UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS AGRÁRIAS
DEPARTAMENTO DE ZOOTECNIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

ANTÔNIO CARLOS DE ALBUQUERQUE TELES FILHO

PROTEOMIC CHARACTERIZATION OF EIGHT-DAY-OLD EQUINE BLASTOCYSTS PRODUCED IN VIVO

FORTALEZA
2022

ANTÔNIO CARLOS DE ALBUQUERQUE TELES FILHO

PROTEOMIC CHARACTERIZATION OF EIGHT-DAY-OLD EQUINE BLASTOCYSTS PRODUCED IN VIVO

Dissertação apresentada ao Programa de Pós-Graduação em Zootecnia da Universidade Federal do Ceará como requisito parcial à obtenção do título de Mestre em Zootecnia. Área de concentração: Reprodução Animal.

Orientador: Prof. Dr. Arlindo de Alencar Araripe Noronha Moura.
Coorientador: Dr. Fábio Roger Vasconcelos.

FORTALEZA

2022

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

T272p Teles Filho, Antonio Carlos de Albuquerque.
PROTEOMIC CARACTERIZATION OF EIGHT-DAY-OLD EQUINE BLASTOCYST PRODUCED IN VIVO / Antonio Carlos de Albuquerque Teles Filho. – 2023.
38 f. : il. color.

Dissertação (mestrado) – Universidade Federal do Ceará, Centro de Ciências Agrárias, Programa de Pós-Graduação em Zootecnia, Fortaleza, 2023.
Orientação: Prof. Dr. Arlindo de Alencar Araripe Noronha Moura.
Coorientação: Prof. Dr. Fábio Roger Vasconcelos.

1. proteina. 2. embrião. 3. equino. I. Título.

CDD 636.08

ANTÔNIO CARLOS DE ALBUQUERQUE TELES FILHO

PROTEOMIC CHARACTERIZATION OF EIGHT-DAY-OLD EQUINE BLASTOCYSTS PRODUCED IN VIVO

Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Zootecnia da Universidade Federal do Ceará como requisito parcial para obtenção do Título de Mestre em Zootecnia. Área de Concentração: Reprodução Animal.

Aprovada em: 07/02/2022

BANCA EXAMINADORA

_____

Prof. Dr. Arlindo de Alencar Araripe Noronha Moura (Orientador)

Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Dárcio Ítalo Alves Teixeira

Universidade Estadual do Ceará (UECE)

_____

Dr. Gustavo Carneiro Ferrer

Universidade Federal Rural de Pernambuco (UFRPE)

_____

Drª. Mariana Machado Neves

Universidade Federal de Minas Gerais (UFMG)

_____

Drª. Fernanda Saules Ignácio

Faculdade EDUVALE de Avaré (EDUVALE)

AGRADECIMENTOS

Aos equinos, pela sua delicadeza, gentileza e entrega incondicional, sendo minha mais formidável escola sobre a vida e o ser humano, além de fontes de inspiração e de equilíbrio fino entre força, sabedoria e resiliência.

À Universidade Federal do Ceará e ao Departamento de Zootecnia pela estrutura e pelas inúmeras oportunidades de aprendizados oferecidas.

À Faculdade de Veterinária da UECE, na qual orgulhosamente me graduei em Medicina Veterinária em 2012.

Ao Regimento de Policiamento Montado do Estado do Ceará (RPMONT), por dar a oportunidade de utilizar os seus nobres animais para execução deste trabalho, principalmente na pessoa do Dr. Miguel Marcos Oliveira de Melo, ao qual devo também ter me tornado Médico Veterinário de equinos.

Ao meu orientador do Laboratório de Fisiologia Animal, Prof. Dr. Arlindo de Alencar Araripe Moura, por sua orientação, paciência e confiança. Ao meu coorientador, Dr. Fábio Roger Vasconcelos, pela confiança e pelo apoio dado em todo momento.

Ao Dr. Dárcio Ítalo Alves Teixeira, o qual foi o meu "primeiro orientador" ainda na minha graduação e me abriu as portas para a Ultrassonografia e a Reprodução Animal.

À Dra. Fernanda Saules, profissional que me apresentou de forma responsável, humana e inspiradora os nobres embriões equinos e segue inspirando a mim e uma legião de Médicos Veterinários brasileiros.

Ao restante dos membros da minha Banca, por terem tido disponibilidade e paciência, dando-me a honra de avaliar meu trabalho e de me sabatinar neste processo: Dr. Gustavo Carneiro Férrer e Drª Mariana Machado.

Aos pesquisadores da Universidade de Brasília, Drª Aline Martins, Dr. Marcelo Sousa e Dr. Wagner Fontes, bem como à colega Drª Arabela Guedes Viana, pela realização das análises de espectrometria de massa e bioinformática e pelo auxílio na interpretação dos dados e escrita.

Ao pesquisador Dr. Ivan Bustamante-Filho, da Universidade do Vale do Taquari, pela realização e revisão das análises de miRNA, tão importantes a este trabalho.

Aos colegas do Laboratório de Fisiologia Animal que me auxiliaram compartilhando momentos bons e conhecimentos. Em especial, a Mayra, a Arabela, a Moêmia, a Eduardo, a Jorge, a Paula, a Júlia, a Maurício, a Thiago, a Eduardo, a Ronaldo, a Aderson, a Kamila, a Taciana, a Bruna, a Mônica e a Catalina.

Aos amigos à época estudantes, hoje brilhantes Médicos Veterinários e Zootecnistas, que tanto me auxiliaram e me deram a honra de lhes ensinar um pouco: Maria Eduarda Magalhães de Souza, Juliana Gomes Vasconcelos, José Cordeiro Neto, Pedro Emídio, Jander Fabrício.

À querida Drª Deisy Johana Diaz Sanchéz, meu "braço direito" na idealização e realização deste trabalho até os dias de hoje, passando ao meu lado todos os percalços desta trajetória.

À Minha família, representada principalmente nas figuras de minha mãe, a Profª Ritacy Azevedo, meu modelo de fortaleza; de minhas irmãs Milena e Melissa; de meus avós Ritomar, Aracy, Maria José e Avany; de meus tios Rivana, Rivanílson e Gerdal.

Aos grupos Ukilombo e Caravana Cultural, que me ajudaram a curar feridas e a "sustentar a gira" com mais leveza.

À Corrida de Rua, que me ajudou a manter a sanidade mental (ou não).

A uma molécula chamada Cafeína, etc...

“O pobre sozinho, sem um cavalo, fica no seu, permanece, feito numa crôa ou ilha, em sua beira de vereda. Homem a pé, esses Gerais comem.” — João Guimarães Rosa, livro
Grande Sertão: Veredas

RESUMO

O desenvolvimento embrionário inicial é dependente de assinaturas moleculares trazidas pelos gametas, expressão gênica e interações materno-embrionárias. O proteoma do embrião reflete seus estado e capacidade de desenvolvimento. O presente estudo teve como objetivo decifrar o proteoma de blastocistos equinos de 8 (oito) dias de idade produzidos in vivo. A dinâmica folicular de 8 (oito) éguas foi monitorada por ultrassonografia. Na presença de um folículo pré-ovulatório, as éguas receberam GnRH e foram inseminadas. Após 8 (oito) dias, 4 (quatro) embriões foram recuperados. As proteínas foram extraídas dos embriões (pool) e foram submetidas à técnica de espectrometria de massa. Diferentes mecanismos de busca foram usados para identificação das proteínas: Peaks, Proteome Discoverer, SearchGUI e PepExplorer. A espectrometria de massa e ferramentas de bioinformática permitiram a identificação de 1.977 (mil, novecentas e setenta e sete) proteínas nos embriões. Os processos biológicos das proteínas embrionárias foram processos celulares e metabólicos. Os componentes celulares foram células, organelas e complexos contendo proteínas. As funções moleculares das proteínas embrionárias estão envolvidas na ligação, atividades catalíticas e atividade estrutural da molécula. Seis vias relevantes para o desenvolvimento embrionário (FDR < 0,05, plataforma Panther) foram relacionadas ao ciclo do TCA, metabolismo do piruvato, glicólise, metabolismo das purinas e via das pentose fosfato. Foram identificadas vias envolvidas na comunicação célula-célula e no remodelamento da matriz extracelular (MEC), além de vias relacionadas ao remodelamento intracelular, como, por exemplo, a regulação do citoesqueleto pela Rho GTPase. As análises foram realizadas em um pool de embriões de diferentes éguas, fornecendo uma visão abrangente sobre o proteoma de embriões de 8(oito) dias de idade. No entanto, não foi possível fazer comparações entre as sínteses de proteínas nas fases iniciais do desenvolvimento do embrião equino. O presente estudo utilizou espectrometria de massa e uma combinação de mecanismos de busca para a identificação de 1.977 (mil, novecentas e setenta e sete) proteínas em embriões equinos de 8(oito) dias de idade produzidos in vivo. Este é o maior atlas de proteínas de embriões da espécie equina. O conhecimento da expressão de proteínas pelo embrião ajuda a entender sua fisiologia e eventos metabólicos.

Palavras-chave: proteína; embrião; equino.

ABSTRACT

Early embryo development is dependent on molecular signatures brought by the gametes, gene expression and maternal-embryo interactions. The proteome of the embryo reflects its development state and defines its fate. The present study aimed to describe the proteome of 8-day old equine blastocysts produced *in vivo*. Follicular dynamics of eight mares were monitored by ultrasonography. When a preovulatory follicle was confirmed, mares received GnRH and were inseminated. After eight days, four embryos were recovered. Proteins were extracted from the embryos (pool) and subjected to mass spectrometry. Different search engines were used for protein identification: Peaks, Proteome Discoverer, SearchGUI and PepExplorer. Mass spectrometry and tools of bioinformatics allowed the identification of 1,977 proteins in the embryos. Biological processes of embryo proteins were cellular and metabolic processes. Cellular components were cell, organelle and protein-containing complex. Molecular functions of embryo proteins were involved in binding, catalytic activities and structural molecule activity. Six pathways relevant to embryo development (FDR < 0.05, Panther platform) related to TCA cycle, pyruvate metabolism, glycolysis, purine metabolism, and pentose phosphate pathway. There were pathways involved in cell-cell communication and extracellular matrix (ECM) remodeling and pathways related to intracellular remodeling, like cytoskeleton regulation by Rho GTPase. Analyses were conducted in a pool of embryos from different mares, providing a comprehensive view about the proteome of 8-day old embryos. However, comparisons could not be made among protein syntheses in the phases of early developmental states of the equine embryo. The current study used mass spectrometry and a combination of search engines for the identification of 1,977 proteins in 8-day-old equine embryos generated in vivo. This is the largest atlas of proteins from embryos of the equine species. Knowledge of protein expression by the embryo helps to understand its physiology and metabolic events.

Keywords: protein; embryo; equine.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

AGE	Embryonic Genome Activation
AKR1B1	Aldose Reductase
AN	Activated Nathenogenetically
ANXA6	Anexin A6
ARTs	Assisted Reproductive Technologies
DDA	Data-Dependent Acquisition
ET	Embryo Transfer
ECM	Extracellular Matrix
HCD	Higher-Energy Collisional Dissociation
ICM	Internal Cell Mass
IVF	Fertilized In Vitro
MZT	Zygotic Maternal Transition
PPP	Pentose Phosphate Pathway
PSM	Peptide-Spectrum Matches (PSM)
PZ	Parthenogenetic Zygotes
TP	Trophectoderm

SUMÁRIO

1 INTRODUCTION

Early embryo development is highly dependent on molecular signals brought by the male and female gametes, gene expression in the newly formed being and interactions between the embryo and the environment in which it is inserted (Ginther, 1992; Allen, 2000). Genomic activation and protein synthesis start during oviduct transit, and it is intensified when the embryo enters the uterus (Latham & Schultz, 2001). Thus, the proteomic signature of the embryo certainly reflects its development states and defines its fate as well. In the equine species, the first stages of embryonic development are not completely understood (Swegen *et al.*, 2017) and the equine embryo itself has peculiar characteristics, such as the presence of an acellular glycoproteic capsule, late entry into the uterus and the migration on the endometrial surface, and complex maternal-fetal recognition (Ginther, 1992). In recent decades, transcriptomics studies have enabled important discoveries about molecular events of embryo's life (Swegen *et al.*, 2017; Demant *et al.*, 2015). Despite great results achieved, studies focused solely on mrna expression have limitations, once a single gene can encode different proteins which, in turn, may undergo post-translational modifications, each associated with diverse biological functions.

The characterization of the equine embryo proteome sets the foundation for discovery of markers of their developmental competence and viability (Katz-Jaffe & McReynolds, 2013; Katz-Jaffe *et al.*, 2006). Also, it allows a better comprehension about the physiology of early embryo development is determinant for successful application of assisted reproductive technologies (ARTs), such as artificial insemination and embryo transfer (ET) (Ginther, 1992; Allen, 2000). In commercial et programs, embryos are typically collected from donor mares on day 8 after ovulation (Galli *et al.*, 2013). Equine embryos can be transferred with a significant degree of uterine assyncrony, however, this assyncrony is not balanced. Transferring embryos into recipients that ovulated up to 5 days after the donor do not reduce pregnancy rates (Jacob *et al.*, 2012). On the other hand, transferring embryos into mares that ovulated more than 2 days earlier the donor does negatively affect embryo survival (Wilsher *et al.*, 2012). These data suggests that the crosstalk between embryo and the endometrium is tightly controlled, and a precisely orchestrated interaction embryo and the uterine environment, if successful, guarantees the continuous progestin support, resulting in a uterine environment adequate for embryo

development (Klein, 2011).

As expected, the proteins expressed by the equine embryo have pivotal role in the events involved in maternal recognition of pregnancy (Camacho, 2020; Camacho, 2018; Camozzato, 2018). Recently, Swegen *et al*. (2017) reported 732 proteins in the equine blastocoel fluid and 11 proteins in the embryo capsule. It is clear, thus, that mammalian embryos go through remarkable changes in protein synthesis in their early development and that such proteins probably define their functional status and survival. However, there is a gap in our understanding of the global proteome of the equine embryo. In this regard, the present study aimed to describe the global proteomic pattern of 8-day old equine blastocysts produced in vivo.

2 LITERATURE REVIEW

The horse is an animal widely used in Brazil and in the world due to its ability to perform various activities (URTIGA, 2022). The main ones stand out: sport (equestrian, running and vaquejada), leisure (walking animals) and work, these being responsible for containing about 5,850,154 horses in Brazil in 2019 (IBGE, 2019).

With the advancement of the technique of embryo transfer in horses, a growing increase in the number of animals has been observed over the last decades, evidenced mainly by the size of the equine herd in Brazil. According to the Ministry of Agriculture, Livestock and Supply (MAPA) the country has the largest herd in Latin America and the third worldwide. Being highlight for the movement of R $ 7,3 billion only with the production of horses. According to data from the International Society for Embryo Transfer, Brazil recovered around 12,000 embryos in the period 2008-2009 and the market has been increasing at an average rate of 20% per year in the last 5 years. The country has about 40 centers specialized in embryo transfer, located mainly in the Southeast region (ALVARENGA, 2010) and together with the United States and Argentina leads the production of embryos in the world. The so-called Horse Agribusiness Complex is responsible for the generation of approximately 3.2 million direct and indirect jobs reaching more than 30 different segments between inputs, breeding and final destination.

2.1 Early embryonic development in mammals

The preimplantation embryonic period in mammals involves the free life period of the embryo in the oviduct and uterus, where the development of the zygote to the blastocyst stage (Watson & Barcroft, 2001). This period is characterized by successive cleavages, embryonic genome activation and morphogenetic events, such as compaction and cavitation, which culminate in blastocyst (Watson *et al.*, 2004).

2.1.1 Embryonic genome activation

After fertilization, the zygote goes through successive cleavages, which are crucial for the subsequent development of the embryo. In ruminants, the first cleavages still occur in the oviduct and under the influence of maternal transcripts and proteins accumulated during oogenesis (Memili *et al.*, 1998). The transition from oocyte control to embryonic control is known as zygotic maternal transition (MZT), in which the majority of maternal mRNA accumulated during oogenesis is degraded and replaced by new embryonic mRNA. In ruminants, in the 8-16 cell phase, the embryo begins to produce its own transcripts and proteins in a process called embryonic genome activation (AGE) (Crosby *et al.*, 1988).

2.1.2 Embryonic compaction and cavitation

As cleavages happen, the number of blastomeres increases, which in turn decrease in size along the cleavages, forming a cell mass called the morula. Compaction is characterized by increased adement and the beginning of cell polarization, which initiates the first event of cell differentiation in embryonic development (Jedrusik, 2015). During compaction, dramatic changes occur in the shape of blastomeres, which gain more contact with each other and lose their spherical shape to acquire a flattened shape, which makes it difficult to distinguish their cellular contour (Watson & Barcroft, 2001). Additionally, the embryo's external cells begin to develop its junctional complexes, consisting of occlusive zonulas (tight junctions), adherent zonulas (adherent junctions), adherent macules (desmosomes) and connecting junctions (gap junctions) (Fleming *et al.*, 2000).

The E-cadherin protein is the main component of adherent joints and is

responsible for cell adherent during compaction (Eckert & Fleming, 2008; Modina *et al.*, 2010). E-cadherin interacts with other proteins, forming a complex with catenine proteins and binding to the actin cytoskeleton (Aberle *et al.*, 1996). Thus, catenins act by forming a plate of proteins that reinforce cell adeation during the compaction process (Watson & Barcroft, 2001). The importance of the role of E- cadherin during compaction processes has been widely demonstrated since embryos of mice treated with Antibodies to E-cadherin showed flaws in the compaction and cavitation process (Watson, 1992).

Another important event during compaction is the beginning of cell polarization, an event characterized by the reorganization of blastomeres, which results in the establishment of the apical and basolateral domains (Fleming & Pickering, 1985). Occlusion joints are an important component of epithelial polarity located on the basal lateral surface consisting of a complex of at least five proteins: occlusive zonula protein 1 and 2 (ZO-1, ZO-2), 7H6, cingulin and ocludine. Ocludine is a transmembrane protein that interacts with ZO-1, ZO-2 and cingulin to form a bridge between the tight joints and the actin cytoskeleton (Barcroft *et al.*, 1998).

Following compaction, the reorganization of blastomeres is initiated, and external cells develop into trophectoderm (ET) cells. A group of internal cells forms the internal cell mass (ICM), and develops a fluid-filled cavity called blastocele. These events form a structure called blastocyst (Watson & Barcroft, 2001). Trophectoderm is the blastocyst wall and is required for the transport of fluids that carry blastocele formation in a process known as cavitation (Barcroft *et al.*, 1998). In particular, the enzyme Na+/K+-ATPase, located in the basolateral membranes of the trophectoderm, is responsible for establishing an ionic gradient through the trophectoderm, promoting osmatic accumulation of water. This enzyme promotes the active transport of Na+ into the blastocele, thus allowing the entry of water by osmotic processes (Watson &Barcroft, 2001).

2.1.3 Proteomics applied to the study of physiology and molecular markers in embryos

Despite advances in proteomics in the study of embryonic physiology and in the identification of molecular markers, studies in this field still remain scarce. This may be due to the limited amount of protein present in embryos, which may contain only 100 ng of protein (Thompson *et al.*, 1988).

In cattle, a study conducted by Demant *et al.* (2015) analyzed the protein

profile during the transition of in vitro-produced grinding stones and blastocysts using an LC-MS/MS iTRAQ-based approach. The authors identified a total of 560 proteins, of which 140 were significantly different between the stages of morula and blastocysts. In particular, the proteins anexin A6 (ANXA6) and aldose reductase (AKR1B1) were more expressed in blastocysts and can be considered important markers of morphula-blastocyst transition. These proteins can play important roles by regulating transient interactions of the cytoskeleton.

Gupta *et al.* (2009) compared the proteome of swine zygotes activated nathenogenetically (NG) and fertilized in vitro (IVF) using SDS-PAGE 1-D, combined with LC-MS/MS. In this study, proteins of 6000 IVF zygotes and 6000 parthenogenetic zygotes (AP) were analyzed. A total of 735 proteins were identified, of which only 51.3% (377) were observed in both groups, indicating major changes in their protein composition. The authors report that these differences may be related to the electroactivation process in the case of nathenogenetic zygotes, in which there are no spermatic factors. These results identified potential marker proteins of embryonic quality and reinforced the hypothesis that aberrant expression of proteins in AP embryos could be a reason for their failure in development.

A study conducted in cattle by Jensen *et al.* (2014) described blastocele fluid proteome and expanded blastocyst cell material, evidencing 23 proteins identified in blastocele fluid, while 803 were identified in the rest of the cellular material. Some functions of these proteins were related to cytoskeletal reorganization processes involving cell support. Examples of these proteins were drebrin, desmoplakin, fibronectin, filamin-A, integrin beta-1, placoglobin, lactaderin, transgelin-2.

Swegen *et al.* (2017) evaluated the perfil of proteins secreted after 24h and 48h of cultivation together with blastocele and embryonic capsule proteins in the equine species. The analyses revealed 72 proteins in 24 hours and 97 proteins in 48 hours of cultivation. 732 were also found in blastocele fluid and 11 in the embryonic capsule. Some found only in the embryonic capsule were transglutaminase 3, chitobiase, uterocalin, phospholipase A2 and amino oxidase. Among them, chitobiase and phospholipase A2 were associated with capsule degradation events, while uterocallin was associated with its formation.

3 MATERIAL AND METHODS

3.1 Experimental *animals and local of experiment*

The experiment was conducted at the Cavalry of the Ceará State Police Department, in Fortaleza, Brazil (3°49'08.3"S 38°29'05.6"W) from September to November of 2018. The weather in that region is defined as Aw (Köppen & Geiger, 1928) and at that latitude, there are no changes in day length throughout the year. Eight adults, healthy and cycling crossbred mares (423.3 ± 35.3 kg) with proven fertility and optimal body condition were used in this study. The mares were fed Tifton hay (*Cynodon dactylon*) and Elephant grass (*Pennisetum purpureum*) and supplemented with concentrate (18% of crude protein), with free access to water.

3.2 Follicular monitoring and artificial insemination

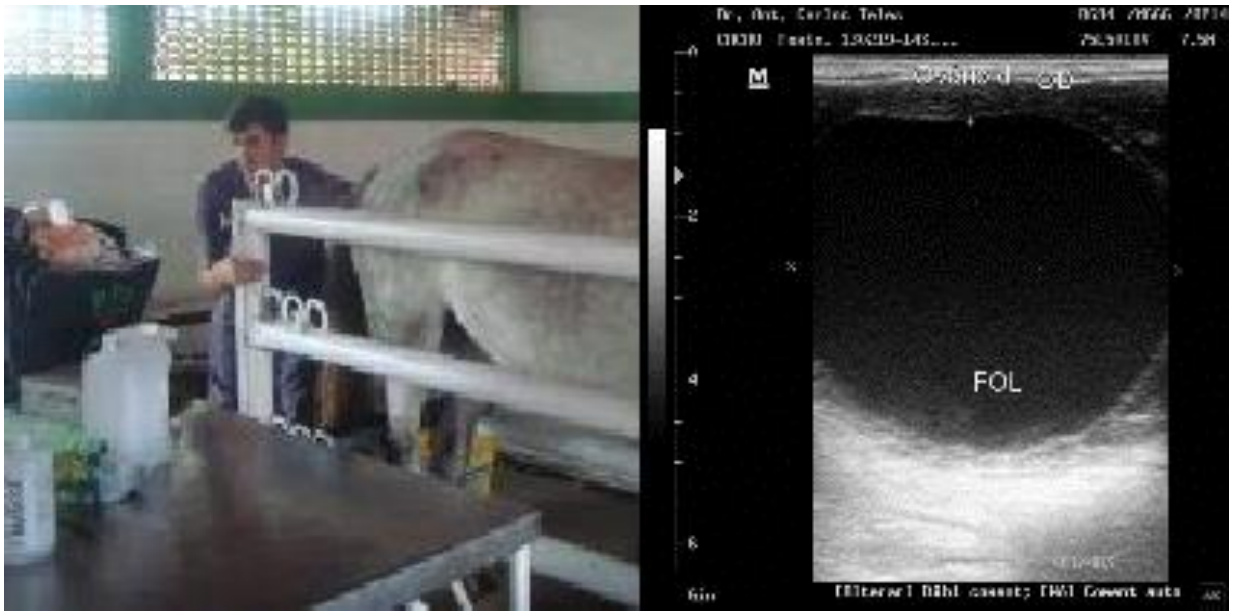
The estrous cycles of mares were monitored by transrectal ultrasonography (DP4900 Vet, Mindray®, Shenzhen, China). When a preovulatory follicle (35-38 mm) and uterine edema were detected, ovulations were induced using 0.075 mg deslorelin acetate i. m. (Sincrorrelin, Ourofino®, Brazil). Twenty-four hours after ovulation was induced, mares were inseminated, placing semen in the middle of the uterine body with a flexible pipette (Minitub®, Porto Alegre, Brazil).

Figure 1. Artificial insemination

Fonte: Author, 2022.

Then, mares were monitored every 6 hours by ultrasonography to detect the exact moment of ovulation (D0). For inseminations, sperm was collected from a single fertile "Brasileiro de Hipismo" stallion using artificial vagina and diluted (1:1) in Botusemen® milk-based extender (Botupharma®; Botucatu, Brazil). Each inseminating dose had at least 10^9 motile sperm.

Figure 2. Follicular monitoring

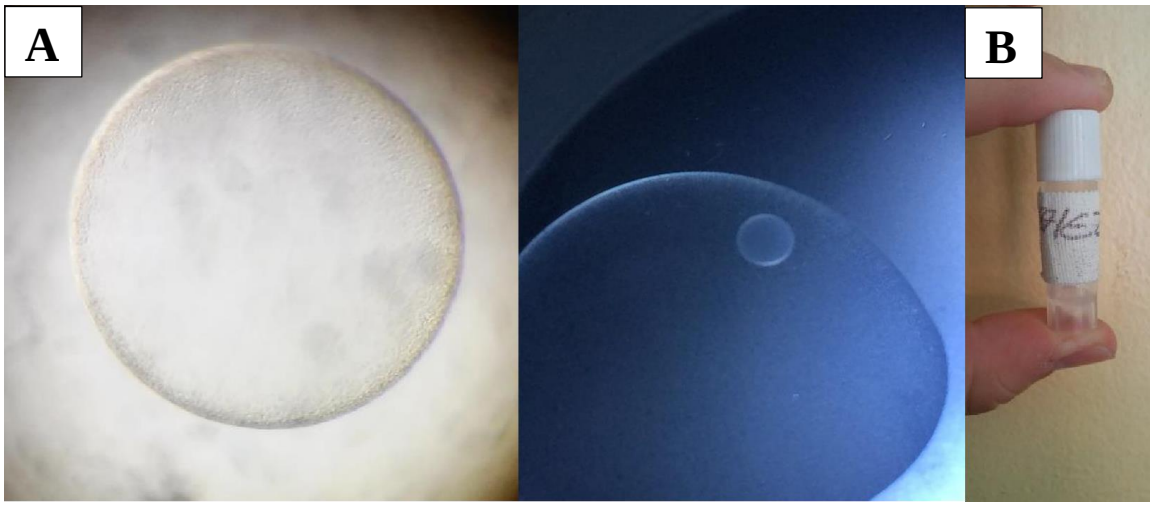


Fonte: Author, 2022.

3.3 Embryo recovery

Mares were submitted to uterine flushing eight days after insemination (D8) using Ringer Lactate solution (JP®; Ribeirão Preto, Brazil) and a uterine catheter (Bioniche®; Washington, USA) [10]. Right after the flushing, the content of the collection filter (WTA®; Cravinhos, Brazil) was placed in a Petri dish for embryo search and evaluation in stereomicroscope (Bel-photonics®; Piracicaba, Brazil). All eight mares ovulated (based on ultrasound analysis) but only four expanded blastocysts were recovered after the flushings (one per mare). These embryos were graded (GI-3; GII-1) based on quality and viability (Stringfellow & Givens, 2009), gently rinsed in ringer lactate solution in a sterile Petri dish, had their size measured, placed in sterile cryovials containing 50 uL ringer lactate solution and frozen in liquid nitrogen.

Figure 3. Visualization and packaging of the recovered embryo. A) Equine recovered embryo; B) Packaging of the recovered embryo.



Fonte: Author, 2022.

3.4 Extraction of embryo proteins

For protein extraction, all four embryos were pooled and sonicated in cold water bath for 30 min (Velez et al., 2016). Then, embryos were frozen in liquid nitrogen and macerated (this procedure was repeated four times). Afterwards, proteins were precipitated with cold acetone and 0.1 M NaCl (1:4), incubated overnight at −20 ºC and then centrifuged (16,000 x g, 70 min, 4 °C) (Crowell et al., 201). The supernatant was pipetted out and the residual acetone was dried under vacuum (SpeedVac® Concentrator, Thermo Fisher Scientific™, EUA).

3.5 Protein digestion and desalinization

Proteins were alkylated with a volume of iodoacetamide to reach a final concentration of 0.014 M. The mixture was maintained at 21 °C and 400 rpm for 40 min in a dark room. For protein reduction, a volume of dithiothreitol was added to reach a final concentration of 0.005 M and a volume of $CaCl_2$ solution was added to the samples to reach a final concentration of 0.001 M. Following this, 0.02 M TEAB was added to reach a 75 µl final volume. Samples were digested with trypsin (Promega®, Madison, USA) with a proportion of 1/50 (enzyme/substrate) and incubated at 37 °C for 18 h. Trifluoroacetic acid (1%) was added to block trypsin digestion (VIANA *et al.*, 2018).

Finally, peptides were added to stage-tip C18 columns for desalting and centrifuged (900 x g, 5 min), followed by two washings with 0.5% acetonitrile (1,000 x g, 3 min). To elute peptides, columns were centrifuged (600 x g, 3 min) with 0.5% acetic acid and increasing acetonitrile concentrations (25% to 80%) (Viana *et al.*, 2018).

3.6 Liquid chromatography and mass spectrometry

Chromatographic and mass spectrometry analyzes were performed as previously described (Viana *et al.*, 2018). Briefly, digested peptides were injected into a chromatographic system (Dionex Ultimate 3000 RSLC nano UPLC, Thermo Fisher ScientificTM, USA), configured with a 3 cm x 100 µm trap column containing 5 µm C18 particles, serially connected to the 24 cm x 75 µm analytical column containing C18 3 µm particles. The samples were injected to obtain 1µg in the column, subjected to linear gradient elution between solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) from 2% B to 35% B over 155 min. Peptide fractions were eluted directly into the ionization source of an Orbitrap Elite mass spectrometer (Thermo Fischer ScientificTM, USA), configured to operate in data-dependent acquisition (DDA) mode. MS1 spectra were acquired at 120,000 resolutions ranged between 300 and 1,650 m/z. The 15 most intense ions above 3,000 intensity limits were conducted to the higher-energy collisional dissociation (HCD) fragmentation chamber and their fragments were also analyzed in the Orbitrap analyzer, generating spectra of MS2 with 15,000 resolutions (Kalli *et al.*, 2013). Reanalysis of already fragmented ions was inhibited by dynamic exclusion (Andrews *et al.*, 2016), favoring the identification of less abundant peptides.

3.7 Data analysis and protein identification

The complete set of spectra generated by mass spectrometry were analyzed using Peaks 7.0 software (BSI, USA), searching in Uniprot and NCBI repositories in September 2019, filtered for *Equus caballus* species (Tanca *et al.*, 2013). The search was performed based on *de novo* sequencing and peptide-spectrum matches (PSM), with precursor ion mass error tolerance of 10 ppm, MS/MS mass tolerance of 0.05 Da, up to 2 missed cleavages, cysteine carbamidomethylation as fixed modification and oxidation of methionine as variable modification. Unimod database modification search modules based on fragmentation patterns and point mutation search were activated

(Zhang *et al.*, 2012). As the combination of search algorithms yields more comprehensive and robust results (Kremer *et al.*, 2016), itegrative searches were also performed using SEQUEST, OMSSA, X! Tandem, Myrimatch, MS Amanda, MS-GF+, Comet, Tide, and Andromeda mechanisms, including Uniprot database, obtained on the same date and filtered to the same taxonomic classification and settings as described above. Additional searches were performed using Proteome Discoverer and SearchGui and consolidated by Peaks InChorus module and SearchGUI (Barsnes *et al.*, 2018). Sequences obtained again by Peaks, but not identified in the database, were submitted to additional search for similarity, using PepExplorer (Leprevost *et al.*, 2014) and the same database. The results were consolidated by excluding protein and peptide redundancies.

3.8 In silico analysis of equine embryo proteins

Proteins identified in the equine embryos were analyzed as regard to pathways and GO terms. The combination of Blast2GO, Revigo (Supek *et al.*, 2011) and g:Profiler (Reimand *et al.*, 2016) platforms were used to calculate significant overrepresentation of grouped terms. To conduct a proper analysis of pathway representation, protein access codes were matched to gene codes in databases of the prediction programs. A workflow using Uniprot (UNIPROT CONSORTIUM, 2019), DB2DB in BioDBnet (Mudunuri *et al.*, 2009), and BLAST Koala (Kanehisa *et al.*, 2016) mapping platforms were used iteratively. The representation of component and pathways were analyzed using Panther (Kanehisa *et al.*, 2016), BlastKoala (MI *et al.*, 2017) and String (Szklarczyk, *et al.*, 2017) platforms.

4 RESULTS

The combination of four different search engines allowed the identification of 4,014 proteins. After elimination of redundancies (at 3 levels, based on identifiers, peptides and cross-bank mapping), 1,977 proteins were reliably identified in the 8-day old equine embryos, with 1,413 proteins identified by Peaks, 316 by Proteome Discoverer, 165 by SearchGUI and 83 by PepExplorer. The low number of overlaps observed for PepExplorer is due to the nature of that particular search, which used peptides sequenced not identified by Peaks program.

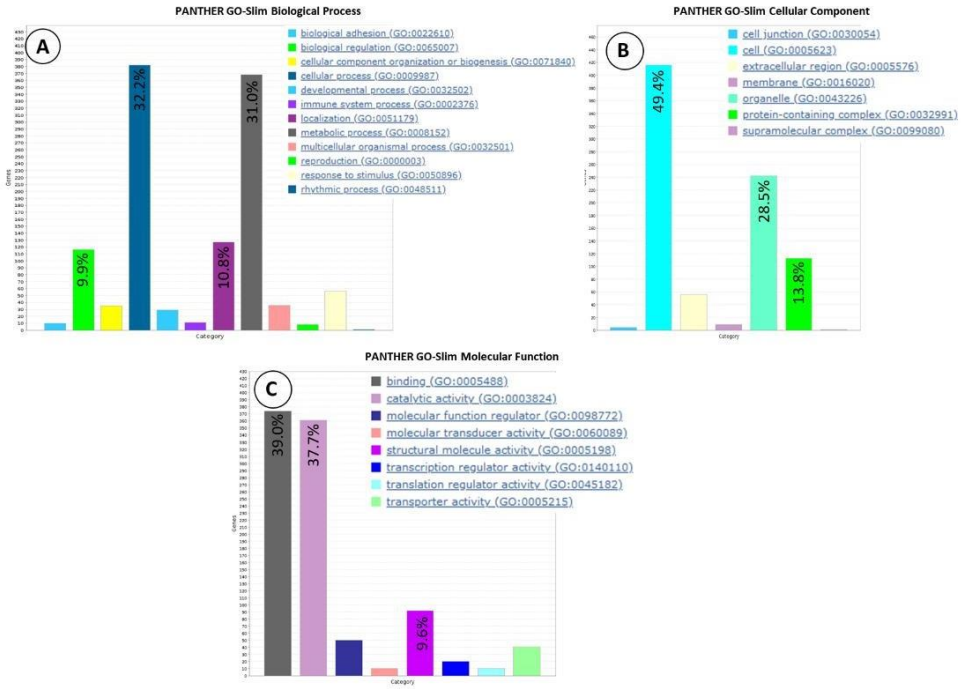
As determined by Panther platform, biological processes of equine embryo

proteins were cellular (32.2%) and metabolic (31%) processes, followed by localization (10.8%) and reproduction (9.9%). Prominent cellular components were cell (49.4%), organelle (28.5%) and protein-containing complex (13.8%). Regarding molecular function, proteins were mainly involved in binding (39.0%), catalytic activities (37.7%) and structural molecule activity (9.6%).

Table 1. Results from PANTHER GO-Slim A) Biological Process; B) Cellular Component; C) Molecular Function

Panther Pathways	Equus Caballus (Ref)	#	Expected	Fold Enrichment	P Value	Fdr
Unclassified	17982	888	962.4	0.92	4.42e-10	3.62e-08
Tca Cycle	8	6	0.43	14.01	3.58e-05	1.47e-03
ATP Synthesis	7	5	0.37	13.35	1.97e-04	5.38e-03
Asparagine And Aspartate Biosynthesis	6	4	0.32	12.46	1.09e-03	1.78e-02
Pentose Phosphate Pathway	9	5	0.48	10.38	4.57e-04	1.07e-02
Pyruvate Metabolism	10	5	0.54	9.34	6.56e-04	1.35e-02
Glycolysis	25	10	1.34	7.47	6.30e-06	3.44e-04
De Novo Purine Biosynthesis	28	7	1.5	4.67	1.65e-03	2.08e-02
Cytoskeletal Regulation By Rho Gtpase	83	16	4.44	3.6	3.74e-05	1.23e-03
Integrin Signalling Pathway	186	22	9.95	2.21	1.17e-03	1.74e-02

Figure 4. Identification of significantly enriched pathways (FDR <0.05, Panther platform) in equine D8 embryo.



Font: Author, 2022

The combination of Blast2GO, Revigo and g: Profiler platforms showed significant overrepresentation of the displayed and grouped terms. Revigo allowed us to employ a multidimensional view, where GO terms' semantic similarities and their closeness are played in two-dimensional space: x and y. The representation of components and pathways analyzed using Panther, BlastKoala and String platforms showed 13 overrepresented pathways. After exclusion of seven pathways related to human behavior, the six pathways relevant to embryo development (FDR < 0.05, Panther platform) related to TCA cycle, pyruvate metabolism, glycolysis, purine metabolism, and pentose phosphate pathway. Also, there were pathways involved in cell-cell communication and extracellular matrix (ECM) remodeling and pathways related to intracellular remodeling, like cytoskeleton regulation by Rho GTPase.

All data from the early stages of chromatography, mass spectrometry, protein quantitation, protein identification, statistical and bioinformatics were deposited in the Spectrometry Center of Mass Computing (University of California, San Diego) using the Virtual Environment Interactive Platform Spectrometry (MassIVE), with MassIVE ID = MSV000083725.

5 DISCUSSION

In the present study, a strategy based on chromatography coupled to mass spectrometry and an interactive database search using four different platforms (Proteome Discoverer, SearchGui and PepExplorer, Peaks 7.0 software) allowed the identification of 1,977 proteins in 8-day old equine embryos produced *in vivo*. The search engine currently employed allowed us to describe 28.5% more proteins than using only one method. To our knowledge, this is the first comprehensive description of the proteome of pre-implantation equine embryos. The number of proteins identified in our experiment exceeds that reported in other studies focused on embryonic structures of the equine species. In fact, Swegen *et al.* described 72 and 97 proteins in D9 and D10 embryo secretomes, respectively, 732 proteins in blastocoel fluid and 11 proteins in the acellular capsule of the equine.

Gene ontology terms related to equine embryo proteins were analyzed by REVIGO and Panther platforms. Such analysis was possible as whole-genome sequences from several organisms become available, contributing to the evolution of biological research. It also allows us to comprehend the data generated beyond individual genes or proteins and assimilate how multiple proteins act together in a complex biological system (Zhou *et al.*, 2015; Gardner, 2015). The analytical tools and interpretation of high-throughput biological experiments have significantly increased with the advance of computational engines and algorithms. Based on the list of equine embryo proteins, the two biological processes with the highest number of genes were cellular and metabolic processes. Most genes of cellular process are related to cellular metabolic process, followed by cellular component organization, secretion, and signal transduction. As regard to cellular processes, our study identified several ribosomal proteins such as S12, S21, L19, L15, L6, L27, L29, S3A, S4, S8 and acidic ribosomal protein P2. These ribosome proteins are crucial for cell growth and differentiation (Zhou *et al.*, 2015) and studies indicate that the number of ribosomes increases remarkably during cell division of the bovine blastocyst (Bilodeau-Goeseels & Schultz, G.A., 1997).

A detailed overview of genes indicated that most of them were related to cellular metabolic processes and organic substance metabolic processes. The latter involved molecular entities containing carbon, such as those molecules presented in the pathways overrepresented involved in embryo energy supply. Regarding molecular functions of equine embryo components, most genes are involved in binding and catalytic

activity, followed by structural molecule activity. The main GO terms detailed in binding activity are heterocyclic compound binding and protein binding, while GO terms related to the catalytic activity are mainly hydrolase activity, followed by transferase and oxidoreductase activities. Panther GO-Slim analysis of cellular process indicates a high number of proteins found in the cell (cell surface, cell division site, and cell periphery), followed by organelle and protein-containing complex localization.

According to Panther pathway analysis, most of the overrepresented pathways are involved in energy supply for biomolecule synthesis and cell proliferation (TCA cycle, ATP synthesis, asparagine and aspartate biosynthesis, pentose phosphate pathway, pyruvate metabolism, glycolysis, and de novo purine biosynthesis). The major energy source of living cells is glucose, which is converted to ATP, NADH and pyruvate. In the presence of oxygen, pyruvate is converted to Acetil-CoA and metabolized in the tricarboxylic acid (TCA) cycle. The final products, electrons carriers NADH and $FADH_2$ will be delivered to the electron transport chain (ETC) that contains an ATP synthase that converts ADP to ATP. In the other hand, without oxygen, pyruvate is converted to lactate. While preimplantation stem cell requires a huge amount of energy substrate, the post-implanted embryo will generate energy using less mitochondrial activity and use more glucose (Mathieu & Ruohola-Baker, 2017). In fact, Gardner and Leese (1989) demonstrated signs of switch of ATP generation from oxidation of TCA cycle to glycolysis in mitochondria of mouse blastocysts.

Oxidative phosphorylation is still functional during early embryo development, but primarily utilizes fatty acids rather than glucose to provide ATP (Krisher & Prather, 2012). This metabolic adaptation is known as the Warburg Effect and supports rapid cell proliferation (Warburg, 1956). Although this is typically an anaerobic process and less efficient way to generate ATP, it is commonly used for proliferating cell types, such as cancer (López-Lázaro, 2008) and embryo cells (Mathieu & Ruohola-Baker 2017).

When embryonic cells decrease mitochondrial activity to produce energy, they can ensure mitochondrial quality control, eliminating damaged mitochondria to guarantee cell proliferation (Khacho *et al.*, 2016). “The quiet embryo hypothesis” mentioned by Leese (2012) states that preimplantation embryos keep a quiet metabolism from zygote to morula stage, limiting the formation of reactive oxygen species. Such a hypothesis is supported by the facT (Houghton *et al.*, 1996) that oxygen consumption is low during cleavage but increases with blastocyst formation. The same authors associated

the requirement of high ATP levels of post-implantation mouse embryos for proper functionality of Na^+, K^+ ATPase activity during blastocoel cavity formation. Most of the glucose is converted to lactate, which decreases the pH around the embryo, supporting the endometrium digestion for embryo implantation (Gardner, 2015). A sharp increase in glucose consumption of viable human embryos also occurs at the blastocyst stage (Gardner, 20).

Proliferating cells have critical metabolic requirements for synthesis of proteins and lipids. Pentose phosphate pathway (PPP) was enriched in 8-day equine embryo and, according to Varum *et al.* (Varum, 2011), PPP generates metabolites for biosynthesis of nucleotides and lipids. Pathways associated with the equine embryo proteins were involved in cell-cell communication, intra and extracellular matrix remodeling, integrin signaling pathway and cytoskeleton regulation by Rho GTPase. Integrins are cell surface receptors, mediating adhesion between extracellular matrix (ECM) and the cytoskeleton (Hynes, 1992). In addition, integrins interact with ECM and mediate intracellular signals, changing cellular morphology and cell cycle progression (Schwartz & SHATTIL, 2000). Both integrin signaling pathway and cytoskeleton regulation by Rho GTPase pathways contribute to regulation of gene expression, cytoskeletal organization, cell growth and migration. This close relationship explains how ECM, growth factors or other extracellular stimuli control cell functions and proliferation (Saoncella *et al.*, 1999). In the present work, the behavior of the cytoskeleton, as well as cell growth and migration were visualized as described as well in the literature.

The extracellular matrix comprises a variety of glycoproteins, collagens, glycosaminoglycans and proteoglycans (Adams & Watt, 1993). In the embryo, cell-ECM interactions regulate migration, adhesion, cell proliferation, differentiation and morphogenesis (Zagris, 2001), as well as the activity of growth factors (Zagris, 2001). ECM glycoproteins include collagens and non-collagenous proteins such as fibronectin (Rozario & Desimone, 2010) which have important roles during embryonic development (Rozario & Desimone, 2010; Gomes de Almeida *et al.*, 2016). In fact, bovine embryos cultivated in collagen gel substrates show better hatching and blastocysts development (Rivera & Rinaudo, 2013) and studies show that almost all types of collagens are important for embryo survival in the mouse (Liu *et al.*, 1995; Wenstrup *et al.*, 2004). In addition, absence of certain types of collagens causes postnatal death in mice (Sumiyoshi *et al.*, 2004) and damages in zebrafish embryo (Gansner & Gitlin, 2008). Fibronectin, in turn, is an ECM glycoprotein found in early embryonic stages (Gomes de Almeida *et al.*,

2016; Richoux *et al.*, 1989) and play roles in cell migration (Richoux *et al.*, 1989; Kurosaka, & Kashina, 2008) Fibronectin binds to integrins, helping to connect ECM adhesion proteins to cytoskeletal components (Shiokawa *et al.*, 1996). Several authors described death of embryonic in mice (George *et al.*, 1993), zebrafish (Trinh & Stainier, 2004) and chicken (Linas & Lash, 1988) due to absence of fibronectin.

6 CONCLUSION

In summary, the current study used mass spectrometry and a combination of search engines for the identification of 1,977 proteins in 8-day-old equine embryos generated in vivo. This is, to date, the largest atlas of proteins from embryos of the equine species. Moreover, knowledge of protein expression by the embryo helps to understand its physiology, metabolic events and future identification of potential biomarkers of its health.

REFERENCES

ABERLE, H.; SCHWARTZ, H.; KEMLER, R. Cadherin-catenin complex: protein interactions and their implications for cadherin function. **Journal of Cellular Biochemistry,** [s.l.], v. 61, p. 514–523, 1996.

ADAMS, J.C.; WATT, F.M. Regulation of development and differentiation by the extracellular matrix. **Development**, v. 117, p. 1183-1198, 1993.

ALLEN, W.R. The Physiology of Early Pregnancy in the Mare. **AAEP Proceedings**. v. 46, p. 338-354, 2000.

ALVARENGA MA. Problems and solutions in equine embryo transfer programs in Brazil. **Acta Sci Vet**, v.38, suppl.1, p. s319-s333, 2010.

ANDREWS, G.L.; DEAN, R.A.; HAWKRIDGE, A.M.; MUDDIMAN, D.C. Improving proteome coverage on a LTQ-Orbitrap using design of experiments. **J Am Soc Mass Spectrom.** v. 22, p. 773-783, 2011

BARCROFT, L.C.; HAY-SCHMIDT, A.; CAVENEY, A.; GILFOYLE, E.; OVERSTROM, E.W.; HYTTEL, P.; WATSON, A.J. trofectoderm differentiation in the bovine embryo: characterization of a polarized epithelium. **Journal of reproduction and fertility**, [s.l.], v. 114, p. 327-339, 1988.

BARSNES, H.; VAUDEL, M. SearchGUI: A Highly Adaptable Common Interface for Proteomics Search and de Novo Engines. **J Proteome Res**. v. 17, 2552-2555, 2018

BILODEAU-GOESEELS, S.; SCHULTZ, G.A. Changes in ribosomal ribonucleic acid content within in vitro-produced bovine embryos. **Biol Reprod**. v. 56, p. 92-100, 1997.

BLEIL, J. D.; GREVE, J. M.; WASSARMAN, P.M. Identification of a secondary sperm receptor in the mouse egg zona pellucida: Role in maintenance of binding of acrosome-reacted sperm to eggs. **Developmental Biology**, s.l., v.128, p.376-385, 1988.

BURKART, D.; XIONG, B.; BAIBAKOV, B.; JIMÉNEZ-MOVILLA, M.J. Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. **Journal of Cell Biology**, s.l., v.197, p. 37–44, 2012.

CALLEBAUT, I.; MORNON, J.P.; MONGET, P. Isolated ZP-N domains constitute the N-terminal extensions of zona pellucida proteins. **Bioinformatics**, [s.l.], v. 23, n. 15, p.1871–1874, 2007.

CAMACHO, C.A.; SANTOS, G.O.; CABALLEROS, J.E.; CAZALES, N.; RAMIREZ. C.J.; VIDIGAL, P.M.P.; RAMOS, H.J.O.; BARROS, E.; MATTOS, R.C. Uterine infusion of conceptus fragments changes the protein profile from cyclic mares. **Anim Reprod,** v.17, n.4, 2020.

CAMACHO, C.A.; CABALLEROS, J.E.; CAZALES, N.; MATTOS, R.C. Endometrial Alterations After the Transfer of Embryonic Remains. **Journal of Equine Veterinary Science,** v. 66, 2018.

CAMOZZATO, G.C.; MARTINEZ, M.N.; BASTOS, H.B.A.; FIALA-RECHSTEINER, S.; MEIKLE, A., JOBIM, M.I.M.; … MATTOS, R. C. Ultrastructural and histological characteristics of the endometrium during early embryo development in mares. **Theriogenology**. 2018

CHRISTENSEN S.; ANDERSEN, C. Proteomic analysis of bovine blastocoel fluid and blastocyst cells. **Systems Biology in Reproductive Medicine**, [s.l.], v. 60, p. 127–135. 2014.

CHRISTENSON, L.K.; STOUFFER, R.L. Follicle-stimulating hormone and luteinizing hormone/chorionic gonadotropin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre- and periovulatory follicles. **The Journal of Clinical Endocrinology and Metabolism**, [s.l.], v. 82, p.2135–42, 1997.

COGNIE, Y.; BARIL, G.; POULIN, N.; MERMILLOD, P. Current status of embryo technologies in sheep and goat. **Theriogenology**, s.l., v. 59, p. 171–88, 2003.

CROSBY, I.; GANDOLFI, F.; MOOR, R. Control of Protein-Synthesis during Early Cleavage of Sheep Embryos. **Journal of reproduction and fertility**, [s.l.], v.82, p.769–775, 1988.

CROWELL, A.M.J.; WALL, M.J.; DOUCETTE, A.A. Maximizing recovery of water-soluble proteins through acetone precipitation**. Anal. Chim. Acta**. v. 796, p. 48-54, 2013.

DEMANT, M.; DEUTSCH, D.R.; FRÖHLICH, T.; WOLF, E.; ARNOLD, G.J. Proteome analysis of early lineage specification in bovine embryos. **Proteomic**, v. 15, p. 688-701, 2015.

DEMANT, M.; DEUTSCH, D.; FRÖHLICH, T.; WOLF, E.; ARNOLD, G. Proteome analysis of early lineage specification in bovine embryos. **Proteomics**, [s.l.], v.15, p. 688–701, 2015.

DEUTSCH, D.R.; FRÖHLICH, T.; OTTE, K.A.; BECK, A.; HABERMANN, F.A.; WOLF, E. e ARNOLD, G.J. Stage-specific proteome signatures in early bovine embryo development. **J Proteome Res**. v. 13, p. 4363- 4376, 2014.

ECKERT, J.J; FLEMING, T.P. Tight junction biogenesis during early development. **Biochimica et Biophysica Acta**, [s.l.], v. 1778, p. 717-728, 2008.

FÁTIMA, L.A.; EVANGELISTA, M.C.; SILVA, R.S.; CARDOSO, A.P.M.; BARUSELLI, P.S.; PAPA, P.C. FSH up-regulates angiogenic factors in luteal cells of buffaloes. **Domestic Animal Reproduction,** [s.l.], v.45, p. 224–237, 2013b.

FAMILIARI, G.; RELUCENTI, M.; HEYN, R.; MICARA, G.; CORRER, S. Three-dimensional structure of the zona pellucida at ovulation. **Microscopy Research and Technique**, s.l., v. 69, p. 415–426, 2006.

FÁTIMA, L.A.; BARUSELLI, P.S.; GIMENES, L.U.; BINELLI, M.; RENNO, F.P.; MURPHY, B.D.; PAPA, P.C. Global gene expression in the bovine corpus luteum is altered after stimulatory and superovulatory treatments. **Reproduction, Fertility and Development**, s.l., v.25, p.998–1011, 2013a.

FERRARA, N.; DAVIS-SMYTH, T. The biology of vascular endothelial growth factor. **Endocrine Reviews**, [s.l.], p.18, p.4-25, 1997.

FLEMING, T.P.; GHASSEMIFAR, M.R.; SHETH, B. Junctional complexes in the early mammalian embryo. **Seminars in Reproductive Medicine**, New York, v.18, n. 2, p.185-93, 2000.

FLEMING, T.P.; PICKERING, S.J. Maturation and polarization of the endocytotic system in outside blastomeres during mouse preimplantation development. **Journal of Embryology and Expimental Morphology**, [s.l.], v. 89, p.1 75-208, 1985.

FRASER, H.M.; WULFF, C. Angiogenesis in the corpus luteum. **Reproduction Biology Endocrinology**, [s.l.], v.10, p.1:88, 2003.

GALLI, C.; COLLEONI, S.; DUCHI, R.; LAGUTINA, I.; LAZZARI, G. Equine assisted reproduction and embryo technologies. **Animal Reproduction**, v. 10, n. 3, p. 334-343. 2018.

GANSNER, J.M.; GITLIN, J.D. Essential role for the alpha 1 chain of type VIII collagen in zebrafish notochord formation. **Dev. Dyn**. v. 237, p. 3715-3726, 2008.

GARDNER, D.K. Lactate production by the mammalian blastocyst: manipulating the microenvironment for uterine implantation and invasion? **BioEssays**. v. 37, p. 364-37, 2015.

GARDNER, D.K.; LEESE, H.J. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. **Hum. Reprod.**, v. 1, p. 25-27, 1989.

GARDNER, D.K.; WALE, P.L.; COLLINS, R.; LANE, M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. **Hum Reprod**. v. 26, p.1981-1986, 2011.

GEORGE, E.L.; GEORGES-LABOUESSE, E.N.; PATEL-KING, R.S.; RAYBURN, H.; HYNES, R.O. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. **Development**. v. 119, p. 1079-1091, 1993.

GINTHER, O.J. **Reproductive biology of the mare: basic and applied aspects**. 2ª ed. Equiservices: Madison, Wisconsin, EUA., 1992. pp 642.

GOMES de ALMEIDA, P.; PINHEIRO, G.G.; NUNES, A.M.; GONCALVES, A.B.; THORSTEINSDOTTIR, S. Fibronectin Assembly During Early Embryo Development: A Versatile Communication System Between Cells and Tissues. **Dev. Dyn**. v. 245, p. 520-535, 2016.

GUPTA, M.; JANG, J.; JUNG, J. Proteomic analysis of parthenogenetic and in vitro fertilized porcine embryos. **Proteomics**, [s.l.], v.9, p.2846–2860, 2009.

GUPTA, S.K.; BHANDARI, B. Acrosome reaction: Relevance of zona pellucida glycoproteins. **Asian Journal of Andrology**, [s.l.], v.1 3, p. 97–105, 2011.

GUPTA, S.K.; CHAKRAVARTY, S.; SURAJ, K.; BANSAL, P.; GANGULY, A.;

JAIN, M.K.; BHANDARI, B. Structural and functional attributes of zona pellucida glycoproteins. **Society of Reproduction and Fertility Supplement**, [s.l.], v. 63, p. 203-16, 2007.

HEDRICK, J.L.; WARDRIP, N.J. On the macromolecular composition of the zona pellucida from porcine oocytes. Developmental Biology, [s.l.], v. 121, p. 478–488, 1987.

HOUGHTON, F.D.; THOMPSON, J.G.; KENNEDY, C.J.; LEESE, H.J. Oxygen and energy metabolism of the early mouse embryo. **Mol Reprod Develop**. v. 44, p.476-485, 1996.

HUNTER, R.H. Components of oviduct physiology in eutherian mammals. **Biological reviews of the Cambridge Philosophical Society**, [s.l.], v.87, p. 244– 255, 2012.

HYNES, R.O Integrins: versatility, modulation, and signaling in cell adhesion. **Cell**, v. 69, p.11-25, 1992.

JACOB, J.C.F.; HAAG, K.T.; SANTOS, G.O.; OLIVEIRA, J.P.; GASTAL, M.O.; GASTAL, E.L. Effect of embryo age and recipient asynchrony in pregnancy rates in a commercial equine embryo transfer program. **Theriogenology**, v. 7, p. 1159–66, 2012.

JEDRUSIK, A. Making the first decision: lessons from the mouse. **Reproductive Medicine and Biology**, [s.l.], v. 14, p. 135–150, 2015.

JENSEN, P.; GRONDAHL, M.; BECK, H.; PETERSEN, J.; STROEBECH, L.; KALLI, A., SMITH, G.T., SWEREDOSKI, M.J.; HESS, S. Evaluation and optimization of mass spectrometric settings during data-dependent acquisition mode: focus on LTQ-Orbitrap mass analyzers. **J Proteome Res**. v. 12, p. 3071-3086, 2013.

KANEHISA, M., SATO, Y. e MORISHIMA, K. BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. **J Mol Biol**. v. 428, p. 726-731, 2016.

KATZ-JAFFE, M.G.; MCREYNOLDS, S. Embryology in the Era of Proteomics**. Fertil Steril.,** v. 99, p. 1073-1077, 2013.

KATZ-JAFFE, M.G.; GARDNER, D.K.; SCHOOLCRAFT, W.B. Proteomic analysis of individual human embryos to identify novel biomarkers of development and viability. **Fertil Steril.** v. 85, p. 101-107, 2006.

KHACHO, M.; CLARK, A.; SVOBODA, D.S.; AZZI, J.; MACLAURIN, J.G.; MEGHAIZEL, C.; SESAKI, H.; LAGACE, D.C.; GERMAIN, M.; HARPER, M.E. Mitochondrial dynamics impacts stem cell identity and fate decisions by regulating a nuclear transcriptional program. **Cell Stem Cell.** v. 19, p. 232-247, 2016.

KLEIN, C.; TROEDSSON, M.H. Transcriptional profiling of equine conceptus reveals new aspects of embryo-maternal communication in the horse. **Biol Repro**, v. 84, p. 872-85, 2011.

KÖPPEN, W.; GEIGER, R. Klimate der Erde. Gotha: Verlag Justus Perthes. Wall-map 150cmx200cm, 1928.

KREMER, L.P.M.; LEUFKEN, J.; OYUNCHIMEG, P.; SCHULZE, S; FUFEZAN, C. Universal Python Module Combining Common Bottom-Up Proteomics Tools for Large-Scale Analysis. **J. Proteome Res**., v. 15, p. 788-794, 2016.

KRISHER, R.L.; PRATHER, R.S. A role for the warburg effect in preimplantation embryo development: metabolic modification to support rapid cell proliferation. **Mol Reprod Dev**. v. 79, p. 311-320, 2012.

KUROSAKA, S.; KASHINA, A. Cell biology of embryonic migration. **Birth Defects Res C Embryo Today**. v. 84, p. 102-122, 2008.

LATHAM, K.E.; SCHULTZ, R.M. Embryonic genome activation. **Front Biosci.** v.1, D748-59, 2001.

LEESE, H.J. Metabolism of the preimplantation embryo: 40 years on. **Reproduction**. v. 143, p. 417-427, 2012.

LEESE, H.J. The formation and function of oviduct fluid. **Journal Reproduction Fertility**, [s.l.], v. 82, p. 843-856, 1988.

LEFIEVRE, L.; CONNER, S.J.; SALPEKAR, A.; OLUFOWOBI, O.; ASHTON, P.; PAVLOVIC, B.; LENTON, W.; AFNAN, M.; BREWIS, IA.; MONK, M.; HUGHES, D.C.; BARRATT, CL. Four zona pellucida glycoproteins are expressed in the human. **Human Reproduction**, [s.l.], v. 19, p. 1580–1586, 2004.

LEPREVOST, F.V.; VALENTE, R.H.; LIMA, D.B.; PERALES, J. PepExplorer: a similarity-driven tool for analyzing de novo sequencing results. Mol. **Cell. Proteomics**. v. 13, p. 2480-2489, 2014.

LI, S.; WINUTHAYANON, W. Oviduct: roles in fertilization and early embryo development. **Journal of Endocrinology**, [s.l.], v. 232, p.1-26, 2017.

LINAS, K.K.; LASH, J.W. A role for fibronectin in the migration of avian precardiac cells. II. Rotation of the heart-forming region during different stages and its effects. **Dev. Biol.** v. 129, p. 324-3, 1988.

LITSCHER, E.S.; WILLIAMS, Z.; WASSARMAN, P. M. Zona pellucida glycoprotein ZP3 and fertilization in mammals. **Molecular Reproduction and Development,** [s.l.], v. 76, p. 933-941, 2009.

LIU, X.; WU, H.; BYRNE, M.; JEFFREY, J.; KRANE, S.; JAENISCH, R. A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. **J. Cell. Biol**. v. 130, p. 227-237, 1995.

LÓPEZ-LÁZARO, M. The warburg effect: why and how do cancer cells activate glycolysis in the presence of oxygen? **Anti-Cancer Agent ME**. v. 3, p. 305-312, 2008.

MATHIEU, J.; RUOHOLA-BAKER, H. Metabolic remodeling during the loss and acquisition of pluripotency. **Development**., v. 144, p. 541-551, 2017.

MEIRA, C.; HENRY, M. Evolution of two non-surgical equine embryo transfer methods**. J.Reprod. Fertil**., v. 44, p. 712-713, 1991.

MEMILI, E.; DOMINKO, T.; FIRST, N.L. Onset of transcription in bovine oocytes and preimplantation embryos. **Molecular Reproduction and Development**, [s.l.], v.51, p.36-41, 1998.

MI, H., HUANG, X., MURUGANUJAN, A., TANG, H., MILLS, C., KANG, D. e THOMAS, P.D. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. **Nucleic Acids Res.**, v. 45, p. D183-D189, 2017.

MOURA, C.E.B.; CAMPOS, D.B.; PEREIRA, F.T.V.; OLIVEIRA, M.F.; MIGLINO, M.A.; PAPA, P.C. Microvascularization and capillary density of corpus luteum from superovulated and control buffalo cows. **Acta Microscopica**, [s.l.], v.12, p. 83–4, 2003.

MUDUNURI, U.; CHE, A.; YI M.; STEPHENS, R.M. bioDBnet: the biological database network**. J. Bioinform**. v. 25, p. 555-556, 2009.

NISWENDER, G.D.; JUENGEL, J.L.; SILVA, P.J.; ROLLYSON, M.K.; MCINTUSH, E.W. Mechanisms controlling the function and life span of the corpus luteum. **Physiological Reviews,** [s.l.], v. 80, p.1–29, 2000.

PAPA, P.C.; MOURA, C.E.B.; ARTONI, L.P.; FATIMA, L.A.; CAMPOS, D.B.; MARQUES, J.E.B.; BARUSELLI, P.S.; BINELLI, M.; PFARRER, C.; LEISER, R. VEGF. system expression in different stages of estrous cycle in the corpus luteum of non-treated and superovulated water buffalo. **Domestic Animal Endocrinology**, [s.l.], v.33, p.379–389, 2007.

REIMAND, J; ARAK, T.; ADLER, P.; KOLBERG, L. g:Profiler—a web server for functional interpretation of gene lists. **Nucleic Acids Res**. v. 44, W83-W89, 2016.

REYNOLDS, LP.; GRAZUL-BILSKA, A.T.; REDMER, D.A. Angiogenesis in the corpus luteum. **Endocrine**, [s.l.], v.12, n. 1, p.1-9, 2000.

RICHOUX, V.; DARRIBERE, T.; BOUCAUT, J.C.; FLECHON, J.E.; THIERY, J.P. Distribution of fibronectins and laminin in the early pig embryo. **Anat. Rec**. v. 233, p. 72-81, 1989.

RIGOGLIO, N.N.; FÁTIMA, L.A.; HANASSAKA, J.Y.; PINTO, G.L.; MACHADO, A.S.; GIMENES, L.U.; BARUSELLI, P.S.; RENNÓ, F.P.; MOURA, C.E.; WATANABE, I.S.; PAPA, P.C. Equine chorionic gonadotropin alters luteal cell morphologic features related to progesterone synthesis. **Theriogenology**, [s.l.], v.79, p. 673-679, 2013.

RIVERA, R.M.; RINAUDO, P. Bovine preimplantation embryo development is affected by the stiffness of the culture substrate. **Mol. Reprod. Dev**., v. 80, p. 184, 2013.

ROZARIO, T.; DESIMONE, D.W. The extracellular matrix in development and morphogenesis: a dynamic view. **Dev Biol.** v. 341, p. 126-140, 2010.

SAONCELLA, S., ECHTERMEYER, F., DENHEZ, F., NOWLEN, J.K., MOSHER,

D.F., ROBINSON, S.D., HYNES, R.O. e GOETINCK, P.F. Syndecan-4 signals cooperatively with integrins in a Rho-dependent manner in the assembly of focal adhesions and actin stress fibers. **Proc. Natl. Acad. Sci. U. S. A**. v. 96, p. 2805-2810, 1999.

SCHWARTZ, M.A.; SHATTIL, S.J. Signaling networks linking integrins and rho family GTPases. **Trends Biochem. Sci**. 25, 388-391, 2000.

SHIKONE, T.; YAMOTO, M.; NAKANO, R. Follicle-stimulating hormone induces functional receptors for basic fibroblast growth factor in rat granulosa cells. **Endocrinology, Wakayama**, v. 131, n. 3, p. 1063-1068, 1992.

SHIOKAWA, S.; YOSHIMURA, Y.; NAGAMATSU, S.; SAWA, H.; HANASHI, H., KOYAMA, N., KATSUMATA, Y., NAGAI, A. e NAKAMURA, Y. Function of β1 Integrins on Human Decidual Cells during Implantation. **Biol. Reprod.** v. 54, p. 745-752, 1996.

STRINGFELLOW, D.A; GIVENS, M.D. A procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures In: Manual of the International Embryo Transfer Society, editor. **International Embryo Transfer Manual**. 4th Edition. Savoy, IL, USA: IETS Publish. v. 155, 2009.

SUMIYOSHI, H.; MOR, N.; LEE, S.Y.; DOTY, S.; HENDERSON, S.; TANAKA, S., YOSHIOKA, H.; RATTAN, S.; RAMIREZ, F. Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX-rich basement membrane zone. **J. Cell. Biol**., v. 166, p. 591-600, 2004.

SUPEK, F.; BOŠNJAK, M.; ŠKUNCA, N.; ŠMUC, T. REVIGO summarizes and visualizes long lists of gene ontology terms. **PLoS One**. V. 6, e21800, 2011.

SWEGEN, A.; GRUPEN, C.G.; GIBB, Z.; BAKER, M.A.; DE RUIJTER-VILLANI; M., SMITH; N.D.; STOUT, T.A.E.; AITKEN, R.J. From peptide masses to pregnancy maintenance: a comprehensive proteomic analysis of the early equine embryo secretome, blastocoel fluid, and capsule. **Proteomics**., v. 17, p. 17-18, 2017.

SWEGEN, A.; GRUPEN, C; GIBB, Z.; BAKER, M.; RUIJER-VILLANI M.; SMITH, N.; STOUT, T.; AITKEN, R. From peptide masses to pregnancy maintenance: a comprehensive proteomic analysis of the early equine embryo secretome, blastocoel fluid, and capsule. Proteomics, [s.l.], v. 17, p. 1600-1633, 2017.

SZKLARCZYK, D.; MORRIS, J.H.; COOK, H.; KUHN, M. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. **Nucleic Acids Res**. v. 45, D362-D368, 2017.

TANCA, A.; PALOMBA, A.; DELIGIOS, M.; CUBEDDU, T.; FRAUMENE, C., BIOSA, G.; PAGNOZZI e D.; ADDIS, M.F. Evaluating the impact of different sequence databases on metaproteome analysis: insights from a lab-assembled microbial mixture. **PLoS One**, v. 8, e82981, 2013.

TESARIK, J; TRAVNIK, A.P. Zona pellucida resistance to sperm penetration before the completion of human oocyte maturation. **Reproduction, Fertility and Development**, [s.l.], v. 83, p. 487-495, 1988.

THOMPSON, J.G.; SHERMAN, A.N.; ALLEN, N.W.; MCGOWAN, L.T.; TERVIT, H.R. Total protein content and protein synthesis within pre-elongation stage bovine embryos. **Molecular Reproduction and Development**, Ribeirão Preto, v. 50, p.139-45, 1998.

TRINH, L.A.; STAINIER, D.Y. Fibronectin regulates epithelial organization during myocardial migration in zebrafish. **Dev. Cell**. v. 6, p. 371-382, 2004.

TROUNSON, A.O.; PUGH, A.; MOORE, N.W. Studies on the zona pellucida of sheep ova: immune lysis and localization of antibodies in ova with and without zonae. Australian journal of biological sciences, [s.l.], v. 29, p. 237-244, 1976.

UNIPROT CONSORTIUM. UniProt: a worldwide hub of protein knowledge**. Nucleic Acids Res**. v. 47, D506–D515, 2019.

URTIGA, Bruno da Costa. A utilização de laserterapia no tratamento de feridas em equinos. 2022.

VARUM, S.; RODRIGUES, A.S.; MOURA, M.B.; MOMCILOVIC, O.; EASLEY, C.A.T.; RAMALHO-SANTOS, J.; VAN HOUTEN, B.; SCHATTEN, G. Energy metabolism in human pluripotent stem cells and their differentiated counterparts. **PLoS One**., v. 6, e20914, 2011.

VELEZ, I.; RAMIREZ, M;, CHICA, A.; URREGO, R.; MOURA, A.; VASCONCELOS, F.; NAGANO, C.; RODRIGUEZ-VILLAMIL, P.; JIMENEZ-ESCOBAR, C.; ZAMBRANO-VARON, J. Proteome of bovine cumulus cells as related to oocyte morphology and in vitro embryo production. **Reprod Fert Develop**., v. 29, p. 183-183, 2016.

VIANA, A.G.A.; MARTINS, A.M.A.; PONTES, A.H.; FONTES, W.; CASTRO, M.S.; RICART, C.A.O.; SOUSA, V.; KAYA, A.; TOPPER, E.; MEMILI, E; MOURA, A.A. Proteomic landscape of seminal plasma associated with dairy bull fertility. **Sci. Rep.**, v. 8, 16323, 2018.

WARBURG, O. On the origin of cancer cells. Science. v. 123, p. 309–314, 1956.

WASSARMAN, P. M.; MORTILLO, S. Structure of the mouse egg extracellular coat, the zona pellucida. International Review of Cytology, [s.l.], v.130, p. 85–110, 1991.

WASSARMAN, P.M. Zona Pellucida Glycoproteins. Journal of Biological Chemistry, [s.l.], v.283, p. 24285–24289, 2008.

WATSON A. The cell biology of blastocyst development. Molecular Reproduction and Development, [s.l.], v.33, p.492-504, 1992.

WATSON, A.; BARCROFT, L. Regulation of blastocyst formation. Frontiers in Bioscience, [s.l.], v.6, p. 708-730, 2001.

WATSON, A.J.; NATALE, D.R.; BARCROFT, L.C. Molecular regulation of blastocyst formation. Animal Reproduction Science, [s.l.], v.82–83, p. 583-592, 2004.

WENSTRUP, R.J.; FLORER, J.B.; BRUNSKILL, E.W.; BELL, S.M.; CHERVONEVA, I.; BIRK, D.E. Type V collagen controls the initiation of collagen fibril assembly. **J. Biol. Chem.**, v.279, p.53331-53337, 2004.

WILSHER, S.; LEFRANC, A.C.; ALLEN, W.R. The effect of an advanced uterine environment on embryonic survival in the mare. **Equine Vet J**, v. 44, p. 432–439, 2012.

WOAD, K.J; ROBINSON, R.S. Luteal angiogenesis and its control. **Theriogenology**, [s.l.], v.86, p.221–228, 2016.

YANAGIMACHI, R. Mammalian sperm acrosome reaction: where does it begin before fertilization?. **Biology of Reproduction**, [s.l.], v. 85, p. 4-5, 2011.

ZAGRIS, N. Extracellular matrix in development of the early embryo. **Micron**. v. 36, p. 427-438, 2001.

ZHANG, J.; XIN, L.; SHAN, B; CHEN, W. PEAKS DB: de novo sequencing assisted database search for sensitive and accurate peptide identification. **Mol Cell Proteomics**. v. 11, p. 1110-1118, 2012.

ZHOU, X.; LIAO, W.J.; LIAO, J.M.; LIAO, P.; LU, H. Ribosomal proteins: functions beyond the ribosome. **J Mol Cell Biol**. v. 7, p. 92-104, 2015.