROS e GSH nos COCs, mas não aumentou o tempo de solubilidade da *zonna-pellucida* (ZP) ou alterou a porcentagem

de monospermia, mas a rHSPA5 na MIV foi capaz de modular positivamente a expressão gênica dos embriões produzidos. Já a inclusão da proteína no CIV, apesar de haver uma pequena modulação na expressão gênica, não foi capaz de aumentar a taxa de embriões produzidos. Com os presentes achados, concluímos que, a HSPA5 pode interagir com o COC e ser capaz de exercer funções ainda não determinadas, assim como o uso da rHSPA5 nas últimas 4 horas da MIV pode otimizar a PIVE e potencializar a qualidade embrionária.

Palavras-chave: GRP78, BiP, heat shock protein, MIV, CIV, PIVE

ABSTRACT

The HSPA5 protein (heat shock 70 kDa protein 5), also known as GRP78 (78 kDa glucose-regulated protein) and BiP (immunoglobulin-binding protein), is a chaperone from the endoplasmic reticulum with an important role in cellular homeostasis. Relatively recent, this protein was described as an oviductal fluid component as well as, its ability to hardly bind the spermatozoa, and then, increase the intracellular calcium abundance. The oviduct provides an appropriate environment for gametes fusion and early embryo development. Once the HSPA5 is one of the main heat shock proteins from oviductal fluid, we have a hypothesis that this protein is able to be beneficial on *in vitro* embryo production. Hypothesizing that HSPA5 can interact with *cumulus*-oocyte complex (COC) during the *in vitro* maturation (MIV) and, also with developing early embryo on *in vitro* culture (CIV), the primordial thematic of this thesis tested the HSPA addition on *in vitro* embryo production. First of all, we analyzed the HSPA5 effect on the last 4 hours of MIV under reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) levels and on the subsequent embryos. The glutathione (GSH) levels on COC and culture medium, the *zona-pellucida* (ZP) solubility, sperm penetration, and last, the rate of embryo production and transcript profile of them. The second point was tested the HSPA5 effect on the eight days of CIV to compare with MIV treatments and an association of both treatments (on the last 4 hours of MIV and 8 days of CIV). We assessed the embryo rate and transcriptome profile of them. In conclusion, the results indicated that HSPA5 was able to interact with the COC and the developing early embryo. This protein was capable to increase the embryo rate on the MIV treatment and maintaining the ROS and GSH levels on COC, but it did not increase the ZP solubility or monospermy rate. Mainly, the HSPA5 was able to positively modulate the gene expression of embryos. On the other hand, the HSPA5 on the CIV did not alter the embryo rate and found a lower modulation on the transcript profile. We conclude that HSPA5 interacts with COC and develops an unknown role and also, the inclusion

of rHSPA5 on the last 4 hours of MIV can improve the *in vitro* embryo production and enhance the embryo quality.

Keywords: GRP78, BiP, heat shock protein, MIV, CIV, PIVE

SUMÁRIO

PREFÁCIO

A presente tese a ser defendida pelo programa de Pós-graduação em Farmacologia e Biotecnologia está dividida em quatro sessões. A primeira, denominada **Capítulo I**, compreende uma Revisão Integrativa que traz um compilado de publicações que introduz a relação das *Heat Shock proteins* com os gametas e embriões, e mostra como essas proteínas, que estão presentes no oviduto, estão envolvidas com o processo reprodutivo. Essa revisão além de ser a base para uma futura revisão sistemática, foi utilizada como uma introdução à temática desta tese. Das duas partes subsequentes, o **Capítulo II - "Evidences that heat shock protein A5 (HSPA5) plays role during bovine in vitro embryo production"** e **Capítulo III – "As novas estratégias do uso da rHSPA5 humana na produção *in vitro* de embriões"**, correspondem um como uma proposta de manuscrito a ser submetido como artigo no periódico *"Reproduction in Domestic Animals"*, e o outro a descrição dos resultados recém obtidos pelo grupo, que será utilizado como base para o próximo artigo. Por fim, a sessão **"Considerações finais"** compreende as conclusões gerais (descrevendo de forma sucinta os resultados obtidos).

Conforme normas do programa de Pós-graduação, o presente exemplar consiste na versão definitiva da tese a ser encaminhada à biblioteca e fará parte do conjunto de publicações do Instituto de Biociências de Botucatu da UNESP. No entanto, o manuscrito aqui apresentado está no prelo, mas não comprometido com a publicação em quaisquer periódicos, estando, assim, passível de correções e modificações por parte da banca de defesa.

CAPÍTULO I

THE FUNCTIONALITY OF OVIDUCTAL HEAT SHOCK PROTEINS ON GAMETES AND EMBRYOS: AN INTEGRATIVE REVIEW

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Running title: How the HSPs from oviductal fluids operate on gametes and embryos.

ABSTRACT

The fusion of gametes to generate an embryo takes place on the oviduct. For a long period, this tubal-like organ was forgotten once the *in vitro* embryo production reaches a great success rate. After it become a study target, their oviductal fluid (OF) was assessed and a range of proteins with an important role on gametes and embryos became known. Some of them were the Heat-Shock proteins (HSPs), a chaperone kind protein responsible to maintain viability and cellular homeostasis. Different HSPs can be found at OF and in the oviductal epithelial cells (OEC) at different portions of the oviduct but few of them were assessed about their possible role on gametes or embryos. This integrative review was conducted examining 323 publications from three databases, to merge relevant publications about HSPs localization on OF and OEC, the interaction, and effects on gametes and early embryos. This study reveals that even HSPs are present on OF, only three proteins have more than one publication about a direct effect on spermatozoa capacity or fertilization process (HSPA5, HSPA8, and HSP60), but a range of them were predicted as actively participating in the reproduction process and a possible additive

on a culture medium to improve the *in vitro* embryo production and quality. After all, more studies are necessary to completely understand the OF HSPs` role on gametes and embryos.

INTRODUCTION

In the *in vivo* embryo development, a range of coordinated processes occurs. Sperm capacitation, fertilization, and embryo development take place in the oviduct during the peri-ovulatory period of the estrous cycle and to each step, the oviduct provides an optimal environment. The oviduct is a dynamic structure of the reproductive system that connects the ovary to the uterus and is divided into three anatomical regions where each physiological process occurs: the infundibulum, ampulla, and isthmus (Avilés, 2010; Pillai *et al.*, 2017).

When the spermatozoa enter into the oviduct in mammalians, they form a sperm reservoir on the isthmus portion. It is possible because the sperm cells bind to the oviductal-cell membranes and can remain adhered for hours to be released only near the time of ovulation. In the isthmus portion, the spermatozoa undergo hyperactivation and early acrosome reaction. As soon as the complex oocyte-*cumulus* (COC) is picked-up by adhesion at the cilia of the infundibulum its cumulus matrix becomes compacted to enter into the oviduct. When the oocyte reaches the ampulla, it immediately attaches to the oviductal epithelium and its zona-*pellucida* becomes more accessible to the OF and the spermatozoa are released from the reservoir and migrate driving to the oocyte (Kolle *et al.*, 2009; Kolle *et al.*, 2010).

From the gamete's passage to the fusion until the early embryo development, the structures maintain contact with the OF, which is essential to these physiological processes (Avilés, 2010). The OF is a complex mixture of components produced by secretions from OEC and plasm transudate with a spatiotemporally dynamic process (Mondejar *et al.*, 2013; Pillai *et al.*, 2017). Multiple proteins are secreted by the oviductal cells, including HSPs (Killian, 2011; Yanagimachi, 2015; Soleilhavoup *et al.*, 2016).

The HSPs are widely expressed and present in the genome of all cellular organisms with the main role in protein degradation, breakdown, and transmembrane transport. There are about ten types of these molecular chaperones and they are divided into seven families according to molecular mass (sHSP, HSP40, HSP60, HSP70, HSP90, HSP110, and ubiquitin) (Shan *et al.*, 2020). For a long period, the HSPs were reported to be an exclusive intracellular component, but later the HSPs were described at the plasmatic membrane and secreted, including in OEC and OF (Buhi *et al.*, 2000; Boilard *et al.*, 2004; Marín-Briggiler, 2010).

Studies report the influence and the essential role of HSPs on OF. Some of these proteins can interact with gametes and improve fertilization (Mondejar *et al.*, 2013), the spermatozoa capacitation and viability (Lloyd *et al.*, 2009; Lamy *et al.*, 2018), and possibly, play a role in early embryo development (Alminana *et al.*, 2017). Although embryo production is possible without the oviduct, this environment provides factors that a simple *in vitro* culture cannot supply and that improve the embryo quality (Mahdavinezhad *et al.*, 2019). Then, better knowledge about the interaction of HSPs and gametes and embryos allows benefits on *in vitro* embryo production efficiency and enhances the embryo quality. This integrative review aimed to merge relevant publications about HSPs localization on OF and OEC, the interaction, and effects on gametes and early embryos.

METHODOLOGY

This article is an integrative review that was conducted following the steps below: 1. identification of the theme and formulation of the guiding question; 2. establishment of criteria to include or exclude the studies; 3. definition of keywords to research in databases; 4. evaluation and categorization of data; 5. interpretation of the results obtained; 6. presentation of results.

The search was carried out in December 2021 using the PubMed, Scopus, and Web of Science databases and involved an extensive review to address the main question: What is the function of heat shock protein from oviductal fluid on gametes and embryos? For it, the search was electronically performed for the following terms in the titles, abstracts, and keywords: 'heat shock protein' and 'oviduct'. I*n vitro* or *in vivo* research with mammals that demonstrate the interaction of HSP with gametes or embryos were included. Studies that used proteomic or immunolocalization analyses that demonstrate the presence of HSP on the oviduct were also included. There was no restriction about the period of publication. First of all, duplicated articles were removed, and then publications were selected by title and abstract. Exclusion criteria included publication not in English language, use of no mammalian as a model, investigations of environmental effect and pathologies on HSP levels, or induction of higher levels of HSP as thermal stress. A total of 32 studies met the criteria, but once five publications were a kind of revision that cited studies already included in this work, they were removed. Then, twenty-seven studies were eligible for this integrative review at the end of the selection process (fig 1).

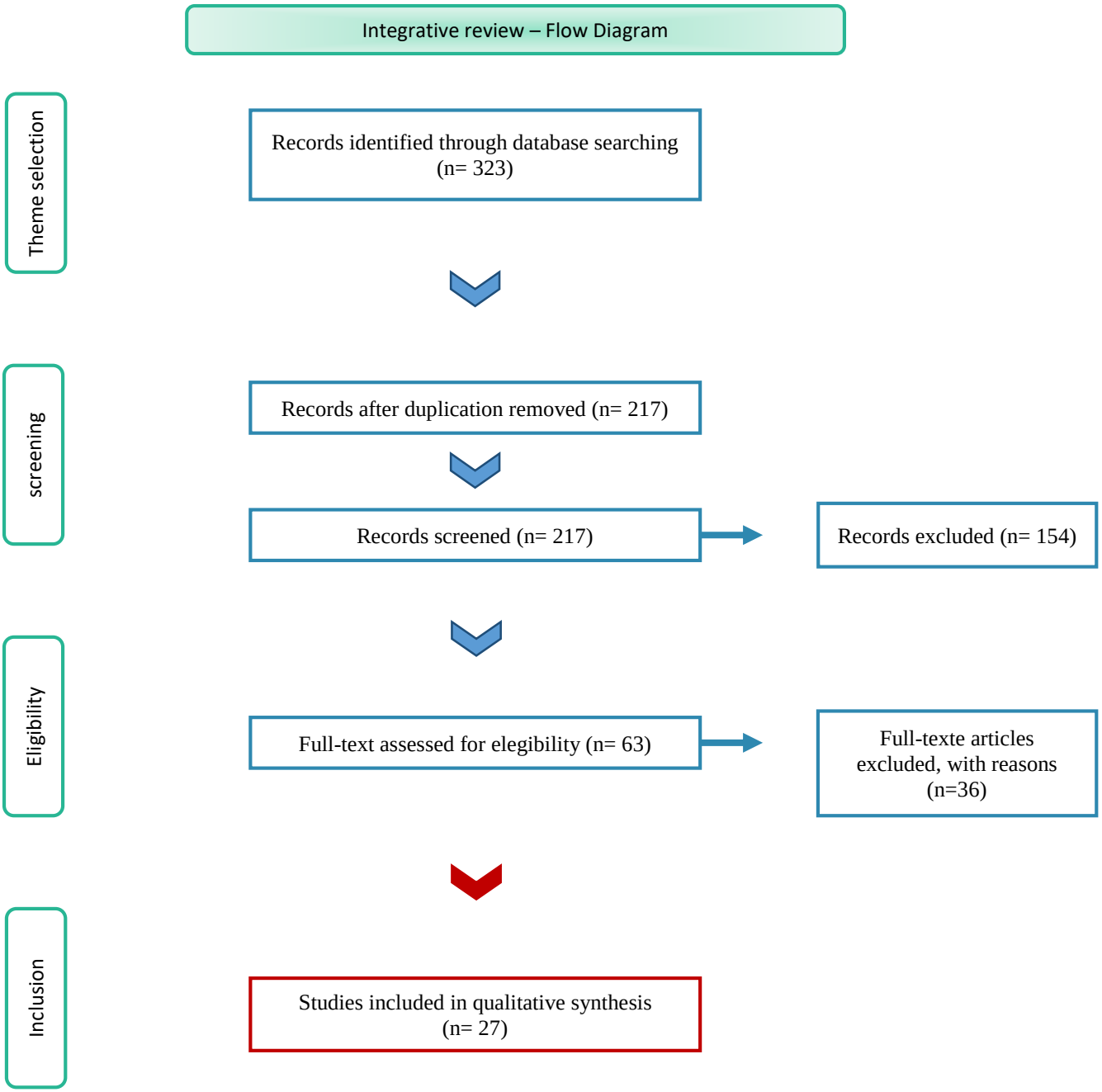

Fig 1. Flowchart of search and selection process. Thirty-seven studies met the criteria and investigate HSPs on the oviduct or its function and were used to review.

RESULTS

Of twenty-seven studies that met the criteria (Table 1), twelve were studies that addressed two or more HSPs. Five mainly focused or at least quote HSPA8, three the HSPA5, two the HSP70, and five studies approached another HSP. About it, in eight of the studies, one

or more proteins was found by proteomic, transcriptomic, or localization analysis and, besides implying a functionality of the proteins on gametes and/or embryo, it did not show data to clarify.

Table 1. Articles that met the criteria and were selected for the integrative review.

Article	Article Title	Authors	Protein
1	Constitutive expression of heat-shock proteins hsp25 and hsp70 in the rat oviduct during neonatal development, the oestrous cycle and early pregnancy	Mariani *et al.*, 2000	HSP25 and HSP70
2	Localization of the chaperone proteins GRP78 and Hsp60 on the luminal surface of bovine epithelial cells and their association with spermatozoa	Boilard *et al.*, 2004	HSPA5 and HSP60
3	Expression of Hsp60 and GRP78 in the human endometrium and oviduct, and their effect in sperm functions	Lachance *et al.*, 2007	HSP60 and HSPA5
4	Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals	Mondéjar *et al.*, 2013	HSP90A, HSPA5, HSP90B and HSP70
5	Viable and morphological normal boar spermatozoa alter the expression of heat-shock protein genes in oviductal epithelial cells during co-culture *in vitro*	Yeste *et al.*, 2014	HSPA8, HSP90AA1 and HSPA5
6	Proteomes of the female genital tract during the oestrous cycle	Soleilhavoup *et al.*, 2016	HSPA8, HSP90B1 and HSP70
7	Regulation of the bovine oviductal fluid proteome	Lamy *et al.*, 2016	HSPA5, HSP90AA1, HSPA8, HSP90AB1, HSP90B1, HSPA6, HSPA4 and HSP70
8	Oviductal extracellular vesicle protein content and their role during oviduct-embryo cross-talk	Almiñana *et al.*, 2017	HSPA1A, HSP60, HSPA4, HSPA5, HSPA8, HSP90AA1, HSP90AB1 and HSP90B1
9	Semen modulated secretory activity of oviductal epithelial cells is linked to cellular proteostasis network remodeling: Proteomic insights into the early phase of interaction in the oviduct *in vivo*	Steinberger *et al.*, 2017	HSPA5 and HSP90B1
10	Identification by proteomics of oviductal sperm-interacting proteins	Lamy *et al.*, 2018	HSPA5, HSP27 and HSP90B1

11	Addition of exogenous proteins detected in oviductal secretions to *in vitro* culture medium does not improve the efficiency of *in vitro* fertilization in pigs	García-Martinez *et al.*, 2020	HSP90α and HSP70
12	Oviductal fluid during IVF moderately modulates polyspermy *in vitro*-produced goat embryos during the non-breeding season	Bragança *et al.*, 2021	HSPA8 and HSPA1A
13	Effects of oviductal proteins, including heat shock 70kDa protein 8, on survival of ram spermatozoa over 48 h *in vitro*	Lloyd *et al.*, 2009	HSPA8
14	Effects of HSPA8, an evolutionarily conserved oviductal protein, on boar and bull spermatozoa	Elliott *et al.*, 2009	HSPA8
15	The oviductal protein, heat-shock 70-kDa protein 8, improves the longterm survival of ram spermatozoa during storage at 17°C in a commercial extender	Lloyd *et al.*, 2011	HSPA8
16	Heat-shock protein A8 restores sperm membrane integrity by increasing plasma membrane fluidity	Moein-Vaziri *et al.*, 2014	HSPA8
17	Heat Shock protein A8 stabilizes the bull sperm plasma membrane during cryopreservation: Effect of breed, protein concentration, and mode of use	Holt, Valle and Fazeli 2015	HSPA8
18	Glucose-regulated protein 78 (Grp78/BiP) is secreted by human oviduct epithelial cells and the recombinant protein modulates sperm-zona pellucida binding	Marín-Briggiler *et al.*, 2010	HSPA5
19	GRP78 expression and the immunohistochemical localization in the female reproductive tract of the mice	Lin *et al.*, 2012	HSPA5
20	Equine chorionic gonadotropin increases estradiol levels in the bovine oviduct and drives the transcription of genes related to fertilization in superstimulated cows	Fontes *et al.*, 2019	HSPA5
21	Expression of the testis-specific HSP70-related gene (HST70 gene) in somatic non-testicular rat tissue revealed by RT-PCR and transgenic mice analysis	Scieglinska *et al.*, 1997	HSP70
22	Gametes alter the oviductal secretory proteome	Georgiou *et al.*, 2005	HSP70
23	Expression and cellular localization of the mRNA for the 25-kDa heat-shock protein in the mouse	Wakayama and Iseki 1998	HSP25
24	An immunohistochemical study of the *rete ovarii* and epoophoron	Woolnough *et al.*, 2000	HSP27
25	The expression pattern of the 70-kDa heat shock protein Hspa2 in the mouse tissues	Vydra *et al.*, 2009	HSPA2

26	The effect of HSP60 on fertilization and pre-implantation embryo development in mice: an *in vitro* study	Abdi *et al.*, 2019	HSP60
27	Which low-abundance proteins are present in the human milieu of gamete/embryo maternal interaction?	Canha-Gouvea *et al.*, 2019	HSP90A

Among the selected article, ten studies only demonstrated the localization or the expression of one or more HSP in the oviduct, three reports an altered expression of HSP on epithelial cells or oviductal secretory proteome in a presence of spermatozoa, and one only demonstrated the interaction of oviductal proteins with spermatozoa after co-incubation, and not the effect of proteins. So, from twenty-seven selected studies, thirteen reports some effect and interaction of HSP on the gametes. From this, seven studies showed the effect of HSP on spermatozoa and four on *in vitro* fertilization. Only one study showed a possible effect of HSP on the embryo through extracellular vesicles, and one publication suggests the effect of HSP on the oocyte pre IVF (Table 2; Fig 2).

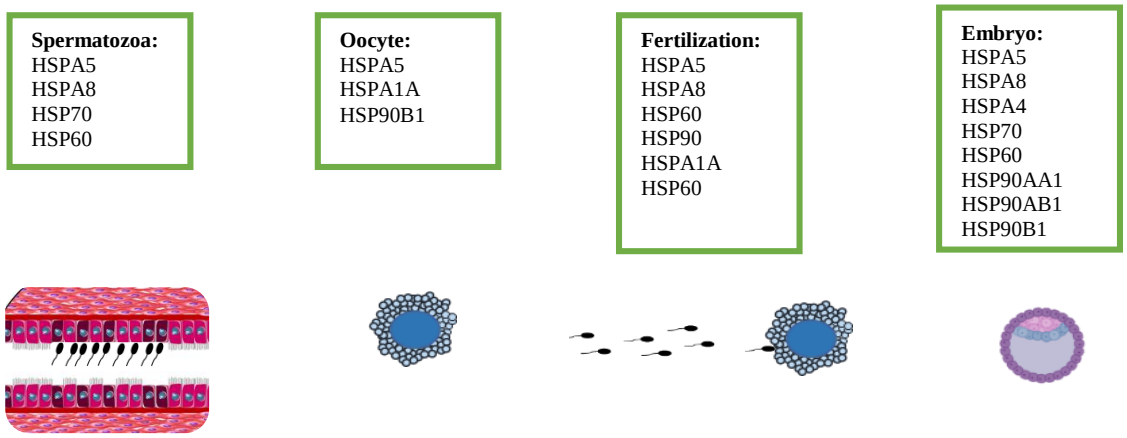

Figure 2: The oviductal HSPs that interact, were predicted, or play some effect on spermatozoa, oocyte, fertilization process, or on the embryo, described by studies included in this integrative review.

Table 2. Objectives of included studies, animal models, and a summary of main results.

Article	Objective	Animal model	Main results
1	Identify hsp25 and hsp70 in the development of the oviduct; to compare and study hsp25 and hsp70 during the estrous cycle and early pregnancy, and examine hsp25 and hsp70 association with estrogen receptor α regulation.	Rats	HSP70 is modulated by the estrous cycle and is more responsive to hormonal changes.
2	Identify oviduct originating factors that associate with spermatozoa and putatively affect their cellular activity.	Bovine	This demonstrates that both HSP60 and GRP78 are expressed on the surface of oviduct epithelial cells and they strongly associate with spermatozoa.
3	Determine whether HSP60 and HSPA5 are expressed by the epithelial cells from the endometrium and oviducts with a cellular localization that would allow interaction with the sperm surface. And determine their effect of them on human sperm functions and investigate whether they modulate the acquisition of fertilizing ability of human spermatozoa.	Human	Both proteins are present in the oviduct epithelial cells, bind to spermatozoa, and increased the sperm intracellular calcium concentration.
4	Investigate whether variations in oocyte hardening in different oviductal fluids are correlated with variations in levels of monospermy after IVF and identify the factors responsible for this effect.	Porcine	The mechanism of pre fertilization ZP hardening in OF is directly related to monospermy levels and suggest a role of proteins from the HSP family.
5	Determine if boar spermatozoa influence the expression of proteins in oviductal epithelial cells (OECs) during in vitro co-culture.	Porcine	Bind spermatozoa induced the upregulation of the HSPs after co-culture.
6	Provide an integrated analysis of the luminal proteomes of the female genital tract by a proteomic study of inner cervical mucus, uterine fluid, and oviduct fluid and quantify the abundance of these luminal proteins throughout the estrous cycle.	Sheep	-
7	Investigate the regulation of the proteome in the bovine oviductal fluid according to the stage of the estrous cycle, to the side relative to ovulation, and local concentrations of steroid hormones	Bovine	-

8	Deciphering the oviduct EVs protein content from both sources and analyzing their functional effect on embryo development.	Bovine	Showed that *in vitro*-produced embryos were able to internalize *in vivo* EVs during culture with a functional effect in the embryo development.
9	Assess the effect of semen on the cellular and molecular mechanisms in rabbit OECs	Rabbit	-
10	Develop a strategy to identify and quantify oviductal proteins that interact with spermatozoa and study the regulation of these interactions in three stages of the estrous cycle: the post-, pre-ovulatory, and luteal phases.	Bovine	-
11	Study whether proteins previously identified in porcine oviductal secretions have a role in zona pellucida resistance to enzymatic digestion, in vitro fertilization, and sperm viability.	Porcine	Sperm penetration and polyspermy rates decreased in presence of HSP proteins.
12	Determined the presence of proteins known to be involved in early reproduction in the oviduct fluid of anestrous goats; and the functional effect of IVF on polyspermy modulation and embryonic development.	Goat	The supplementation with OF during IVF may modulate the polyspermy incidence and enhance IVF efficiency.
13	Decipher which component(s) of the ewe oviduct actively participates in maintaining the viability of ram spermatozoa.	Sheep	Recombinant HSPA8 had a beneficial effect on the viability of ram spermatozoa during coincubation.
14	Assess the effects of HSPA8 on boar and bull spermatozoa.	Boar and bovine	The HSPA8 has an important role in the maintenance of boar and bull sperm survival in vitro implying that the mechanism of action could be highly conserved across species.
15	Determine whether supplementing extenders with HSPA8 would improve their performance in maintaining freshly collected ram sperm viability and sperm nuclear DNA integrity during storage over 48 h at 178C.	Ram	The extender supplemented with HSPA8 maintained sperm viability significantly better than alone
16	Characterize the immediate impact of exogenous HSPA8 on spermatozoa.	Boar	The HSPA8 rapidly promotes the viability of uncapacitated spermatozoa, the ability of spermatozoa to bind to oviductal epithelial cells, enhances IVF

			performance, and decreases sperm mitochondrial activity.
17	Explore the possibility that bovine recombinant HSPA8 might therefore protect bull spermatozoa during cryopreservation through its beneficial effects on the sperm plasma membrane.	Bovine	The study underlined the potential benefits of controlling plasma membrane permeability, especially in the more fragile semen samples from the beef cattle.
18	Determine the secretion of GRP78 by human epithelial cells, its association to spermatozoa, and its involvement in gamete interaction	Human	Soluble GRP78 is present in OF in the periovulatory period and recombinant GRP78 was able to bind to the sperm acrosomal cap.
19	Determine the distribution and cyclic variations of GRP78 in the mouse oviduct and uterus throughout the estrous cycle.	Mouse	-
20	Evaluate the effect of two superstimulatory protocols on the oviductal levels of E2 and P4 and their outcome on oviductal cells.	Bovine	The FSH/eCG treatment showed a higher concentration of E2 and a higher abundance of mRNA HSPA5 in the infundibulum and ampulla.
21	Detecting the hst70 gene transcripts in various male and female non-spermatogenic rat tissues.	Rat	-
22	Test the hypothesis that the presence of gametes in the oviduct alters the oviductal secretory proteomic profile.	Porcine	The oviductal response to spermatozoa was different from its response to oocytes and both of them altered the secretion of specific proteins.
23	Examine the expression and cellular localization of Hsp25 mRNA in mice under physiological and unstressed conditions.	Mouse	-
24	Compare the immunohistochemical profile of the human *rete ovarii*, and epoophoron, with the Fallopian tube and ovarian surface epithelium.	Human	-
25	Assess the extra-testicular expression pattern of mouse Hspa2.	Mouse	-
26	Assess the importance of HSP60 in sperm capacitation and the effect of HSP60 on the	Mouse	HSP60 in low doses had a positive effect on

	rate of *in vitro* fertilization and the cleavage rate in the mouse.		cleavage embryos. But it had adverse effects at higher doses.
27	Performer a comparative study of the low abundance proteins in plasma, uterine, and oviductal fluid collected, simultaneously, from healthy and fertile women that underwent a salpingectomy.	Human	-

The most employed animal model was bovine with 25.92% of studies followed by mouse, human and porcine (14.81%; Table 3), and the most studied protein on the effect on the gamete was HSPA8 (25.92%) followed by HSPA5 (18.51%) and HSP60 (14.81%) on publications. All other protein that exhibits some effect on gamete or embryo was cited once.

When we add publications about interactions and expression/localization of HSP, seventeen proteins were cited in the selected studies (Fig 3), with two belonging to small HSPs (sHSP; HSP25 and HSP27), seven to HSP70 (HSP70, HSPA1A, HSPA2, HSPA4, HSPA5, HSPA6, HSPA8), one to HSP60 and six to HSP90 family when four is from HSP90-alpha (HSP90A, HSPA1A, HSP90AA1, and HSPAB1) and two from HSP90-beta (HSP90B and HSP90B1).

Table 3: Species approached on selected publications

Specie	Study (n)	*Percent* (%)
Bovine*	7	25.92
Mouse	4	14.81
Human	4	14.81
Porcine	4	14.81
Rat	2	7.40
Sheep	2	7.40
Boar*	2	7.40
Rabbit	1	3.70
Goat	1	3.70
Ram	1	3.70

* One publication approaches both species.

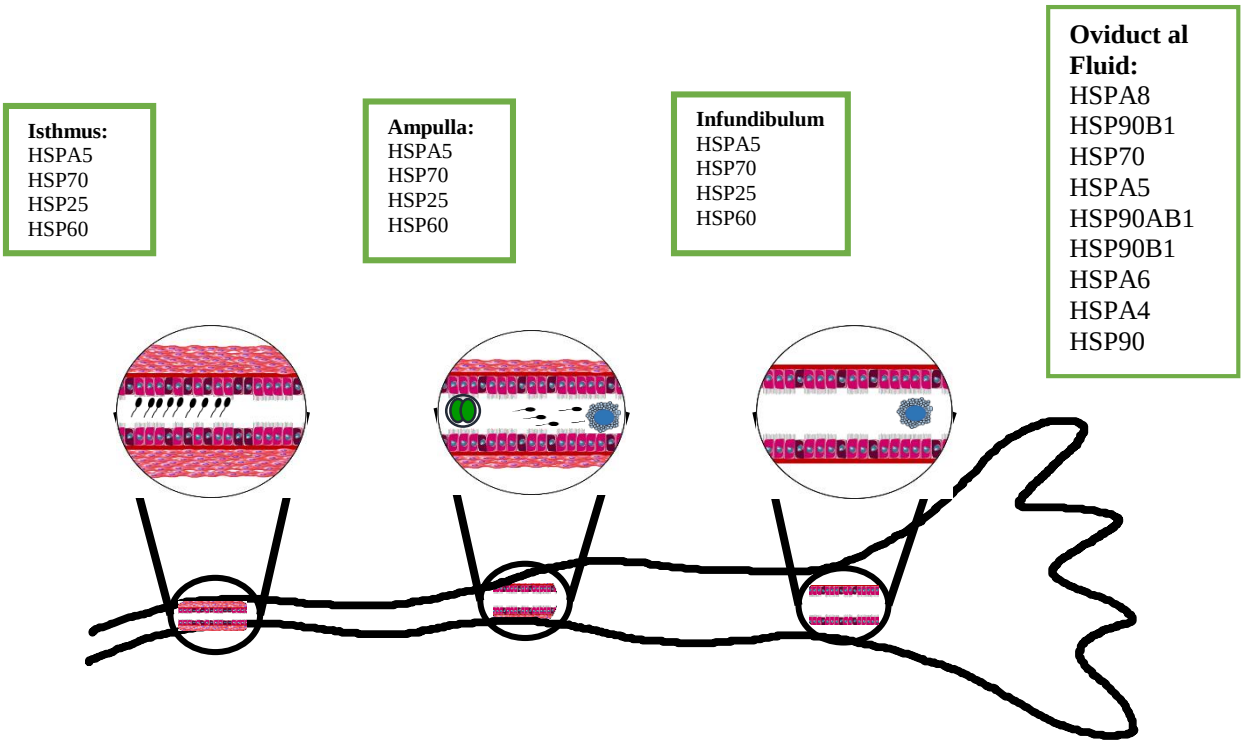

Figure 3: The localization of oviductal HSPs from mammals according to publications included in this integrative review. Figure adapted from Patricia Fontes

DISCUSSION

The HSPs protein family is a well-established intra-cellular protein that develops a fundamental role in the maintenance of cellular homeostasis (Shan *et al.*, 2020). Relatively recently the HSPs have been demonstrated to be released under nonpathological conditions and exert protective roles (Holt e Fazeli, 2016). On the oviduct, a range of HSP was reported expressed on OEC and present on oviductal fluid, and mainly, that the abundance of these HSP as other proteins from the oviduct, can be different into the estrous cycle. This approach leads to the study of the possible interaction of HSP with embryos and gametes, and, its possible role on fertilization and early embryo development. Our integrative review gathers for the first time publications that demonstrated the interaction and effects of HSP on gametes and embryos.

The HSPs are widely expressed and present in the genome of all cellular organisms with the main role in protein degradation, breakdown, and transmembrane transport. There are about ten types of these molecular chaperones and they are divided into seven families according to molecular mass (sHSP, HSP40, HSP60, HSP70, HSP90, HSP110, and ubiquitin) (Shan *et al.*, 2020). In this review, four families of HSP were cited: sHSP, HSP70, HSP60, and HSP90. From this, HSP70 and HSP60 families were the most cited proteins.

Among the selected publications, ten executed studies about localization or expression study on OEC or OF and founded HSPs. Five of them demonstrated that the abundance of HSPs is higher on estrus (Mariani *et al.*, 2000; Lin *et al.*, 2012; Lamy *et al.*, 2016; Soleilhavoup *et al.*, 2016; Fontes *et al.*, 2019), and more abundant on the ipsilateral side (Lamy *et al.*, 2016). In all five studies, the HSP70 family is present in all of them, closely with proteins from the HSP90 family and sHSP. The rest of localization and expression publications were not studies that aimed to compare the estrous cycle HSPs abundance, and presented results about specifics proteins as HSP70 (Scieglinska *et al.*, 1997), HSP25 (Wakayama e Iseki, 1998), HSP27 (Woolnough *et al.*, 2000), HSPA2 (Vydra *et al.*, 2009) and HSP90 (Canha-Gouveia *et al.*, 2019).

Besides studies about localization, some authors performed proteomic or transcriptomic analysis reporting alterations on HSP levels on OEC or OF after gamete OEC interaction. It was unanimous that the presence of gametes influences proteins abundance. Authors demonstrate that *in vitro* co-incubation of OEC (Yeste *et al.*, 2014) and oviduct (Georgiou *et al.*, 2005) with spermatozoa can alter the expression and abundance of proteins, among them the HSPs, HSP70, HSPA8, and HSPA5 belonging HSP70 family and HSP90AA1. About the oocyte, it was also showed that it is also able to alter the secretory proteome of the oviduct, but not about HSPs expression (Georgiou *et al.*, 2005). In *in vivo* study, Steinberger and colleagues identified two HSP on cellular surface 2 hours after intrauterine inseminations, the HSPA5 and HSP90B1 (Steinberger *et al.*, 2017). Besides the studies that demonstrate the influence of gametes on oviductal proteins, Lamy et al (2018) also reveal that when spermatozoa were co-incubated with OF, an interaction occurs between HSPs proteins and spermatozoa as HSPA5 and HSP90B1, from all estrous phases OF (Lamy *et al.*, 2018). The HSPA5 was also found interacting with spermatozoa by Boilard (2004) along with HSP60 (Boilard *et al.*, 2004). These results point to the importance of HSPs on reproductive processes and allow studies to clarify the impact of HSPs on gametes and embryos.

When the oocyte is incubated for 30 minutes in undiluted OF, the levels of monospermy increase, as well as the time of membrane digestion. These effects were related to the protein content of OF, which includes HSP70 (HSPA5 and HSPA1A) and HSP90 family - HSP90B1 (Mondejar *et al.*, 2013). Besides the presence of HSPs on OF, little is known about the interaction of oocyte and HSPs. This is the unique publication included in this work that demonstrates a probable direct effect of oviductal chaperones on the oocyte, having a lack about the theme.

Another point that needs more studies, is the interaction of HSPs and the embryo. The oviduct supplies biochemical substrates (carbohydrates, ions, proteins, enzymes, etc) and

physical requirements (pH, viscosity, osmolarity) to the early embryo development (Besenfelder *et al.*, 2020). Relative recently, extracellular vesicles (EVs) have been described to participate in maternal-embryo communication transferring their molecular cargo (proteins, mRNA, miRNA) from cell-to-cell (Valadi *et al.*, 2007). From this, Alminana and colleagues (2017) demonstrate that EVs from OF content HSPs (HSPA8, HSPA5, HSP70, HSP60, HSP90AA1, HSP90AB1, and HSP90B) (Alminana *et al.*, 2017), and mainly, that it was internalized by the embryo during *in vitro* culture and enhance their ability to reach the blastocyst stage. Although the EVs have a range of proteins, the data support the hypothesis of interaction and effect of HSP from OF under the embryo.

Some HSPs have a well-established role in the reproductive process, specifically on spermatozoa. The HSPA8 was the most cited protein on publications included at this review, on data presented above describing proteins present on oviduct or interacting on gametes and others publication, that will be discussed next, that present their direct effect on spermatozoa. Lloyd et al (2009) showed through preincubation of spermatozoa and the soluble OEC apical plasma membrane from the ampullar region, that HSPA8 is an active component and supports the survival of ram spermatozoa, enhancing its viability (Lloyd *et al.*, 2009). The same results on sperm viability were assessed by different authors, in a varied way as on 17°C (Lloyd *et al.*, 2012), on 39°C (Moein-Vaziri *et al.*, 2014), or during cryopreservation (Holt e Fazeli, 2016). The HSPA8 protein also showed effects on polyspermy modulation, increasing the membrane fluidity and enhancing the IVF efficiency and embryo rate (Elliott *et al.*, 2009; Lloyd *et al.*, 2012; Moein-Vaziri *et al.*, 2014; Holt e Fazeli, 2016; Braganca *et al.*, 2021). These studies demonstrate the presence of HSPA8 on OF is not only an effect for stress as were believed, but there are reasons to oviduct cells released these HSPs.

The HSPA5 proteins were the second most cited protein on publications eligible to our work, are present at all oviduct segments, and seem to be related to all stages of reproductive

processes. As cited above this protein interact with spermatozoa and was found increased at the estrous phase (Boilard *et al.*, 2004; Lin *et al.*, 2012; Fontes *et al.*, 2019). Lachance et al., (2007) did not find an increase in sperm viability after sperm were incubated with the protein but demonstrated that exogenous HSPA5 alters the spermatic physiology in the process of acquisition of fertilizing ability, as increased intracellular calcium concentration (Lachance *et al.*, 2007). In the same way, Marin-Briggiler (2010) found similar results and demonstrate that the protein seems to facilitate sperm binding to *zona pellucida*, influencing fertilization (Marín-Briggiler, 2010).

The other two publications reached the effects of specific HSPs proteins on fertilization. Garcia-Martinez (2020) assessed the effect of HSP90 and HSPA1A on ZP digestion (Garcia-Martinez *et al.*, 2020). Both proteins increased the time of ZP digestion but only HSPA1A increased the monospermy rate. On the other hand, none of them enhance the IVF efficiency. The exogenous HSP60 protein was not able to enhance the fertilization ability of spermatozoa but seems to elevate the two-cell embryo rate in mice (Abdi *et al.*, 2019).

In a general way, the results found here demonstrate that HSPs play an active and fundamental role in the reproductive process, and these proteins seem to influence some specific aspects of spermatozoa capacitation and in the fertilization that could directly impact embryo development. In addition, the use of exogenous HSPs on *in vitro* embryo production can enhance the IVF efficiency and embryo rate but there is a lot to clarify about their mechanism of action of them.

CONCLUSION

Fertilization involves a range of processes on gametes and the oviduct should be able to receive them and provide the required substrate as carbohydrates, ions, enzymes, and proteins. The HSPs are expressed on OEC and present on OF and their concentration fluctuates into the

estrous cycle. These chaperones can bind on spermatozoa and exert a fundamental role in the fertilization process but their action mechanism is not clear. Studies are necessary to completely understand the OF HSPs.

REFERENCES

ABDI, Z. et al. The Effect of Hsp60 on Fertilization and Pre-Implantation Embryo Development in Mice: An in Vitro Study. **Acta Endocrinol (Buchar),** v. 15, n. 2, p. 153-157, Apr-Jun 2019. ISSN 1841-0987 (Print)

1841-0987 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31508170 >.

ALMINANA, C. et al. Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. **Reproduction,** v. 154, n. 3, p. 153-168, Sep 2017. ISSN 1741-7899 (Electronic)

1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/28630101 >.

AVILÉS, M. G.-A., A; COY, PILAR. Oviductal secretions: will they be key factors for the future of ARTs? **Molecular Human Reproduction,** v. 16, n. 12, p. 896-906, 2010.

BESENFELDER, U.; BREM, G.; HAVLICEK, V. Review: Environmental impact on early embryonic development in the bovine species. **Animal,** v. 14, n. S1, p. s103-s112, Mar 2020. ISSN 1751-732X (Electronic)

1751-7311 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/32024564 >.

BOILARD, M. et al. Localization of the chaperone proteins GRP78 and HSP60 on the luminal surface of bovine oviduct epithelial cells and their association with spermatozoa. **Biol Reprod,** v. 71, n. 6, p. 1879-89, Dec 2004. ISSN 0006-3363 (Print)

0006-3363 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15286042 >.

BRAGANCA, G. M. et al. Oviduct fluid during IVF moderately modulates polyspermy in in vitro-produced goat embryos during the non-breeding season. **Theriogenology,** v. 168, p. 59-65, Jul 1 2021. ISSN 1879-3231 (Electronic)

0093-691X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/33857909 >.

BUHI, W. C.; ALVAREZ, I. M.; KOUBA, A. J. Secreted proteins of the oviduct. **Cells Tissues Organs,** v. 166, n. 2, p. 165-79, 2000. ISSN 1422-6405 (Print)

1422-6405 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10729726 >.

CANHA-GOUVEIA, A. et al. Which Low-Abundance Proteins are Present in the Human Milieu of Gamete/Embryo Maternal Interaction? **Int J Mol Sci,** v. 20, n. 21, Oct 24 2019. ISSN 1422-0067 (Electronic)

1422-0067 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31653120 >.

ELLIOTT, R. M. et al. Effects of HSPA8, an evolutionarily conserved oviductal protein, on boar and bull spermatozoa. **Reproduction,** v. 137, n. 2, p. 191-203, Feb 2009. ISSN 1741-7899 (Electronic)

1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18996976 >.

FONTES, P. K. et al. 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,** v. 86, n. 11, p. 1582-1591, Nov 2019. ISSN 1098-2795 (Electronic)

1040-452X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31353672 >.

GARCIA-MARTINEZ, S. et al. Addition of exogenous proteins detected in oviductal secretions to in vitro culture medium does not improve the efficiency of in vitro fertilization in pigs. **Theriogenology,** v. 157, p. 490-497, Nov 2020. ISSN 1879-3231 (Electronic)

0093-691X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/32898824 >.

GEORGIOU, A. S. et al. Gametes alter the oviductal secretory proteome. **Mol Cell Proteomics,** v. 4, n. 11, p. 1785-96, Nov 2005. ISSN 1535-9476 (Print)

1535-9476 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16105986 >.

HOLT, W. V.; FAZELI, A. Sperm Storage in the Female Reproductive Tract. **Annu Rev Anim Biosci,** v. 4, p. 291-310, 2016. ISSN 2165-8110 (Electronic)

2165-8102 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26526545 >.

KILLIAN, G. Physiology and endocrinology symposium: evidence that oviduct secretions influence sperm function: a retrospective view for livestock. **J Anim Sci,** v. 89, n. 5, p. 1315-22, May 2011. ISSN 1525-3163 (Electronic)

0021-8812 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20935135 >.

KOLLE, S. et al. Ciliary transport, gamete interaction, and effects of the early embryo in the oviduct: ex vivo analyses using a new digital videomicroscopic system in the cow. **Biol Reprod,** v. 81, n. 2, p. 267-74, Aug 2009. ISSN 1529-7268 (Electronic)

0006-3363 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19299315 >.

KOLLE, S.; REESE, S.; KUMMER, W. New aspects of gamete transport, fertilization, and embryonic development in the oviduct gained by means of live cell imaging. **Theriogenology,** v. 73, n. 6, p. 786-95, Apr 1 2010. ISSN 1879-3231 (Electronic)

0093-691X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20080295 >.

LACHANCE, C.; BAILEY, J. L.; LECLERC, P. Expression of Hsp60 and Grp78 in the human endometrium and oviduct, and their effect on sperm functions. **Hum Reprod,** v. 22, n. 10, p. 2606-14, Oct 2007. ISSN 0268-1161 (Print)

0268-1161 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17670764 >.

LAMY, J. et al. Regulation of the bovine oviductal fluid proteome. **Reproduction,** v. 152, n. 6, p. 629-644, Dec 2016. ISSN 1741-7899 (Electronic)

1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/27601716 >.

LAMY, J. et al. Identification by proteomics of oviductal sperm-interacting proteins. **Reproduction,** v. 155, n. 5, p. 457-466, May 2018. ISSN 1741-7899 (Electronic)

1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29540510 >.

LIN, P. et al. GRP78 expression and immunohistochemical localization in the female reproductive tract of mice. **Theriogenology,** v. 78, n. 8, p. 1824-9, Nov 2012. ISSN 1879-3231 (Electronic)

0093-691X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22968032 >.

LLOYD, R. E. et al. Effects of oviductal proteins, including heat shock 70 kDa protein 8, on survival of ram spermatozoa over 48 h in vitro. **Reprod Fertil Dev,** v. 21, n. 3, p. 408-18, 2009. ISSN 1031-3613 (Print)

1031-3613 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19261218 >.

LLOYD, R. E. et al. The oviducal protein, heat-shock 70-kDa protein 8, improves the long-term survival of ram spermatozoa during storage at 17 degrees C in a commercial extender. **Reprod Fertil Dev,** v. 24, n. 4, p. 543-9, 2012. ISSN 1031-3613 (Print)

1031-3613 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22541542 >.

MAHDAVINEZHAD, F. et al. In vitro versus In vivo: Development-, Apoptosis-, and Implantation-Related Gene Expression in Mouse Blastocyst. **Iran J Biotechnol,** v. 17, n. 1, p. e2157, Jan 2019. ISSN 1728-3043 (Print)

1728-3043 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31457046 >.

MARIANI, M. L. et al. Constitutive expression of heat shock proteins hsp25 and hsp70 in the rat oviduct during neonatal development, the oestrous cycle and early pregnancy. **J Reprod Fertil,** v. 120, n. 2, p. 217-23, Nov 2000. ISSN 0022-4251 (Print)

0022-4251 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11058436 >.

MARÍN-BRIGGILER, C. I. G.-E., M.F.; MUNUCE, M.J.; GHERSEVICH, S.; CAILLE, A.M.; HELLMAN, U.; CORRIGALL, V.M.; VAZQUEZ-LEVIN, M.H. Glucose-regulated protein 78 (GRP78/BiP) is secreted by human oviduct epithelial cells and the recombinantprotein modulates sperm-zona pellucida binding. **Fertil Steril,** v. 93, n. 5, 2010.

MOEIN-VAZIRI, N. et al. Heat-shock protein A8 restores sperm membrane integrity by increasing plasma membrane fluidity. **Reproduction,** v. 147, n. 5, p. 719-32, May 2014. ISSN 1741-7899 (Electronic)

1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24501193 >.

MONDEJAR, I. et al. Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals. **Biol Reprod,** v. 89, n. 3, p. 67, Sep 2013. ISSN 1529-7268 (Electronic)

0006-3363 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23863406 >.

PILLAI, V. V. et al. Profiling of proteins secreted in the bovine oviduct reveals diverse functions of this luminal microenvironment. **PLoS One,** v. 12, n. 11, p. e0188105, 2017. ISSN 1932-6203 (Electronic)

1932-6203 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29155854 >.

SCIEGLINSKA, D. et al. Expression of the testis-specific HSP70-related gene (hst70 gene) in somatic non-testicular rat tissues revealed by RT-PCR and transgenic mice analysis. **Cell Biol Int,** v. 21, n. 12, p. 813-21, Dec 1997. ISSN 1065-6995 (Print)

1065-6995 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9812345 >.

SHAN, Q. et al. Physiological Functions of Heat Shock Proteins. **Curr Protein Pept Sci,** v. 21, n. 8, p. 751-760, 2020. ISSN 1875-5550 (Electronic)

1389-2037 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31713482 >.

SOLEILHAVOUP, C. et al. Proteomes of the Female Genital Tract During the Oestrous Cycle. **Mol Cell Proteomics,** v. 15, n. 1, p. 93-108, Jan 2016. ISSN 1535-9484 (Electronic)

1535-9476 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26518761 >.

STEINBERGER, B. et al. Semen modulated secretory activity of oviductal epithelial cells is linked to cellular proteostasis network remodeling: Proteomic insights into the early phase of interaction in the oviduct in vivo. **J Proteomics,** v. 163, p. 14-27, Jun 23 2017. ISSN 1876-7737 (Electronic)

1874-3919 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/28495501 >.

VALADI, H. et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. **Nat Cell Biol,** v. 9, n. 6, p. 654-9, Jun 2007. ISSN 1465-7392 (Print)

1465-7392 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17486113 >.

VYDRA, N. et al. The expression pattern of the 70-kDa heat shock protein Hspa2 in mouse tissues. **Histochem Cell Biol,** v. 132, n. 3, p. 319-30, Sep 2009. ISSN 1432-119X (Electronic)

0948-6143 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19462178 >.

WAKAYAMA, T.; ISEKI, S. Expression and cellular localization of the mRNA for the 25-kDa heat-shock protein in the mouse. **Cell Biol Int,** v. 22, n. 4, p. 295-304, 1998. ISSN 1065-6995 (Print)

1065-6995 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10101046 >.

WOOLNOUGH, E. et al. An immunohistochemical study of the rete ovarii and epoophoron. **Pathology,** v. 32, n. 2, p. 77-83, May 2000. ISSN 0031-3025 (Print)

0031-3025 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10840824 >.

YANAGIMACHI, C. A. The common and species-specific roles of oviductal proteins in mammalian fertilization and embryo development. **Oxford University Press**, 2015.

YESTE, M. et al. Viable and morphologically normal boar spermatozoa alter the expression of heat-shock protein genes in oviductal epithelial cells during co-culture in vitro. **Mol Reprod Dev,** v. 81, n. 9, p. 805-19, Sep 2014. ISSN 1098-2795 (Electronic)

1040-452X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24945888 >.

CAPÍTULO II

EVIDENCE THAT HEAT SHOCK PROTEIN A5 (HSPA5) PLAYS A ROLE DURING BOVINE IN VITRO EMBRYO PRODUCTION

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Grants: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 and by São Paulo Research Foundation (FAPESP; grant numbers: 2018/06674-7; 2013/11480-3; 2015/04505-5.

ABSTRACT

Proteins derived from oviductal fluid (OF) have shown an essential role in reproductive physiology. Some studies demonstrate that heat shock proteins as HSPA5 are secreted on OF and participate in zona *pellucida* (ZP)-sperm interaction. Hypothesizing that HSPA5 can interact with the oocyte, we assessed its effect during the last four hours of bovine *in vitro* complex *cumulus*-oocyte maturation and subsequence embryo yield/quality. The COCs were aspirated from cow ovaries obtained in a slaughterhouse (n= 20 COC/group) and matured for 20 hours in base medium (BM). After that, the medium was part exchanged to BM (control) or BM + recombinant HSPA5 (100 ng/ml) and continued *in vitro* maturation to complete 24 hours. *In vitro* matured COCs were mechanically separated from cumulus cells or followed to the *in vitro* fertilization (IVF) and *in vitro* culture (IVC) until 8 days to obtain expanded blastocyst and its rate. To gain insight into the presumptive role of HSPA5, we investigated reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) from oocytes and

expanded blastocysts, the GSH content from COCs and IVM medium, the oocyte penetration, the *zona pellucida* solubility, the blastocyst rate, and its gene expression analysis. The rHSPA5 modulated mitochondrial activities from oocytes (p= 0.0006) and increased blastocyst yield (p= 0.04). Besides that, in blastocysts derived from oocytes matured with rHSPA5, we figure out an upregulation of 19 genes involved in embryo quality, DNA methylation, cell growth, cellular development, and oxidative stress. Taken all data together, we infer that rHSPA5 during the final time of oocyte *in vitro* maturation, even though it did not change monospermic fertilization and oocyte oxidative stress, plays a role in oocyte mitochondrial function, improves blastocyst development, and modulates low-scale embryonic transcriptional profile.

Keywords: GRP78, heat shock protein, *in vitro* maturation, bovine, embryo production

INTRODUCTION

During the ovulation, the complex *cumulus*-oocyte (COC) is released from the ovary and attached by cilia of the infundibulum, an initial portion of the oviduct (P. Coy, Garcia-Vazquez, Visconti, & Aviles, 2012; Kolle et al., 2009; Li & Winuthayanon, 2017). The oviduct is a tubal-like structure that connects the ovary to the uterus. It is divided into three main regions, the infundibulum, the ampulla, and the isthmus (ordered from the ovary to uterus), where each region differs in morphology and fluid composition according to function during the gamete and embryo transport (Li & Winuthayanon, 2017). The oviducts play a crucial role in ensuring the gametes' transport, fertilization, development, and transport of early embryos. Once in the oviduct, the ovulated COC directly contacts oviductal epithelial cell (OEC) and oviductal fluid (OF).

The OF is a complex mixture of components produced by secretions from OEC and plasm transudate (M. G.-A. Avilés, A; Coy, Pilar, 2010; Mondejar, Martinez-Martinez, Aviles,

& Coy, 2013). In contact of gametes, some contents of OF can be modified to support all gametes necessities and, besides that, the regions tend to have a different pattern of secretion (Alminana et al., 2017; M. G.-A. Avilés, A; Coy, Pilar, 2010; Buhi, Ashworth, Bazer, & Alvarez, 1992). The ampulla is the region that fertilization takes place. The passage of bovine COC through the oviduct was estimated at 72 hours, but the arrival to ampulla seems to last only a few minutes (Croxatto, 2002). In the ampulla, the COC is firmly attached to OEC for a variable time (Kolle et al., 2009). The ZP becomes more accessible to the OF during this process, permitting its modification by different molecules, including proteins (P. Coy et al., 2012).

Among a range of proteins in OF, there is heat shock protein A5 (HSPA5). The HSPA5, also known as glucose-regulated protein (GRP78), is a member of the HSP70 family of proteins and share partial amino acid sequence identity with the 70-kDa heat shock protein (HSP70) that is also present in the oviduct (C. Zhang, 2017). The HSPA5 is a well-characterized chaperone from endoplasmic reticulum (ER) that play an essential role in maintaining cellular homeostasis (Gonzalez-Gronow, Selim, Papalas, & Pizzo, 2009). The protein plays a crucial role in the unfolded protein response (UPR), which is activated when proteins are misfolded and accumulate in the ER (Hebert-Schuster, Rotta, Kirkpatrick, Guibourdenche, & Cohen, 2018). However, as a member of the HSP family, the HSPA5 performs a diverse array of functions in multiple cellular compartments (Hebert-Schuster et al., 2018), including on cell surface (Gonzalez-Gronow et al., 2009).

Studies have shown that HSPA5 is present on the cell surface where it acts as a receptor-like function and regulates cell proliferation and survival (Gonzalez-Gronow et al., 2009; Sato, Yao, Arap, & Pasqualini, 2010). Besides that, (Boilard et al., 2004; Lachance, Bailey, & Leclerc, 2007; Lin et al., 2012; Marín-Briggiler, 2010) have shown that HSPA5 is located at OEC, is secreted on OF (Boilard et al., 2004; Lamy et al., 2016; Marín-Briggiler, 2010)

promoting sperm capacitation and sperm-*zona pellucida* (ZP) binding (Bromfield & Nixon, 2013; Mondejar et al., 2013). Moreover, the presence of HSPA5 protein in the oolemma from mature mouse oocytes was described by (Calvert, Digilio, Herr, & Coonrod, 2003), but how the protein can modulate the final maturation of COC is until unknown.

Considering that the studies about secreted HSPA5 on OF demonstrate their role on sperm membrane and that COCs co-cultured with ampullary cells is beneficial to the oocyte (Azari, Kafi, Asaadi, Pakniat, & Abouhamzeh, 2021), as well as the subsequence embryo quality (Kidson et al., 2003), the influence of oviductal proteins on oocyte should be investigated. Based on that, to gain insight into the effect of HSPA5 on the final maturation of oocytes, we add the protein on the last 4 hours of *in vitro* maturation (IVM), and we hypothesized that protein affects bovine oocyte maturation and influences embryo production and blastocyst quality. Specifically, we aimed to investigate the impact of this heat shock protein on oocyte maturation, oxidative stress, polyspermy control, mitochondrial functionality, and embryonic gene expression.

RESULTS

Effect of HSPA-5 protein on COCs IVM on ROS and mitochondrial activities oocyte and embryo and GSH content determination from COCs and IVM medium.

The HSPA5 is a protein-related to maintaining cellular homeostasis under stress conditions. To gain insight into the effect of rHSPA5 protein on COC stress during IVM, we assessed ROS and MMP levels on oocyte and GSH content from culture medium and COC. The ROS and MMP were also verified from expanded blastocysts to assess the effect of rHSPA5 on the stress of subsequence embryos. The presence of protein for the last 4 hours of maturation decreased the MMP levels in oocytes ($p = 0.0006$, Figure 2A). Nevertheless, HSPA-

5 did not affect ROS levels on treated oocytes (p= 0.539, Figure 2A), even changing the GSH contents on COCs and IVM medium (Table 1). The rHSPA5 also did not modulate the ROS and MMP from subsequence embryos (p= 0.88 and p= 0.98, respectively) as demonstrated on Figure 2B.

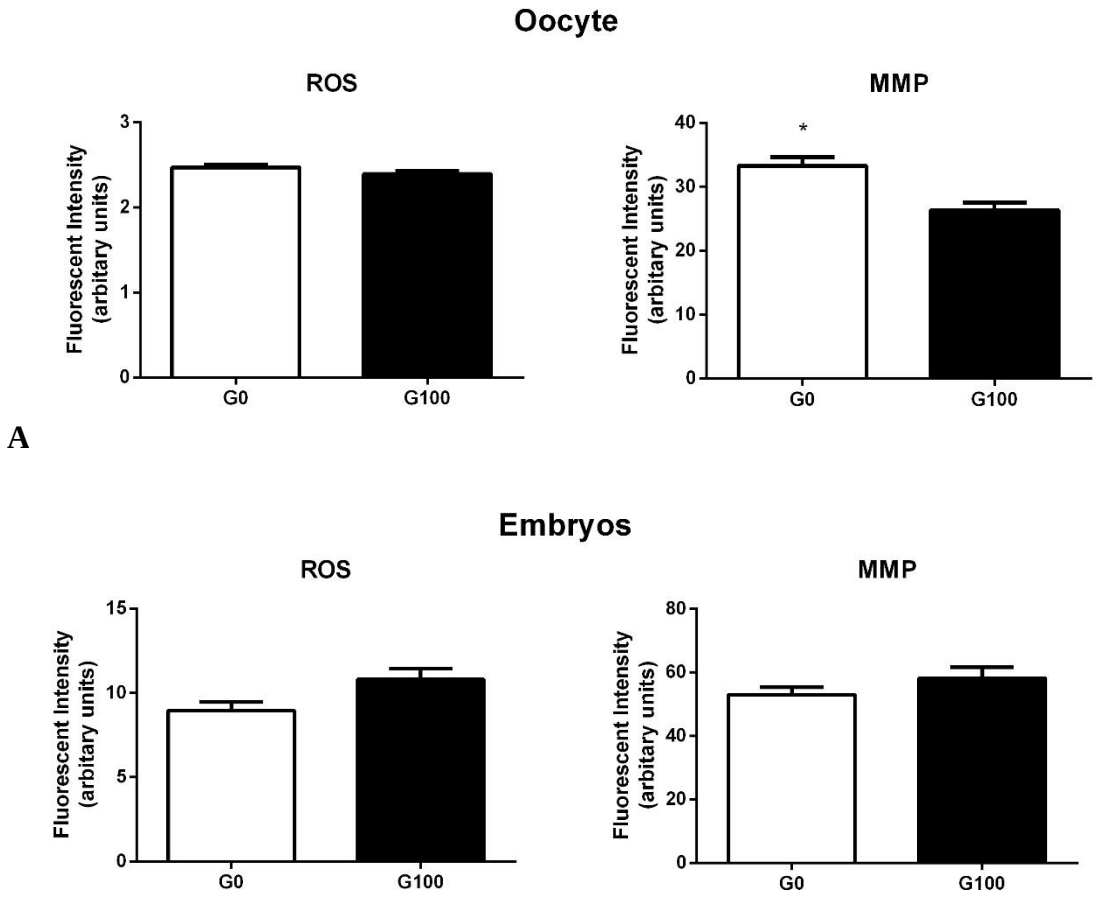


Figure 2. Effects of addiction of HSPA-5 protein on the last 4 hours of bovine COCs IVM on **A)** MMP from oocytes (n= 92-109 oocytes/group; n= 5 replicates; p= 0.0006) detected using MitoTracker® red CMXRox dye; and ROS levels from oocytes (n= 94-111 oocytes/group; 5 replicates; p= 0.539) detected using CellRox™ Green dye. **B)** MMP from embryos (n= 36 embryos/group; n= 5 replicates; p= 0.9880) detected using MitoTracker® red CMXRox dye; and ROS levels from embryos (n= 36 embryos/group; 5 replicates; p= 0.8839) detected using CellRox™ Green dye. Data are means ± SEM of pixel intensity, and differences were considered significant when $p \leq 0.05$. Different letters indicate statistical difference.

TABLE 1. Total GSH content (mean ± SEM) in COCs and IVM medium from COC maturation treated with HSPA-5 on the last 4 hours or not.

	TOTAL GSH CONTENT		
Group	IVM medium (pMol/ml)	Cumulus cells (pMol/pool)	Oocytes (pMol/pool)
Control	0.069 ± 0.02	0.87 ± 0.20	0.11 ± 0.01
rHSPA5	0.039 ± 0.0092	0.87 ± 0.03	0.08 ± 0.02
p value	0.37	0.33	0.85

Cumulus cells pool: cumulus cells from 20 COCs;
Oocytes pool: 20 oocytes.

Effect of HSPA-5 during the last 4 hours of COCs IVM on zona pellucida solubility and oocyte penetration

The oviduct contains a range of proteins, but some have a lacking or an unknown role. Studies have been demonstrated that the HSPA5 can bind to the sperm membrane and possible is related to sperm-ZP interaction. So, to figure out the effect of protein on ZP and fertilization, we tested ZP solubility and oocyte penetration. The presence of HSPA-5 protein on COCs IVM did not modulate the time of digestion of *zona pellucida* on treated oocytes (259.58 sec ± 12.29) compared to control (276.09 sec ± 11.16; p= 0.4093; Figure 3). Conversely, the protein did not alter the standard of sperm block on *zona pellucida*, once the means of polyspermy zygote (p= 0.9669) was not different between the group and the monospermy zygote (p= 0.9692).

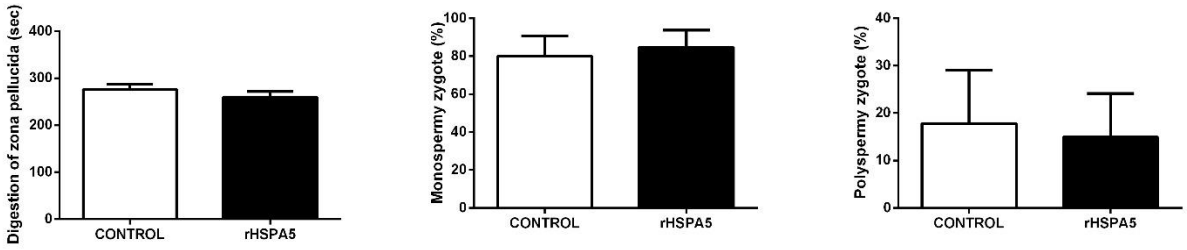


Figure 3. Effects of addiction of HSPA-5 protein on the last 4 hours of bovine COCs IVM on *zona pellucida* digestion time (n=16-20 oocytes/group; n= 4 replicates); monospermy and polyspermy rate (n=15-21 zygotes/group; n=4 replicates). Digestion of *zona pellucida* by pronase was continued observed and counted the time (in seconds). Data are means ± SEM, and differences were considered significant when $p \leq 0.05$.

Effect of HSPA-5 on IVM on embryo production and transcript profile of embryo produced

An adequate COC maturation is necessary to obtain a competent oocyte to be fertilized and a subsequence development of an embryo of good quality. The rHSPA5 in the last 4 hours of IVM was able to increase the blastocysts yield (*p= 0.04*; Figure 4). Regarding the transcript profile of embryos derived from the treated oocyte, 19 genes were up-regulated on the embryo, as demonstrated in Table 2.

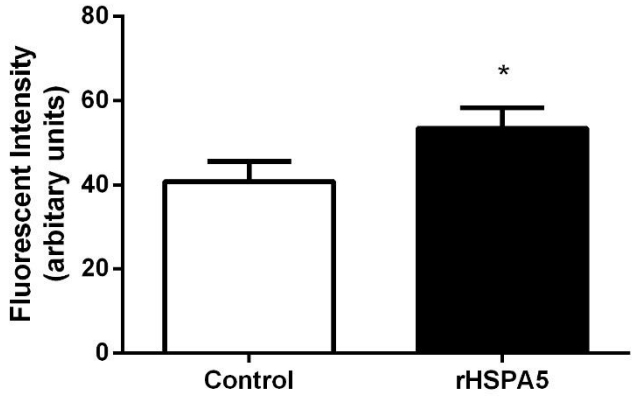


Figure 4. Effects of addiction of HSPA-5 protein on the last 4 hours of bovine COCs IVM on subsequence embryo development. Percentage of blastocysts produced at 8 days of culture (7 replicates). Data are means ± SEM. Differences were considered significant when $p \leq 0.05$.

TABLE 2. Relative mRNA abundance of differently expressed genes on blastocyst ($p \leq 0.05$). Target genes were normalized with the reference genes (*HMBS, PPIA*, and *RPL30*) geometric means using the $2^{(-\Delta Ct)}$ method. Data are means ± SEM of four biological replicates.

Gene symbol	Control	Treated	*p*-Value	Function	Gene ID
ATP5L	2.30±0.08	2.56±0.10	0.04	Transmembrane transport	Bt03210836_g1
IGFBP2	1.97±0.12	2.79±0.28	0.02	Cell growth regulation	Bt01040719_m1
IMPDH1	0.04±0.004	0.06±0.005	0.04	Cell growth regulation	Bt00995384_m1
IMPDH2	0.23±0.01	0.31±0.03	0.03	Cell growth regulation	Bt03226238_g1
MAPK1	0.18±0.01	0.25±0.01	0.01	Cell differentiation	Bt03216718_g1
HSP90AA1	4.46±0.23	5.15±0.09	0.002	Cellular survive	Bt0.218068_g1
NANOG	0.04±0.01	0.10±0.01	0.006	Pluripotency	Bt03220541_m1
ATF4	0.29±0.06	0.47±0.02	0.02	ER stress	Bt03221057_m1
GFPT2	0.04±0.001	0.05±0.005	0.04	Cellular Stress	Bt03223996_m1
PRDX3	0.10±0.01	0.17±0.021	0.01	Oxidative Stress	Bt03214402_m1
XBP1	0.32±0.05	0.45±0.01	0.04	Oxidative Stress	Bt03227621_g1
SLC2A3	0.82±0.11	1.16±0.14	0.05	Energetic metabolism	Bt03259514_g1
SLC2A1	1.66±0.28	2.32±0.15	0.05	Energetic Metabolism	Bt03215314_m1
SREBF2	0.55±0.06	0.81±0.09	0.03	Lipid metabolism	Bt04283467_m1
DNMT3A	0.63±0.09	0.87±0.06	0.04	Active DNA methylation	Bt01027164_m1
REST	0.37±0.04	0.61±0.08	0.02	Gene expression control	Bt03278318_s1
GLRX2	0.31±0.02	0.39±0.01	0.03	Oxidative Stress	Bt03250351_m1
HPRT1	0.26±0.02	0.37±0.04	0.04	Others	Bt03225311_g1
S100A10	0.72±0.10	1.12±0.11	0.02	Others	Bt03215645_m1

DISCUSSION

The HSPA-5 protein is secreted on OF and has been demonstrated an interaction and develops an essential role in capacitation and activation of sperm. In our study, for the first time, we showed evidence that this oviductal secreted protein seems to interact with bovine COC. The rHSPA5 protein in the last 4 hours of COCs IVM medium, in general, increased blastocyst yield and could improve blastocyst quality modulating target genes involved with keys cellular pathways.

The HSPA5 protein is an ER chaperone well-known for the unfolded proteins response (UPR). First, we need to clarify that cells can transcript specific genes during stress conditions to maintain homeostasis. In normal conditions, the HSPA5 from ER is binding to an intraluminal portion of IRE1 (endoplasmic reticulum to nucleus signaling-1), PERK (protein kinase RNA-like endoplasmic reticulum kinase), and ATF6 (activating transcription factor 6) that are membrane receptors from ER. Under stress conditions, the HSPA5 dissociates from receptors to bind to the unfolded proteins. The IRE1, PERK, and ATF6 trigger UPR vias to maintain the cell or reach apoptosis (J. Y. Zhang, Diao, Kim, & Jin, 2012; X. Zhang, Zhang, Qi, Huang, & Zhang, 2016). Besides the HSPA5 is best known as an ER luminal protein, the HSPA5 is also found on cytoplasm, mitochondria, the nucleus, plasma membrane, and secreted (Casas, 2017; Delpino & Castelli, 2002; Gonzalez-Gronow et al., 2009), including on OF (Marín-Briggiler, 2010).

Then, why did we test HSPA5 protein during oocyte IVM? First of all, during *in vitro* embryo production, COCs are exposed to a range of stressors, resulting in a high level of ROS (Goto, Noda, Mori, & Nakano, 1993; Nasr-Esfahani, Aitken, & Johnson, 1990). Although the HSPA5 is responsible for maintaining the cellular homeostasis on stress (Basar et al., 2014; Latham, 2016), in the present study, we demonstrated that HSPA-5 neither influences the total GSH content of ROS concentration but modulate MMP levels. Based on these findings, we could propose, as previously described (Dumollard, Carroll, Duchen, Campbell, & Swann, 2009; Quinlan, Perevoshchikova, Hey-Mogensen, Orr, & Brand, 2013), that basal ROS production, on control or HSPA-5 groups can act as a natural regulatory mechanism for the cell, involved in general housekeeping and regulation of redox state, which no cause excess of ROS production, no increased oxidative stress ROS production must be kept to a minimum. On the other hand, oocytes treated with HSPA-5 showed lower levels of MMP but did not influence oocyte competence to be fertilized and promote embryo development. Indeed, we know that

mitochondria have diverse activities in the regulation of cellular homeostasis, including ATP production, calcium control homeostasis, redox regulation, etc. (Demant, Trapphoff, Frohlich, Arnold, & Eichenlaub-Ritter, 2012) and that ROS induces mitochondrial damages, resulting in low values of MMP (Hao et al., 2011; Loor et al., 2010). So, how to explain lower values of MMP and no negative impact of oocyte competence to reach more blastocysts? First, we need to reinforce that there is may also discrepancies in results due to variations in MMP measurement techniques. Further, it is interesting that we showed no ROS production changes despite an apparent decrease in mitochondrial activity, as usually, a reduced MMP coincides with lowered ROS generation as described (Korshunov, Skulachev, & Starkov, 1997).

Some studies have evaluated the role of HPA5 protein on OF. An important finding regarding fertilization controlling was that HSPA5 protein did not influence ZP hardening and not changes rates of polyspermy. It is known that the hardening of ZP is directly related to monospermy fertilization (P. Coy et al., 2008). Studies described a difference between oocytes from the ovary compared to that transit through the oviduct, and that OF influence in ZP bindings and develop the role on ZP in the control of polyspermy (P. Coy et al., 2012). Together, these results caused us an astonishment once HSPA5 protein was described to be on the surface of mature mouse oolema (Calvert et al., 2003) and seems to be involved in preparing oocytes for fusion with spermatozoa (Bromfield & Nixon, 2013). In humans, (Marín-Briggiler, 2010) and colleagues demonstrated that the protein is secreted by OEC. It is associated with sperm-ZP binding modulation and proposed that HSPA5 bind to the gametes modulates their interaction in a calcium-dependent manner. Moreover, (Mondejar et al., 2013) suggested that this binding could modify the ZP chemical properties such as resistance to enzymatic hardening. Even though our findings did not corroborate with previous studies, we need to highlight that these works always used the HSPA-5 during fertilization or previously on spermatozoa, which could modify interactions and modulation of ZP hardening during oocyte IVM.

As know, oviduct takes place the gametes meeting to fertilization. According to (Croxatto, 2002), the COC transport through the oviduct can spend approximately 72 hours in cows. During this time, COCs maintain contact with the OF and its proteins. The OF has been shown a fundamental role in the reproduction process because the presence of OEC during IVM improves the quality of cytoplasmic maturation of the oocyte and subsequence quality of embryos produced, probably because of their secretions (Azari et al., 2021; Kidson et al., 2003). Indeed, here we found that HSPA5 protein could increase the blastocyst yield and further alter the transcriptional pattern of embryos. Also, we would like to highlight that all genes modulated by HSPA5 was up-regulated in the blastocyst, e.g., genes related to embryo quality as *ATP5L, IGFBP2, IMPDH1, IMPDH2, MAPK, HSP90AA1* and, *NANOG*; genes related to DNA methylation (*DNMT3B*), glucose transporter (*SLC2A3* and *SLC2A1*), gene expression control (*REST*) lipid metabolism (*SREBF2*), RE stress (*ATF4, XBP1*) oxidative stress (*PDRX3*) and others functional genes (*GLRX2* and *HPRT1*).

Although in the present study we demonstrated that HSPA5 did not affect some parameters of oxidative stress on oocytes, we verify that HSPA5 protein up-regulated as Activating transcription factor-4 (*ATF4*), Glutaredoxin-2 (*GLRX2*), Peroxiredoxin-3 (*PRDX3*), Glutamine-fructose-6-phosphate transaminase-2 (*GFPT2*), and X-box binding protein-1 (*XBP1*). Based on that, we believe that upregulation of ATF4 and XBP1 by HSPA5 could indicate a possible protective mechanism to prevent ER stress in the embryos derived from oocytes treated with HSPA5 once we reached a higher yield of embryo produced in this group. To summarizing, even though we did not find any phenotypical evidence of HSPA5 on oxidative stress (ROS levels, GSH), indeed, proteins from OF protect gametes from environmental stress to ensure embryo quality and future pregnancy outcome and in our case, these internal anti-stress activities are being performed throughout mechanism controlling transcription of target genes as mentioned above.

The other genes up-regulated by treatment have been found in embryos as a marker of quality. The up-regulation of the DNA methyltransferase 3B enzyme could results in well-driven embryo development and implantation rate (Gujar, Weisenberger, & Liang, 2019) once DNA methylation is essential in the embryonic stem cells and post-implantation embryo development periods and early embryos. In the same way, the NANOG enzyme is also essential to embryo development. It is a crucial regulator of pluripotency during early development (Chambers et al., 2003; Karantzali et al., 2011), leaving us to think this upregulation caused by HSPA5 could be critical for normal embryonic development (Carey et al., 2015).

Regarding other genes related to multiple pathways to guarantee a better embryo development, we could summarize and suggest that blastocysts derived from oocytes treated with HSPA5 could demonstrate a positive transcriptional panel which would lead to preimplantation embryo development, up-regulating genes as SLC2A1 and SLC2A3 (Solute carrier family 2, facilitated glucose transporter member 1 and 3) to energy support (Kramer et al., 2020), IMPDH1 and IMPDH 2 (monophosphate dehydrogenase 1 and 2) to embryo development and cell growth (Lake, Avetisyan, Zimmermann, & Heuckeroth, 2016), IGFBP2 (Insulin-like growth factor-binding protein-2) to cell proliferation and differentiation (Heyner, Shi, Garside, & Smith, 1993; Yang, Brown, Robcis, Rechler, & de Pablo, 1993), SREBF2 (Sterol regulatory element-binding transcription factor 2) to embryo development (Vergnes et al., 2016).

As HSPA5, some other oviductal proteins have been studied to identify their role in fertilization and embryo development (P. Y. Coy, R., 2017). However, there is a lack of these proteins' importance on final oocyte maturation. The co-culture with OEC was demonstrated to be beneficial to oocyte resulting embryos with good quality (Kidson et al., 2003) and indicate that proteins participate in the process of final maturation and preparation to fertilization and embryo development (possible a molecular modulation). In all, our study demonstrated that

the rHSPA5 decreased the MMP levels and possibly modulate the final oocyte maturation in a good perspective once it was able to increase the *in vitro* embryo rate and up-regulated transcripts of genes related to embryo quality.

Taken all data together, we conclude that the HSPA5 protein modulates the mitochondrial functionality, possible participation of final oocyte maturation and that rHSPA-5 on *in vitro* maturation modulates the final oocyte maturation once it increases the blastocyst rate and the quality of the embryo (Figure 6).

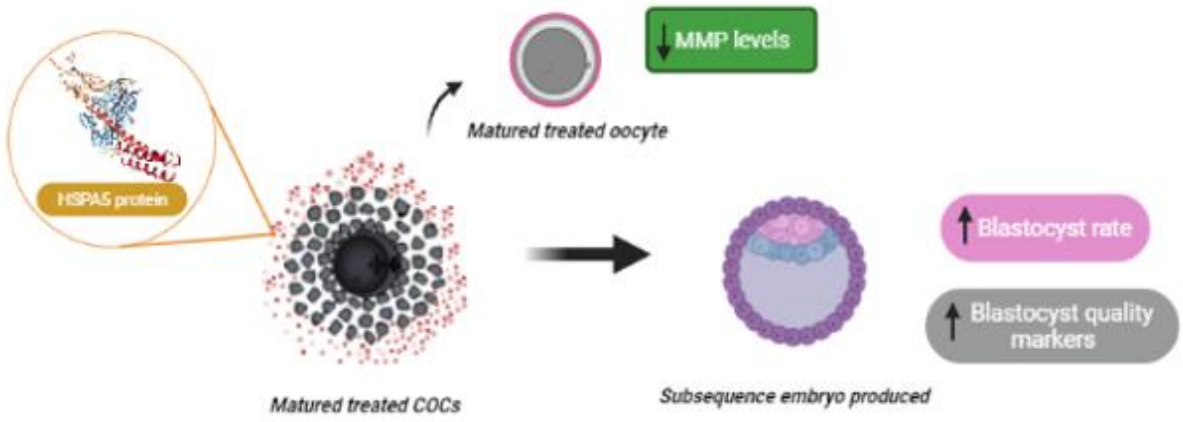


Figure 5. Effects of rHSPA-5 on the last 4 hours of bovine COCs IVM on final maturation and subsequence embryo development. The rHSPA5 decreased the MMP levels on oocytes and resulted in a higher blastocyst rate and an up-regulation of genes markers of blastocyst quality. rHSPA5, recombinant HSPA5; COC, cumulus-oocytes complex; IVM, *in vitro* maturation.

MATERIALS AND METHODS

Experimental design

This study was designed to reach how the oviductal protein HSPA5 can interact with bovine oocyte and its influence on subsequence initial embryo development on *in vitro* embryo production. The final maturation of oocyte occurs on the oviduct (C. Avilés, and Rizos, 2015; M. G.-A. Avilés, A; Coy, Pilar, 2010; P. Coy et al., 2012), but the precise time that the oocyte

can be firmly attached to the OEC is not defined. The passage of bovine COC through the oviduct is about 72 hours (Croxatto, 2002) and, considering that in 20 hours of *in vitro* maturation, 89% of COC achieve the MII stage (Khatir, Lonergan, & Mermillod, 1998), we proposed to include the HSPA5 protein at this moment. The protein concentration was previously tested for our group (data not published), comparing the 100 ng/ml with 1 ng/ml concentrations and no addition of rHSPA5. The concentration of 100 ng/ml was more effective on embryo production than another one and was not detrimental to the oocyte maturation.

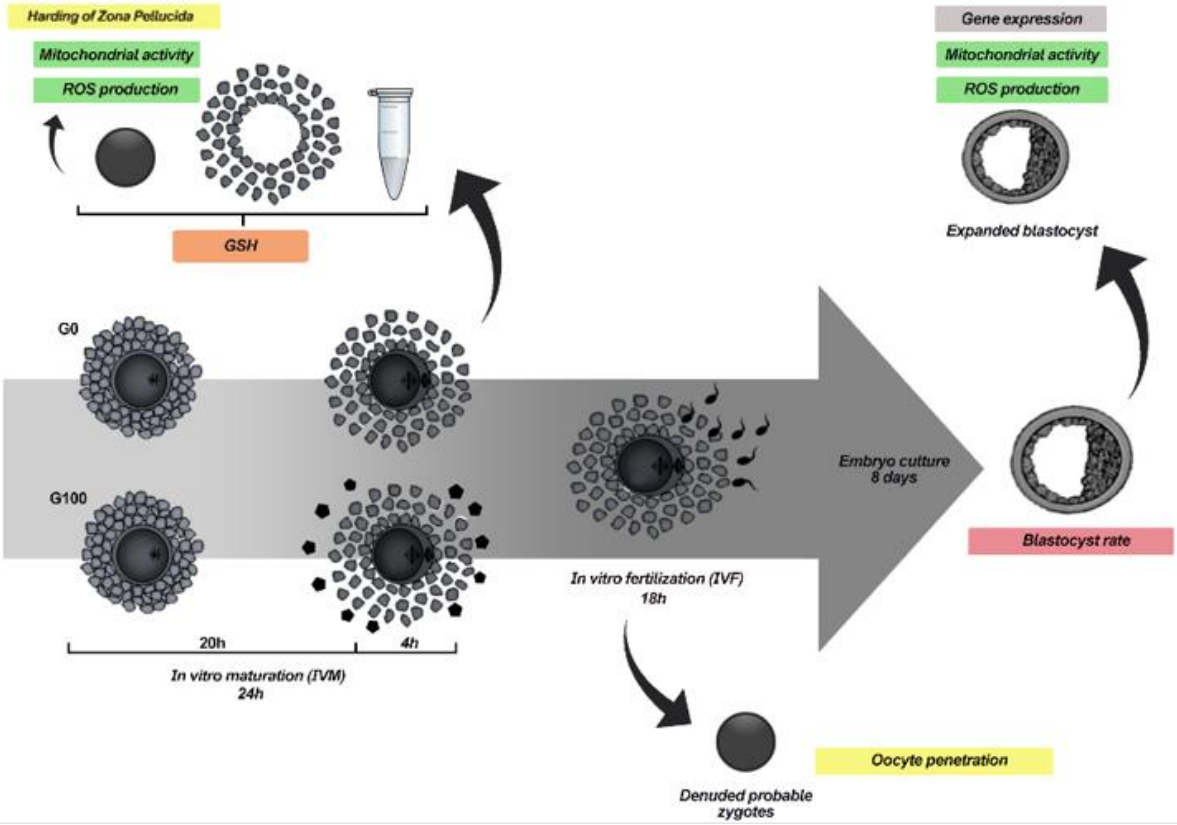


Figure 1. Effects of addiction of HSPA-5 protein on the last 4 hours of bovine COCs IVM. The immature COCs (20 COCs/group) were selected and randomly distributed among the groups: control - basic maturation medium (BM); and treated - BM + human rHSPA5 (100 ng/ml). In the first 20 hours of maturation, COCs from both groups were matured in the BM medium. After that, the medium was partially exchanged, and the rHSPA5 protein was added to the specific group. The IVM maturation continued to complete 24 hours. Matured COCs were separated to cumulus cells mechanically or followed to the *in vitro* fertilization (IVF) and *in vitro* culture (IVC) until 8 days to obtain expanded blastocyst. The IVM medium was collected to assess the total glutathione (GSH) content.

COC collection

Ovaries from abattoir at Torrinha, São Paulo, Brazil (22º 25' 34" S; 48º 10' 09" W) were collected and transported to the laboratory in saline solution (0.9%) at 36 °C. Follicles in 2-8 mm were aspirated with an 18-gouged needle and the follicular fluid pooled in a conical tube to sedimentation. COCs with homogenous cytoplasm and compacted multilayers of *cumulus* cells (grade 1 and 2; (Stojkovic et al., 2001) were recovered using a stereomicroscope and randomly divided into experimental groups.

In vitro maturation

All COCs selected were randomly divided into each experimental group. It was transferred to a 4-well plate with 10 µL/COC of primary maturation medium (BM) containing TCM199 with bicarbonate and Earle's slats, follicular stimulating hormone (0.1 IU/ml solution, Gonal-f, Merck Serono, Bari, Italy), 22 µg/ml of sodium pyruvate, 75 µg/ml of amikacin and 4 mg/ml of fatty acid-free bovine serum albumin –BSA). The groups were cultured for 20 hours at 38.5 °C in humidified air containing 5.5% CO_2. After that, both experimental groups had a partially medium exchanged to add BM (control) or BM plus human recombinant HSPA5 (treated; 100 ng/ml; Abcam© plc, Cambridge, MA, USA) of the same volume, and continued the culture until complete 24 hours at identical conditions. The matured COC was propriety collected to subsequent experiments or continue to embryo development.

In vitro fertilization

The matured COCs were washed in IVF medium (Progest, Botucatu, São Paulo, Brazil) and were placed in plates with 90 µl of IVF medium drops (in a proportion of 25 COCs/drops) and covered with silicone oil (Quimesp Química, Guarulhos, São Paulo, Brazil).

Cryopreserved semen samples from different ejaculates of same Nelore bull (CRV Lagoa, Sertãozinho, São Paulo, Brazil) were used in the whole experiment. The sample of semen was thawed at 37 °C for the 30s, enriched for motility using density-gradient centrifugation (BotuFIV Select SPERM – Botupharma, Botucatu, São Paulo, Brazil) at 3,000 rpm for 5 min. After that, the pellet was recovered and extended with the fertilization medium to a final concentration of 1×10^6 spermatozoa ml^{-1}. Co-incubation of oocytes and spermatozoa was performed for 18 h at 38 °C under 5% CO_2 in humidified air.

In vitro culture

After the insemination, the presumptive zygotes were denuded by vortexing, washed, and transferred to four-well plates containing 500 µl of IVC medium (SOF medium, 0.2 mM of sodium pyruvate, 5 mg/ml of BSA, and 2.5% (v/v) of fetal bovine serum). The cultured occurred at 38.5 °C in 5.5% CO_2 and low oxygen concentration (5% O_2) for 8 days. Embryos in expanded blastocyst stages were appropriately collected for analyses on day seven and day 8 of incubation.

Nuclear progression, mitochondrial activity, and intracellular ROS in oocytes and embryos

A combined fluorescence staining technique using Hoechst 33342, MitoTracker® Red CMXRos, and CellROX™ Green Reagent (Invitrogen, São Paulo, Brazil) was used to estimate nuclear progression, indirect mitochondrial inner membrane potential (MMP), and oxidative stress (ROS), respectively, in the oocytes. For the embryos, blastocysts were used on the expanded stage, and only MMP and ROS were analyzed.

Matured oocytes were denuded by vortex and washed in Phosphate-Buffered Saline pH 7.2 with 1 mg/ml polyvinylpyrrolidone (PBS-PVP). Denuded oocytes were incubated in drops with Hoechst (1µg/ml), CellROX Green (5µM), and MitoTracker Red (0.5µM), while embryos

were incubated in drops with only CellROX and MitoTracker, for 30 minutes at 5.5% CO_2 and 38.5 °C. Then, both samples were washed thrice in PBS-PVP and fixed in 4% paraformaldehyde for 15 min. After they were washed three times in PBS-PVP again and placed in glycerol micro drops on a blade covered by a coverslip and analyzed using Leica DM4500 epifluorescence microscope to detect DNA nuclear progression, ROS, and MMP. Images were acquired by LAS software using a camera attached to the microscope. The quantification of average pixel intensity was performed using Image J (NIH, MD, USA) software for ROS and MMP analysis.

Zona Pellucida solubillity

The *zona pellucida* (ZP) hardening was estimated according to (P. Coy et al., 2008). Matured COCs (n= 20 oocytes per group in 4 replicated) were denuded in vortex (13,000 rpm for 1 min), washed in PBS-PVP. To assess the hardening, the oocytes were added in 25 µl of pronase (0.5% v/v in PBS; Sigma-Aldrich Co., St Louis, MO), and the ZP was continuously observed using a stereomicroscope and the time digestion was clocked and registered as the time between the placement of samples in pronase solution and time at which the ZP was no longer visible.

Detection of oocyte penetration: polyspermatic assay

The polyspermy was assessed as demonstrated by (Ferraz et al., 2017). Semen samples from the same Nelore bull previously described were thawed at 37 °C for 30 seconds and washed in a discontinuous gradient of BotuFIV Select SPERM (Botupharma, Botucatu-SP) at 3,000 rpm for 5 min. The spermatozoa pellet was recovered and was added 350 µl of MitoTracker™ Green FM solution (Invitrogen, São Paulo, Brazil) – 200 nM in IVF medium, and incubated for 30 min at 37 °C. The mitotracker stained spermatozoa were washed three

400 times in IVF medium at 700 × g for 5 min. The spermatozoa pellet was resuspended in an IVF medium and used for *in vitro* fertilization in the same conditions previously described.

After 18 hours of *in vitro* fertilization with mitotracker stained spermatozoa, presumptive zygotes were fixed in 4% paraformaldehyde for 15 min, washed three times in PBS-PVP, then incubated with Hoechst (5µg/ml) for 30 min. Later, the presumptive zygotes
405 were washed three times in PBS-PVP again and then mounted into glycerol micro drops in a blade covered by a coverslip and analyzed using laser scanning confocal microscopy (Leica TCS SP8) attached to an inverted microscope with a 40× using LAS X software. Polyspermy was identified by the detection of 2 or more sperm mid-pieces into the ooplasm.

410 *GSH content determination*

Total glutathione (GSH) content was assessed according to (Anderson, 1985). Denuded oocytes (n= 20 oocytes per pool in 5 replicates), their cumulus cells, and were kept in trichloroacetic acid (TCA) at -80 °C. The MIV medium used in the cultured (20 µl) was also collected and stored at -80 °C, and TCA was added immediately before the GSH assay. The
415 samples were mixed in vortex (except medium samples) for 20 sec at 12,000 rpm. Standards containing 0 to 200 pmol of GSH in 50 ml were prepared in water, simultaneously with the samples, and both were kept on ice until be allocated in a microtiter plate. Each sample and standard was added in a volume of 20 µl in a 96-well microtiter plate with 0.1 ml of reaction mixture newly-prepared (6 mM of DTNB, 0.2 mM of NADPH, and 1.0 IU of GSH reductase/ml
420 (final concentrations) in 0.1M phosphate buffer supplemented with 1 mM of EDTA, pH 7.8). Immediately the plate was analyzed at 405 nm (SpectraCount, Packard, Meriden, CT, USA), first only with the mixing reaction, and then repeated-reads functions at 2 min intervals for 30 min.

Transcript profile of blastocysts

Embryos that reach the expanded blastocyst stage at day 7 of development were collected (n= 5 blastocysts per pool in 4 replicates) and stored at -80 °C until the RNA extraction. Total RNA from samples was extracted using PicoPure™ RNA Isolation Kit (Applied Biosystem®, Foster City, CA, USA) according to the manufacturer's protocol. After the purification, the RNA samples were eluted in 12µL of RNAse free water, and all volume of sample was used in the analyze.

The total RNA was incubated with DNAse I (1IU/µg; Invitrogen, São Paulo, Brazil) and then reverse-transcribed using random primers according to the protocol provided with the High Capacity kit (Applied Biosystem®, Foster City, CA, USA).

Then, the mRNA abundance of 96 genes was determined to analyze the transcripts profile of blastocysts produced. All analyses were performed using Biomark HD assay (Fluidigm, South San Francisco, CA, USA) as previously described (Fontes, Castilho, Razza, & Nogueira, 2020; Franchi et al., 2019; Giroto et al., 2019). The relative expression values for each gene were calculated with the ΔCt method. Data were normalized using geometric means of the most stable reference genes for each cellular type (NormFinder software tested). The mRNA abundance geometric mean of *HMBS*, *PPIA*, and *RPL30* was used to normalize the blastocysts from the IVM treatment experiment (Fontes et al., 2020).

Statistical analysis

Blastocyst yield and polyspermatic assays were calculated as percentages and were arcsine-transformed. All data were tested for distribution normality by the Shapiro-Wilk test, and non-normal distribution was log-transformed. Follow, *t*-test or ANOVA was performed followed by Tukey-test (to parametric data) or Kruskal-Wallis followed by Wilcoxon-test (to non-parametric means) were performed to compared means. All data are presented as means ±

SEM, and statistical significance was defined as $p \leq 0.05$. To analyses JMP software (SAS Institute, Cary, NC) was used.

REFERENCES

Alminana, C., Corbin, E., Tsikis, G., Alcantara-Neto, A. S., Labas, V., Reynaud, K., . . . Mermillod, P. (2017). Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. *Reproduction, 154*(3), 153-168. doi:10.1530/REP-17-0054

Anderson, M. E. (1985). Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol, 113*, 548-555. doi:10.1016/s0076-6879(85)13073-9

Avilés, C., and Rizos. (2015). The oviduct: A key organ for the success of the early reproductive events. *Animal Frontiers, 5*(1). doi:10.2527/af.2015-0005

Avilés, M. G.-A., A; Coy, Pilar. (2010). Oviductal secretions: will they be key factors for the future of ARTs? *Molecular Human Reproduction, 16*(12), 896-906.

Azari, M., Kafi, M., Asaadi, A., Pakniat, Z., & Abouhamzeh, B. (2021). Bovine oocyte developmental competence and gene expression following co-culturing with ampullary cells: An experimental study. *Int J Reprod Biomed, 19*(4), 371-380. doi:10.18502/ijrm.v19i4.9063

Basar, M., Bozkurt, I., Guzeloglu-Kayisli, O., Sozen, B., Tekmen, I., Schatz, F., . . . Kayisli, U. A. (2014). Unfolded protein response prevents blastocyst formation during preimplantation embryo development in vitro. *Fertil Steril, 102*(6), 1777-1784. doi:10.1016/j.fertnstert.2014.09.004

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

Bromfield, E. G., & Nixon, B. (2013). The function of chaperone proteins in the assemblage of protein complexes involved in gamete adhesion and fusion processes. *Reproduction, 145*(2), R31-42. doi:10.1530/REP-12-0316

Buhi, W. C., Ashworth, C. J., Bazer, F. W., & Alvarez, I. M. (1992). In vitro synthesis of oviductal secretory proteins by estrogen-treated ovariectomized gilts. *J Exp Zool, 262*(4), 426-435. doi:10.1002/jez.1402620409

Calvert, M. E., Digilio, L. C., Herr, J. C., & Coonrod, S. A. (2003). Oolemmal proteomics--identification of highly abundant heat shock proteins and molecular chaperones in the mature mouse egg and their localization on the plasma membrane. *Reprod Biol Endocrinol, 1*, 27. doi:10.1186/1477-7827-1-27

Carey, T. S., Cao, Z., Choi, I., Ganguly, A., Wilson, C. A., Paul, S., & Knott, J. G. (2015). BRG1 Governs Nanog Transcription in Early Mouse Embryos and Embryonic Stem Cells via Antagonism of Histone H3 Lysine 9/14 Acetylation. *Mol Cell Biol, 35*(24), 4158-4169. doi:10.1128/MCB.00546-15

Casas, C. (2017). GRP78 at the Centre of the Stage in Cancer and Neuroprotection. *Front Neurosci, 11*, 177. doi:10.3389/fnins.2017.00177

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., & Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell, 113*(5), 643-655. doi:10.1016/s0092-8674(03)00392-1

Coy, P., Canovas, S., Mondejar, I., Saavedra, M. D., Romar, R., Grullon, L., . . . Aviles, 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., Garcia-Vazquez, F. A., Visconti, P. E., & Aviles, M. (2012). Roles of the oviduct in mammalian fertilization. *Reproduction, 144*(6), 649-660. doi:10.1530/REP-12-0279

Coy, P. Y., R. (2017). The common and species-specific roles of oviductal proteins in mammalian fertilization and embryo development. *Bioscience, 65*, 973-984.

Croxatto, H. B. (2002). Physiology of gamete and embryo transport through the fallopian tube. *Reprod Biomed Online, 4*(2), 160-169. doi:10.1016/s1472-6483(10)61935-9

Delpino, A., & Castelli, M. (2002). The 78 kDa glucose-regulated protein (GRP78/BIP) is expressed on the cell membrane, is released into cell culture medium and is also present in human peripheral circulation. *Biosci Rep, 22*(3-4), 407-420. doi:10.1023/a:1020966008615

Demant, M., Trapphoff, T., Frohlich, T., Arnold, G. J., & Eichenlaub-Ritter, U. (2012). Vitrification at the pre-antral stage transiently alters inner mitochondrial membrane potential but proteome of in vitro grown and matured mouse oocytes appears unaffected. *Hum Reprod, 27*(4), 1096-1111. doi:10.1093/humrep/der453

Dumollard, R., Carroll, J., Duchen, M. R., Campbell, K., & Swann, K. (2009). Mitochondrial function and redox state in mammalian embryos. *Semin Cell Dev Biol, 20*(3), 346-353. doi:10.1016/j.semcdb.2008.12.013

Ferraz, M., Henning, H. H. W., Costa, P. F., Malda, J., Melchels, F. P., Wubbolts, R., . . . Gadella, B. M. (2017). Improved bovine embryo production in an oviduct-on-a-chip system: prevention of poly-spermic fertilization and parthenogenic activation. *Lab Chip, 17*(5), 905-916. doi:10.1039/c6lc01566b

Fontes, P. K., Castilho, A. C. S., Razza, E. M., & Nogueira, M. F. G. (2020). Bona fide gene expression analysis of samples from the bovine reproductive system by microfluidic platform. *Anal Biochem, 596*, 113641. doi:10.1016/j.ab.2020.113641

Franchi, F. F., Satrapa, R. A., Fontes, P. K., Santos, P. H., Razza, E. M., Emanuelli, I. P., . . . de Souza Castilho, A. C. (2019). Equine chorionic gonadotropin drives the transcriptional profile of immature cumulus-oocyte complexes and in vitro-produced blastocysts of superstimulated Nelore cows. *Mol Reprod Dev, 86*(11), 1639-1651. doi:10.1002/mrd.23251

Giroto, A. B., Fontes, P. K., Franchi, F. F., Dos Santos, P. H., Razza, E. M., Nogueira, M. F. G., . . . Castilho, A. C. S. (2019). 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. *Mol Reprod Dev, 86*(11), 1694-1704. doi:10.1002/mrd.23259

Gonzalez-Gronow, M., Selim, M. A., Papalas, J., & Pizzo, S. V. (2009). GRP78: a multifunctional receptor on the cell surface. *Antioxid Redox Signal, 11*(9), 2299-2306. doi:10.1089/ARS.2009.2568

Goto, Y., Noda, Y., Mori, T., & Nakano, M. (1993). Increased generation of reactive oxygen species in embryos cultured in vitro. *Free Radic Biol Med, 15*(1), 69-75. doi:10.1016/0891-5849(93)90126-f

Gujar, H., Weisenberger, D. J., & Liang, G. (2019). The Roles of Human DNA Methyltransferases and Their Isoforms in Shaping the Epigenome. *Genes (Basel), 10*(2). doi:10.3390/genes10020172

Hao, C. N., Geng, Y. J., Li, F., Yang, T., Su, D. F., Duan, J. L., & Li, Y. (2011). Insulin-like growth factor-1 receptor activation prevents hydrogen peroxide-induced oxidative

stress, mitochondrial dysfunction and apoptosis. *Apoptosis, 16*(11), 1118-1127. doi:10.1007/s10495-011-0634-9

Hebert-Schuster, M., Rotta, B. E., Kirkpatrick, B., Guibourdenche, J., & Cohen, M. (2018). The Interplay between Glucose-Regulated Protein 78 (GRP78) and Steroids in the Reproductive System. *Int J Mol Sci, 19*(7). doi:10.3390/ijms19071842

Heyner, S., Shi, C. Z., Garside, W. T., & Smith, R. M. (1993). Functions of the IGFs in early mammalian development. *Mol Reprod Dev, 35*(4), 421-425; discussion 425-426. doi:10.1002/mrd.1080350417

Karantzali, E., Lekakis, V., Ioannou, M., Hadjimichael, C., Papamatheakis, J., & Kretsovali, A. (2011). Sall1 regulates embryonic stem cell differentiation in association with nanog. *J Biol Chem, 286*(2), 1037-1045. doi:10.1074/jbc.M110.170050

Khatir, H., Lonergan, P., & Mermillod, P. (1998). Kinetics of nuclear maturation and protein profiles of oocytes from prepubertal and adult cattle during in vitro maturation. *Theriogenology, 50*(6), 917-929. doi:10.1016/s0093-691x(98)00196-4

Kidson, A., Schoevers, E., Langendijk, P., Verheijden, J., Colenbrander, B., & Bevers, M. (2003). The effect of oviductal epithelial cell co-culture during in vitro maturation on sow oocyte morphology, fertilization and embryo development. *Theriogenology, 59*(9), 1889-1903. doi:10.1016/s0093-691x(02)01291-8

Kolle, S., Dubielzig, S., Reese, S., Wehrend, A., Konig, P., & Kummer, W. (2009). Ciliary transport, gamete interaction, and effects of the early embryo in the oviduct: ex vivo analyses using a new digital videomicroscopic system in the cow. *Biol Reprod, 81*(2), 267-274. doi:10.1095/biolreprod.108.073874

Korshunov, S. S., Skulachev, V. P., & Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett, 416*(1), 15-18. doi:10.1016/s0014-5793(97)01159-9

Kramer, A. C., Steinhauser, C. B., Gao, H., Seo, H., McLendon, B. A., Burghardt, R. C., . . . Johnson, G. A. (2020). Steroids Regulate SLC2A1 and SLC2A3 to Deliver Glucose Into Trophectoderm for Metabolism via Glycolysis. *Endocrinology, 161*(8). doi:10.1210/endocr/bqaa098

Lachance, C., Bailey, J. L., & Leclerc, P. (2007). Expression of Hsp60 and Grp78 in the human endometrium and oviduct, and their effect on sperm functions. *Hum Reprod, 22*(10), 2606-2614. doi:10.1093/humrep/dem242

Lake, J. I., Avetisyan, M., Zimmermann, A. G., & Heuckeroth, R. O. (2016). Neural crest requires Impdh2 for development of the enteric nervous system, great vessels, and craniofacial skeleton. *Dev Biol, 409*(1), 152-165. doi:10.1016/j.ydbio.2015.11.004

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

Latham, K. E. (2016). Stress signaling in mammalian oocytes and embryos: a basis for intervention and improvement of outcomes. *Cell Tissue Res, 363*(1), 159-167. doi:10.1007/s00441-015-2124-9

Li, S., & Winuthayanon, W. (2017). Oviduct: roles in fertilization and early embryo development. *J Endocrinol, 232*(1), R1-R26. doi:10.1530/JOE-16-0302

Lin, P., Chen, F., Yang, Y., Song, Y., Li, X., Lan, X., . . . Wang, A. (2012). GRP78 expression and immunohistochemical localization in the female reproductive tract of mice. *Theriogenology, 78*(8), 1824-1829. doi:10.1016/j.theriogenology.2012.07.020

Loor, G., Kondapalli, J., Schriewer, J. M., Chandel, N. S., Vanden Hoek, T. L., & Schumacker, P. T. (2010). Menadione triggers cell death through ROS-dependent mechanisms involving PARP activation without requiring apoptosis. *Free Radic Biol Med, 49*(12), 1925-1936. doi:10.1016/j.freeradbiomed.2010.09.021

Marín-Briggiler, C. I. G.-E., M.F.; Munuce, M.J.; Ghersevich, S.; Caille, A.M.; Hellman, U.; Corrigall, V.M.; Vazquez-Levin, M.H. (2010). Glucose-regulated protein 78 (GRP78/BiP) is secreted by human oviduct epithelial cells and the recombinantprotein modulates sperm-zona pellucida binding. *Fertil Steril, 93*(5).

Mondejar, I., Martinez-Martinez, I., Aviles, 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

Nasr-Esfahani, M. H., Aitken, J. R., & Johnson, M. H. (1990). Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. *Development, 109*(2), 501-507.

Quinlan, C. L., Perevoshchikova, I. V., Hey-Mogensen, M., Orr, A. L., & Brand, M. D. (2013). Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox Biol, 1*, 304-312. doi:10.1016/j.redox.2013.04.005

Sato, M., Yao, V. J., Arap, W., & Pasqualini, R. (2010). GRP78 signaling hub a receptor for targeted tumor therapy. *Adv Genet, 69*, 97-114. doi:10.1016/S0065-2660(10)69006-2

Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Goncalves, P. B., & Wolf, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod, 64*(3), 904-909. doi:10.1095/biolreprod64.3.904

Vergnes, L., Chin, R. G., de Aguiar Vallim, T., Fong, L. G., Osborne, T. F., Young, S. G., & Reue, K. (2016). SREBP-2-deficient and hypomorphic mice reveal roles for SREBP-2 in embryonic development and SREBP-1c expression. *J Lipid Res, 57*(3), 410-421. doi:10.1194/jlr.M064022

Yang, Y. W., Brown, D. R., Robcis, H. L., Rechler, M. M., & de Pablo, F. (1993). Developmental regulation of insulin-like growth factor binding protein-2 in chick embryo serum and vitreous humor. *Regul Pept, 48*(1-2), 145-155. doi:10.1016/0167-0115(93)90343-7

Zhang, C. (2017). Roles of Grp78 in Female Mammalian Reproduction. *Adv Anat Embryol Cell Biol, 222*, 129-155. doi:10.1007/978-3-319-51409-3_7

Zhang, J. Y., Diao, Y. F., Kim, H. R., & Jin, D. I. (2012). Inhibition of endoplasmic reticulum stress improves mouse embryo development. *PLoS One, 7*(7), e40433. doi:10.1371/journal.pone.0040433

Zhang, X., Zhang, X., Qi, Y., Huang, D., & Zhang, Y. (2016). 2,4-dichlorophenol induces ER stress-mediated apoptosis via eIF2alpha dephosphorylation in vitro. *Environ Toxicol, 31*(2), 245-255. doi:10.1002/tox.22039

CAPÍTULO III

AS NOVAS ESTRATÉGIAS DO USO DA rHSPA5 HUMANA NA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

RESUMO

As *heat-shock proteins* (HSPs) estão presentes no fluido ovidutal e suas concentrações variam de acordo o ciclo estral. Recentemente, essas proteínas têm sido relacionadas à capacitação espermática e na interação *zona-pellucia*-espermatozoide, mas pouco se sabe sobre sua função na maturação final oocitária e no desenvolvimento inicial embrionário. Estudos prévios do nosso grupo demonstraram que a adição de HSPA5 recombinante humana (rHSPA5) nas últimas horas de MIV é capaz de melhorar a eficiência da produção embrionária e, principalmente, modular a expressão de genes relacionados a qualidade embrionária destes embriões. A fim de verificar um possível efeito desta proteína no desenvolvimento embrionário, nós comparamos o efeito da adição da rHSPA5 nas últimas 4 horas da maturação *in vitro* (grupo 4 h) com 8 dias de cultivo embrionário (grupo 8 dias) e a associação desses dois tratamentos (4h + 8 dias) na produção *in vitro* e na transcrição gênica desses embriões. A taxa de embriões produzidos *in vitro* não foi diferente entre os grupos estudados, mas 37 genes foram diferentemente expressos ($p \geq 0,05$). Na comparação entre os grupos, os embriões do grupo 4h apresentaram uma maior abundancia de genes sugestivos de melhor qualidade, com 19 genes mais expressos quando comparados aos demais grupos (*IMPDH, IMPDH2, MAPK1, EGFR, IMPDH, IMPDH2, MAPK1, EGFR, SRBP2, FADS2, ELOVL6, SLC2A1, SLC2A3, NANOG, POU5F1, Dnmt3B* e *Dnmt3A, S100A10, HSP90AA1, GFBP2, CAT* e *CASP9*). Embriões do grupo 8 dias e 4h + 8 dias apesar de apresentar diferença em alguns poucos genes, não tiveram tanta influência quando comparados ao grupo controle e 4h. Tais resultados sugerem que a rHSPA5 apesar de modular a expressão de determinados genes quando presente nos 8 dias de cultivo, não parece trazer grandes benefícios à produção embrionária como o tratamento nas

últimas 4 horas de maturação oocitária, que mostra um grande impacto na qualidade molecular embrionária.

INTRODUÇÃO

Por muito tempo o oviduto foi visto apenas como um órgão de passagem e fusão de gametas e como um ambiente propício para o desenvolvimento inicial do embrião antes de sua chegada no útero e implantação. Atualmente sabemos que o oviduto apresenta um importante papel no processo reprodutivo, e que o seu fluido ovidutal (FO) contém fatores essenciais para cada etapa que precede o desenvolvimento embrionário (Avilés, 2010; Binelli *et al.*, 2018; Banliat *et al.*, 2019).

O oviduto é dividido em três partes: fímbrias, infundíbulo e istmo, que conectam o ovário e o útero (Avilés, 2015; Li e Winuthayanon, 2017). Suas células epiteliais ovidutais (CEO) secreta fatores que, juntamente aos secretados do plasma sanguíneo, formam o FO. Apesar da composição deste fluido não ser completamente conhecida, é sabido que ele contém diversos fatores importantes para a maturação final dos gametas e desenvolvimento do embrião, como substratos energéticos, íons, aminoácidos, lipídios e proteínas com função estrutural, catalítica e regulatória (Buhi *et al.*, 2000; Avilés, 2010; Avilés, 2015; Li e Winuthayanon, 2017).

Entre as proteínas que compõem o FO, as HSPs aparecem em grande concentração e em todas as fases do ciclo estral (Lamy *et al.*, 2016; Lamy *et al.*, 2018), e entre essas, uma das proteínas mais citadas é a proteína HSPA5, que está presente em grande concentração no FO, principalmente no período peri-ovulatório (Lamy *et al.*, 2018). Essa proteína é uma chaperona presente no retículo endoplasmático de todas as células eucarióticas, participando ativamente

da manutenção da homeostase e proteção celular (Gonzalez-Gronow *et al.*, 2009; Hebert-Schuster *et al.*, 2018).

No oviduto, a HSPA5 exerce efeitos nos gametas, especificamente na capacitação e viabilidade espermática (Boilard *et al.*, 2004; Lachance *et al.*, 2007) e está diretamente relacionada com o processo de fertilização, sendo associada como um dos fatores ligados ao enrijecimento da *zona-pellucida* (ZP) e promoção da fusão dos gametas (Marín-Briggiler, 2010; Bromfield e Nixon, 2013; Mondejar *et al.*, 2013). Apesar de não haver dados publicados sobre o efeito da proteína no oócito, a presença da proteína foi encontrada no oolema de oocitos maduros de ratas (Bromfield e Nixon, 2013) e, dados não publicados do nosso grupo, mostraram que a adição da rHSPA5 nas últimas 4 horas de MIV aumentaram a eficiência da produção embrionária e tiverem um efeito benéfico no perfil de expressão gênica dos embriões produzidos.

A HSPA5 também foi encontrada nas vesículas extracelulares (VEs) do FO (Alminana *et al.*, 2017; Harris *et al.*, 2020). Alminana e colegas (2017) demonstraram que estas VEs são internalizadas pelos embriões produzidos *in vitro* e, principalmente, são capazes de aumentar a taxa de blastocistos, de eclosão e sobrevivência desses embriões (Alminana *et al.*, 2017). A presença da HSPA5 nestas vesículas nos leva a pensar, que assim como no processo de fertilização e a ação nos espermatozoides, a HSPA5 possivelmente pode ter efeitos nos embriões em desenvolvimento no oviduto.

Com isso, este trabalho teve como objetivo verificar a influência da rHSPA5 nas últimas 4 horas de MIV, nos 8 dias de CIV e na associação dos tratamentos (4 horas de MIV e 8 dias de CIV), sobre a taxa de embriões produzidos e o perfil de expressão gênica destes embriões. E também, testar a hipótese de que a proteína HSPA5 tem efeito direto no embrião em desenvolvimento inicial e que a adição da rHSPA5 nas últimas 4 horas da MIV e nos 8 dias de

CIV pode impactar de forma positiva a produção e qualidade embrionária. Este trabalho traz dados recém recebidos, que nos possibilita diferentes visões dos tratamentos, mas que pode nos levar à novas perspectivas para a produção *in vitro* de embriões.

MATERIAL E MÉTODO

Desenho experimental

Este trabalho foi desenhado para avaliar o efeito da proteína recombinante humana HSPA5 na produção de embriões bovinos *in vitro*. Estudos anteriores (dados não publicados) demonstraram que a adição da rHSPA5 nas últimas 4 horas da MIV foi capaz de aumentar a taxa de embriões e modular a expressão de genes relacionados a qualidade embrionária. Considerando esses resultados, este trabalho propôs avaliar a adição da HSPA5 nos 8 dias de cultivo embrionário provenientes de oócitos maturados na presença da proteína ou não, quanto a taxa de embriões produzidos e a transcrição gênica (Fig 1).

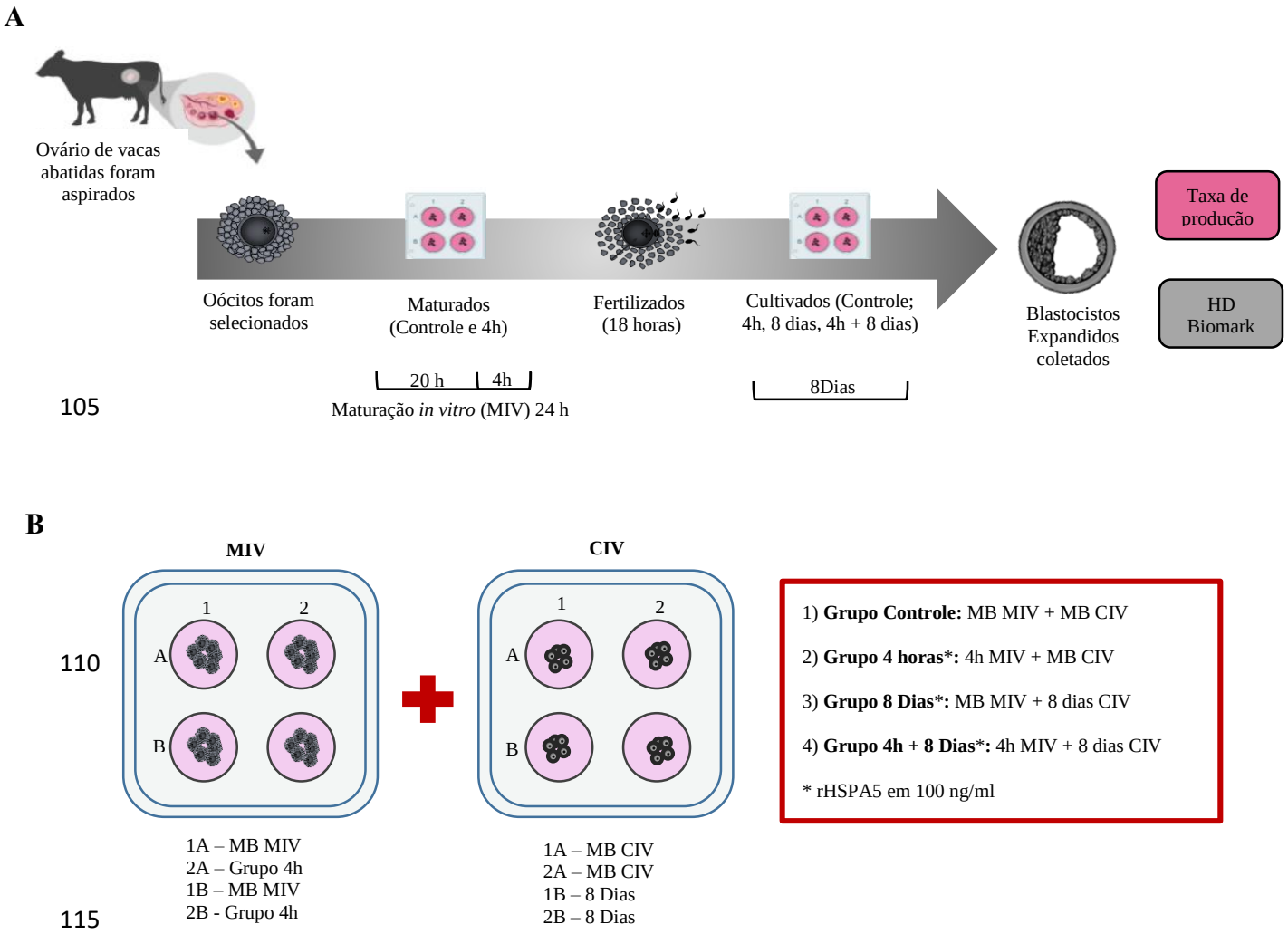


Figura 1: Efeito da adição da proteína rHSPA5 nas últimas 4 horas da MIV e/ou no cultivo embrionário. **A)** Ovários provenientes de vacas de abatedouro foram aspirados e os complexos *cumulus*-oócitos (COC) foram coletados, selecionados (40 COC/grupo) e distribuídos aleatoriamente entre os grupos: Controle - meio base de maturação (MB); 4h – MB + rHSPA5 humano (100 ng/ml) nas últimas 4 horas de MIV. Nas primeiras 20 horas de maturação os COCs de ambos os grupos foram cultivados em MB. Após as 20 h iniciais da MIV, o meio de cultivo foi parcialmente trocado e a proteína rHSPA5 adicionada ao grupo 4h. Após a troca parcial do meio, os COCs retornaram para MIV para completar as 24 horas de maturação. Os COCs maturados seguiram para a fertilização *in vitro* (FIV) por 18 horas e para o cultivo *in vitro* (CIV), onde os prováveis zigotos (PZ) foram divididos da seguinte forma divididos para cultivo em Meio Base (MB) CIV – CIV comum, e grupo 8 dias – com adição da rHSPA5 (100 ng/ml) nos 8 dias de cultivo (20 PZ/grupo). O experimento foi realizado em 7 réplicas. **B)** Divisão dos grupos experimentais: **controle** (sem adição da proteína rHSPA5), **4h** (presença da proteína nas últimas 4 h de MIV), **8 dias** (adição da proteína no CIV) e **4h + 8 dias** (adição da proteína na MIV e no CIV).

Coleta dos COCs

Ovários do abatedouro localizado em Bariri, São Paulo, Brasil (22° 4' 28" S; 48º 44' 26" O) foram coletados e transportados em solução salina (0,9%) à 36 °C para o laboratório. Os

folículos com diâmetro de 2-8 mm foram aspirados com auxílio de agulha (18G) acoplada à seringa de 10 ml e o fluido folicular aspirado foi acondicionado em tubo cônico para sedimentação. Os COCs com citoplasma homogêneo e múltiplas camadas de células do *cumulus* compactadas (grau 1 e 2) (Stojkovic *et al.*, 2001) foram selecionados sob uso de esteromicroscópio e divididos randomicamente nos grupos experimentais para a MIV.

Maturação in vitro dos COCs

Todos os COCs selecionados e divididos entre os grupos foram transferidos para placa de 4-poços com 10 µL/COC de meio base (BM) de maturação, contendo TCM199 com bicarbonato e sais de Earle, hormônio folículo estimulante (FSH, solução de 0,1 IU/ml, Gonal-f, Merk Serono, Bari, Itália), 22 µg/ml de piruvato de sódio, 75 µg/ml de amicacina e 4 mg/ml de albumina sérica bovina (BSA) livre de ácidos graxos. Os grupos foram então cultivados à 38,5 °C em ambiente úmido contendo 5,5% de CO_2. Após as primeiras 20 h de cultivo, os meios de ambos os grupos foram parcialmente trocados para a adição de MB (grupo controle) ou MB + rHSPA5 humana (concentração final no poço de 100 ng/ml; Abcam© plc, Cambridge, MA, USA) para completar as 24 h de cultivo, com as mesmas condições iniciais. Após a MIV, os COCs seguiram para a FIV.

Fertilização in vitro

Os COCs maturados foram lavados três vezes em meio de FIV (Progest, Botucatu, São Paulo, Brazil) e alocados em gotas de 90 µl de meio FIV (proporção de 20 COCs/gota) recobertas com óleo de silicone (Quimesp Química, Guarulhos, São Paulo, Brazil).

Amostras de sêmen criopreservado de diferentes ejaculados do mesmo touro Nelore (CRV Lagoa, Sertãozinho, São Paulo, Brazil) foi utilizado para todo o experimento. A amostra

de sêmen foi descongelada à 37 °C por 30 segundos, centrifugada em meio com gradiente de densidade (BotuFIV Select SPERM – Botupharma, Botucatu, São Paulo, Brazil) à 3.000 rpm por 5 min. Após a centrifugação, o pellet formado foi recuperado e meio FIV adicionado para concentração final de 1×10^6 espermatozoides/ml^{-1}. Os COCs e espermatozoides foram co-cultivados por 18 h à 37,5 °C sob 5,5% CO_2 em ambiente úmido.

Cultivo in vitro

Após a fertilização, os prováveis zigotos foram denudados com auxílio de vórtex, lavados e transferidos para placas 4-poços contendo 500 µl de meio CIV (fluido ovidutal sintético – SOF, 0,2 mM de piruvato de sódio, 5 mg/ml de BSA e 2,5% (v/v) de soro fetal bovino). O cultivo embrionário foi realizado à 38,5 °C em ambiente úmido e controlado (5% CO_2, 5% O_2 e 90% N_2). A taxa de blastocisto foi avaliada no oitavo dia de cultivo e os blastocistos expandidos (do dia 7 e do dia 8 de cultivo) foram coletados para as análises subsequentes.

Perfil de transcritos dos blastocistos

Blastocistos expandidos foram coletados no sétimo dia de cultivo (n= 5 blastocistos por *pool* em 4 réplicas) e armazenados em ultrafreezer (-80 °C) até a extração do RNA. O RNA total das amostras foi extraído usando o kit PicoPure™ RNA Isolation Kit (Applied Biosystem®, Foster City, CA, USA) de acordo com o protocolo do fabricante. Após o processo de purificação, as amostras de RNA foram eluídas em 12 µl de água livre de RNAse e todo o volume da amostra foi utilizada para a análise.

Após a aquisição da amostra, esta foi incubada com DNAse I (1 IU/μg; Invitrogen, São Paulo, Brasil) e realizada a sua transcrição reversa com uso de *random primers*, seguindo o protocolo fornecido com o Kit High Capacity (Applied Biosystem®, Foster City, CA, USA).

Os níveis de mRNA de 96 genes foi determinado para avaliar o perfil de transcritos dos blastocistos produzidos. As análises foram realizadas com equipamento Biomark HD (Fluidigm, South San Francisco, CA, USA) como descrito anteriormente (Franchi *et al.*, 2019; Giroto *et al.*, 2019; Fontes *et al.*, 2020). O valor da expressão relativa para cada gene foi calculado usando o método de ΔCt e os dados foram normalizados usando a média geométrica dos genes de referência mais estáveis (NormFinder software tested). A média geométrica do mRNA dos genes *GAPDH*, *PPIA* e *RPL30* foi utilizada para normalizar os dados obtidos neste experimento.

Análises estatísticas

A taxa dos blastocistos produzidos foi calculada como porcentagem e transformada em arco seno. Todos os dados obtidos foram testados para distribuição normal pelo teste de Shapiro-Wilk e os dados com distribuição não-normal foi transformado em *log*. Em seguida, para a comparação das médias, foi realizado ANOVA seguido de teste Tukey (dados paramétricos) ou Kruskal-Wallis seguido de Wilcoxon (para os dados não paramétricos). Todos os dados deste trabalho estão apresentados em média ± erro padrão da média e a diferença estatística foi definida como $p \leq 0,05$. As análises foram realizadas no *software* JMP (SAS Institute, Cary, NC).

RESULTADOS E DISCUSSÃO

Efeito da adição da proteína rHSPA5 humana nos meios de maturação e cultivo durante a produção de embriões in vitro.

A presença das proteínas HSP varia de acordo com o ciclo estral (Mariani *et al.*, 2000; Lamy *et al.*, 2016; Soleilhavoup *et al.*, 2016; Lamy *et al.*, 2018). A HSPA5 está presente em todas as fases do ciclo, mesmo que em menores concentrações, tendo sua maior expressão na fase peri ovulatória (Lin *et al.*, 2012; Lamy *et al.*, 2016), o que justifica a adição desta proteína nos meios de maturação e cultivo.

A proteína ovidutal HSPA5 tem demonstrado atuar sobre os processos reprodutivos no oviduto, como na manutenção do enrijecimento da *zona pelúcida* e polispermia (Mondejar *et al.*, 2013) e na viabilidade espermática (Boilard *et al.*, 2004; Lachance *et al.*, 2007; Marín-Briggiler, 2010). Dados mais recentes (dados não publicados), demonstraram que a proteína é capaz de interagir com os COCs nas últimas 4 horas da maturação, aumentando a taxa de embriões produzidos *in vitro.* Com isso, a adição desta proteína no meio de cultivo para uma possível interação com os embriões foi avaliada, com a associação de seu uso na maturação oocitária ou não. A porcentagem dos embriões produzidos em 8 dias de cultivo não foi diferente entre os 4 grupos avaliados: controle (45,11±5,00), 4h (55,54±5,00), 8dias (46,81±5,00) e 4h+8dias (47,11±5,40) com $p = 0,5035$ (Fig. 2).

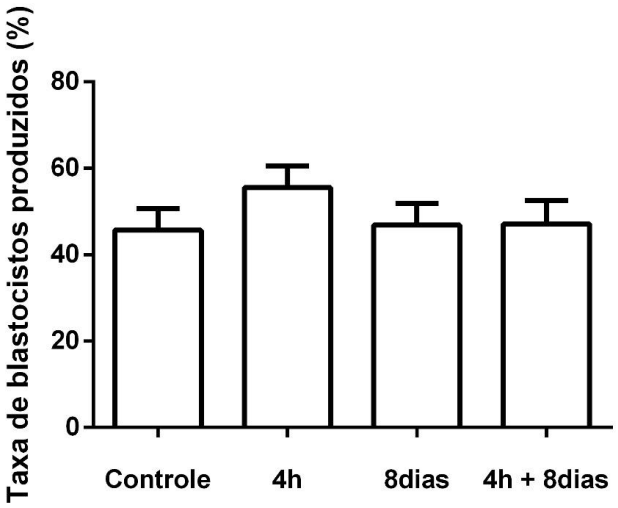


Figura 2: Efeito da adição da proteína rHSPA5 nas últimas 4 horas de MIV, em 8 dias de cultivo embrionário e na associação dos tratamentos sob a taxa de blastocistos produzidos. Os dados foram apresentados em média ± EPM. Diferenças foram consideradas significativas quando $p \leq 0,05$.

Estudos de pré-incubação de espermatozoides com proteínas HSPs demonstraram efeito direto das proteínas no gameta, como aumento da viabilidade espermática (Elliott *et al.*, 2009; Lloyd *et al.*, 2009; Lloyd *et al.*, 2012; Moein-Vaziri *et al.*, 2014; Holt e Fazeli, 2016). Já na produção *in vitro* de embriões, García-Matinez et al (2020) adicionou as proteínas HSP90α e HSP70 no processo de fertilização *in vitro* (Garcia-Martinez et al., 2020). Os autores mostraram que ambas as proteínas parecem exercer um efeito sobre a ZP, resultando em um aumento no tempo de digestão da ZP, mas somente a HSP70 aumentou a taxa de monospermia. De qualquer forma, a adição das proteínas não aumentou a eficiência da produção de embriões suíno. Além disso, as proteínas não exerceram efeito nos espermatozoides. A adição da proteína HSP60 também foi anteriormente na fertilização *in vitro*, mas também não aumentou significativamente a eficiência da produção de blastocistos ou promoveu um aumento de viabilidade espermática, mas foi capaz de elevar a taxa de embriões de 2 células de maneira dose-dependente (Abdi *et al.*, 2019).

Tais achados, juntamente com os nossos resultados de produção embrionária após a adição da rHSPA no meio MIV e/ou CIV, nos leva a pensar que as proteínas não necessariamente aumentam a eficiência da produção embrionária, mas podem intervir em outros aspectos, e além disso, a escolha da proteína a ser incluída no meio de cultivo deve ser precisa quanto à etapa em que ela será adicionada.

Efeito da adição da proteína rHSPA5 humana nos meios de maturação e cultivo sob o perfil de transcritos dos embriões produzidos

O FO fornece as condições ideias para a fertilização e desenvolvimento inicial embrionário como metabólitos (p. ex. glicose, lactato, piruvato e amino ácidos), ions, enzimas e proteínas em concentrações propícias para cada etapa do processo reprodutivo (Li e Winuthayanon, 2017). As proteínas HSPs secretadas no FO parecem ser capazes de auxiliar na modulação do enrijecimento da ZP (Mondejar *et al.,* 2013) e mais recentemente, dados não publicados do nosso grupo demonstrou que a proteína HSPA5 adicionada nas últimas 4 horas da maturação *in vitro* é capaz de modular a atividade mitocondrial do oócito e de alguma forma aumentar a taxa de embriões produzidos provenientes dos oócitos tratados, assim como modular sua expressão gênica. Considerando que embriões produzidos *in vitro* são capazes de internalizar VEs provenientes do fluido ovidutal que contém proteínas como as HSPs e ainda, que estas proteínas podem influenciar na qualidade embrionária e eficiência da produção (Alminana *et al.*, 2017), neste experimento nós investigamos o possível efeito na expressão gênica dos embriões produzidos com a adição da proteína rHSPA5 no meio CIV associado ou não ao tratamento na maturação.

Os tratamentos utilizados neste experimento foram capazes de modular o perfil transcricional dos embriões de uma maneira não homogênea entre os grupos experimentais,

como apresentado no gráfico *heatmap* (Fig 3A), mas apesar disso, os tratamentos foram capazes de alterar vias de enriquecimento, como apresentado no GO *slim* (Fig 3B).

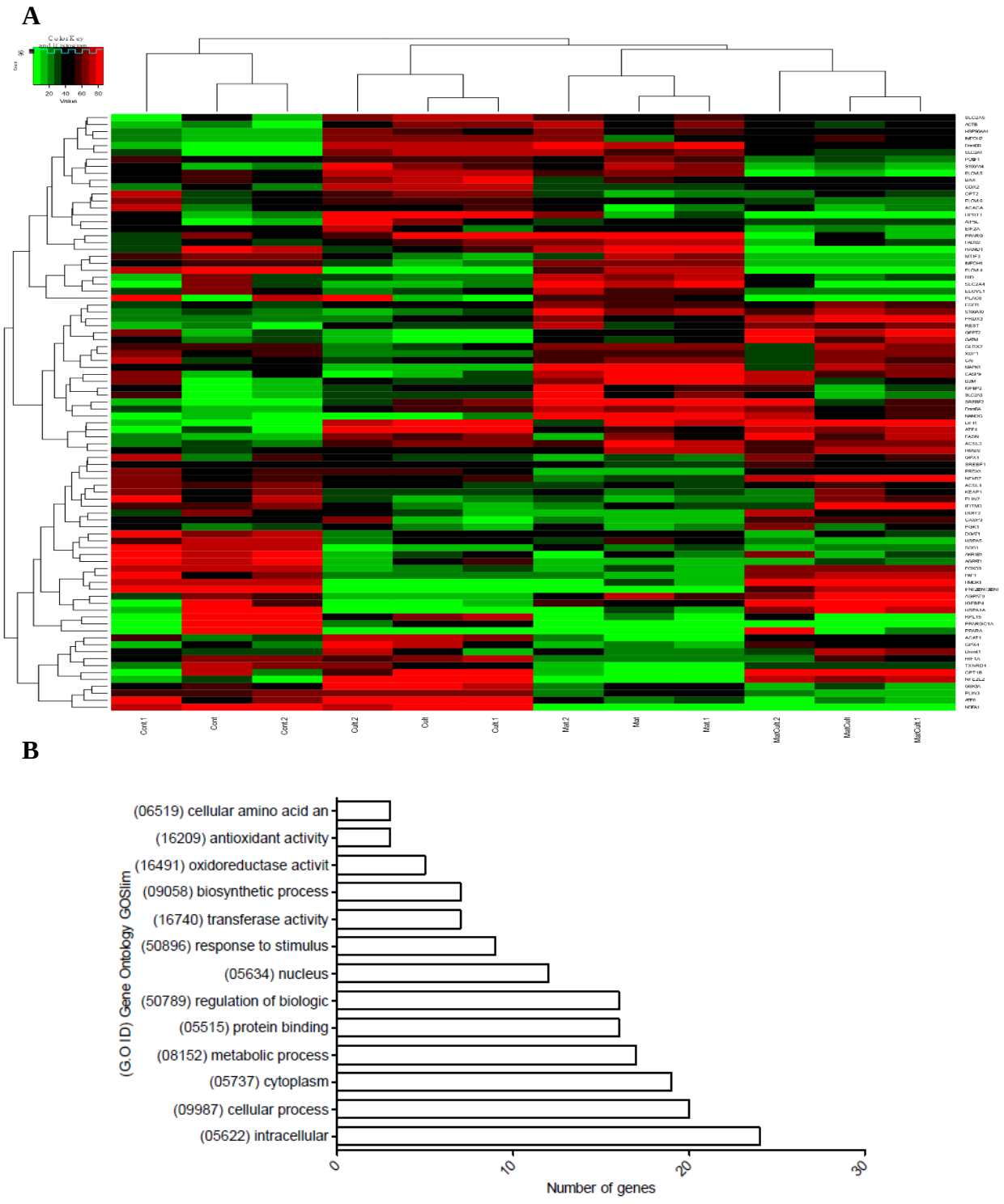


Figura 3: A) Gráfico *heatmap* mostraando os genes diferencialmente expressos nos embriões produzidos de acordo com os grupos experimentais: Controle, 4h, 8 dias, 4h + 8 dias. As fileiras e colunas foram agrupadas pela distância Euclediana e pelo método de ligação Ward. A intensidade de cor representa o nível de diferença entre os grupos (baseado na correlação de Speaman). **B)** Análise de *Gene Ontology* demostrando as principais categorias

reguladas pelos tratamentos. A classificação foi realizada pela categorização da expressão gos genes. Todas as categorias apresentadas foram reguladas pelos tratamentos.

Dos 96 genes avaliados (Tabela 1), 37 apresentaram diferença estatística. Para uma melhor apresentação e descrição dos resultados, os gráficos foram organizados de acordo com os perfis da expressão gerados entre os grupos.

Tabela 1: Genes analisados em amostras blastocistos por RT-qPCR (8 genes referências e 88 genes alvo) nos 4 grupos experimentais – Controle, 4h, 8 dias, 4h + 8 dias.

Símbolo do gene	Nome	Categoria	ID da amostra
ACTB	Actin, beta	Referência	Bt03279174_g1
B2M	Beta-2-microglobulin	Referência	Bt03251628_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Referência	Bt03210912_g1
HMBS	hydroxymethylbilane synthase	Referência	Bt03234763_m1
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Referência	Bt03225311_g1
PPIA	Peptidylprolyl isomerase A	Referência	Bt03224617_g1
RPL15	Ribosomal protein L15	Referência	Bt03288449_g1
RPL30	Ribosomal protein L30	Referência	Bt03226330_g1
DNMT1	Dna (cytosine-5-)-methyltransferase 1	Metilação de DNA	Bt03224737_m1
DNMT3A	Dna (cytosine-5-)-methyltransferase 3A	Metilação de DNA	Bt01027164_m1
DNMT3B	Dna (cytosine-5-)-methyltransferase 3B	Metilação de DNA	Bt03259810_m1
BAX	Bcl2-associated X protein	Apoptose	Bt03211777_g1
BCL2	B-cell CLL/lymphoma 2	Apoptose	Bt04298952_m1
BID	BH3 interacting domain death agonist	Apoptose	Bt03241255_m1
CASP3	Caspase 3	Apoptose	Bt03250954_g1
CASP9	Caspase 9	Apoptose	Bt04282453_m1
EGFR	Epidermal growth factor - receptor	Regulação do crescimento celular	AJT96D7
MAPK1	Mitogen-Activated Protein Kinase	Cell cycle/ apoptose	Bt03216718_g1
IMPDH1	IMP (inosine 5'-monophosphate) dehydrogenase 1	Regulação do crescimento celular	Bt00995384_m1
IMPDH2	IMP (inosine 5'-monophosphate) dehydrogenase 2	Regulação do crescimento celular	Bt03226238_g1
GATM	Glycine amidinotransferase mitochondrial	Diferenciação celular	Bt03210912_g1
S100A10	S100 calcium binding protein A10	Diferenciação celular/ proliferação	Bt03215645_m1
S100A14	S100 calcium binding protein A14	Diferenciação celular/ proliferação	Bt03230771_g1
IGF1	Insulin-like growth factor 1	Proliferação celular	Bt03252282_m1
IGF2	Insulin-like growth factor 2	Proliferação celular	Bt03259225_m1
HSP90AA1	Heat Shock Protein 90kDa Alpha	Sobrevivência celular	Bt03218068_g1
HSPA1A	Heat shock protein family A member 1A	Sobrevivência celular	Bt03292670_g1
HSPA5	Heat shock protein family A member 5	Sobrevivência celular	Bt03244880_m1

HAND1	Heart and neural crest cell derivative 1	Diferenciação/ implantação	Bt04318733_g1
LIF	Leukemia inhibitory factor	Implantação embrionária	Bt03211910_m1
LIFR	Leukaemia inhibitory factor receptor	implantação embrionária	Bt04310863_m1
ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex subunit E	Qualidade embrionária	Bt03210836_g1
CDX2	Caudal type homeobox transcription factor 2	Qualidade embrionária	Bt03649157_m1
IFNT/IFNT2/IFNT3	Interferon tau-1,2 and 3	Qualidade embrionária	Bt03210589_g1
MTIF3	Mitochondrial translational initiation factor 3	Qualidade embrionária	Bt03231844_m1
NANOG	Nanog homeobox	Qualidade embrionária	Bt03220541_m1
PLAC8	Placenta-specific 8	Qualidade embrionária	Bt03211579_m1
POU5F1	POU class 5 homeobox 1 (OCT4)	Qualidade embrionária	Bt03223846_g1
REST	RE1-silencing transcription factor	Qualidade embrionária	Bt03278318_s1
IGFBP2	Insulin-like growth factor binding protein 2	Regulação transcricional	Bt01040719_m1
IGFBP4	Insulin-like growth factor binding protein 4	Regulação transcricional	Bt03259500_m1
GFPT2	Glutamine-fructose-6-phosphate transaminase 2	Metabolismo de glicose	Bt03223996_m1
SLC2A1	Solute carrier family 2 member 1	Transportador de glicose	Bt03215314_m1
SLC2A3	Solute carrier family 2 member 3	Transportador de glicose	Bt03259514_g1
SLC2A4	Solute carrier family 2 member 4	Transportador de glicose	Bt03215316_m1
SLC2A5	Solute carrier family 2 member 5	Transportador de glicose	Bt03258296_m1
GSK3A	Glycogen Synthase Kinase 3 alpha	Síntese de gliconeogênese	Bt03273695_m1
PGK1	Phosphoglycerate Kinase 1	Metabolismo glicolítico	Bt03225854_mH
ACACA	Acetyl-Coa carboxylase	Metabolismo lipídico	Bt03213389_m1
ACAT1	Acetyl-Coa acetyltransferase 1	Metabolismo lipídico	Bt03238649_g1
ACSL1	Acyl-Coa synthetase long-chain family member 1	Metabolismo lipídico	Bt03248469_m1
ACSL3	Acyl-Coa synthetase long-chain family member 3	Metabolismo lipídico	Bt04282138_m1
AGPAT1	1-acylglycerol-3-phosphate O-acyltransferase 1	Metabolismo lipídico	Bt03224587_g1
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	Metabolismo lipídico	Bt04292093_m1
CPT1B	Carnitine palmitoyltransferase 1B	Metabolismo lipídico	Bt03244645_m1
CPT2	Carnitine palmitoyltransferase 2	Metabolismo lipídico	Bt03233823_m1
DGAT1	Diacylglycerol O-acyltransferase 1	Metabolismo lipídico	Bt03251719_g1
ELOVL1	Elongation of very long chain fatty acids protein 1	Metabolismo lipídico	Bt03286627_s1
ELOVL4	Elongation of very long chain fatty acids protein 4	Metabolismo lipídico	Bt03270721_m1
ELOVL5	Elongation of very long chain fatty acids protein 5	Metabolismo lipídico	Bt03235956_m1
ELOVL6	Elongation of very long chain fatty acids protein 6	Metabolismo lipídico	Bt00907566_m1
FADS2	Fatty acid desaturase 2	Metabolismo lipídico	Bt03256255_g1
FASN	Fatty acid synthase	Metabolismo lipídico	Bt03210485_m1
PLIN2	Perilipin 2	Metabolismo lipídico	Bt03212182_m1
PLIN3	Perilipin 3	Metabolismo lipídico	Bt03230537_m1
PPARA	Peroxisome proliferator-activated receptor alpha	Metabolismo lipídico	Bt03220821_m1
PPARG	Peroxisome proliferator-activated receptor gamma	Metabolismo lipídico	Bt03217547_m1
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Metabolismo lipídico	Bt01016720_m1

SREBF1	Sterol regulatory element-binding transcription F1	Metabolismo lipídico	Bt03276370_m1
SREBF2	Sterol regulatory element-binding transcription F2	Metabolismo lipídico	Bt04283467_m1
AKR1B1	Aldo-keto reductase family 1 member B1	Estresse oxidativo	Bt03218049_g1
CAT	Catalase	Estresse oxidativo	Bt03228713_m1
DDIT3	DNA-damage-inducible transcript 3	Estresse oxidativo	Bt03251320_g1
FOXO3	Forkhead box O3	Estresse oxidativo	Bt03649334_s1
GLRX2	Glutaredoxin 2	Estresse oxidativo	Bt03250351_m1
GPX1	Glutathione peroxidase 1	Estresse oxidativo	Bt03259217_g1
GPX4	Glutathione peroxidase 4	Estresse oxidativo	Bt03259611_m1
HMOX1	Heme oxygenase (decycling) 1	Estresse oxidativo	Bt03218632_m1
KEAP1	Kelch-like ECH-associated protein 1	Estresse oxidativo	Bt03817661_m1
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2	Estresse oxidativo	Bt03251880_m1
NDUFA1	NADH:ubiquinone oxidoreductase subunit A1	Estresse oxidativo	Bt03216720_g1
PRDX1	Peroxiredoxin-1	Estresse oxidativo	Bt03223684_m1
PRDX3	Peroxiredoxin-3	Estresse oxidativo	Bt03214402_m1

Entre os tratamentos, o grupo 4h foi o que apresentou um maior número de genes diferentemente expressos nos embriões. Dezenove genes apresentaram uma maior abundância de mRNA quando comparado com pelo menos um dos outros grupos experimentais (Fig 4). Entre esses, 4 estão relacionados à regulação do crescimento celular ou ciclo celular (*IMPDH, IMPDH2, MAPK1* e *EGFR*), 3 com as vias do metabolismo lipídico (*SRBP2, FADS2* e *ELOVL6*), 2 transportadores de glicose (*SLC2A1* e *SLC2A3*), 2 marcadores de qualidade embrionária (*NANOG* e *POU5F1*), 2 relacionados à ativação de metilação do DNA (*Dnmt3B* e *Dnmt3A*), 1 de diferenciação e proliferação celular (*S100A10*), 1 de sobrevivência (*HSP90AA1*), 1 de regulação de transcrição (*IGFBP2*), 1 relacionado ao estresse oxidativo (*CAT*) e 1 relacionado à apoptose (*CASP9*).

De forma geral, as vias das quais os genes *up-regulated* fazem parte, demonstram que o tratamento no COC tem uma influência positiva nestes embriões, e que esta pode ser uma ótima estratégia para um aumento na qualidade e no número de embriões produzidos *in vitro*. Essa maior expressão dos genes no grupo 4h também corrobora com dados prévios (dados não

publicados), que mostram que a adição da proteína nas últimas 4 horas de MIV influência a maturação oocitária e, além disso, modula os genes relacionados a qualidade embrionária.

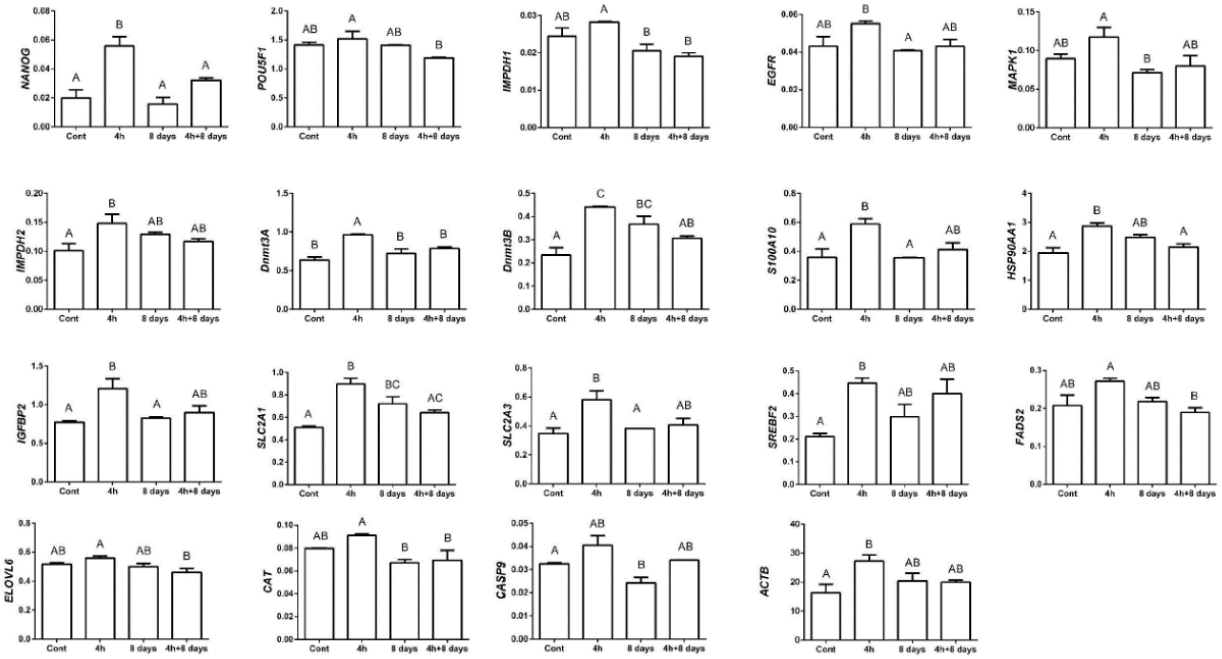


Figura 4: Efeito da adição da proteína rHSPA5 nas últimas 4 horas da maturação oocitária (grupo 4h) na abundancia relativa do mRNA de embriões. O tratamento na MIV elevou a expressão de 19 genes (*NANOG, POU5F1, IMPDH1, EGFR, MPK1, IMPDH2, Dnmt3A. Dnmt3B, S100A10, HSP90AA1, IGFBP2, SLC2A1, SLC2A3, SREBF2. FADS2, ELOVL6, CAT, CASP9* e *ACTB*) quando comparado com pelo menos um dos outros grupos experimentais. Os genes alvo foram normalizados com a média geométrica dos genes de referência (*GAPDH*, *PPIA* e *RPL30*) pelo método $2^{(-\Delta Ct)}$. Os dados foram apresentados com média ± EPM e as diferenças foram consideradas significativas quando $p \leq 0{,}05$. Letras diferentes indicam a diferença estatística.

Na análise entre os grupos, 8 genes apresentam uma menor expressão de mRNA nos grupos tratados, quando comparados ao grupo controle (Figura 5). Destes, 4 são genes marcadores de estresse oxidativo (*FOXO3, SOD1*, *AKR1B1* e *PRDX1*), 3 de metabolismo lipídico (*ELOVL4, DAGT1* e *AGPAT1*) e 1 marcador de qualidade embrionária (*IFNT2*). Estudos prévios do nosso grupo demonstraram que a adição da rHSPA5 nas últimas 4 horas de MIV manteve os níveis de glutationa (GSH) e a concentração de espécies reativas de oxigênio (ROS – do inglês *reactive oxigen species*). A menor expressão dos genes relacionados ao estresse oxidativo neste trabalho, associado aos dados anteriores, nos leva a pensar que adição

da proteína rHSPA5 pode influenciar na homeostase do embrião durante o desenvolvimento *in vitro*. A HSPA5 é uma proteína chaperona, presente no retículo endoplasmático de todas as células eucariontes. Ela é responsável por manter a homeostase celular e por ativar vias antiestressoras para manter a sobrevivência celular (Zhang *et al.*, 2012; Zhang *et al.*, 2016).

Apesar da menor expressão dos genes relacionados ao metabolismo lipídico nos grupos tratados, essa modulação não afetou o desenvolvimento embrionário. Os lipídios são essenciais para o desenvolvimento embrionário, uma vez que são fonte de energia celular, mas também por estar envolvido na fisiologia e estrutura celular (Tsujii *et al.*, 2001). Os três genes relacionados ao metabolismo lipídicos alterados participam direta ou indiretamente da síntese lipídica (Rieger, 1992; Tsujii *et al.*, 2001). Apesar de uma importante fonte de energia, um maior conteúdo lipídico leva ao aumento na produção de ROS em embriões produzidos *in vitro* (Bragança *et al*., 2019). Dessa forma, nossos resultados mostram que os tratamentos com a rHSPA5 parecem diminuir a síntese lipídica e por consequência estar ligado aos níveis basais de ROS encontrado.

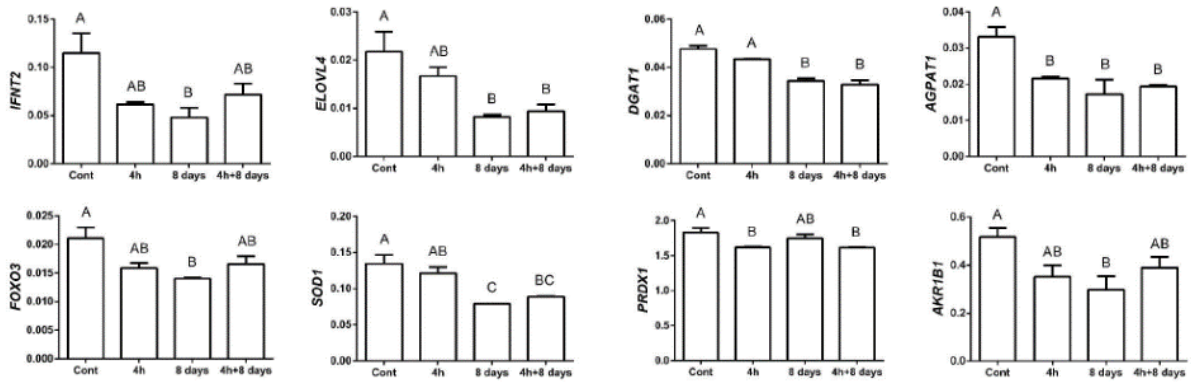


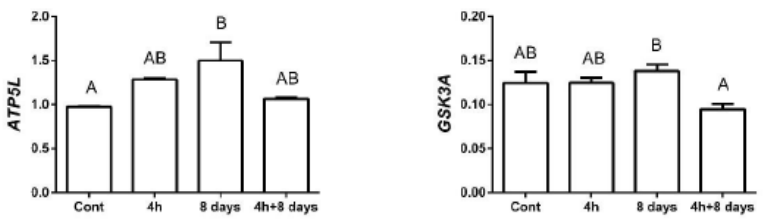
Figura 5: Abundancia relativa do mRNA de embriões dos grupos controle, 4h, 8 dias e 4h + 8 dias. O grupo controle apresentou uma maior expressão dos genes quando comparado com pelo menos um dos grupos experimentais (*IFNT2, ELOVL4, DGAT1, AGPAT1, FOXO3, SOD1, PRDX1* e *AKR1B1*). Os genes alvo foram normalizados com a média geométrica dos genes de referência (*GAPDH*, *PPIA* e *RPL30*) pelo método $2^{(-\Delta Ct)}$. Os

dados foram apresentados com média ± EPM e as diferenças foram consideradas significativas quando $p \leq 0,05$. Letras diferentes indicam a diferença estatística.

Quando a adição da proteína aconteceu nos 8 dias de cultivo embrionário, dois genes apresentaram *up-regulation* (Figura 6A), sendo um gene marcador de qualidade embrionária (ATPL5) e outro de síntese de glicogênio (*GSK3A*), e 4 genes com *downregulation* (Figura 6B) quando comparados aos outros grupos. Os genes com menor abundância estão relacionados à diferenciação celular (*GATM*), metabolismo de glicose (*GFPT2F*), regulação transcricional (*PAF1*) ou ao estresse oxidativo (*HMOX1*). A presença dos genes diferencialmente expressos no grupo 8 dias mostra que de alguma forma a proteína pode estar interagindo com os embriões em cultivo, mesmo sem o auxílio das VEs, como ocorre com a sua internalização demonstrada anteriormente (Alminana *et al.*, 2017).

Mesmo com a modulação na expressão de alguns genes no embrião, a adição da rHSPA5 durante o cultivo embrionário não parece ter grandes efeitos. Além de não haver diferença na taxa de embriões produzidos entre os grupos, os genes que apresentaram menor expressão neste grupo participam de vias importantes para o desenvolvimento do embrião (Sandell *et al.*, 2003; Zhang *et al.*, 2013; E. Orozco-Lucero, 2014; Frank *et al.*, 2014), mostrando que, possivelmente, o principal papel da proteína HSPA5 seja na maturação final oocitária e fertilização.

A



B

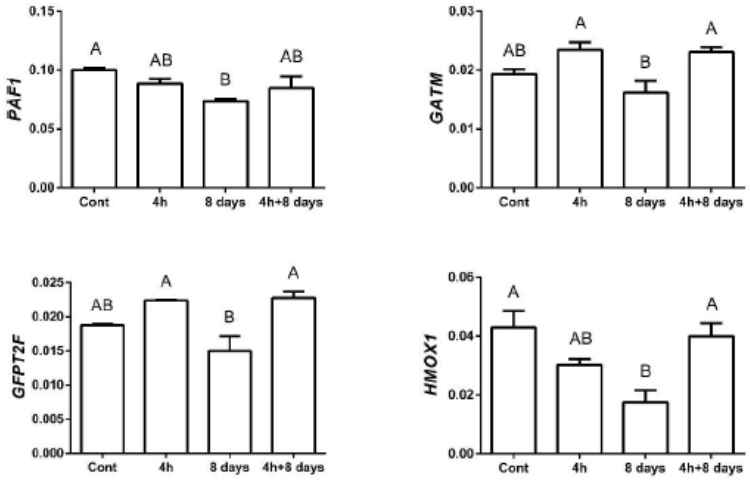


Figura 6: Efeito da adição da rHSPA5 nos 8 dias de cultivo embrionário (grupo 8 dias) na abundância relativa de mRNA de embriões. O grupo 8 dias apresentou uma *up-regulation* em dois genes (6A; *ATPL5* e *GSK3A*) e reduziu a expressão de 4 genes (6B; *PAF1, GATM, GFPT2F* e *HMOX1*) quando comparado à um ou mais grupos experimentais. Os genes alvo foram normalizados com a média geométrica dos genes de referência (*GAPDH, PPIA* e *RPL30*) pelo método $2^{(-\Delta Ct)}$. Os dados foram apresentados com média ± EPM e as diferenças foram consideradas significativas quando $p \leq 0,05$. Letras diferentes indicam a diferença estatística.

Outro perfil de expressão entre os grupos analisados mostrou uma redução na expressão de 4 genes no grupo 4h + 8 dias, onde 2 genes estão relacionados ao estresse oxidativo (*ATF6* e *NDUFA1*), 1 ao metabolismo lipídico (*ELOVL5*) e 1 com a diferenciação celular (*S100A14*; Figura 7). A associação dos tratamentos, diferente do que tivemos como hipótese, não aumentou a eficiência da produção embrionária. Além disso, o perfil de expressão gênica destes embriões nos leva a acreditar que o uso da rHSPA5 nos 8 dias de cultivo, quando comparado ao uso na MIV, não é uma boa estratégia para a PIVE, uma vez que genes relacionados à qualidade embrionária que são mais abundantes quando há o tratamento na MIV, não foram encontrados nos embriões provenientes da associação dos tratamentos.

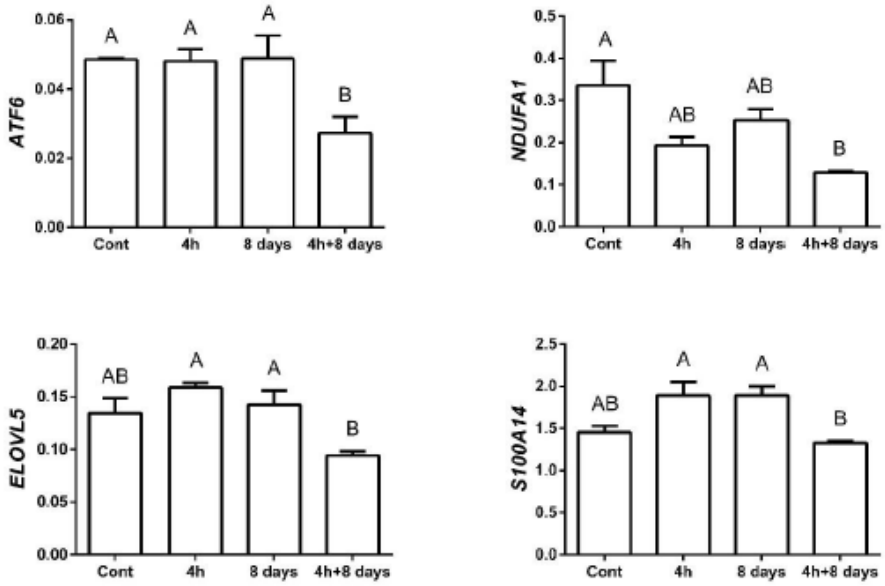


Figura 7: Efeito da adição da rHSPA5 na maturação *in vitro* e cultivo *in vitro* embrionário (grupo 4h + 8 dias) sob abundância relativa de mRNA de embriões. O grupo 4h + 8 dias diminuiu o nível de expressão de 4 genes (*ATF6, NDUFA1, ELOVL5* e *S100A14*) quando comparado à um ou dois dos grupos experimentais. Os genes alvo foram normalizados com a média geométrica dos genes de referência (*GAPDH*, *PPIA* e *RPL30*) pelo método $2^{(-\Delta Ct)}$. Os dados foram apresentados com média ± EPM e as diferenças foram consideradas significativas quando $p \leq 0,05$. Letras diferentes indicam a diferença estatística.

CONCLUSÃO

Apesar das HSPs presente no fluido ovidutal desenvolver um papel crucial nos gametas e na fertilização, pouco se sabe sobre sua função no desenvolvimento inicial do embrião. A proteína rHSPA5 adicionada no cultivo embrionário *in vitro* apesar de parecer ser apta a interagir com o embrião, não parece ter grande influência no seu desenvolvimento, tanto por não aumentar a eficiência da produção embrionária, quanto por não modular positivamente o perfil de transcritos. Diferente da ação no cultivo e corroborando com dados prévios, a rHSPA5 parece trazer benefícios à produção *in vitro* quando adicionada as últimas 4 horas da maturação, aumentando a expressão de genes marcadores de qualidade embrionária.

REFERÊNCIAS

ABDI, Z. et al. The Effect of Hsp60 on Fertilization and Pre-Implantation Embryo Development in Mice: An in Vitro Study. **Acta Endocrinol (Buchar),** v. 15, n. 2, p. 153-157, Apr-Jun 2019. ISSN 1841-0987 (Print)1841-0987 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31508170 >.

ALMINANA, C. et al. Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. **Reproduction,** v. 154, n. 3, p. 153-168, Sep 2017. ISSN 1741-7899 (Electronic)1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/28630101 >.

AVILÉS, C., AND RIZOS. The oviduct: A key organ for the success of the early reproductive events. **Animal Frontiers,** v. 5, n. 1, Jan 2015 2015.

AVILÉS, M. G.-A., A; COY, PILAR. Oviductal secretions: will they be key factors for the future of ARTs? **Molecular Human Reproduction,** v. 16, n. 12, p. 896-906, 2010.

BANLIAT, C. et al. Stage-dependent changes in oviductal phospholipid profiles throughout the estrous cycle in cattle. **Theriogenology,** v. 135, p. 65-72, Sep 1 2019. ISSN 1879-3231 (Electronic) 0093-691X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31203089 >.

BINELLI, M. et al. Sex Steroid-Mediated Control of Oviductal Function in Cattle. **Biology (Basel),** v. 7, n. 1, Feb 2 2018. ISSN 2079-7737 (Print)

2079-7737 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29393864 >.

BOILARD, M. et al. Localization of the chaperone proteins GRP78 and HSP60 on the luminal surface of bovine oviduct epithelial cells and their association with spermatozoa. **Biol Reprod,** v. 71, n. 6, p. 1879-89, Dec 2004. ISSN 0006-3363 (Print)0006-3363 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15286042 >.

BROMFIELD, E. G.; NIXON, B. The function of chaperone proteins in the assemblage of protein complexes involved in gamete adhesion and fusion processes. **Reproduction,** v. 145, n. 2, p. R31-42, Feb 2013. ISSN 1741-7899 (Electronic)1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23166368 >.

BUHI, W. C.; ALVAREZ, I. M.; KOUBA, A. J. Secreted proteins of the oviduct. **Cells Tissues Organs,** v. 166, n. 2, p. 165-79, 2000. ISSN 1422-6405 (Print)1422-6405 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10729726 >.

E. OROZCO-LUCERO, M.-A. S. Molecular markers of fertility in cattle oocytes and embryos: progress and challenges. **Anim. Reprod,** v. 11, n. 3, p. 183-194, 2014.

ELLIOTT, R. M. et al. Effects of HSPA8, an evolutionarily conserved oviductal protein, on boar and bull spermatozoa. **Reproduction,** v. 137, n. 2, p. 191-203, Feb 2009. ISSN 1741-7899 (Electronic)1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18996976 >.

FONTES, P. K. et al. Bona fide gene expression analysis of samples from the bovine reproductive system by microfluidic platform. **Anal Biochem,** v. 596, p. 113641, May 1 2020. ISSN 1096-0309 (Electronic)0003-2697 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/32087128 >.

FRANCHI, F. F. et al. Equine chorionic gonadotropin drives the transcriptional profile of immature cumulus-oocyte complexes and in vitro-produced blastocysts of superstimulated Nelore cows. **Mol Reprod Dev,** v. 86, n. 11, p. 1639-1651, Nov 2019. ISSN 1098-2795 (Electronic)

1040-452X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31389116 >.

FRANK, L. A. et al. The effect of peri-conception hyperglycaemia and the involvement of the hexosamine biosynthesis pathway in mediating oocyte and embryo developmental competence. **Mol Reprod Dev,** v. 81, n. 5, p. 391-408, May 2014. ISSN 1098-2795 (Electronic)1040-452X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24415135 >.

GARCIA-MARTINEZ, S. et al. Addition of exogenous proteins detected in oviductal secretions to in vitro culture medium does not improve the efficiency of in vitro fertilization in pigs. **Theriogenology,** v. 157, p. 490-497, Nov 2020. ISSN 1879-3231 (Electronic)0093-691X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/32898824 >.

GIROTO, A. B. et al. 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. **Mol Reprod Dev,** v. 86, n. 11, p. 1694-1704, Nov 2019. ISSN 1098-2795 (Electronic)1040-452X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/31468638 >.

GONZALEZ-GRONOW, M. et al. GRP78: a multifunctional receptor on the cell surface. **Antioxid Redox Signal,** v. 11, n. 9, p. 2299-306, Sep 2009. ISSN 1557-7716 (Electronic)1523-0864 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19331544 >.

HARRIS, E. A.; STEPHENS, K. K.; WINUTHAYANON, W. Extracellular Vesicles and the Oviduct Function. **Int J Mol Sci,** v. 21, n. 21, Nov 5 2020. ISSN 1422-0067 (Electronic)1422-0067 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/33167378 >.

HEBERT-SCHUSTER, M. et al. The Interplay between Glucose-Regulated Protein 78 (GRP78) and Steroids in the Reproductive System. **Int J Mol Sci,** v. 19, n. 7, Jun 22 2018. ISSN 1422-0067 (Electronic)1422-0067 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29932125 >.

HOLT, W. V.; FAZELI, A. Sperm Storage in the Female Reproductive Tract. **Annu Rev Anim Biosci,** v. 4, p. 291-310, 2016. ISSN 2165-8110 (Electronic)2165-8102 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26526545 >.

LACHANCE, C.; BAILEY, J. L.; LECLERC, P. Expression of Hsp60 and Grp78 in the human endometrium and oviduct, and their effect on sperm functions. **Hum Reprod,** v. 22, n. 10, p. 2606-14, Oct 2007. ISSN 0268-1161 (Print)0268-1161 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17670764 >.

LAMY, J. et al. Regulation of the bovine oviductal fluid proteome. **Reproduction,** v. 152, n. 6, p. 629-644, Dec 2016. ISSN 1741-7899 (Electronic)1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/27601716 >.

LAMY, J. et al. Identification by proteomics of oviductal sperm-interacting proteins. **Reproduction,** v. 155, n. 5, p. 457-466, May 2018. ISSN 1741-7899 (Electronic)1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29540510 >.

LI, S.; WINUTHAYANON, W. Oviduct: roles in fertilization and early embryo development. **J Endocrinol,** v. 232, n. 1, p. R1-R26, Jan 2017. ISSN 1479-6805 (Electronic)0022-0795 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/27875265 >.

LIN, P. et al. GRP78 expression and immunohistochemical localization in the female reproductive tract of mice. **Theriogenology,** v. 78, n. 8, p. 1824-9, Nov 2012. ISSN 1879-3231 (Electronic)0093-691X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22968032 >.

LLOYD, R. E. et al. Effects of oviductal proteins, including heat shock 70 kDa protein 8, on survival of ram spermatozoa over 48 h in vitro. **Reprod Fertil Dev,** v. 21, n. 3, p. 408-18, 2009. ISSN 1031-3613 (Print)1031-3613 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19261218 >.

LLOYD, R. E. et al. The oviducal protein, heat-shock 70-kDa protein 8, improves the long-term survival of ram spermatozoa during storage at 17 degrees C in a commercial extender. **Reprod Fertil Dev,** v. 24, n. 4, p. 543-9, 2012. ISSN 1031-3613 (Print)1031-3613 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22541542 >.

MARIANI, M. L. et al. Constitutive expression of heat shock proteins hsp25 and hsp70 in the rat oviduct during neonatal development, the oestrous cycle and early pregnancy. **J Reprod Fertil,** v. 120, n. 2, p. 217-23, Nov 2000. ISSN 0022-4251 (Print)0022-4251 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11058436 >.

MARÍN-BRIGGILER, C. I. G.-E., M.F.; MUNUCE, M.J.; GHERSEVICH, S.; CAILLE, A.M.; HELLMAN, U.; CORRIGALL, V.M.; VAZQUEZ-LEVIN, M.H. Glucose-regulated protein 78 (GRP78/BiP) is secreted by human oviduct epithelial cells and the recombinantprotein modulates sperm-zona pellucida binding. **Fertil Steril,** v. 93, n. 5, 2010.

MOEIN-VAZIRI, N. et al. Heat-shock protein A8 restores sperm membrane integrity by increasing plasma membrane fluidity. **Reproduction,** v. 147, n. 5, p. 719-32, May 2014. ISSN 1741-7899 (Electronic)1470-1626 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24501193 >.

MONDEJAR, I. et al. Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals. **Biol Reprod,** v. 89, n. 3, p. 67, Sep 2013. ISSN 1529-7268 (Electronic)0006-3363 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23863406 >.

RIEGER, D. Relationships between energy metabolism and development of early mammalian embryos. **Theriogenology,** v. 37, n. 1, p. 75-93, 1992. ISSN 0093-691X. Disponível em: < https://www.sciencedirect.com/science/article/pii/0093691X9290248P >.

SANDELL, L. L. et al. Gatm, a creatine synthesis enzyme, is imprinted in mouse placenta. **Proc Natl Acad Sci U S A,** v. 100, n. 8, p. 4622-7, Apr 15 2003. ISSN 0027-8424 (Print)0027-8424 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12671064 >.

SOLEILHAVOUP, C. et al. Proteomes of the Female Genital Tract During the Oestrous Cycle. **Mol Cell Proteomics,** v. 15, n. 1, p. 93-108, Jan 2016. ISSN 1535-9484 (Electronic)535-9476 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26518761 >.

STOJKOVIC, M. et al. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. **Biol Reprod,** v. 64, n. 3, p. 904-9, Mar 2001. ISSN 0006-3363 (Print)0006-3363 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11207207 >.

TSUJII, H.; KHANDOKER, M. A. M. Y.; HAMANO, K.-I. Lipid in Mammalian Embryo Development. **Journal of Mammalian Ova Research,** v. 18, n. 3, p. 73-80, 2001.

ZHANG, A. et al. The effect of human cumulus cells on the maturation and developmental potential of immature oocytes in ICSI cycles. **J Assist Reprod Genet,** v. 29, n. 4, p. 313-9, Apr 2012. ISSN 1573-7330 (Electronic)058-0468 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22354726 >.

ZHANG, K.; HAVERSAT, J. M.; MAGER, J. CTR9/PAF1c regulates molecular lineage identity, histone H3K36 trimethylation and genomic imprinting during preimplantation development. **Dev Biol,** v. 383, n. 1, p. 15-27, Nov 1 2013. ISSN 1095-564X (Electronic)0012-1606 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24036311 >.

ZHANG, X. et al. 2,4-dichlorophenol induces ER stress-mediated apoptosis via eIF2alpha dephosphorylation in vitro. **Environ Toxicol,** v. 31, n. 2, p. 245-55, Feb 2016. ISSN 1522-7278 (Electronic)1520-4081 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25160872 >.

CONSIDERAÇÕES FINAIS

Testamos aqui se a proteína HSPA5 é capaz de interagir com COC e com os embriões e, além disso, se a adição da HSPA5 recombinante humana nos meios de MIV e CIV melhora a eficiência da produção *in vitro* e a qualidade dos embriões produzidos.

Na abordagem da interação da rHSPA5 com o COC, nos adicionamos a proteína nas últimas 4 horas de MIV. Nós avaliamos os níveis de ROS e MMP destes oócitos e dos subsequentes embriões produzidos, os níveis de GSH nos oócitos, células do *cumulus* e no meio MIV. Além disso, nós avaliamos o efeito da proteína sobre a solubilidade da ZP e a taxa de monospermia, a taxa e a expressão gênica dos embriões produzidos. A proteína não alterou o tempo de digestão da ZP e também não alterou a taxa de monospermia, demonstrando que aparentemente não produz efeito no enrijecimento da ZP. A rHSPA5 também não influenciou os níveis de ROS e GSH dos oócitos e embriões, mas diminuiu a atividade mitocondrial dos oócitos. Apesar desse resultado, a adição da proteína nas últimas 4 horas de MIV aumentou a taxa de embriões produzidos e foi capaz de modular positivamente a expressão de genes relacionados com a qualidade embrionária, demonstrando um efeito benéfico na produção *in vitro* e um forte potencial de inclusão no meio de MIV.

Em um segundo momento nós adicionamos a rHSPA5 nos 8 dias de cultivo embrionário para comparar com o tratamento na MIV e com a associação destes (últimas 4 horas de MIV mais 8 dias de CIV). Foi avaliada a taxa de embriões produzido com os tratamentos, assim como o perfil de transcritos destes embriões. O tratamento no cultivo e associação dos tratamentos não aumentou a taxa de embriões produzidos e apresentou uma pequena modulação nos genes expressos, comparado ao grupo controle e tratamento nas últimas 4 horas de MIV.

Com esses resultados, nós acreditamos que apesar da HSPA5 conseguir interagir com os embriões, ela não parece ter um efeito essencial nestes, diferente do oócito, A proteína parece exercer uma função nos oócitos durante as últimas horas de maturação e foi eficaz quando sua forma recombinante humana foi adicionada ao meio de MIV, melhorando a produção embrionária e impactando a qualidade molecular destes embriões.

ATIVIDADES ACADÊMICAS DESENVOLVIDAS DURANTE O DOUTORADO

Artigos completos publicados em periódicos

1. **P.H. SANTOS;** S.G. NUNES; F.F. FRANCHI; A.B. GIROTO; P.K. FONTES; A.C.S. CASTILHO. Expression of bta-miR-222 and LHCGR in bovine cultured granulosa cells: Impact of follicle deviation and regulation by FSH/insulin in vitro. Theriogenology. v. 182, p. 71-77, 2022.

2. ELISA MARIANO PIOLTINE; CAMILA BORTOLIERO COSTA; LAÍS BARBOSA LATORRACA; FERNANDA FAGALI FRANCH; **PRISCILA HELENA DOS** SANTOS; GISELE ZOCCAL MINGOTI; FABÍOLA FREITAS DE PAULA LOPES; MARCELO FÁBIO GOUVEIA NOGUEIRA. Treatment of *in vitro*-Matured Bovine Oocytes With Tauroursodeoxycholic Acid Modulates the Oxidative Stress Signaling Pathway. Front. Cell dev. Biol. v. 9, 2021.

3. FRANCHI, FERNANDA FAGALI; SATRAPA, RAFAEL AUGUSTO; FONTES, PATRÍCIA KUBO; **SANTOS, PRISCILA HELENA**; RAZZA, EDUARDO MONTANARI; EMANUELLI, ISABELE PICADA; ERENO, RONALDO LUIZ; MARECO, EDSON ASSUNÇÃO; NOGUEIRA, MARCELO FÁBIO GOUVEIA; BARROS, CIRO MORAES; SOUZA CASTILHO, ANTHONY CÉSAR. Equine chorionic gonadotropin drives the transcriptional profile of immature cumulus-oocyte complexes and in vitro-produced blastocysts of superstimulated Nelore cows. Molecular Reproduction and Development, v. 86, p. 1639, 2019.

4. GIROTO, ALAN B; FONTES, PATRÍCIA K; FRANCHI, FERNANDA F; DOS **SANTOS, PRISCILA H**; RAZZA, EDUARDO M; NOGUEIRA, MARCELO F. G; MAIOLI, MARCOS A; NOGUEIRA, GUILHERME P; NUNES, GIOVANA B; MINGOTI, GISELE Z; MARECO, EDSON A; CASTILHO, ANTHONY C. S. 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, v. 86, p. 1694, 2019.

5. PAOLA FRANCINI FÁVERO; VICTOR AUGUSTO VIEIRA DE LIMA; **PRISCILA HELENA DOS SANTOS**; ANA PAULA MARQUES ANDRADE; LEONARDO OLIVEIRA MENDES; FRANCIS LOPES PACAGNELLI; ANTHONY CÉSAR DE SOUZA CASTILHO. Differential fractal dimension is associated with extracellular matrix remodeling in developing bovine corpus luteum. Biochemical and Biophysical Research Communications. v. 516, 3, p. 888-893. 2019.

6. GOUVEIA NOGUEIRA, MARCELO; BERTOGNA GUILHERME, VITÓRIA; PRONUNCIATE, MICHELI; **DOS SANTOS, PRISCILA**; LIMA BEZERRA DA SILVA, DIOGO; ROCHA, JOSÉ Artificial Intelligence-Based Grading Quality of Bovine Blastocyst Digital Images: Direct Capture with Juxtaposed Lenses of Smartphone Camera and Stereomicroscope Ocular Lens. SENSORS. v.18, p.4440 - , 2018.

7. **SANTOS, P.H.**; SATRAPA, R.A.; FONTES, P.K.; FRANCHI, F.F.; RAZZA, E.M.; MANI, F.; NOGUEIRA, M.F.G.; BARROS, C.M.; CASTILHO, A.C.S. Effect of superstimulation on the expression of microRNAs and genes involved in steroidogenesis and ovulation in Nelore cows. THERIOGENOLOGY. , v.110, p.192 - 200, 2018.

8. RAZZA, EDUARDO M.; SUDANO, MATEUS J.; FONTES, PATRICIA K.; FRANCHI, FERNANDA F.; BELAZ, KATIA ROBERTA A.; **SANTOS, PRISCILA H.**; CASTILHO, ANTHONY C. S.; ROCHA, DANIELE F. O.; EBERLIN, MARCOS N.; MACHADO, MARIANA F.; NOGUEIRA, MARCELO F. G. 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. , v.31, p.xx - , 2018.

9. **SANTOS, P.H.**; FONTES, P.K.; FRANCHI, F.F.; NOGUEIRA, M.F.G.; BELAZ, K.R.A.; TATA, A.; EBERLIN, M.N.; SUDANO, M.J.; BARROS, C.M.; CASTILHO, A.C.S. Lipid profiles of follicular fluid from cows submitted to ovarian superstimulation.

THERIOGENOLOGY. v.94, p.64 - 70, 2017.

Capítulos de livros publicados

1. Castilho, Anthony C.S.; Fontes, Patrícia K.; FRANCHI, FERNANDA F.; **SANTOS, PRISCILA H.**; RAZZA, EDUARDO M.
Renin-Angiotensin System on Reproductive Biology In: Renin-Angiotensin System - Past, Present and Future.1 ed.: InTech, 2017, p. 71-81.

Trabalhos publicados em anais de eventos (resumo)

1. PIOLTINE, E. M.; COSTA, CAMILA BORTOLIERO; FRANCHI, F. F ; **SANTOS, P. H**.; NOGUEIRA, M. F. G. Molecular effects of TUDCA addition during in vitro maturation of bovine oocytes. In: Sociedade Brasileira de Tecnologia de Embriões, 2020, Online. XXXIV Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, 2020.

2. VIEIRA DE LIMA, VICTOR AUGUSTO; DELLAQUA, T. T; GIROTO, A. B; FRANCHI, F. F; **SANTOS, P. H**; CASTILHO, A. C. S. Impacto da adição da proteína sérica-a associada à prenhez (papp-a) durante a maturação oocitária in vitro sobre o perfil e metabolismo lipídico de embriões bovinos. In: Encontro Nacional de Ensino, Pesquisa e Extensão (ENEPE), 2020, Online. Encontro Nacional de Ensino, Pesquisa e Extensão (ENEPE), 2020.

3. DELLAQUA, T. T.; RAZZA, E. M.; GUILHERME, V. B.; GIROTO, A. B.; NEVES, S.; **SANTOS, P.H.**; SUDANO, M. J.; MULLER, D. B.; CASTILHO, A. C. S.
Addition of pregnancy-associated plasma proteins-A during in vitro maturation did not impact the lipid content of in vitro-matured bovine oocytes In: 10 International remunants reproduction symposium, 2018, Foz do Iguaçu. Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018). 2018. v.15. p.1055 -

4. CHAVES, M. P.; **SANTOS, P.H.**; MENDES, L. O.; CASTILHO, A. C. S.

Differential expression of LH receptor, LHR mRNA binding protein (LRBP) and bta-miR- 222 in the developing bovine ovary In: 10th International Ruminant Reproduction Symposium, 2018, Foz do Iguaçu. Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018). 2018. v.15. p.1066 -

5. FRANCHI, F. F.; **SANTOS, P.H.**; FONTES, P. K.; RAZZA, E. M.; MARECO, E.; NOGUEIRA, M. F. G.; CASTILHO, A. C. S.
Equine chorionic gonadotropin (eCG) impacts the transcriptional profile of genes involved with competence of cumulus-oocyte complexes and embryo quality in superstimulated Nelore cows. In: 10th International Ruminant Reproduction Symposium, 2018, Foz do Iguaçu. Proceedings of the 10th International Ruminant Reproduction Symposium. 2018. v.15. p.1122 -

6. GIROTO, A. B.; AMORIS, J. V.; FRANCHI, F. F.; FONTES, P. K.; **SANTOS, P.H.**; MAIOLI, M. A.; NOGUEIRA, G. O.; NUNES, G. B.; MINGOTI, G. Z.; NOGUEIRA, M. F. G.; CASTILHO, A. C. S.
Evidence that pregnancy-associated plasma protein A (PAPP-A) plays a role in bovine in vitro-embryo production In: 10th International Ruminant Reproduction Symposium, 2018, Foz do Iguaçu. Proceedings of the 10th International Ruminant Reproduction Symposium. 2018. v.15. p.1123 -

7. **SANTOS, P.H.**; FRANCHI, F. F.; MENDES, L. O.; FONTES, P. K.; PINHEIRO, V. G.; CASTILHO, A. C. S.
Expression of bta-miR-222 in bovine granulosa cells: effect of follicle deviation and regulation by FSH/insulin in vitro. In: XXXII Annual Meeting of the Brazilian Embryo Technology Society (SBTE), 2018, Florianópolis. Proceedings of the 32nd Annual Meeting of the Brazilian Embryo Technology Society. 2018. v.15. p.453 -

8. **SANTOS, P.H.**; FONTES, P. K.; FRANCHI, F. F.; GIROTO, A. B.; NUNES, S. G.; GUILHERME, V. B.; CASTILHO, A. C. S.
HSPA5 and in vitro embryo production: effect of addition of the protein during in vitro embryo culture in cattle. In: 10th International Ruminant Reproduction Symposium, 2018, Foz do Iguaçu. Proceedings of the 10th International Ruminant Reproduction Symposium. 2018. v.15.

p.1070 -

9. **SANTOS, P. H.**; FONTES, P. K.; FRANCHI, F. F.; GUILHERME, V. B.; NOGUEIRA, M. F. G.; BARROS, C. M.; CASTILHO, A. C. S.
Changes in MicroRNAs expression in granulosa cells from superstimulated cows In: XXXI Annual Meeting of the Brazilian Embryo Technology Society, Cabo de Santo Agostinho. Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society. 2017. v.15. p.786 -

10. FRANCHI, F. F.; FONTES, P. K.; **SANTOS, P.H.**; RAZZA, E. M.; RODRIGUES, R. B.; CARVALHO, A.; NOGUEIRA, M. F. G.; CASTILHO, A. C. S.
Pregnancy-associated plasma protein-A (PAPP-A) added during in vitro maturation: effects on meiosis progression, oocyte DNA fragmentation and gene expression in bovine cumulus-oocyte complexes. In: XXXI Annual Meeting of the Brazilian Embryo Technology Society, 2017, Cabo de Santo Agostinho. Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society. , 2017. v.15. p.830 -

Demais produções bibliográficas

1. GUILHERME, V. B.; PRONUNCIATE, M.; **SANTOS, P.H.**; CINICIATO, D.S.; TAKAHASHI, M.B; ROCHA, J. C.; NOGUEIRA, M. F. G. **Distinct Sources of a Bovine Blastocyst Digital Image do not Produce the Same Classification by a Previously Trained Software using Artificial Neural Network**. preprint. Cold Spring Harbor, NY, USA:Cold Spring Harbor Laboratory, 2018. (Outra produção bibliográfica)

Demais produções técnicas

1. SANTOS, PRISCILA H. **Obtenção dos ovócitos a partir do líquido folicular, 2021.** Aula ministrada para alunos da Pós-graduação em Reprodução Assistida Núcleo Semear

2. SANTOS, PRISCILA H. **Cultivo e Seleção embrionária, 2021.** Aula ministrada para alunos

da Pós-graduação em Reprodução Assistida Núcleo Semear

3. SANTOS, PRISCILA H.**A atuação do biomédico na Reprodução Humana Assistida.** Palestra ministrada para a V Jornada acadêmica de Biomedicina e Farmácia da Faculdade Marechal Rondon

4. GIROTO, A. B.; PIOLTINE, E. M.; **SANTOS, P.H. Produção in vitro de embriões bovinos: propósito da técnica**, 2017. Curso de curta duração ministrado.

Participação em banca examinadora

1. SANTOS, P. H.. Participação em banca de Aline Gonçalves Sawa. Efeito do TUDCA sobre a progressão da meiose e a concentração de espécies reativas de oxigênio na maturação in vitro de complexos cumulus-oócito bovinos. 2018. Trabalho de Conclusão de Curso (Graduação em Engenharia de Biotecnologia) - Universidade Estadual Paulista Júlio de Mesquita Filho.

2. SANTOS, P. H.. Participação em banca de Thaisy Tino Dellaqua. Efeitos da adição da proteína sérica A associada à prenhez (PAPP-A) sobre o perfil e metabolismo lipídico de oócitos bovinos maturados in vitro. 2018. Trabalho de Conclusão de Curso (Graduação em Engenharia de Biotecnologia) - Universidade Estadual Paulista Júlio de Mesquita Filho.

3. SANTOS, P. H.; GARCIA, P. C.; FONTES, P. K.. Participação em banca de Natalia Pereira.Adição da proteína GRP78 na etapa de fertilização in vitro de embriões bovino: benefícios na produção in vitro de embriões bovino. 2017. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Universidade Paulista.

4. SANTOS, P. H.. Participação em banca de Suellen Neves. Trabalho de Conclusão de Curso e Relatório de Estágio Supervisionado. 2017. Trabalho de Conclusão de Curso (Graduação em Medicina Veterinária) - UniCesumar.

5. SANTOS, P. H.. Participação em banca de Alexandre Mendes de Almeida Junior.Efeitos da utilização do ácido tauroursodesoxicólico sobre a progressão meiótica na maturação in vitro de oócitos bovinos. 2017. Trabalho de Conclusão de Curso (Graduação em Engenharia de Biotecnologia) - Universidade Estadual Paulista Júlio de Mesquita Filho.

6. SANTOS, P. H.. Participação em banca de Camila Motta Boverio. Caracterização da cinética de expressão dos genes dos receptores de FSH, LH e AREG durante a maturação in vitro de complexos cumulus-oócito bovinos. 2017. Trabalho de Conclusão de Curso (Graduação em Engenharia de Biotecnologia) - Universidade Estadual Paulista Júlio de Mesquita Filho.