LETÍCIA SIGNORI DE CASTRO

Estudo do perfil proteico e epigenético do núcleo espermático bovino com influência na produção *in vitro* de embriões

São Paulo
2018

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

Departamento:
Reprodução Animal

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Profa. Dra. Mayra Elena Ortiz D'Ávila Assumpção

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CERTIFICADO

Certificamos que a proposta intitulada "Estudo do perfil proteico e epigenético do núcleo espermático bovino com influência na produção in vitro de embriões", protocolada sob o CEUA nº 1291280115 (ID 001608), sob a responsabilidade de **Mayra Elena Ortiz D' Ávila Assumpção** *e equipe; Letícia Signori de Castro* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 13/10/2015.

We certify that the proposal "Study of protein and epigenetic profile of bovine sperm nucleus with impact on in vitro embryo production", utilizing 10 Bovines (10 males), protocol number CEUA 1291280115 (ID 001608), under the responsibility of **Mayra Elena Ortiz D' Ávila Assumpção** *and team; Letícia Signori de Castro* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/13/2015.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de Fev/201 a Fev/201 Área: Reprodução Animal

Origem:	Amostras biológicas estocadas						
Espécie:	Bovinos	sexo:	Machos	idade:	2 a 5 anos	N:	10
Linhagem:	Bos taurus			Peso:	400 a 500 kg		

Local do experimento: Laboratório de Biologia do Espermatozoide

São Paulo, 28 de novembro de 2018

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretária
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FOLHA DE AVALIAÇÃO

Nome: CASTRO, Letícia Signori de

Título: Estudo do perfil proteico e epigenético do núcleo espermático bovino com influência na produção *in vitro* de embriões

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Prof. Dr. _____

Instituição: _____ Julgamento: _____

Prof. Dr. _____

Instituição: _____ Julgamento: _____

Prof. Dr._____

Instituição: _____ Julgamento: _____

Prof. Dr. _____

Instituição: _____ Julgamento: _____

Dedico esta tese aos meus pais, que me inspiram e incentivam a busca constante pelo conhecimento.

Meus sinceros agradecimentos...

Fazer doutorado não é fácil. São 4 anos se dedicando exclusivamente para uma tese que enquanto está sendo construída nunca parece ser o bastante, o instinto científico quer sempre mais. Mas ao coloca-la no papel, você se depara quão imenso foi o seu trabalho. Aqui ficam meus sinceros agradecimentos a todas as pessoas envolvidas direta ou indiretamente, que contribuíram com parte do seu tempo e experiência para que eu fosse capaz de concluir mais essa etapa.

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"Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less."

Marie Curie

RESUMO

CASTRO, L. S. de. **Estudo do perfil proteico e epigenético do núcleo espermático bovino com influência na produção in vitro de embriões**. [Study of protein and epigenetic profile of bovine sperm nucleus with impact on in vitro embryo production] 2018. 98 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

A produção *in vitro* de embriões (PIVE) vem se destacado como biotecnologia reprodutiva nos rebanhos bovinos, porém ainda com algumas limitações. A diferença de fertilidade entre touros é uma delas e dentre os atributos espermáticos que podem impactar na PIVE, pouco se sabe sobre a composição do núcleo espermático. Sendo assim, este trabalho teve como objetivo principal identificar quais aspectos nucleares do espermatozoide caracterizam a diferença nos índices de PIVE. Para tanto, o estudo foi dividido em 3 experimentos nos quais foi utilizado o banco de dados da empresa In Vitro Brasil para seleção de touros de alta (AF) e baixa (BF) taxa de desenvolvimento embrionário (taxa de blastocisto/taxa de clivados; n=5 touros/grupo). No primeiro experimento, foi realizado a avaliação da cromatina para susceptibilidade ao desafio ácido (SCSA modificado), deficiência de protaminação (CMA3) e fragmentação do DNA (COMETA). Apenas a deficiência de protaminação apresentou diferença entre os grupos, entretanto a porcentagem de células positivas para o CMA3 foi baixa, não sendo possível atribuir a esta alteração as diferenças de fertilidade encontradas. No segundo experimento, foi realizada a avaliação de proteômica do núcleo espermático destes touros. Para tanto, as amostras foram incubadas com detergente CTAB para remoção de parte da membrana e cauda, extração das proteínas, digestão com tripsina e análise por espectrometria de massas. A quantificação das proteínas foi avaliada pelo índice iBAQ obtido após a identificação dos peptídeos e a média deste índice comparado entre os grupos para identificação das proteínas diferencialmente expressas. Foram identificadas 196 proteínas, 21 diferencialmente expressas, sendo 17 hiperexpressas no grupo AF e 4 hiperexpressas no grupo BF. Dentre elas, proteínas relacionadas ao processo de espermatogênese, fecundação e motilidade. No terceiro experimento, com objetivo de identificar as marcações epigenéticas relacionadas com a diferença de fertilidade *in vitro*, foi realizado a avaliação das estruturas de nucleossomos utilizando a enzima MNase para posteriormente utilização na técnica de imunoprecipitação das marcações de

histona. Entretanto, não foi identificado a presença de nucleossomos no DNA espermático bovino, inviabilizando análises posteriores. Sendo assim, a avalição da metilação de DNA foi escolhida como marcação epigenética para ser estudada. Neste caso, foi utilizada a técnica de RRBS (*reduced representation of bisulfite sequencing*) para identificar as citosinas diferencialmente metiladas (CDM) entre os grupos AF e BF. Foram identificadas 440 CDM, sendo 327 hipometiladas e 113 hipermetiladas no grupo AF. Além disto, 184 genes contendo estas CDM foram identificados, entretanto nenhum processo biológico foi enriquecido por estes genes. Foi possível concluir com este trabalho que a fertilidade *in vitro* pode ser considerada de caráter multifatorial, sendo que alterações pequenas de integridade de cromatina não afetam diretamente, já proteínas relacionadas a espermatogênese, motilidade e fecundação podem ter relação com o fenótipo fertilidade estudado. Dentre as marcações epigenética estudadas, as estruturas de nucleossomo pareceram estar ausentes no espermatozoide bovino, e a metilação de DNA não apresenta impacto significativo no perfil de fertilidade.

Palavras-chave: Espermatozoide. Fertilidade. Proteômica. Epigenética.

ABSTRACT

CASTRO, L. S. de. **Study of protein and epigenetic profile of bovine sperm nucleus with impact on in vitro embryo production**. [Estudo do perfil proteico e epigenético do núcleo espermático bovino com influência na produção in vitro de embriões]. 2018. 98 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

In vitro embryo production (IVP) has been highlighted as reproductive biotechnique in bovine herds, but still with some limitations. The fertility difference between bulls is one of them and among the sperm attributes that can impact on IVP, little is known about the composition of the bovine sperm nucleus. Therefore, this work had as main goal to identify which sperm nuclear aspects characterize the difference in the IVP index. Then, the study was divided in 3 experiments in which the database of the company In Vitro Brasil was used for selection of bulls with high (HF) and low (LF) embryo development rate (blastocyst rate/cleavage rate; n=5 bulls/group). In the first experiment, chromatin analysis of susceptibility to acid denaturation (modified SCSA), protamine deficiency (CMA3) and DNA fragmentation (COMET assay) were performed. Only protamine deficiency presented difference between groups, however the percentage of CMA3 positive cells was low and it was not possible to attribute to this alteration the fertility difference between groups. In the second experiment, proteomic analysis of the sperm nucleus of these bulls was performed. For that, samples were incubated with CTAB detergent to remove part of the membrane and tail, submitted to protein extraction, trypsin digestion and mass spectrometry analysis. The amount of the proteins was evaluated by the iBAQ index obtained after the identification of peptides and the average of this index compared to identify the differentially expressed proteins. In this experiment, 196 proteins were identified, 21 differentially expressed, being 17 overexpressed in the HF and 4 overexpressed in LF group. Among them, proteins related to the spermatogenesis, fertilization and motility. In the third experiment, in order to identify the epigenetic marks related to *in vitro* fertility differences it was performed the evaluation of the nucleosome structures using the MNase enzyme to later utilization of immunoprecipitation of the histone marks technique. However, the presence of nucleosomes in bovine sperm DNA was not identified, unfeasible further analysis. Thus, the evaluation of DNA methylation was chosen as epigenetic mark to be studied. In this case, the RRBS (reduced representation of bisulfite sequencing) technique was used to identify the differentially

methylated cytosines (DMC) between the two groups. In this experiment, it was identified 440 DMC, being 327 hypomethylated and 113 hypermethylated in HF group. In addition, 184 genes containing these DMCs were identified, however no biological process enrichment was identified with these genes. In conclusion, this study indicated that *in vitro* fertility characterization can be multifactorial; small changes in chromatin integrity do not directly affect, but proteins related to spermatogenesis, motility and fertilization may be related to the fertility phenotype studied. Among the epigenetic marks studied, nucleosome structures were absent in bovine spermatozoa, and DNA methylation did not have a significant impact on the fertility profile.

Keywords: Spermatozoa. Fertility. Proteome. Epigenetic.

CONTENTS

1 INTRODUCTION

The worldwide food demand should increase until 2050 around 70% according to ONU projections (FAO, 2009). In livestock, many countries are already at the limit of production. Australia, for example, has increased by 0.9% per year in the carcass weight of cattle since 1980; the United States 0.8%, while Brazil still has a rate of 0.4% per year. These numbers show that Brazil has great possibilities to advance in this area (DE ZEN AND SANTOS, 2015). The beef market demand has evolved in the way that only those producers that reach high levels of productivity will remain competing in the market. For that, the production gain will only be achieved combining genetic breeding and reproductive biotechniques improvement.

Between reproduction biotechniques available for cattle production, *in vitro* embryo production (IVP) gains market every year and Brazil leads the world ranking (PERRY, 2017). However, no biotechnique is perfect; IVP still have some issues that limits the process with an embryo production rate around 30% (GALLI et al., 2014; LONERGAN et al., 2016). When thinking about how to improve IVP results, many researchers focus their studies in the oocyte, because they consider the female gamete as the main contributor for initial embryo development; or the *in vitro* system, which still has some differences regarding to culture environment when compared to the physiological process. Certainly, they are important factors, but there is another protagonist not only responsible for delivery half of genome, but also contributes to start and sustain the embryo development – the sperm.

When looking to the overall IVP, it is possible to see clearly the presence of bull effect with individuals that produce more blastocyst than other, independently of oocyte donor or the *in vitro* system. Then, considering that IVP process has the same production cost regardless of animal donors used, embryos derived from bulls with lower blastocyst production will cost more than the embryos produced by the average or higher embryo production bulls. Therefore, identify and characterize bulls with low *in vitro* fertility is an easy and cheap way to improve IVP without necessary changing or including new steps in the process.

In the race to identify which sperm traits impair or improve bull fertility, many studies tried to describe the gold standard of sperm analysis. However, sperm is a complex cell and the uniquely chromatin compaction turns difficult to access and to understand the paternal aspects during embryo development. In addition, the breed

selection over the years turns the bovine species well improved compared to others like human and mice, requiring some adaptations in the common approaches, to study the spermatozoa. Still, there are different aspects of bovine sperm chromatin that have not been elucidate yet, like the sperm nuclear proteins and the epigenetic profile, that could explain different embryo production among bulls.

2 LITERATURE REVIEW

2.1 THE SMALLEST CELL IN THE BODY: SPERM HISTORY

In 1677, Anton van Leeuwenhoek was the first person to see a sperm cell in the microscope. He examined his own ejaculate and discovered the smallest cell in the body, publishing his results about the “animalcules” that he found and open the doors to a new field of sperm biology (VAN LEEUWENHOEK, 1678). Before that, the creation of life disagreed between scientists. There were two theories: The Preformationism, which believed the organisms develop from miniature versions, and the Epigenesis, which believed that each organism was gradually formed. Hippocrates (470-350 BC) believed that the generation took place with equal contribution of two kinds of semen, the male sperm and female menstrual blood, supported by the Preformationism theory. While Aristotle published around 350 BC the *Generatione Animalium* where he described the theory of reproduction mechanism in many species and observed that the organs develop gradually in the embryo, agreeing with the Epigenesis theory. He believed that only the male semen or “seed” contributes to form the fetus, imprinting in the form provided by the menstrual blood from the female (revised by COBB, 2012).

Years later, around 1500, theories about fertilization site and how reproductive organs contribute to generate a new organism started to show up, after human and animal dissections, confirming Aristotle theory. In 1651, Willian Harvey published *Exercitationes de generatione animalium*, in which he described the egg as fundamental for life generation, and not the sperm. In 1677, Nicolaas Hartsoeker, a student of van Leeuwenhoek, postulated that the sperm carried a tiny man inside, coming again the Preformationism theory. After that, van Leeuwenhoek confirmed the presence of “animalcules” structures with tail on the microscope and concluded that sperm was like a seed, and the female track just provided the nutrition soil for the new life (revised by CLARKE, 2006).

However, the function of sperm for the generation of an organism was not clear. Only in 1784, Lazarro Spallanzani published the first successful artificial insemination in the dog, after evidences that physical contact between the sperm and the egg is necessary to embryo development. In 1824, Jean-Louis Prevost and Jean-Baptiste Dumas affirmed that sperm was the active agent of fertilization and they proposed that sperm entered inside the egg to form the embryo. In 1840, Rudolph Albert von Köllicker

described the formation of spermatozoa in the testis. With the advance of staining and microscopy during 19th century, studies started to show the different shapes of sperm cell between species, including the description of Sertoli cells by Enrico Sertoli and the interstitial cells of testis by Franz Leydig's, who gave the name for these cells in the testis. The discovery of seminiferous cycles occurred around 1950. Yves Clermont and collaborators described the pattern of how germ cells are organized in the seminiferous tubules, and identified the cell stages from spermatogonia to mature sperm, detailing the kinetic of spermatogenesis in different species (revised by LONERGAN, 2018).

2.2 THE BIGGEST MORPHOLOGICAL CHANGE: SPERMATOGENESIS

The sperm suffers one of the most significant morphology changes known in biology, and the process to acquire its specific shape is divided into two stages: spermatogenesis and spermiogenesis. The spermatogenesis started with spermatogonia, the sperm stem cell, which suffers changes in its shape, switching the round and global form to the typical sperm form. During this process, mitosis and meiosis occur to self-renew germ stem cells or to divide the paternal DNA in half, starting the differentiation process respectively (ZINI; AGARWAL, 2011). Dramatically changes in the nuclear shape occurs to allow the paternal DNA to fit inside the tinny sperm head. This exclusively sperm head shape, which is 1/13th of the size of an oocyte nucleus, acquires a transcriptionally inactive state, making its DNA protected against nucleases, oxidative stress, and any possible DNA damage while passing through female tract (revised by JOHNSON et al., 2010).

On the contrary of oocyte that carries cell machinery, RNA and proteins enough to sustain the first cell cycles before embryo genome activation, sperm during spermatogenesis need to selectively eliminate organelles that are not necessary during sperm transport and remodeling sperm nucleus to protect paternal DNA. Then, Golgi complex changes its shape and enzymatic content to form the acrosome, vesicle essential for fertilization. Centrioles and microtubes migrate to the opposite side to form the flagellum and mid-piece cover by mitochondria that will produce ATP during sperm transport. Losing almost all the cytoplasm and organelles, spermatozoa is considered a compact and quiescent cell, carrying exclusively paternal DNA and the oocyte the main protagonist to sustain early embryo development.

2.3 SPERM NUCLEAR REMODELING DURING SPERMATOGENESIS

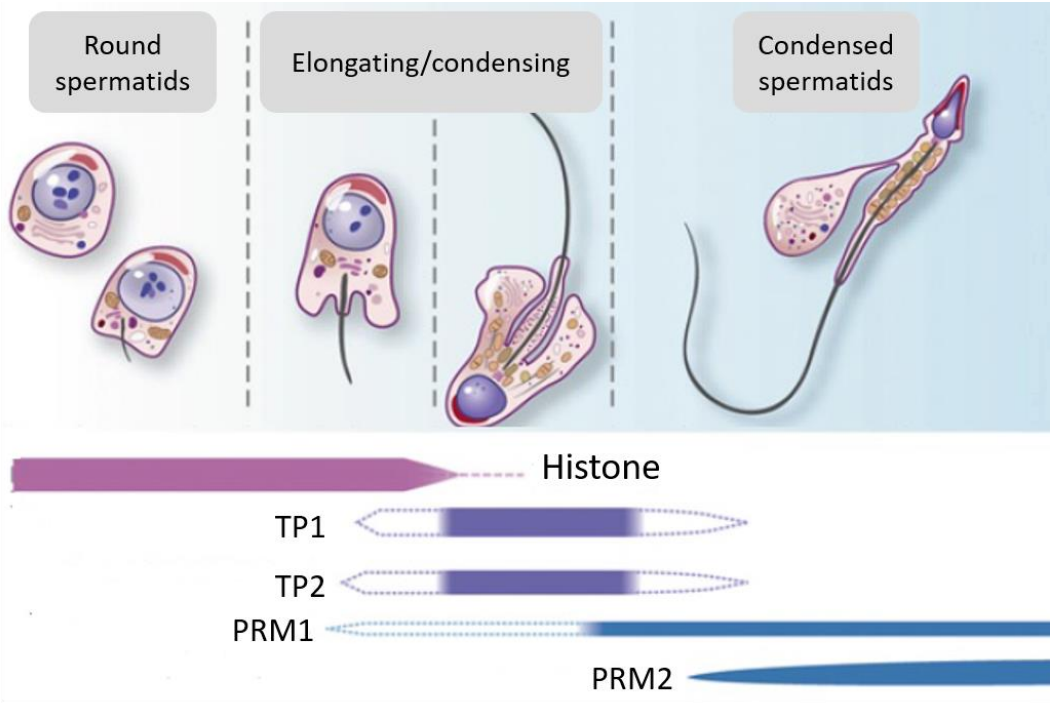
Mature sperm is considered as a transcriptional inactive cell, once it loses most part of the machinery necessary to transcribe RNA and synthesize proteins during spermatogenesis and the chromatin acquires a quiescent feature due to the tightly packed DNA. In this new shape, the haploid DNA will fit inside the sperm head and will be protect during the transport inside the female tract. The protein responsible for this high level of compaction is protamine.

2.3.1 Histone to protamine exchange

As the cell proceeds though meiosis, several DNA-binding proteins are synthesized. They are testis specific histones and appears in early stages of spermatogenesis (CHURIKOV; ZALENSKAYA; ZALENSKY, 2004). After meiosis, most histones are replaced by three transition proteins (TP1, TP2 and TP4), that remained attached to DNA for a short period. By this point, the chromatin begins to condensate progressively from the apical to caudal direction (OKO et al., 1996). The transition proteins help to prevent the DNA to bend, initiate the termination of gene transcription by TP2 binding to CpG sites and facilitate the repair of DNA strand break (CARON; VEILLEUX; BOISSONNEAULT, 2001; PRADEEPA; RAO, 2007).

As the final chromatin organization step, the transition proteins are replaced by protamine in spermatids (Figure 1). Protamine is a small protein rich in cysteine, basic amino acids and positively charged. There are two variants called protamine 1 (PRM1) and protamine 2 (PRM2). In general, the sperm DNA is mainly packaged by protamine, being PRM1 expressed in all mammals (OLIVA; DIXON, 1991) and PRM2 found only in human and mice (CARRELL; EMERY; HAMMOUD, 2007). However, in human sperm, around 10 to 15% of DNA remains attached to histones (CARRELL; HAMMOUD, 2010) and recently studies try to understand the hole of these retained histones on sperm function and embryo development.

Figure 1. Histone to protamine transition.



Legend: Histones are replaced by transition proteins (TP1 and TP2) in the round spermatids during the elongating and condensing phase. Protamine 1 and 2 (PRM1 and PRM2 respectively) present at final stages of elongating and in the condensed spermatids (ZINI; AGARWAL, 2011 modifided).

Sperm nuclear proteins, besides structuring the chromatin and compacting the genetic material, have chemical alterations that personalize the mode of association with the genetic material. This association between DNA and proteins is one of the main mechanisms of gene expression control, called epigenetics (JAENISCH; BIRD, 2003).

2.3.2 Epigenetic profile establishment

Epigenetics is the study of heritable changes in gene expression not related to DNA bases alterations or mutations. These changes can activate or silence specific genes through two main pathways: histones modifications and DNA methylation (GOLDBERG; ALLIS; BERNSTEIN, 2007). Recently, both have been described and characterized in sperm in attempt to understand the epigenetic male effect on embryo development.

2.3.2.1 Histone modifications

As mentioned before, part of sperm DNA remains attached to a few histones as nucleosome structure. Nucleosome is assembled from two H3/H4 and two H2A/H2B histone-fold heterodimers wrapping 145 to 147 bp DNA (MCGINTY; TAN, 2015). Its structure serves as signaling hub for chromatin-templated processes by providing a scaffold for chromatin enzymes. This template is a combination of post-translational modifications (PTMs) in the histones tails that controls the recruitment of chromatin enzymes to start gene transcription (TAVERNA et al., 2007). These PTMs can be acetylation, methylation, phosphorylation, ubiquitination and many others modifications that promote the activation or silencing depending on which histone and amino acid residue they occur (CAMPOS; REINBERG, 2009).

So far, it is not clear the function of retained histones in sperm nucleus. They could be product of an inefficient protamine replacement, being randomly retained over the genome or they could be enriched in specific genes. Hammoud and colleagues (2009) demonstrated that nucleosome are enriched at loci of developmental genes like imprinted genes and microRNA clusters, HOX gene and the promoters of stand-alone developmental transcription and signaling factors in human sperm. After that, many studies came out using immunoprecipitation and DNA sequencing (ChIP-Seq) approach to describe the localization of nucleosome and histones modifications in sperm DNA, mainly in human and mice.

However, studies indicate two distinct lines for histones in sperm: One of them agrees with Hammoud et al (2009) findings, confirming the nucleosome and retained histones overrepresented in promoter regions and exons (ERKEK et al., 2013), enriched around transcription start sites (TSSs) (BRYKCZYNSKA et al., 2010) or gene regulatory regions, including many promoter sequences and sequences recognized by CCCTC-binding factor (CTCF) (ARPANAHI et al., 2009). On the other hand, studies using similar approaches found nucleosome enrichment in intergenic regions and introns (SAMANS et al., 2014) or depleted in promoter regions (CARONE et al., 2014) suggesting that histones could be localized in “gene deserts”. It is reasonable to find nucleosome structures close to repetitive sequences throughout the genome, e.g., retrotransposon like SINE (short interspersed nuclear elements) and LINE (long interspersed nuclear elements) (SAMANS et al., 2014) and immunostaining studies corroborate with these findings, localizing histones in peripherical regions of sperm

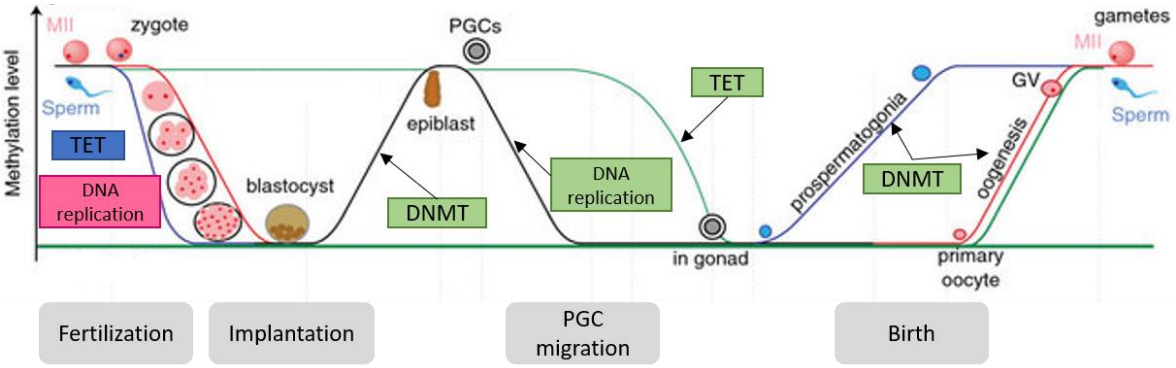
nucleus (GOVIN et al., 2007; PITTOGGI et al., 1999). Also, reanalysis of sequencing data by other groups indicates that inappropriate computational methodology could create wrong conclusions about nucleosome localization and enrichment (ROYO; STADLER; PETERS, 2016). Then, the results about the epigenetic role of retained histones are still controversial and more studies are needed, including in other species, to clarify and confirm an interspecies pattern of nucleosome distribution in sperm DNA.

2.3.2.2 DNA methylation

DNA methylation is considered the most stable epigenetic modification of the chromatin structure. It consists by the addition of methyl group (CH_3) in the cytosine residues of the CpG dinucleotides, forming 5-methyl-cytosine (5mC). DNA methylation normally occurs on the fifth position of cytosine bases in the context of cytosine followed by guanine (CpG dinucleotides) and in less extend at cytosine base in non-CpG context (JANG et al., 2017). Several genes have rich regions of GC bases near to promoter regions, called CpG islands, which provides possible sites to be methylated, and once methylated these genes are silenced (AHLUWALIA, 2009). This modification is involved in gene regulation, transposon silencing, genomic imprinting, maintenance of genome integrity and chromosome X inactivation (MESSERSCHMIDT; KNOWLES; SOLTER, 2014). The enzyme responsible to form 5hmC is called DNA methyltransferase (DNMT) and is divided in two classes: DNMT3A, DNMT3B, and DNMT3L which are responsible for *de novo* methylation, and DNMT1 involved in methylation maintenance during cell division (HITT et al., 1988; OKANO; XIE; LI, 1998).

During the embryo/fetus development, the DNA methylation is established in two specific moments: in zygote stage after fertilization and in primordial germ cells stage (PGCs), before the migration to the gonadal tissues (Figure 2). In the first moment, the paternal DNA suffers active DNA demethylation at the same time of protamine-histone exchange (Figure 2, blue line) while maternal DNA remains unaffected to be passive demethylated later (Figure 2, pink line). The second moment occurs with primordial germ cells (PGCs) when genome-wide DNA demethylation leads to erasure of parental imprints and X reactivation in females, losing 90% of their methylation patterns (LEE; HORE; REIK, 2014).

Figure 2. DNA methylation establishment during embryo/fetus development.



Legend: The paternal (blue line) and maternal (pink line) genome undergoes genome-wide demethylation after fertilization, primarily via TET enzymes (paternal DNA) and DNA replication (maternal DNA). The *de novo* methylation establishment occurs around implantation for both genomes (black line) caused by DNMT enzymes. In the germ line, primordial germ cells (PGCs) lose their DNA methylation during early (black – embryo genome) and late (green – imprinted regions) demethylation stages, by DNA replication and TET enzymes depending of DNA context. The methylation will be re-established for germ cells during spermatogenesis and oogenesis, in the first case complete before birth and in the second case after puberty during their final growth stage (GV: germinal vesicle oocyte) being activated prior to ovulation (MII: metaphase II oocytes) (SAADEH; SCHULZ, 2014 modified).

The demethylation in PGCs occurs in two phases: first during their migration to the gonadal tissue, at this point the demethylation happens by inactivation of the maintenance of methylation pattern during cells divisions (Figure 2, black line – DNA replication) (KAGIWADA et al., 2013). This phenomenon decreases methylation levels in 30% by the time PGCs arrives to the gonadal region. The second phase includes differentially methylated regions (DMRs) in imprinted loci, CpG islands, the X chromosome and germline-specific genes (Figure 2, green line) (SEISENBERGER et al., 2012). At this phase, the demethylation seems to require TET1 and TET2 proteins in a process similar to the zygote stage (HACKETT et al., 2013).

Both events are determinant to establish pluripotent features to lineage of cells derived from zygotes and PGCs. In mice, the *de novo* methylation is established at embryonic day E13.5 in pro-spermatogonia and the methylome is complete prior to birth, and sustained through all spermatogenesis stages until mature spermatozoa (OAKES et al., 2007). Once established the methylation profile, the spermatozoa is consider highly methylated, with almost 90% of methylated CpGs versus 40% in the oocyte in mice (KOBAYASHI et al., 2012).

Although during the zygote stage and PGCs migration the paternal DNA suffers active demethylation process, some sequences in the genome are resistant to demethylation. They include intra-cisternal A particles (IAP), retrotransposon family,

CpG islands and adjacent regions (HACKETT et al., 2013; SEISENBERGER et al., 2012). These regions could be sites of potential mechanism of transgenerational epigenetic inheritance, meaning the paternal life-history experiences could have an influence on the health of the future offspring. Recently studies, using mice as a model, showed the effect of unbalance diet (DE CASTRO BARBOSA et al., 2016; WEI et al., 2014), pesticide (SKINNER et al., 2018), fungicide (BEN MAAMAR et al., 2018) and age of father (MILEKIC et al., 2015) on DNA methylation pattern impairing the next generation health. Therefore, the well establishment of methylation profile during PGCs differentiation and zygote stages is important to further generations of cells, which includes not only mature sperm, but also the next generation.

2.4 SPERM NUCLEAR REMODELING AFTER FERTILIZATION

An interesting study performed by Ward, Kimura and Yanagimachi (1999) found that it is possible to obtain viable offspring of murine oocytes injected with only the sperm nucleus, i.e. intact chromatin and nuclear matrix, without the cytoplasm sperm component. Later, Mohar and colleagues (2002) demonstrated that an intact nuclear matrix containing "naked" DNA, which means, after removal of nuclear proteins, is able to maintain the formation of the male pro-nucleus and the normal condensation of the chromosomes, but it was not possible to obtain progress in embryonic development. These studies together indicate that, although the oocyte can repack the sperm chromatin with its own histones after fertilization, nuclear proteins, that probably signalize epigenetic marks present in paternal DNA, are critical for normal subsequent embryonic development.

2.4.1 Establishment of a somatic cell chromatin profile: Protamine to histone exchange

Right after fertilization, oocyte machinery faces the challenge to remodeling paternal DNA. This process occurs in three phases concomitant with oocyte meiosis II ending. First phase, the sperm chromatin increases the area, reaching the maximum while the oocyte finishes the anaphase II and extrudes the second polar body. When the oocyte chromosomes complete the telophase II, the sperm chromatin begins to recondense and decreases the area about one-half. The last phase, the chromatin

extensively decondenses within the male pronucleus and the area increases about 10-fold, in synchrony with the oocyte chromatin decondensation (ADENOT et al., 1991; WRIGHT; LONGO, 1988). At this point, the male pronucleus expand and fuses with female pronucleus to start the first cleavage. In humans, the protamine-histone replacement lasts one hour after intracytoplasmic sperm injection (JONES; ZALENSKY; ZALENSKAYA, 2011) and in porcine, 80% of protamine is removed 3 hours after fertilization (SHIMADA et al., 2000).

The ability of oocyte to reduce disulfide bonds in sperm DNA to start the chromatin decompaction is through glutathione (GSH), which presents higher concentration in metaphase II oocytes when compared to immature germinal vesicles and fertilized pronuclear oocytes, and the block of this pathway prevent or delay pronuclear formation (PERREAULT; BARBEE; SLOTT, 1988). This process is ATP dependent and the use of ATP inhibitors like antimycin A blocks the paternal chromatin remodeling (WRIGHT; LONGO, 1988). The protamine removal is mediated by chaperon nucleoplasmin (NPM), stored in the oocyte and the deficiency of this protein can also block sperm chromatin remodeling (INOUE et al., 2011; OHSUMI; KATAGIRI, 1991).

As described before, sperm DNA can bring to oocyte some paternal histones. If these persistent histones contribute to the embryo development is still a matter of debate. The variants H3.1/H3.2 showed a clear signal in the decondensed human sperm chromatin prior to S-phase, suggesting the permanence of these variants in the zygote (VAN DER HEIJDEN et al., 2008). On the opposite side, the variants H2AL1/2 rapidly disappears after fertilization in mouse (WU et al., 2008). Recently, Kong and colleagues (2018) verified that sperm derived H3.3 is removed from sperm genome shortly after fertilization and extruded from the zygote via the second polar body. These studies together showed that, as well as nucleosome enriched DNA, the function of paternal histones for embryo development it is not completely understood, and further studies are necessary.

2.4.2 Establishment of a pluripotency state: Active and passive demethylation

Another event that needs to happen before the first cleavage is the active demethylation process of paternal DNA. The active paternal DNA demethylation starts after the conversion of 5mC to 5-hydroxymethylcytosine (5hmC), an intermediary

product of demethylation process produced by TET3 enzyme during pronuclear formation (WOSSIDLO et al., 2011). The asynchrony demethylation between paternal and maternal DNA in zygote occurs due to the protection of maternal DNA by PGC7 (also known as Stella or Dppa3). This protein protects 5mC from TET3-mediated conversion to 5hmC by binding to maternal chromatin sites containing histone H3 lysine 9 dimethylation (H3K9me2). However, years later, Guo and colleagues (2014) demonstrated that both genomes suffer passive and active demethylation. In this study, they verified that passive demethylation was blocked using replication inhibitor agent and active demethylation was abolished deleting TET3 in both pronuclei. Others studies corroborate with this, showing active and passive demethylation for both maternal and paternal DNA (SHEN et al., 2014; WANG et al., 2014).

In addition, imprinted loci marked with H3K9me2 in sperm seems also to be protected by PGC7 in early embryogenesis (NAKAMURA et al., 2012). Other studies were able to confirm that imprinting control regions are maintained methylated by different factors like TRIM28 or DNMT1 (HIRASAWA et al., 2008; MESSERSCHMIDT et al., 2012). One of the targets of TRIM28 is the Y-linked gene Rbmy1a1. This gene resists to the demethylation process of paternal DNA and aberrant hypomethylation of it causes embryonic death (SAMPATH KUMAR et al., 2017). This window of reprogramming methylation is crucial to further embryo development and point it out another feature of paternal chromatin that could impair on fertility status.

2.5 EPIGENETIC AND FERTILITY: WHAT HUMAN STUDIES SAY

The advances in global approaches like proteome analysis and genome-wide sequencing allow us to evaluate sperm DNA beyond the chromatin integrity. Recently, studies in human, known as one of the species with highest fertility problems, demonstrated the effects of epigenetic profile and nuclear proteins composition on fertility status. Alterations of epigenetics marks are associated to fertility decrease, embryo quality and pregnancy establishment (DOERKSEN; TRASLER, 1996; KELLY; LI; TRASLER, 2003). Benchaib and colleagues (2005) found that global methylation levels of sperm DNA in humans may not influence fertilization rates, but can interfere on embryo development. More recently, Aston and colleagues (2015) found a different pattern of DNA methylation among fertile and infertile men, suggesting that the methylation profile could be a tool to predict the quality of IVP embryos. The sperm

methylation status was also different between good and poor-quality embryos. The retrospective analysis suggests that altered sperm DNA methylation could compromise embryo development (DENOMME et al., 2017).

Despite the hole of retained histones is still debated, they were also correlated with fertility in humans. Patients with poor-quality embryos or male infertility had reduction in the amount of histones modifications H3K4me3 or H3K27me3 retained at developmental transcription factors and certain imprinted genes. Also, some of the infertile patients presented random histones distribution on the sperm DNA (HAMMOUD et al., 2011). Although in humans, histone retention have been described as a possibility of sperm epigenetic mark for further embryo development, DNA evaluations that measure protamine deficiency correlate this analysis to fertility and pregnancy loss (NASR-ESFAHANI et al., 2005; TALEBI et al., 2016; ZHANG; SAN GABRIEL; ZINI, 2006), raising the discussion about how far histone retention are needed to maintain or impair the next generation.

The relationship and quantity of some nuclear proteins have already been significantly correlated to sperm morphology, DNA fragmentation, embryonic quality and pregnancy rates (INTASQUI et al., 2013; SIMON et al., 2011, 2014). It has also been verified that normozoospermic smoking men present higher percentage of histone-protamine transition protein abnormality when compared to non-smoking patients. This change in sperm nucleus proteins presented negative correlation with motility, vitality and total number of spermatozoa in the ejaculate (YU et al., 2014). For proteome analysis, normozoospermic men submitted to IVP showed that among the proteins differentially expressed, in the samples whose embryos resulted in pregnancy are proteins responsible for the formation of the chromatin structure. This indicates that changes in the protein composition of the sperm nucleus may be responsible for embryo development arrest (AZPIAZU et al., 2014). In the protein characterization of human sperm nucleus by mass spectrometry, 403 proteins were identified, of which only 191 (47.4%) had previously been described (DE MATEO et al., 2011). Castillo and colleagues (2014) reviewed several studies of nuclear proteome and found that of the 581 exclusively nuclear proteins described, 56% of them have function or some relationship with epigenetic signaling and interestingly, 46 histones and histone variants have been identified, reaffirming that the sperm chromatin beyond DNA is equally important as DNA integrity and probably carries important information for next generation.

2.6 BULL FERTILITY: WHAT WE ALREADY KNOW

While for human it is so clear the difference between fertile and sub or unfertile men, for bovine the pathways to characterize the fertility is not clear yet. In addition, the different biotechniques used in cattle turn difficult to apply the discovery in some technique to another. Animals that exhibit high *in vivo* fertility (65%-75% non-return rates) demonstrate variability on *in vitro* fertility (6.9%-51.2% blastocyst rate), with a high and significant bull effect on IVP, without interaction with the female effect (PALMA; SINOWATZ, 2004). Furthermore, negative correlations between *in vivo* fertility (non-return rates) and IVP rates (cleavage and blastocyst rate) have also been reported (VANDAELE et al., 2006). Recently, Ortega and colleagues (2018) verified that low fertility bulls (based on conception rate) presented higher percentage of unfertilized oocyte in superovulation embryo recover when compared to high fertility bulls, but for IVP results no difference was found for cleavage rate, an *in vitro* indicator of fertilization ability. All these data together indicate that it is difficult to extrapolate the reality from the field to the laboratory.

So far, there is no single sperm analysis that consistently predict bull fertility (SELLEM et al., 2015; UTT, 2016; YANIZ et al., 2017). Studies with large database helps the characterization of positive sperm traits that can improve both *in vitro* and *in vivo* embryo production. However, in recent years, with the combination of breeding programs and the increased pressure for good sperm quality, it is hard to find sub or unfertile animals in the Reproduction Centers, but still, it is possible to see bulls with divergent embryo production with no clear reason.

In attempt to find out a possible explanation for individual differences, the study of extreme populations of bulls, clearly distinct for a specific sperm parameter, helps to describe the effect of bull and possible reasons for different fertility status. In an interesting study, Siqueira and colleagues (2018) were able to isolate different sperm traits, like acrosome, membrane and DNA integrity, mitochondrial potential, and motility to study the effect of these characteristics on IVP. The advantage to study sperm parameters on *in vitro* environment is isolate specific aspects of sperm biology to study their possible impact on embryo production. In the *in vitro* environment, studies proved that aspects related to fertilization ability could be compensated, and the bull effect turns evident after the genome embryo activation (KROPP et al., 2017; ORTEGA et al., 2018). That means, once the sperm is inside the oocyte, the main contribution

is the DNA and then, all the aspects described before could contribute or impair on embryo development.

The bull sperm DNA was widely studied regard its integrity. One of the first studies relating bull DNA integrity and fertility was described by Evenson and colleagues (1980). This author is the creator of sperm chromatin structure assay (SCSA), widely used in sperm biology to identify sperm chromatin alterations. Many studies came after that applying sperm DNA assays like COMET, TUNEL, sperm chromatin dispersion, protamine deficiency and others to evaluate chromatin integrity in different species using the human based tests (revised by EVENSON, 2016). However, unlike human species, bovine species was selected all over the years for fertility aspects. Then, animals with clearly fertility problems were excluded either by natural or artificial selection in the breeding programs. Therefore, the bovine sperm chromatin regards its integrity seems to be very different than other species. Villani and colleagues (2010) compared human, mice and bovine sperm chromatin treated with DNAse I or hydrogen peroxide by COMET and TUNEL assay. In this study they verified that bovine sperm chromatin is more resistant to DNAse I treatment suggesting that the low percentage of histone-bound DNA (regions more sensible to endonucleases digestion) could be an explanation for this higher sensitivity. The low percentage of chromatin alterations identified in bull studies, independently of which DNA assay used, corroborating with this theory of greater bovine sperm DNA resistance (D'OCCHIO et al., 2013; SIMÕES et al., 2013; FORTES et al., 2012, 2014; CASTRO et al., 2018). Then, it is possible to assume that DNA integrity should not be a problem in the population of commercial bulls used for reproductive biotechniques nowadays.

As mentioned before, sperm DNA information goes beyond DNA sequencing, or integrity. How DNA is fold – proteins that participate of this process; and how its information is transmitted – epigenetic marks that could contribute to proper embryo development have not been explored yet in bovine. Recently, studies start to explore deeply the epigenetic profile of bull sperm. The analysis of nucleosome retention in bull sperm identified these structures enriched in intergenic regions, introns, centromere repeats and retrotransposons (LINE1 and SINEs) (SAMANS et al., 2014), with no enrichment at genes related to embryo development (SILLASTE et al., 2017). Therefore, these results go to the direction of human studies mentioned before that believe the retained histones are randomly distributed or in gene deserts regions. In

regard DNA methylation, studies comparing sperm DNA with somatic cells verified that bovine sperm, differently of human sperm, is overall hypomethylated (PERRIER et al., 2018; ZHOU et al., 2018a). For both studies, hypomethylated bull sperm DNA targeted processes relevant to germline differentiation and sperm function. It is clear the peculiarity of bovine DNA, but the number of studies is still limited, and they are only descriptive. In a design model of two groups of bulls with distinct fertility, it could be a good way to understand epigenetic differences and better understand the contribution of paternal DNA for this species.

REFERENCES

ADENOT, P. G. et al. Dynamics of paternal chromatin changes in live one-cell mouse embryo after natural fertilization. **Molecular Reproduction and Development**, v. 28, n. 1, p. 23–34, 1991.

AHLUWALIA, K. B. **Genetics**. Second Edi ed. [s.l.] New Age International Limited, 2009.

ARPANAHI, A. et al. Endonuclease-Sensitive Regions of Human Spermatozoal Chromatin Are Highly Enriched in Promoter and CTCF Binding Sequences. **Genome research**, v. 19, n. 8, p. 1338–1349, ago. 2009.

ASTON, K. I. et al. Aberrant sperm DNA methylation predicts male fertility status and embryo quality. **Fertility and Sterility**, v. 104, n. 6, p. 1388–1397.e5, 2015. Disponível em: <http://dx.doi.org/10.1016/j.fertnstert.2015.08.019>.

AZPIAZU, R. et al. High-throughput sperm differential proteomics suggests that epigenetic alterations contribute to failed assisted reproduction. **Human Reproduction**, v. 29, n. 6, p. 1225–1237, 2014.

BEN MAAMAR, M. et al. Alterations in Sperm DNA Methylation, Non-Coding RNA Expression, and Histone Retention Mediate Vinclozolin-Induced Epigenetic Transgenerational Inheritance of Disease. **Environmental epigenetics**, v. 4, n. 2, p. dvy010, abr. 2018.

BENCHAIB, M. et al. Influence of global sperm DNA methylation on IVF results. **Human reproduction (Oxford, England)**, v. 20, n. 3, p. 768–773, mar. 2005. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15640258>. Acesso em: 24 set. 2014.

BRYKCZYNSKA, U. et al. Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. **Nature structural & molecular biology**, v. 17, n. 6, p. 679–87, jun. 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20473313>. Acesso em: 29 nov. 2014.

CAMPOS, E. I.; REINBERG, D. Histones: Annotating Chromatin. **Annual review of genetics**, v. 43, p. 559–599, 2009.

CARON, N.; VEILLEUX, S.; BOISSONNEAULT, G. Stimulation of DNA Repair by the Spermatidal TP1 Protein. **Molecular reproduction and development**, v. 58, n. 4, p. 437–443, abr. 2001.

CARONE, B. R. et al. High-resolution mapping of chromatin packaging in mouse embryonic stem cells and sperm. **Developmental Cell**, v. 30, n. 1, p. 11–22, 2014. Disponível em: <http://dx.doi.org/10.1016/j.devcel.2014.05.024>.

CARRELL, D. T.; EMERY, B. R.; HAMMOUD, S. Altered protamine expression and diminished spermatogenesis: what is the link? **Human Reproduction Update**, v. 13, n. 3, p. 313–27, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17208950>. Acesso em: 21 jul. 2011.

CARRELL, D. T.; HAMMOUD, S. S. The human sperm epigenome and its potential role in embryonic development. **Molecular Human Reproduction**, v. 16, n. 1, p. 37–47, jan. 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19906823>. Acesso em: 14 out. 2014.

CASTILLO, J.; AMARAL, a.; OLIVA, R. Sperm nuclear proteome and its epigenetic potential. **Andrology**, v. 2, n. 3, p. 326–338, 2014.

CASTRO, L. S. et al. Effect of bovine sperm chromatin integrity evaluated using three different methods on in vitro fertility. **Theriogenology**, v. 107, p. 142–148, 2018. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0093691X1730540X>.

CHURIKOV, D.; ZALENSKAYA, I. A.; ZALENSKY, A. O. Male Germline-Specific Histones in Mouse and Man. **Cytogenetic and genome research**, v. 105, n. 2–4, p. 203–214, 2004.

CLARKE, G. N. A.R.T. and history, 1678-1978. **Human Reproduction**, v. 21, n. 7, p. 1645–1650, 2006.

COBB, M. An amazing 10 years: The discovery of egg and sperm in the 17th century. **Reproduction in Domestic Animals**, v. 47, n. SUPPL.4, p. 2–6, 2012.

D'OCCHIO, M. J. et al. Sperm chromatin in beef bulls in tropical environments. **Theriogenology**, v. 79, n. 6, p. 946–52, 1 abr. 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23434358>. Acesso em: 14 jan. 2014.

DE CASTRO BARBOSA, T. et al. High-Fat Diet Reprograms the Epigenome of Rat Spermatozoa and Transgenerationally Affects Metabolism of the Offspring. **Molecular metabolism**, v. 5, n. 3, p. 184–197, mar. 2016.

DE MATEO, S. et al. Proteomic characterization of the human sperm nucleus. **Proteomics**, v. 11, p. 2714–2726, 2011.

DENOMME, M. M. et al. Alterations in the Sperm Histone-Retained Epigenome Are Associated with Unexplained Male Factor Infertility and Poor Blastocyst Development in Donor Oocyte IVF Cycles. **Human reproduction (Oxford, England)**, v. 32, n. 12, p. 2443–2455, dez. 2017.

DOERKSEN, T.; TRASLER, J. Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation development in rats. **Biology of reproduction**, v. 1162, p. 1155–1162, 1996. Disponível em:

<http://www.biolreprod.org/content/55/5/1155.short>. Acesso em: 27 jan. 2015.

ERKEK, S. et al. Molecular Determinants of Nucleosome Retention at CpG-Rich Sequences in Mouse Spermatozoa. **Nature structural & molecular biology**, v. 20, n. 7, p. 868–875, jul. 2013.

EVENSON, D.; DARZYNKIEWICZ, Z.; MELAMED, M. Relation of mammalian sperm chromatin heterogeneity to fertility. **Science**, v. 210, n. December, p. 1978–1980, 1980. Disponível em: <http://www.sciencemag.org/content/210/4474/1131.short>. Acesso em: 19 fev. 2015.

EVENSON, D. P. The Sperm Chromatin Structure Assay (SCSA) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. **Animal Reproduction Science**, v. 169, p. 56–75, 2016.

FORTES, M. R. S. et al. The integrity of sperm chromatin in young tropical composite bulls. **Theriogenology**, v. 78, n. 2, p. 326–33, 333.e1–4, 15 jul. 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22494672>. Acesso em: 15 fev. 2013.

FORTES, M. R. S. et al. Sperm protamine deficiency correlates with sperm DNA damage in Bos indicus bulls. **Andrology**, v. 2, n. 3, p. 370–378, 2014.

GOLDBERG, A. D.; ALLIS, C. D.; BERNSTEIN, E. Epigenetics: A Landscape Takes Shape. **Cell**, v. 128, n. 4, p. 635–638, fev. 2007.

GOVIN, J. et al. Pericentric heterochromatin reprogramming by new histone variants during mouse spermiogenesis. **Journal of Cell Biology**, v. 176, n. 3, p. 283–294, 2007.

GUO, F. et al. Active and passive demethylation of male and female pronuclear DNA in the mammalian zygote. **Cell Stem Cell**, v. 15, n. 4, p. 447–458, 2014.

HACKETT, J. A. et al. Germline DNA Demethylation Dynamics and Imprint Erasure through 5-Hydroxymethylcytosine. **Science (New York, N.Y.)**, v. 339, n. 6118, p. 448–452, jan. 2013.

HAMMOUD, S. S. et al. Distinctive chromatin in human sperm packages genes for embryo development. **Nature**, v. 460, n. 7254, p. 473–478, 23 jul. 2009. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2858064&tool=pmcentrez&rendertype=abstract>. Acesso em: 20 jan. 2014.

HIRASAWA, R. et al. Maternal and Zygotic Dnmt1 Are Necessary and Sufficient for the Maintenance of DNA Methylation Imprints during Preimplantation Development. **Genes & development**, v. 22, n. 12, p. 1607–1616, jun. 2008.

HITT, M. M. et al. De Novo and Maintenance DNA Methylation by a Mouse Plasmacytoma Cell DNA Methyltransferase. **The Journal of biological chemistry**, v. 263, n. 9, p. 4392–4399, mar. 1988.

INOUE, A. et al. Involvement of Mouse Nucleoplasmin 2 in the Decondensation of Sperm Chromatin after Fertilization. **Biology of reproduction**, v. 85, n. 1, p. 70–77, jul. 2011.

INTASQUI, P. et al. Unraveling the sperm proteome and post-genomic pathways associated with sperm nuclear DNA fragmentation. **Journal of Assisted Reproduction and Genetics**, v. 30, p. 1187–1202, 2013.

JAENISCH, R.; BIRD, A. Epigenetic Regulation of Gene Expression: How the Genome Integrates Intrinsic and Environmental Signals. **Nature genetics**, v. 33 Suppl, p. 245–254, mar. 2003.

JANG, H. S. et al. CpG and Non-CpG Methylation in Epigenetic Gene Regulation and Brain Function. **Genes**, v. 8, n. 6, maio 2017.

JOHNSON, G. D. et al. The sperm nucleous: chromatin RNA, and the nuclear matrix. **Reproduction**, v. 141, n. 1, p. 21–36, 2010.

JONES, E. L.; ZALENSKY, A. O.; ZALENSKAYA, I. A. Protamine Withdrawal from Human Sperm Nuclei Following Heterologous ICSI into Hamster Oocytes. **Protein and peptide letters**, v. 18, n. 8, p. 811–816, ago. 2011.

KAGIWADA, S. et al. Replication-Coupled Passive DNA Demethylation for the Erasure of Genome Imprints in Mice. **The EMBO journal**, v. 32, n. 3, p. 340–353, fev. 2013.

KELLY, T.; LI, E.; TRASLER, J. 5-Aza-2′-Deoxycytidine Induces Alterations in Murine Spermatogenesis and Pregnancy Outcome. **Journal of andrology**, v. 24, n. 6, p. 822–830, 2003. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1002/j.1939-4640.2003.tb03133.x/full>. Acesso em: 27 jan. 2015.

KOBAYASHI, H. et al. Contribution of intragenic DNA methylation in mouse gametic DNA methylomes to establish Oocyte-specific heritable marks. **PLoS Genetics**, v. 8, n. 1, 2012.

KONG, Q. et al. Histone variant H3.3–mediated chromatin remodeling is essential for paternal genome activation in mouse preimplantation embryos. **Journal of Biological Chemistry**, v. 293, n. 10, p. 3829–3838, 2018.

KROPP, J. et al. Male fertility status is associated with DNA methylation signatures in sperm and transcriptomic profiles of bovine preimplantation embryos. **BMC Genomics**, v. 18, n. 1, p. 280, 2017. Disponível em: <http://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-017-3673-y>.

LEE, H. J.; HORE, T. A.; REIK, W. Reprogramming the Methylome: Erasing Memory and Creating Diversity. **Cell Stem Cell**, v. 14, n. 6, p. 710–719, jun. 2014. Disponível em: <http://dx.doi.org/10.1016/j.stem.2014.05.008>.

LEE, I. et al. New control of mitochondrial membrane potential and ROS formation - A Hypothesis. **Biological chemistry**, v. 382, n. 12, p. 1629–1636, dez. 2001. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/11843176>. Acesso em: 1 set. 2014.

LONERGAN, P. Review: Historical and futuristic developments in bovine semen technology. **Animal**, p. 1–15, 2018.

MCGINTY, R. K.; TAN, S. **Nucleosome Structure and FunctionChemical Reviews**, mar. 2015. .

MESSERSCHMIDT, D. M. et al. Trim28 Is Required for Epigenetic Stability during

Mouse Oocyte to Embryo Transition. **Science (New York, N.Y.)**, v. 335, n. 6075, p. 1499–1502, mar. 2012.

MESSERSCHMIDT, D. M.; KNOWLES, B. B.; SOLTER, D. DNA Methylation Dynamics during Epigenetic Reprogramming in the Germline and Preimplantation Embryos. **Genes & development**, v. 28, n. 8, p. 812–828, abr. 2014.

MILEKIC, M. H. et al. Age-Related Sperm DNA Methylation Changes Are Transmitted to Offspring and Associated with Abnormal Behavior and Dysregulated Gene Expression. **Molecular psychiatry**, v. 20, n. 8, p. 995–1001, ago. 2015.

MOHAR, I. et al. Sperm nuclear halos can transform into normal chromosomes after injection into oocytes. **Molecular Reproduction and Development**, v. 62, n. January, p. 416–420, 2002.

NAKAMURA, T. et al. PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. **Nature**, p. 2–7, 2012. Disponível em: <http://www.nature.com/doifinder/10.1038/nature11093>.

NASR-ESFAHANI, M. H. et al. Effect of Sperm DNA Damage and Sperm Protamine Deficiency on Fertilization and Embryo Development Post-ICSI. **Reproductive biomedicine online**, v. 11, n. 2, p. 198–205, ago. 2005.

OAKES, C. C. et al. Developmental Acquisition of Genome-Wide DNA Methylation Occurs Prior to Meiosis in Male Germ Cells. **Developmental biology**, v. 307, n. 2, p. 368–379, jul. 2007.

OHSUMI, K.; KATAGIRI, C. Characterization of the Ooplasmic Factor Inducing Decondensation of and Protamine Removal from Toad Sperm Nuclei: Involvement of Nucleoplasmin. **Developmental biology**, v. 148, n. 1, p. 295–305, nov. 1991.

OKANO, M.; XIE, S.; LI, E. **Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases.Nature genetics**United States, jul. 1998. .

OKO, R. J. et al. Chromatin Reorganization in Rat Spermatids during the Disappearance of Testis-Specific Histone, H1t, and the Appearance of Transition Proteins TP1 and TP2. **Biology of reproduction**, v. 54, n. 5, p. 1141–1157, maio 1996.

OLIVA, R.; DIXON, G. H. Vertebrate Protamine Genes and the Histone-to-Protamine Replacement Reaction. **Progress in nucleic acid research and molecular biology**, v. 40, p. 25–94, 1991.

ORTEGA, M. S. et al. Influences of sire conception rate on pregnancy establishment in dairy cattle. **Biology of Reproduction**, v. 0, n. July, p. 1–11, 2018. Disponível em: <https://academic.oup.com/biolreprod/advance-article/doi/10.1093/biolre/ioy141/5040218>.

PALMA, G. A.; SINOWATZ, F. Male and female effects on the in vitro production of bovine embryos. **Anatomia, Histologia and Embryologia**, v. 33, n. 5, p. 257–262, out. 2004. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15352877>.

PERREAULT, S. D.; BARBEE, R. R.; SLOTT, V. L. Importance of glutathione in the

acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. **Developmental Biology**, v. 125, n. 1, p. 181–186, 1988.

PERRIER, J. et al. A multi-scale analysis of bull sperm methylome revealed both species peculiarities and conserved tissue-specific features. **BMC Genomics**, v. 19, n. 1, p. 404, 2018. Disponível em: <https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-018-4764-0>.

PITTOGGI, C. et al. A Fraction of Mouse Sperm Chromatin Is Organized in Nucleosomal Hypersensitive Domains Enriched in Retroposon DNA. **Journal of cell science**, v. 112 (Pt 2, p. 3537–3548, out. 1999.

PRADEEPA, M. M.; RAO, M. R. S. Chromatin Remodeling during Mammalian Spermatogenesis: Role of Testis Specific Histone Variants and Transition Proteins. **Society of Reproduction and Fertility supplement**, v. 63, p. 1–10, 2007.

ROYO, H.; STADLER, M. B.; PETERS, A. H. F. M. Alternative Computational Analysis Shows No Evidence for Nucleosome Enrichment at Repetitive Sequences in Mammalian Spermatozoa. **Developmental Cell**, v. 37, n. 1, p. 98–104, 2016.

SAADEH, H.; SCHULZ, R. Protection of CpG islands against de novo DNA methylation during oogenesis is associated with the recognition site of E2f1 and E2f2. **Epigenetics and Chromatin**, v. 7, n. 1, p. 1–15, 2014.

SAMANS, B. et al. Uniformity of nucleosome preservation pattern in mammalian sperm and Its connection to repetitive DNA elements. **Developmental Cell**, v. 30, n. 1, p. 23–35, 2014. Disponível em: <http://dx.doi.org/10.1016/j.devcel.2014.05.023>.

SAMPATH KUMAR, A. et al. Loss of Maternal Trim28 Causes Male-Predominant Early Embryonic Lethality. **Genes & development**, v. 31, n. 1, p. 12–17, jan. 2017.

SEISENBERGER, S. et al. The Dynamics of Genome-Wide DNA Methylation Reprogramming in Mouse Primordial Germ Cells. **Molecular cell**, v. 48, n. 6, p. 849–862, dez. 2012.

SELLEM, E. et al. Use of combinations of in vitro quality assessments to predict fertility of bovine semen. **Theriogenology**, v. 84, n. 9, p. 1447–1454.e5, 2015.

SHEN, L. et al. Tet3 and DNA replication mediate demethylation of both the maternal and paternal genomes in mouse zygotes. **Cell Stem Cell**, v. 15, n. 4, p. 459–470, 2014.

SHIMADA, A. et al. Protamine Dissociation before Decondensation of Sperm Nuclei during in Vitro Fertilization of Pig Oocytes. **Journal of reproduction and fertility**, v. 120, n. 2, p. 247–256, nov. 2000.

SILLASTE, G. et al. A novel hypothesis for histone-to-protamine transition in Bos taurus spermatozoa. **Reproduction**, v. 153, n. 3, p. 241–251, 2017.

SIMÕES, R. et al. Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo in vitro development outcome. **Reproduction**, v. 146, n. 5, p. 433–441, jan. 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23940385>. Acesso em: 18 mar. 2014.

SIMON, L. et al. Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. **Reproductive BioMedicine Online**, v. 23, n. 6, p. 724–734, 2011. Disponível em: <http://dx.doi.org/10.1016/j.rbmo.2011.08.010>.

SIMON, L. et al. Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment. **Human Reproduction**, v. 29, n. 5, p. 904–917, 2014.

SIQUEIRA, A. F. P. et al. Sperm traits on in vitro production (IVP) of bovine embryos: Too much of anything is good for nothing. **PLoS ONE**, v. 13, n. 7, p. 1–16, 2018.

SKINNER, M. K. et al. Alterations in Sperm DNA Methylation, Non-Coding RNA and Histone Retention Associate with DDT-Induced Epigenetic Transgenerational Inheritance of Disease. **Epigenetics & chromatin**, v. 11, n. 1, p. 8, fev. 2018.

TALEBI, A. R. et al. Relationship between Sperm Protamine Deficiency and Apoptosis in Couples with Unexplained Repeated Spontaneous Abortions. **International journal of reproductive biomedicine (Yazd, Iran)**, v. 14, n. 3, p. 199–204, mar. 2016.

TAVERNA, S. D. et al. How Chromatin-Binding Modules Interpret Histone Modifications: Lessons from Professional Pocket Pickers. **Nature structural & molecular biology**, v. 14, n. 11, p. 1025–1040, nov. 2007.

UTT, M. D. Prediction of bull fertility. **Animal Reproduction Science**, v. 169, p. 37–44, 2016. Disponível em: <http://dx.doi.org/10.1016/j.anireprosci.2015.12.011>.

VAN DER HEIJDEN, G. W. et al. Sperm-derived histones contribute to zygotic chromatin in humans. **BMC developmental biology**, v. 8, p. 34, 2008.

VAN LEEUWENHOEK, A. De natis è semine genitali animalculis. **The Philosophical Transactions of the Royal Society**, v. 12, n. 1040, p. 1040–1043, 1678.

VANDAELE, L. et al. Is apoptosis in bovine in vitro produced embryos related to early developmental kinetics and in vivo bull fertility? **Theriogenology**, v. 65, n. 9, p. 1691–703, jun. 2006. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/16280159>. Acesso em: 26 jun. 2012.

VILLANI, P. et al. Sperm DNA fragmentation induced by DNAse I and hydrogen peroxide: An in vitro comparative study among different mammalian species. **Reproduction**, v. 140, n. 3, p. 445–452, 2010.

WANG, L. et al. Programming and inheritance of parental DNA methylomes in mammals. **Cell**, v. 157, n. 4, p. 979–991, 2014. Disponível em: <http://dx.doi.org/10.1016/j.cell.2014.04.017>.

WARD, W. S.; KIMURA, Y.; YANAGIMACHI, R. An intact sperm nuclear matrix may be necessary for the mouse paternal genome to participate in embryonic development. **Biology of reproduction**, v. 60, p. 702–706, 1999.

WEI, Y. et al. Paternally Induced Transgenerational Inheritance of Susceptibility to Diabetes in Mammals. **Proceedings of the National Academy of Sciences of the United States of America**, v. 111, n. 5, p. 1873–1878, fev. 2014.

WOSSIDLO, M. et al. 5-Hydroxymethylcytosine in the mammalian zygote is linked with

epigenetic reprogramming. **Nature Communications**, v. 2, n. 1, 2011.

WRIGHT, S. J.; LONGO, F. J. Sperm Nuclear Enlargement in Fertilized Hamster Eggs Is Related to Meiotic Maturation of the Maternal Chromatin. **The Journal of experimental zoology**, v. 247, n. 2, p. 155–165, ago. 1988.

WU, F. et al. Testis-Specific Histone Variants H2AL1/2 Rapidly Disappear from Paternal Heterochromatin after Fertilization. **The Journal of reproduction and development**, v. 54, n. 6, p. 413–417, dez. 2008.

YANIZ, J. L. et al. Toward an Integrative and Predictive Sperm Quality Analysis in Bos Taurus. **Animal reproduction science**, v. 181, p. 108–114, jun. 2017.

YU, B. et al. Cigarette smoking is associated with abnormal histone-to-protamine transition in human sperm. **Fertility and Sterility**, v. 101, n. 1, p. 51–57, 2014. Disponível em: <http://dx.doi.org/10.1016/j.fertnstert.2013.09.001>.

ZHANG, X.; SAN GABRIEL, M.; ZINI, A. Sperm Nuclear Histone to Protamine Ratio in Fertile and Infertile Men: Evidence of Heterogeneous Subpopulations of Spermatozoa in the Ejaculate. **Journal of andrology**, v. 27, n. 3, p. 414–420, 2006.

ZHOU, Y. et al. Comparative whole genome DNA methylation profiling of cattle sperm and somatic tissues reveals striking hypomethylated patterns in sperm. **GigaScience**, n. April 2018, 2018. Disponível em: <http://doi.org/10.1093/gigascience/giy039%0Ahttps://academic.oup.com/gigascience/advance-article/doi/10.1093/gigascience/giy039/4965117>.

ZINI, A.; AGARWAL, A. **Sperm Chromatin**. New York, NY: Springer New York, 2011.

3 HYPOTHESIS

Bulls with difference for *in vitro* embryo production do not present difference for chromatin integrity, but they present difference for nuclear protein composition and/or epigenetic modifications in the sperm that reflect the embryo development.

4 GOALS

To identify the nuclear aspects of bull sperm that characterize different rates on *in vitro* embryo production.

Specific goals

Identify between bulls with high and low *in vitro* fertility, evaluated by embryo development rate (blastocyst rate/cleavage rate):

1- If there is difference in chromatin structure related to integrity and protamination level;

2- If there is difference in the protein profile of the sperm nucleus and proteins that could be *in vitro* fertility marks;

3- If there is difference in the histone modifications;

4- If there is difference in the DNA methylation profile.

5 CHAPTER 1: EFFECT OF BOVINE SPERM CHROMATIN INTEGRITY EVALUATED USING THREE DIFFERENT METHODS ON *IN VITRO* FERTILITY

In vitro fertility potential of individual bulls is still relatively uncharacterized. Classical sperm analysis does not include the evaluation of all sperm characteristics and thus, some cell compartments could be neglected. In humans, sperm DNA integrity has already proven to have major influence in embryo development and assisted reproduction techniques successfully. In bovine, some studies already correlated chromatin integrity with field fertility. However, none of those have attempted to relate DNA assessment approaches such as chromatin deficiency (CMA3), chromatin stability (SCSA; AO+) and DNA fragmentation (COMET assay) to predict *in vitro* bull fertility. To this purpose, we selected bulls with high and low *in vitro* fertility (n=6/group), based on embryo development rate (blastocyst/cleavage rate). We then performed CMA3, SCSA test and COMET assay to verify if the difference of *in vitro* fertility may be related to DNA alterations evaluated by these assays. For the three tests performed, our results showed only differences in the percentage of cells with chromatin deficiency (CMA3+; high: 0.19±0.03 *vs* low: 0.04±0.04; p=0.03). No difference for chromatin stability and any of COMET assay categories (grade I to grade IV) was observed between high and low *in vitro* fertility bulls. A positive correlation between AO+ cells and grade IV cells were found. Despite the difference between groups in CMA3 analysis, our results suggest that protamine deficiency in bovine spermatozoa may not have a strong biological impact to explain the difference of *in vitro* fertility between the bulls used in this study[1].

[1]The full version of this article was published in Theriogenology (Castro et al. 2018. Effect of bovine sperm chromatin integrity evaluated using three different methods on *in vitro* fertility, v. 107, p. 142-148. doi: 10.1016/j.theriogenology.2017.11.006).

6 CHAPTER 2: SPERM NUCLEAR PROTEOME BETWEEN HIGH AND LOW *IN VITRO* FERTILITY BULLS

6.1 INTRODUCTION

Sperm nucleus is considered a black box, highly compacted mainly by protamine, with quiescent gene expression activity (WARD; COFFEY, 1991). This cell gets to this point after a major morphological modification called spermatogenesis. During this process, sperm loses most part of its organelles, to keep inside its head just the message that it needs to be send: the paternal genome.

In attempt to compact and protect the paternal DNA, the sperm during spermatogenesis switches its histones to protamines, the main protein attached to paternal DNA. Protamine 1 is presented in all mammals (OLIVA; DIXON, 1991), while protamine 2 was described as active only in human and mice (CARRELL; EMERY; HAMMOUD, 2007). The ratio between P1 and P2 in humans is around 1, and this rate is commonly evaluated to identify fertility problems in men, been in the most cases the cause of infertility related to decrease of P2 expression (NI et al., 2016). For bull, there are few studies evaluating the P1 expression and the presence of P2 in mature sperm is controversial. Maier et al. (1990) demonstrated that P2 is transcribed and translated in low levels in spermatids, but its gene product was not find in mature sperm. Authors believed that P2 is replaced by P1, with more affinity to DNA. Unlike humans, the ratio P1 and P2 and the level of protamination and fertility have not been well established yet in bovine sperm (DOGAN et al., 2015).

However, sperm nucleus is not only composed by protamine. Nuclear proteins, besides structuring the chromatin and compacting the genetic material, have chemical alterations that personalize the mode of association with the genetic material. This association between DNA and proteins is one of the main gene expression controls called epigenetics signals (JAENISCH; BIRD, 2003; BONASIO; TU; REINBERG, 2010). An interesting study performed by Ward, Kimura and Yanagimachi (1999) found that it is possible to obtain viable offspring from murine oocytes injected with only the sperm nucleus, i.e. intact chromatin and nuclear matrix, without the cytoplasm sperm component. Later, Mohar and colleagues (2002) demonstrated that an intact nuclear matrix containing "naked" DNA, which means, after removal of nuclear proteins, is able to maintain the formation of the male pro-nucleus and the normal condensation of the

chromosomes, but it was not possible to obtain progress in embryonic development. These studies together indicate that, although the oocyte can repack the sperm chromatin with its own histones after fertilization, nuclear proteins that probably signalize epigenetic marks present in paternal DNA are critical for the success of embryo development.

Nuclear proteome analysis identified different transcription factors, zinc-finger proteins, modification enzymes that were postulated to be important to paternal epigenetic signals (CASTILLO; AMARAL; OLIVA, 2014). Castillo et al. (2014) revised studies evaluating nuclear proteins and verified that, from 581 exclusively nuclear proteins, 56% of them have function or some relationship with epigenetic signals, being identified 46 histones and variants.

Sperm nuclear composition had also influence in human fertility. The relation and quantity of some nuclear proteins were correlated to morphology, DNA fragmentation, embryo quality and pregnancy rate (INTASQUI et al., 2013; SIMON et al., 2011, 2014). Also, normozoospermic smoker men showed higher percentage of abnormalities in histone-protamine transition when compared to non-smoker. This protein alterations presented negative correlation with motility, viability and sperm concentration (YU et al., 2014). Proteome analysis of normozoospermic men submitted to IVF, with successful pregnancy or not identified differential expression of proteins related to chromatin assembly and lipoprotein metabolism that maybe involved in the embryo development arrest (AZPIAZU et al., 2014). So far, bovine sperm nuclear proteins were only described by target identification protein assays, like western blot or immunofluorescence (DE OLIVEIRA et al., 2013; DOGAN et al., 2015; KUTCHY et al., 2018) and between the global protein analysis of bull sperm, none of them investigate deeply the sperm nucleus in attempt to correlate nuclear proteins with bull fertility status. Then, the aim of this study was to conduct the quantitative comparison of the proteome of high and low *in vitro* fertility bulls, used on IVP procedures. We reasoned that this approach would result in the identification of sperm protein differences that may affect embryonic development or could be study deeply in the future as a possible mark for bull fertility.

6.2 MATERIALS AND METHODS

The present study was based on the database of the reproduction biotechnology company *In Vitro* Brazil (Mogi Mirim, Sao Paulo, Brazil) of 2012 to 2015. This company yielded *in vitro* embryo productions (IVP) from hundreds of bulls to select characteristics according to the interest of the present study.

6.2.1 Reagents and solutions

All chemical reagents and solutions used in this study were purchased from Sigma-Aldrich (St. Louis, MO, E.U.A.) unless otherwise stated.

6.2.2 Selection and ranking of the bulls

First, bulls were screened including only animals with more than 5 IVP manipulations and each of them with more than 50 oocytes/manipulation. Only bulls submitted to the same *in vitro* fertilization protocol and using X sex-sorted semen were included. In this first screening, 140 bulls from different breeds were selected. They were ranked based on embryo development rate, which is the blastocyst rate over cleavage rate. From this ranking, the 5 bulls in the top and the 5 bulls in the bottom of the list with commercially available semen straws were select to form the high (HF) and low (LF) fertility groups, respectively (n=5 per group). This bull selection methodology was previously described in Castro et al. (2018) and the *in vitro* rates from selected bulls were confirmed in other study from our group (ALMEIDA, 2018). They presented similar cleavage rate (HF: 86.66±1.15 *vs* LF: 84.94±0.95; p=0.25) and different blastocyst (HF: 29.41±1.56 *vs* LF: 16.02±0.92; p<0.0001) and embryo development rates (HF: 33.96±1.67 *vs* LF: 18.91±1.09; p<0.0001).

6.2.3 Semen preparation

The straws of each bull were thawed at 37ºC for 30 seconds and washed 2X (400G/5 minutes) in 1 mL of Sp-TALP (PARRISH; SUSKO-PARRISH, 1988) to remove

the extender. The spermatozoa sediment was recovered and diluted in 50 mM Tris-HCl (pH 8) to the nuclei isolation protocol.

6.2.4 Sperm nuclei isolation

Sperm nuclei were isolated using the protocol described by Castillo et al. (2014) with few modifications. Briefly, sperm samples were incubated on ice with 50 mM Tris-HCl (pH 8) and 10 mM DTT for 15 minutes and CTAB (cetyltrimethylammonium bromide) was added to a final concentration of 0.1%. After 30 minutes on ice, samples were washed 2X with 50 mM Tris-HCl (pH 8) (3000G/5 minutes at 4 ^{o}C). The sediment containing sperm nuclei were recovered and submitted to protein extraction.

6.2.5 Evaluation of sperm nuclei in optical and transmission electron microscope

After the sperm nuclei isolation, to verify the effect of CTAB on bovine sperm, one sample exposed to CTAB treatment and a control sample (submitted to the same steps without CTAB) were evaluated in optical microscope (Olympus IX80 Olympus Corporation) with 100x magnification under mineral oil. The same samples were fixed with 2% glutaraldehyde in 0.1M sodium cacodylate buffer. The post-fixation occurred in 1% osmium tetroxide in the same buffer, followed by dehydration in ethanol, immersion in propylene oxide and inclusion in epoxy resin (Spurr, Electron Microscopy Sciences, Hatfield, PA, USA). Samples sections were stained with toluidine blue to select the appropriate areas. Then, ultrathin cuts (70 nm) were obtained and collected on 200 mesh screens covered with formvar film. The cuts were contrasted with lead citrate and uranyl acetate. The samples were analyzed by transmission electron microscopy Jeol 1010, 80Kv (Jeol USA, Peabody, MA, USA).

6.2.6 Nuclei protein extraction

Sperm nuclei were submitted to the protein extraction according to the protocol described by Castillo et al. (2014) with few modifications. Samples were incubated on ice with 650 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 8) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 15 minutes and after centrifugation (3000G/2

minutes at 4 ºC) the supernatant containing the soluble nuclear proteins were recovered. Proteins remained in the pellet (insoluble nuclear proteins) were treated with 500 mM HCl for 5 minutes at 37ºC and then both fractions were put tougher and precipitated with trichloroacetic acid (20% final concentration) for 10 minutes on ice, washed twice with acetone supplemented with 1% β-mercaptoethanol (16.000G/5 minutes at 4 ºC), dried at room temperature and resuspended with ultrapure water to quantify at Qubit®. Due to the low amount of protein extracted from each individual bull, samples were pooled (3 pools per group), and each sample was analyzed in duplicate. To avoid batches variations, the 10 bull samples were processed at the same time.

6.2.7 Protein digestion and MS analysis

Dried proteins were resuspended in 8M urea and submitted to in-solution digestion with trypsin (1:50 of trypsin to protein) overnight, at 30°C according to manufacture instructions. Samples were desalted using ZipTip C18 membranes. Mass spectrometry analysis were performed on LTQ-Orbitrap Velos ETD (ThermoFisher Scientific) coupled with Easy nanoLC II. The peptides were separated on a C18RP column on a 115 min gradient. The search of the peptides was performed using the UniprotKB database (*Bos taurus*) and the quantitation analysis was performed using MaxQuant (v1.6.2.3) software. For the proteins identified in each sample it was calculated the iBAQ ratio (iBAQ value/sum iBAQ value of all proteins in the sample) and these values were used to group comparison. For statistical analysis, we calculated the mean of iBAQ ratio for protein identified in both groups and the fold-change (mean HF/mean LF), considering six replicates per group. Groups were compared using Mann-Whitney test ($p<0.05$).

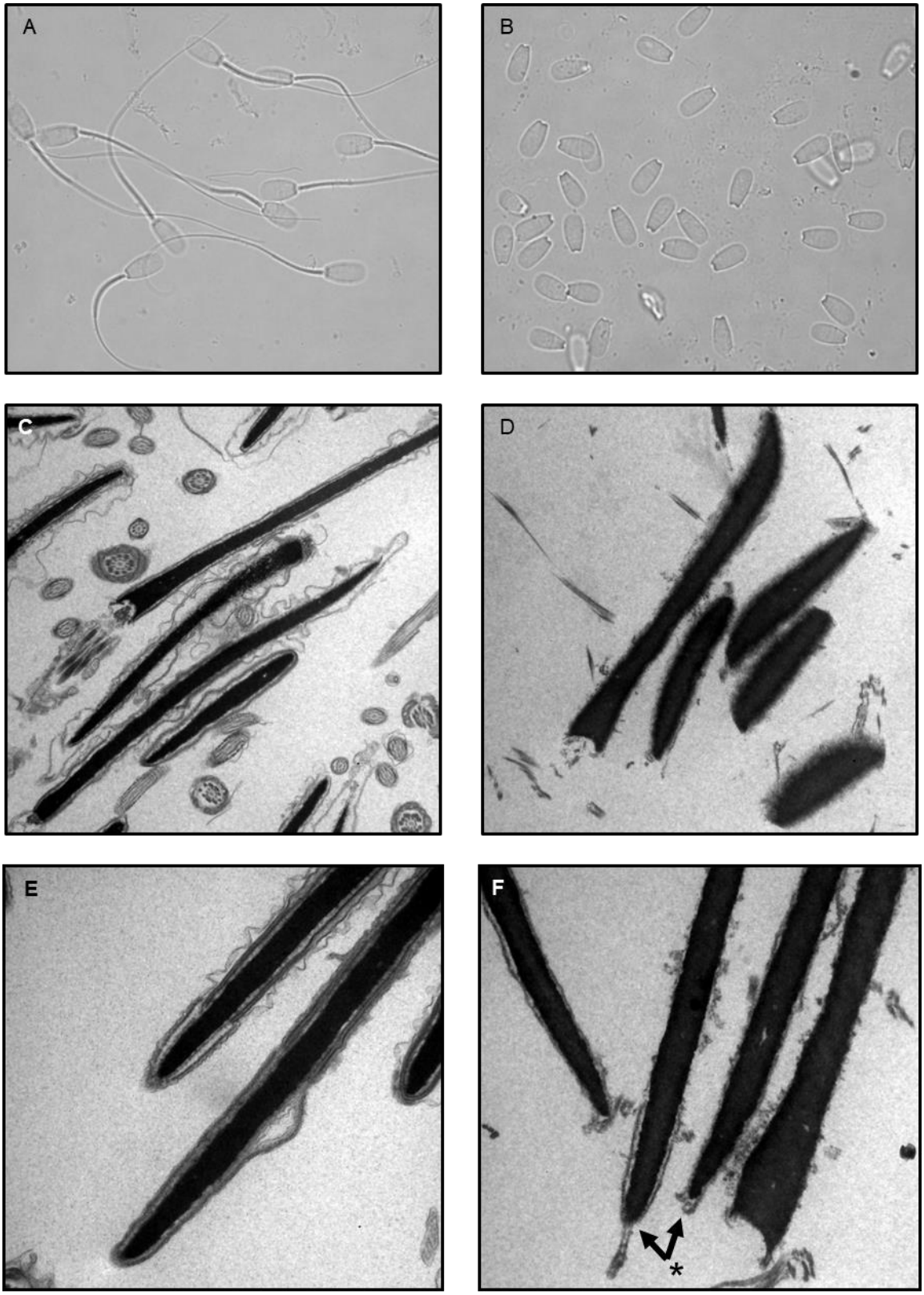
6.3 RESULTS

6.3.1 Evaluation of sperm nuclei in optical and transmission electron microscope

Based on the optical microscopy images (Figure 1A and B), we could verify that most part of sperm (around 90%) had the tail removed after the treatment with 0.1%

CTAB. No change was observed in the morphology of sperm head under optical microscopy. In the transmission electron microscopy (Figure 1C to F), we verified that, besides the tail, part of the sperm plasma membrane was removed. However, some cells remained with acrosome membrane parts. In addition, it is interesting to note a certain degree of nucleus edema in the CTAB-treated sample and apparent decompaction of the chromatin, with loss of definition around the sperm nucleus.

Figure 1. Microscope evaluations of control and CTAB-treated sperm samples.

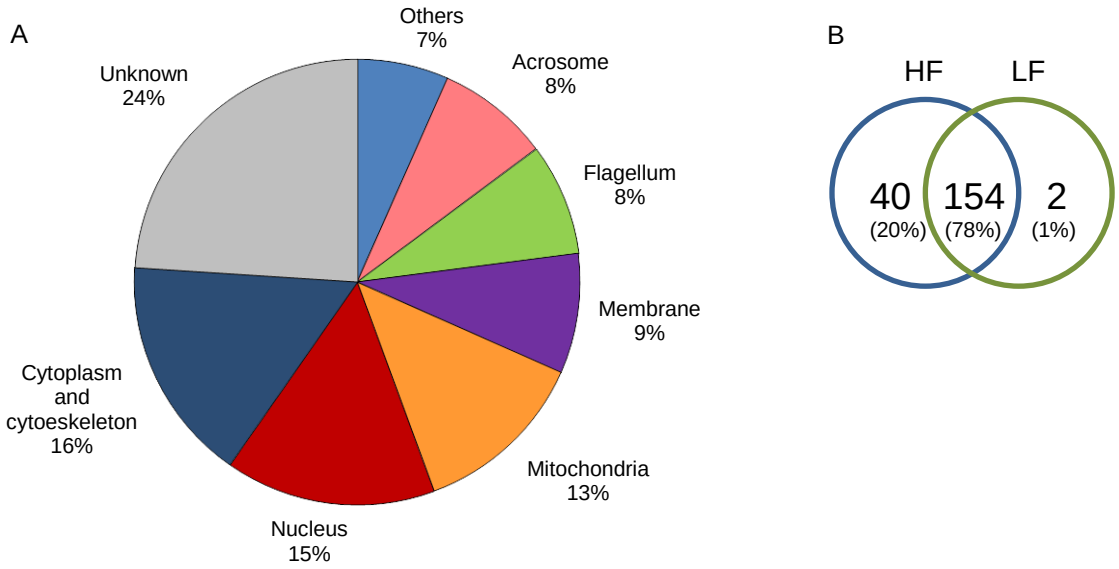


Legend: A and B – optical microscope (100x magnification with mineral oil); C-F – transmission electron microscope (C and D: 12,000X; E and F 25,000X). A, C and E – Control; C, D and F – treated with CTAB. F: arrows with asterisk indicate residual acrosome membrane.

6.3.2 Proteins identified by MS

A total of 196 proteins were identified in high (HF) and low (LF) *in vitro* fertility bull samples. Between them, 154 proteins were presented in both groups, 40 identified only in HF and 2 only in LF groups (Figure 3B, Table 1). The most abundant proteins for both groups were acrosomal vesicle protein (HF=0.60 and LF=0.64), protamine 2 (HF=0.16 and LF=0.19) and protamine 1 (HF=0.07 and LF=0.03). According to Gene Ontology classification for cellular component in the Uniprot database, 15% of proteins identified have nuclear localization, including different histones variants (H2A, H2B, H3.3C, H4) and isoforms (H2A.V, H2A.J and H2B subacrosomal). Proteins from other sperm compartments were also identified and the percentage of each one is presented in Figure 3A. Comparison between groups identified 21 differential expressed proteins, being 4 overexpressed in the LF group and 17 overexpressed in the HF group (Table 2; $p<0.05$). Fold-changes >1 indicated overexpressed proteins in the HF group (bold fold-change values), and < 1 indicated overexpressed proteins in the LF group (italic fold-change values). The cellular component information was indicated according to Uniprot database and when the information was absent for Bovine, it was used the database from Human.

Figure 3. Proteins identified by MS analysis of sperm nuclear proteins enrichment of high and low *in vitro* fertility groups



Legend: A – Percentage of proteins identified for each cell component according to UniprotKB database; B – Number and percentage of proteins identified in each group and commonly present. HF = high *in vitro* fertility; LF = low *in vitro* fertility.

Table 1. Proteins identified by MS analysis of sperm nuclear proteins enrichment exclusively in high or low *in vitro* fertility groups.

Uniprot ID	Protein names	Cellular component	Group
P82908	28S ribosomal protein S36, mitochondrial	Mitochondria	HF
Q2TA43	Actin-related protein T2	Cytoplasm	HF
F1MXA4	Adducin 2	Membrane	HF
O77797	A-kinase anchor protein 3	Acrosome	HF
P19483	ATP synthase subunit alpha	Mitochondria	HF
Q00361	ATP synthase subunit e, mitochondrial	Mitochondria	HF
A5PK67	Chromosome 15 open reading frame 48	Mitochondria	HF
F1MWN8	Cilia and flagella associated protein 58	Flagellum	HF
Q2TBP7	c-Myc-binding protein	Nucleus	HF
Q7YRA7	Cytochrome c-1 (Fragment)	Unknown	HF
Q9MZG3	Diazepam-binding inhibitor-like 5	Cytoplasm	HF
F1N206	Dihydrolipoyl dehydrogenase	Mitochondria	HF
E1BLB4	Dynein axonemal heavy chain 17	Flagellum	HF
E1BMG2	Dynein axonemal heavy chain 5	Flagellum	HF
P79136	F-actin-capping protein subunit beta	Cytoskeleton	HF
Q3ZBY4	Fructose-bisphosphate aldolase	Unknown	HF
F1MUX6	Glutathione S-transferase	Cytoplasm	HF
Q32LA7	Histone H2A.V	Nucleus	HF
E1BK75	Histone H2B	Nucleus	HF
E1B991	Keratin 2	Cytoskeleton	HF
P06394	Keratin, type I cytoskeletal 10	Cytoskeleton	HF
Q9BGH9	NAD(+) dependent isocitrate dehydrogenase subunit 3 (Fragment)	Unknown	HF
Q8SPP7	Peptidoglycan recognition protein 1	Extracellular	HF
E1BF14	POP1 homolog, ribonuclease P/MRP subunit	Nucleus	HF
Q32PB1	Profilin-3	Cytoskeleton	HF
Q2T9Y3	Pyruvate dehydrogenase E1 component subunit alpha	Mitochondria	HF
A8E4N3	Radial spoke head protein 3 homolog	Flagellum	HF
Q3T064	Ropporin-1	Flagellum	HF
P81019	Seminal plasma protein BSP-30 kDa	Extracellular	HF
P02784	Seminal plasma protein PDC-109	Extracellular	HF
Q32PB3	Sperm acrosome membrane-associated protein 4	Acrosome	HF
Q32L54	Sperm associated antigen 6	Cytoskeleton	HF
E1B830	Spermatogenesis-associated protein 6	Unknown	HF
A1A4K9	Succinyl-CoA:3-ketoacid-coenzyme A transferase	Mitochondria	HF
A6H782	Tektin-3	Flagellum	HF
E1BBM9	Uncharacterized protein	Membrane	HF
F1MR62	Uncharacterized protein	Unknown	HF
F1N6K8	Uncharacterized protein	Unknown	HF
F1MJB5	Uncharacterized protein	Unknown	HF
Q2TA11	Uncharacterized protein C1orf158 homolog	Unknown	HF
L8B5P3	Adenylate cyclase type 10 variant I	Nucleus	LF
Q2YDN8	Inactive serine/threonine-protein kinase VRK3	Nucleus	LF

Legend: HF = high *in vitro* fertility; LF = low *in vitro* fertility.

Table 2. Differential expressed proteins identified by MS analysis of sperm nuclear proteins enrichment between HF x LF groups

Uniprot ID	Protein name	Mean (iBAQ ratio)		Fold-change HF/LF	p value	Cellular Component
		HF	LF			
Q2T9N7	Lysozyme-like protein 4 (LYZL4)	4.83E-04	2.25E-04	**2.15**	0.0039	Acrosome (vesicle; secreted)
E1BE64	Testis specific 10 (TSGA10)	2.01E-04	3.27E-05	**6.15**	0.0039	Cytoeskeleton*
P62157	Calmodulin (CaM)	1.79E-03	3.28E-05	**54.50**	0.0062	Cytoplasm
E1BJG2	Radial spoke head 6 homolog A (RSPH6A)	1.29E-04	6.71E-05	**1.92**	0.0065	Intracellular
Q2YDG7	Sperm acrosome membrane-associated protein 1 (SPACA1)	3.97E-04	7.08E-05	**5.62**	0.0065	Acrosome (inner membrane)
Q3MHM7	60S ribosomal protein L35	1.68E-03	6.74E-04	**2.49**	0.0104	Ribosomal
G3N0H2	Chromosome 11 C2orf16 homolog	6.08E-06	1.50E-05	*0.40*	0.0105	-
G3X6J2	Uncharacterized protein	4.27E-06	8.56E-06	*0.50*	0.0143	-
Q3T0Y5	Proteasome subunit alpha type-2	2.80E-05	1.69E-05	**1.66**	0.0143	Nucleus
Q9XS94	Major fibrous sheath protein	1.62E-03	1.96E-05	**83.06**	0.0163	Flagellum*
Q5E956	Triosephosphate isomerase (TIM)	2.44E-04	1.06E-04	**2.30**	0.0163	Cytosol
Q3ZBX9	Histone H2A.J	1.30E-02	6.66E-03	**1.95**	0.0250	Nucleus
Q03763	Desmoglein-1	2.55E-03	2.52E-02	*0.10*	0.0250	Membrane
Q0IIA4	Glycoprotein 2	1.66E-05	4.21E-05	*0.39*	0.0250	Extracellular; secreted
Q32L60	E3 ubiquitin-protein ligase TRIM13	8.34E-03	3.60E-04	**23.16**	0.0250	Endoplasmic reticulum
W0UV03	Ribonuclease A C1	6.89E-05	4.15E-05	**1.66**	0.0339	Endonuclease
Q28068	Calicin	9.03E-05	1.46E-05	**6.21**	0.0339	Cytoeskeleton
Q2T9X5	Uncharacterized protein C7orf61 homolog	9.35E-04	3.30E-04	**2.83**	0.0374	-
Q2TBH0	Outer dense fiber protein 3 (ODF3)	1.95E-03	8.52E-04	**2.29**	0.0374	Flagellum
Q9XSJ4	Alpha-enolase 1 (ENO1)	2.67E-04	4.81E-05	**5.56**	0.0446	Membrane
Q6YFP9	Cytochrome c oxidase subunit 6B2	4.93E-04	6.06E-05	**8.13**	0.0446	Mitochondria

*according to Uniprot database for Human. Bold fold-change value indicates overexpressed proteins in the HF group, italic fold-change value indicates overexpressed proteins in the LF group. HF = high *in vitro* fertility; LF = low *in vitro* fertility.

6.4 DISCUSSION

Proteome studies in human sperm had identified so far 6238 different proteins, being 40% exclusively located to the head or sperm tail (AMARAL et al., 2014a; JODAR; SOLER-VENTURA; OLIVA, 2017). Most part of this studies try to identify proteins that could explain or serve as biological marks for fertility status. The proteomic subcellular fractions help to understand the function of identified protein and their relationship with proposed study model. In the present study we performed the quantitative proteomic analysis of sperm nuclei extraction from high and low *in vitro* fertility bulls. The idea of using the sperm nuclei extraction protocol with CTAB before the protein precipitation was to enrich the samples with nuclear proteins that could be lost in conventional cellular protein extraction protocol due to their different solubility. For that, we based our protocol in the previous described for human sperm (CASTILLO et al., 2014; DE MATEO et al., 2011). To verify the effect of CTAB on bovine spermatozoa, we analyzed treated CTAB samples in optical microscopy and electron transmission microscope. We were able to identify the removal of tail and part of plasma membrane, with a minor degree of nuclear swelling. However, some spermatozoa still presented acrosome membrane residues, which explains some extra-nuclear proteins in the proteome profile. Studies in mice using CTAB-like detergents, such as ATAB (alkyltrimethylammonium bromide) and DTT demonstrated by electron microscopy similar alterations like ours of nuclear swelling (MOHAR et al., 2002; WARD; KIMURA; YANAGIMACHI, 1999). In these studies, the authors denominate this apparent decondensation or swealling as "nuclear halos", that would be the formation of DNA loops adhered to nuclear matrix.

According to our proteome results, we identified 196 proteins between high and low *in vitro* fertility bull samples that were distributed with higher percentage in nuclear (15%), cytoplasm and cytoskeleton (16%) and mitochondrial (13%) compartments. Despite the number of protein found in our study is lower than human studies, the proportion is similar to studies that perform sperm nuclei extraction to proteome analysis (CASTILLO et al., 2014; DE MATEO et al., 2011). As expected, two of the most abundant proteins in our samples were protamine 1 (PRM1) and protamine 2 (PRM2). These proteins are rich in cysteine which forms disulfide bonds that stabilize and sustain the high level of sperm chromatin compaction (DE YEBRA; OLIVA, 1993). Interestingly, PRM2 was described only in human and mice sperm (CARRELL;

EMERY; HAMMOUD, 2007). In bovine, PRM2 was identified as transcribed and translated on low levels and point mutations were identified that altered its affinity to the DNA (MAIER et al., 1990). The authors concluded that PRM2 in bovine is functionally deficient, been replaced by PRM1 during spermatogenesis. As well as histones, protamines can also carry post-translational modifications (PTM) that may modulate protamine-DNA interaction and control the protamine-histone exchange after fertilization (BRUNNER; NANNI; MANSUY, 2014). Although in our study the PRM2 level was similar between HF and LF groups, it is important to notice that this protein had higher levels when compared to PRM1. This finding open new doors to investigate deeply the relationship between both protamines in bovine sperm and the function of PRM2.

The quantitative comparison between HF and LF groups identified 21 different expressed proteins, most of them (17 proteins) overexpressed in the HF group. For some of the 21 proteins there is no clear description or function annotation in the protein database, or they were generic proteins with multiple functions and were not deeply investigate. The main goal of our study was to identify possible nuclear sperm proteins that could explain bull *in vitro* fertility difference. Two of 21 proteins differentially expressed has nuclear localization according to Uniprot database: Proteasome subunit alpha type-2 and histone H2A.J. Histones are well conserved proteins found in eukaryote cells responsible to form nucleosome structures, which is composed by two H3/H4 and two H2A/H2B histone-fold heterodimers wrapping 145 to 147 bp DNA (MCGINTY; TAN, 2015). During spermatogenesis, the nucleosome structures are replaced by toroids formed by protamine that increase the sperm DNA compaction (ZINI; AGARWAL, 2011). For human sperm, around 15% of the chromatin remains associated with histones (CARRELL; EMERY; HAMMOUD, 2007), but for bovine sperm, this number is still unknown. Tovich and Oko (2003) described the presence of extra-nuclear histones, identifying 4 different types of histones (H3, H2B, H2A and H4) in bovine sperm peri-nuclear theca, considering as somatic histones with non-nuclear localization. Other studies verified histones variants in bovine sperm by immunofluorescence or western-blotting techniques (DE OLIVEIRA et al., 2013; KUTCHY et al., 2018). In our study, we identified 7 histones variants (H2A, H2B, H2A.V, H2A.J, H3.3C, H4, H2B subacrosomal), being the H2A.J overexpressed in HF group. This histone variant is only found in mammals cells and differs from canonical H2A by only five amino acids (CONTREPOIS et al., 2017). A comprehensive analysis

of PTM in mouse and human germ cells including mature sperm identified 10 different isoforms of H2A, including H2A.J (LUENSE et al., 2016). The only study describing its function relates H2A.J accumulation in senescent fibroblast with DNA damage, suggesting its potential functional in senescence, aging and cancer pathways (CONTREPOIS et al., 2017). However, it is difficult to extrapolate the same effect for sperm, especially due to the lack of studies for this variant.

The proteasome is a large protein complex, ATP-dependent responsible for degradation of intracellular proteins; misfolded, damaged or no longer required. The proteasome subunit alpha type-2, overexpressed in the HF group, is a component of 20S proteasome complex, which in turn is part of 26S proteasome complex (TANAKA, 2009). In sperm, the proteasome complex localization is normally associated to acrosomal membrane, during acrosomal biogenesis at the spermatids stages (RIVKIN et al., 2009) and regulates fertilization, particularly in the process of sperm penetration of zona pellucida (SAKAI; SAWADA; SAWADA, 2004; SANCHEZ et al., 2011). This complex was also involved in starting the capacitation process and acrosome reaction, extending its participation after sperm incorporation (revised by SUTOVSKY, 2011).

Another acrosome protein overexpressed in HF group was the sperm acrosome membrane-associated protein 1 (SPACA1). SPACA1 proteins are localized in the equatorial segment and along the inner acrosomal membrane of human spermatozoa, remaining in the equatorial segment of acrosome-reacted sperm (HAO et al., 2002). SPACA1 gene disrupted mice lines were infertile. For these animals the disruption of SPACA1 led to disappearance of nuclear plate, that coincides with acrosomal expansion during spermiogenesis, culminating with absence of acrosome in the mature spermatozoa (FUJIHARA et al., 2012). In human, the SPACA1 context was positively correlated with developmental rates of IVF embryos (KISHIDA et al., 2016).

Lysozyme-like protein 4 (LYZL4) was overexpressed in the HF group. Lysozyme proteins have bacteriolytic activity by hydrolyzing peptidoglycan in bacterial walls and they are widely distribute between species including plants and fungus (JOLLES; JOLLES, 1984). These proteins can present different functions according to biochemical assays. Narmadha and colleagues (2016) characterized the lysozyme genes and protein in rat and identified that LYZL4 possess hyaluronan binding ability and free radical scavenging. Also, in this study the presence of mRNA transcripts of LYZL4 seems to be androgen-dependent. In another study in mice LYZL4 was found on acrosome and tail, with no bacteriolytic activity. The immune-neutralization of this

protein significantly decreased *in vitro* fertilization, suggesting its function on fertilization process (SUN et al., 2011). In human sperm proteome analysis, LYZL4 was underexpressed in asthenozospermic men (AMARAL et al., 2014b). In livestock animals, LYZL4 was overexpressed in high littler size boars, and together with calmodulin, another overexpressed protein in HF group, integrate the groups of proteins that could have potential involvement in boar fertility regulation (KWON et al., 2015). It is not completely understood the function of LYZL4 for fertilization, but these studies indicate the participation of LYZL4 in sperm-oocyte binding process and sperm motility and then its participation in the characterization of fertility for different species.

The main scope of our work was to identify nuclear proteins that could explain differences between *in vitro* fertility bulls, considering their possible effect on the subsequent embryo development. However, some of the extranuclear overexpressed proteins in HF point it out to another direction, like calmodulin (CaM) and alpha enolase-1 (ENO1) proteins. Comparative proteome profile of bull sperm with different conception rate verified that the levels of CaM was higher for fertile bulls (SOMASHEKAR et al., 2017). Its overexpression was also presented in high litter size boars (KWON et al., 2015). Calmodulin is a highly conserved protein expressed in all eukaryotic cells that serves as an intermediary intracellular Ca^{2+} receptor (STEVENS, 1983). It is localized in the sperm tail and its function is associated to regulation of motility, once when inhibited, the motility decreases (SCHLINGMANN et al., 2007). ENO1 was also highly expressed in bulls with high conception rates and positively correlated to bull fertility status (MUHAMMAD ASLAM et al., 2018; PARK; KWON, 2012; SOGGIU et al., 2013). It is a metalloenzyme dependent of Mg^{2+} responsible for catalyzes the conversion of 2-phosphogycerate to phosphoenolpyruvate, one of the last steps of glycolysis (PANCHOLI, 2001) but it is also considered a multifunctional protein, found in a variety of tissues and marker for different pathologies like cancer, rheumatoid arthritis and Alzheimer's diseases (DIAZ-RAMOS et al., 2012). ENO1 is more abundant in mature spermatozoa, immunodetected in the sperm tail, and residual cytoplasm bodies from elongating spermatids, primary spermatocytes and round spermatids (GITLITS et al., 2000). In this study, authors verified the interaction of ENO1 with microtubules suggesting a possible regulation of enzyme activity by linking energy production from glycolysis to microtubules utilization. The relationship of ENO1 with motility was later described by its interaction with other proteins to enhance sperm metabolism and then increasing the motility (MOR et al., 2008). Another

overexpressed protein in HF with participation in glycolysis is triosephosphate isomerase (TIM) responsible for interconvert dihydroxyacetone phosphate with glyceraldehyde 3-phosphate (OLIVARES-ILLANA et al., 2017). The overexpression of these proteins in high fertility bulls, either *in vivo* or *in vitro* fertility conditions indicate an important effect of CaM and ENO1 to bull fertility profile.

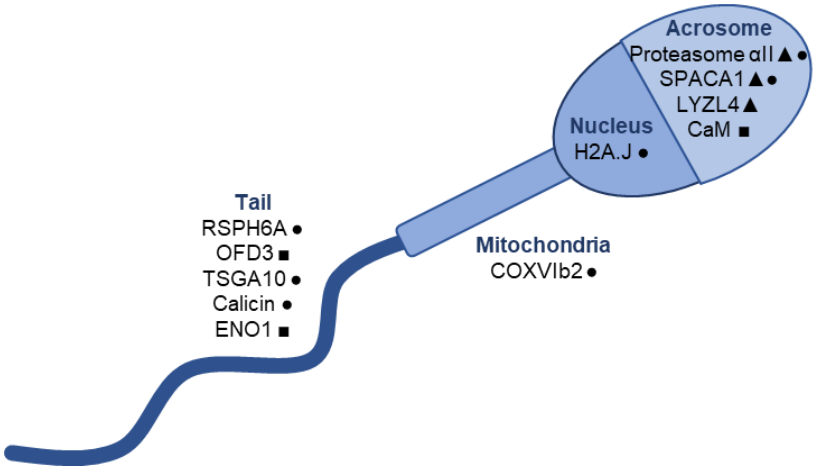
Following the same direction, other proteins related to sperm tail assembly and function were overexpressed in HF group: Radial spoke head 6 (RSPH6A), outer dense fiber 3 (ODF3), testis specific 10 (TSGA10) and calicin. The mammalian sperm flagellum is composed by cytoskeleton proteins that form the axoneme. This structure is formed by microtubes, outer dense fiber, surrounding the axoneme in the midpiece, and the fibrous sheath, which surround the outer dense fiber in the principal piece (OKO, 1988). Both major fibrous sheath and outer dense fiber 3 protein were overexpressed in HF bulls. ODF3 protein is related to sperm motility via stabilization of axoneme. Asthenozoospermic men presented down-regulation of ODFs family and they could be involved in this pathogenesis (ZHAO et al., 2018). TSGA10 is one of the highly expressed genes in testis (OSADA et al., 2002) and it is localized in the midpiece and tail of spermatozoa (BEHNAM et al., 2006). A case report patient with high percentage of acephalic spermatozoa showed homozygotic deletion of TSGA10 gene and completely absence of this protein (SHA et al., 2018). *Rsph6a* knockout male mice are infertile as result of short immotile spermatozoa. The observation of testis tissue from this animals indicated that the axoneme can elongate but is disrupted before the formation of accessory structures, with impair of manchette removal, indicating that the presence of RSPH6A is essential to sperm flagellar assembly (ABBASI et al., 2018). Calicin was identified for the first time by von Bulow and colleagues (1995) in bull and men sperm as a component of the calyx, the cytoskeleton structure that associates the nucleus to the post-acrosomal region. This structure is part of perinuclear theca and appear to contribute to the morphogenesis and stability of sperm head (LECUYER et al., 2000). The degradation of calicin after fertilization seems to be intimately involved with nuclear decondensation (PARANKO; SALONEN, 1995).

Regarding to cellular metabolism, cytochrome c oxidase subunit 6B2 (COXVIb2) was overexpressed in HF group. Cytochrome c oxidase or complex IV is the last complex of mitochondrial electron transfer chain (LI et al., 2006). The COXVIb2 was reported as a testes-specific isoform in human, bull, rat, and mouse. Differences between amino acids sequence for isoform VIb1 and VIb2 suggest interactions with

cytochrome c and sperm-specific energy requirements (HUTTEMANN; JARADAT; GROSSMAN, 2003).

Although we performed sperm nuclei enrichment to increase the changes to identify nuclear proteins that could explain the *in vitro* differences during the embryo development, differentially expressed proteins indicate another pathway to bovine fertility. Overall, most of overexpressed proteins in HF group are associated to acrosome and tail components of sperm cell and their function are intimately related to spermatogenesis, motility and ability to fertilize the oocyte (Figure 4). These proteins cannot be directly associated by their function to embryo development or possible nuclear epigenetic marks delivered from sperm to the embryo as it was our initial hypothesis. However, they could be indicators of a successful spermatogenesis which will form sperm that produce embryos with higher development potential.

Figure 4. Some of the overexpressed identified proteins in high *in vitro* fertility group and their function and localization in spermatozoa.



Legend: Proteins were grouped according to localization (tail, nucleus, acrosome or mitochondria). Symbols indicate their function on spermatozoa: ● Spermatogenesis; ▲ Fertilization and ■ Motility.

The spermatogenesis is a complex process in which a round spermatogonia loses half of its DNA, cytoplasm and organelle, suffers nuclear compaction to form the flagellar shape of spermatozoa. The sperm competence requires the collective activities of several proteins so then the spermatozoa will be capable to fertilize the oocyte and sustain the subsequent embryo development. The overexpressed proteins in the HF group could be indicators of other well-established sperm pathways that would be demonstrated as a later effect, increasing the changes to produce an *in vitro* blastocyst. Futures studies are necessary to study deeply some of these proteins,

primarily because, most of them, there is no report in bovine species. Calmodulin and ENO1 proteins are strong candidates to be studied as fertility markers in bull sperm once they were previous described by other studies, including *in vivo* fertility status.

In conclusion, our study identified 21 differentiated expressed proteins between high and low *in vitro* fertility bulls. These proteins are related to spermatogenesis, motility and ability to fertilize the oocyte, being possible indicator to future studies of bull fertility.

REFERENCES

ABBASI, F. et al. RSPH6A Is Required for Sperm Flagellum Formation and Male Fertility in Mice. **Journal of cell science**, v. 131, n. 19, out. 2018.

ALMEIDA, T. G. Cinética e desenvolvimento embrionário *in vitro* de embriões bovinos de touros de alta e baixa fertilidade. Dissertação de mestrado em Ciência, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2018.

AMARAL, A. et al. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. **Human reproduction update**, v. 20, n. 1, p. 40–62, 2014a. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24082039>. Acesso em: 10 fev. 2014.

AMARAL, A. et al. Identification of Proteins Involved in Human Sperm Motility Using High-Throughput Differential Proteomics. **Journal of proteome research**, v. 13, n. 12, p. 5670–5684, dez. 2014b.

AZPIAZU, R. et al. High-throughput sperm differential proteomics suggests that epigenetic alterations contribute to failed assisted reproduction. **Human Reproduction**, v. 29, n. 6, p. 1225–1237, 2014.

BEHNAM, B. et al. Expression of Tsga10 Sperm Tail Protein in Embryogenesis and Neural Development: From Cilium to Cell Division. **Biochemical and biophysical research communications**, v. 344, n. 4, p. 1102–1110, jun. 2006.

BONASIO, R.; TU, S.; REINBERG, D. Molecular Signals of Epigenetic States. **Science (New York, N.Y.)**, v. 330, n. 6004, p. 612–616, out. 2010.

BRUNNER, A. M.; NANNI, P.; MANSUY, I. M. Epigenetic marking of sperm by post-translational modification of histones and protamines. **Epigenetics & chromatin**, v. 7, n. 1, p. 2, 2014. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3904194&tool=pmcentrez&rendertype=abstract>.

CARRELL, D. T.; EMERY, B. R.; HAMMOUD, S. Altered protamine expression and diminished spermatogenesis: what is the link? **Human Reproduction Update**, v. 13, n. 3, p. 313–27, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17208950>. Acesso em: 21 jul. 2011.

CASTILLO, J. et al. Genomic and proteomic dissection and characterization of the human sperm chromatin. **Molecular human reproduction**, v. 20, n. 11, p. 1041–53, 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/25193639>.

CASTILLO, J.; AMARAL, a.; OLIVA, R. Sperm nuclear proteome and its epigenetic potential. **Andrology**, v. 2, n. 3, p. 326–338, 2014.

CASTRO, L. S. et al. Effect of bovine sperm chromatin integrity evaluated using three different methods on in vitro fertility. **Theriogenology**, v. 107, p. 142–148, 2018. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0093691X1730540X>.

CONTREPOIS, K. et al. Histone variant H2A.J accumulates in senescent cells and promotes inflammatory gene expression. **Nature Communications**, v. 8, n. May, 2017.

DE MATEO, S. et al. Proteomic characterization of the human sperm nucleus. **Proteomics**, v. 11, p. 2714–2726, 2011.

DE OLIVEIRA, R. V et al. Molecular morphology and function of bull spermatozoa linked to histones and associated with fertility. **Reproduction (Cambridge, England)**, v. 146, n. 3, p. 263–72, set. 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23904564>. Acesso em: 24 set. 2014.

DE YEBRA, L.; OLIVA, R. Rapid analysis of mammalian sperm nuclear proteins. **Analytical biochemistry**, v. 209, p. 201–203, 1993. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0003269783711048>. Acesso em: 23 fev. 2015.

DIAZ-RAMOS, A. et al. Alpha-Enolase, a Multifunctional Protein: Its Role on Pathophysiological Situations. **Journal of biomedicine & biotechnology**, v. 2012, p. 156795, 2012.

DOGAN, S. et al. Sperm Protamine-Status Correlates to the Fertility of Breeding Bulls. **Biology of Reproduction**, v. 92, n. August 2014, p. 1–9, 2015. Disponível em: <http://www.biolreprod.org/cgi/doi/10.1095/biolreprod.114.124255>.

FUJIHARA, Y. et al. SPACA1-Deficient Male Mice Are Infertile with Abnormally Shaped Sperm Heads Reminiscent of Globozoospermia. **Development (Cambridge, England)**, v. 139, n. 19, p. 3583–3589, out. 2012.

GITLITS, V. M. et al. The Glycolytic Enzyme Enolase Is Present in Sperm Tail and Displays Nucleotide-Dependent Association with Microtubules. **European journal of cell biology**, v. 79, n. 2, p. 104–111, fev. 2000.

HAO, Z. et al. SAMP32, a Testis-Specific, Isoantigenic Sperm Acrosomal Membrane-Associated Protein. **Biology of reproduction**, v. 66, n. 3, p. 735–744, mar. 2002.

HUTTEMANN, M.; JARADAT, S.; GROSSMAN, L. I. Cytochrome c Oxidase of Mammals Contains a Testes-Specific Isoform of Subunit VIb--the Counterpart to Testes-Specific Cytochrome C? **Molecular reproduction and development**, v. 66, n. 1, p. 8–16, set. 2003.

INTASQUI, P. et al. Unraveling the sperm proteome and post-genomic pathways associated with sperm nuclear DNA fragmentation. **Journal of Assisted**

Reproduction and Genetics, v. 30, p. 1187–1202, 2013.

JAENISCH, R.; BIRD, A. Epigenetic Regulation of Gene Expression: How the Genome Integrates Intrinsic and Environmental Signals. **Nature genetics**, v. 33 Suppl, p. 245–254, mar. 2003.

JODAR, M.; SOLER-VENTURA, A.; OLIVA, R. Semen Proteomics and Male Infertility. **Journal of proteomics**, v. 162, p. 125–134, jun. 2017.

JOLLES, P.; JOLLES, J. What's New in Lysozyme Research? Always a Model System, Today as Yesterday. **Molecular and cellular biochemistry**, v. 63, n. 2, p. 165–189, set. 1984.

KISHIDA, K. et al. Individual Differences in the Distribution of Sperm Acrosome-Associated 1 Proteins among Male Patients of Infertile Couples; Their Possible Impact on Outcomes of Conventional in Vitro Fertilization. **Zygote (Cambridge, England)**, v. 24, n. 5, p. 654–661, out. 2016.

KUTCHY, N. A. et al. Acetylation and methylation of sperm histone 3 lysine 27 (H3K27ac and H3K27me3) are associated with bull fertility. **Andrologia**, v. 50, n. 3, p. 1–11, 2018.

KWON, W.-S. et al. Discovery of Predictive Biomarkers for Litter Size in Boar Spermatozoa. **Molecular & cellular proteomics : MCP**, v. 14, n. 5, p. 1230–1240, maio 2015.

LECUYER, C. et al. Actin-Binding Properties and Colocalization with Actin during Spermiogenesis of Mammalian Sperm Calicin. **Biology of reproduction**, v. 63, n. 6, p. 1801–1810, dez. 2000.

LI, Y. et al. Cytochrome c Oxidase Subunit IV Is Essential for Assembly and Respiratory Function of the Enzyme Complex. **Journal of bioenergetics and biomembranes**, v. 38, n. 5–6, p. 283–291, dez. 2006.

LUENSE, L. J. et al. Comprehensive analysis of histone post-translational modifications in mouse and human male germ cells. **Epigenetics and Chromatin**, v. 9, n. 1, p. 1–15, 2016.

MAIER, W. M. et al. The lack of protamine 2 (P2) in boar and bull spermatozoa is due to mutations within the P2 gene. **Nucleic Acids Research**, v. 18, n. 5, p. 1249–1254, 1990.

MCGINTY, R. K.; TAN, S. **Nucleosome Structure and FunctionChemical Reviews**, mar. 2015. .

MOHAR, I. et al. Sperm nuclear halos can transform into normal chromosomes after injection into oocytes. **Molecular Reproduction and Development**, v. 62, n. January, p. 416–420, 2002.

MOR, I. et al. Acetylcholinesterase-R Increases Germ Cell Apoptosis but Enhances Sperm Motility. **Journal of cellular and molecular medicine**, v. 12, n. 2, p. 479–495, abr. 2008.

MUHAMMAD ASLAM, M. K. et al. Identification of biomarker candidates for fertility in

spermatozoa of crossbred bulls through comparative proteomics. **Theriogenology**, v. 119, p. 43–51, 2018. Disponível em: <https://doi.org/10.1016/j.theriogenology.2018.06.021>.

NARMADHA, G.; YENUGU, S. In silico and biochemical characterization of lysozyme-like proteins in the rat. **PLoS ONE**, v. 11, n. 9, p. 1–32, 2016.

NI, K. et al. The impact of sperm protamine deficiency and sperm DNA damage on human male fertility: a systematic review and meta-analysis. **Andrology**, v. 4, n. 5, p. 789–99, 2016. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/27231200>.

OKO, R. Comparative Analysis of Proteins from the Fibrous Sheath and Outer Dense Fibers of Rat Spermatozoa. **Biology of reproduction**, v. 39, n. 1, p. 169–182, ago. 1988.

OLIVA, R.; DIXON, G. H. Vertebrate Protamine Genes and the Histone-to-Protamine Replacement Reaction. **Progress in nucleic acid research and molecular biology**, v. 40, p. 25–94, 1991.

OLIVARES-ILLANA, V. et al. A Guide to the Effects of a Large Portion of the Residues of Triosephosphate Isomerase on Catalysis, Stability, Druggability, and Human Disease. **Proteins**, v. 85, n. 7, p. 1190–1211, jul. 2017.

OSADA, N. et al. Cynomolgus Monkey Testicular CDNAs for Discovery of Novel Human Genes in the Human Genome Sequence. **BMC genomics**, v. 3, n. 1, p. 36, dez. 2002.

PANCHOLI, V. Multifunctional Alpha-Enolase: Its Role in Diseases. **Cellular and molecular life sciences : CMLS**, v. 58, n. 7, p. 902–920, jun. 2001.

PARANKO, J.; SALONEN, I. Calicin in Human Sperm Fertilizing Zona-Free Hamster Eggs in Vitro. **Reproduction, fertility, and development**, v. 7, n. 1, p. 97–105, 1995.

PARK, Y.; KWON, W. Fertility-related proteomic profiling bull spermatozoa separated by percoll. **Journal of proteome …**, v. 11, p. 4162–4168, 2012. Disponível em: <http://pubs.acs.org/doi/abs/10.1021/pr300248s>. Acesso em: 9 abr. 2014.

PARRISH, J.; SUSKO-PARRISH, J. Capacitation of bovine sperm by heparin. **Biology of Reproduction**, p. 1171–1180, 1988. Disponível em: <http://www.biolreprod.org/content/38/5/1171.short>. Acesso em: 19 jun. 2013.

RIVKIN, E. et al. Rnf19a, a Ubiquitin Protein Ligase, and Psmc3, a Component of the 26S Proteasome, Tether to the Acrosome Membranes and the Head-Tail Coupling Apparatus during Rat Spermatid Development. **Developmental dynamics : an official publication of the American Association of Anatomists**, v. 238, n. 7, p. 1851–1861, jul. 2009.

SAKAI, N.; SAWADA, M. T.; SAWADA, H. Non-Traditional Roles of Ubiquitin-Proteasome System in Fertilization and Gametogenesis. **The international journal of biochemistry & cell biology**, v. 36, n. 5, p. 776–784, maio 2004.

SANCHEZ, R. et al. Participation of the Sperm Proteasome during in Vitro Fertilisation and the Acrosome Reaction in Cattle. **Andrologia**, v. 43, n. 2, p. 114–120, abr. 2011.

SCHLINGMANN, K. et al. Calmodulin and CaMKII in the Sperm Principal Piece: Evidence for a Motility-Related Calcium/Calmodulin Pathway. **Journal of andrology**, v. 28, n. 5, p. 706–716, 2007.

SHA, Y.-W. et al. TSGA10 Is a Novel Candidate Gene Associated with Acephalic Spermatozoa. **Clinical genetics**, v. 93, n. 4, p. 776–783, abr. 2018.

SIMON, L. et al. Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. **Reproductive BioMedicine Online**, v. 23, n. 6, p. 724–734, 2011. Disponível em: <http://dx.doi.org/10.1016/j.rbmo.2011.08.010>.

SIMON, L. et al. Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment. **Human Reproduction**, v. 29, n. 5, p. 904–917, 2014.

SOGGIU, A. et al. Unravelling the bull fertility proteome. **Molecular bioSystems**, v. 9, n. 6, p. 1188–95, jun. 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23392320>. Acesso em: 9 abr. 2014.

SOMASHEKAR, L. et al. Comparative sperm protein profiling in bulls differing in fertility and identification of phosphatidylethanolamine-binding protein 4, a potential fertility marker. **Andrology**, v. 5, n. 5, p. 1032–1051, 2017.

STEVENS, F. C. Calmodulin: An Introduction. **Canadian journal of biochemistry and cell biology = Revue canadienne de biochimie et biologie cellulaire**, v. 61, n. 8, p. 906–910, ago. 1983.

SUN, R. et al. Lyzl4, a Novel Mouse Sperm-Related Protein, Is Involved in Fertilization. **Acta biochimica et biophysica Sinica**, v. 43, n. 5, p. 346–353, maio 2011.

SUTOVSKY, P. Sperm Proteasome and Fertilization. **Reproduction (Cambridge, England)**, v. 142, n. 1, p. 1–14, jul. 2011.

TANAKA, K. The Proteasome: Overview of Structure and Functions. **Proceedings of the Japan Academy. Series B, Physical and biological sciences**, v. 85, n. 1, p. 12–36, jan. 2009.

TOVICH, P. R.; OKO, R. J. Somatic histones are components of the perinuclear theca in bovine spermatozoa. **Journal of Biological Chemistry**, v. 278, n. 34, p. 32431–32438, 2003.

VON BULOW, M. et al. Molecular Nature of Calicin, a Major Basic Protein of the Mammalian Sperm Head Cytoskeleton. **Experimental cell research**, v. 219, n. 2, p. 407–413, ago. 1995.

WARD, W.; COFFEY, D. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. **Biology of Reproduction**, p. 569–574, 1991. Disponível em: <http://www.biolreprod.org/content/44/4/569.short>. Acesso em: 19 ago. 2014.

WARD, W. S.; KIMURA, Y.; YANAGIMACHI, R. An intact sperm nuclear matrix may be necessary for the mouse paternal genome to participate in embryonic development. **Biology of reproduction**, v. 60, p. 702–706, 1999.

YU, B. et al. Cigarette smoking is associated with abnormal histone-to-protamine transition in human sperm. **Fertility and Sterility**, v. 101, n. 1, p. 51–57, 2014. Disponível em: <http://dx.doi.org/10.1016/j.fertnstert.2013.09.001>.

ZHAO, W. et al. Outer Dense Fibers Stabilize the Axoneme to Maintain Sperm Motility. **Journal of cellular and molecular medicine**, v. 22, n. 3, p. 1755–1768, mar. 2018.

ZINI, A.; AGARWAL, A. **Sperm Chromatin**. New York, NY: Springer New York, 2011.

7 CHAPTER 3: THE ABSENCE OF NUCLEOSOME-BINDING DNA IN MATURE SPERM FROM LIVESTOCK SPECIES AND PRIMATE

7.1 INTRODUCTION

Sperm chromatin have a unique composition when compared to other cells types. Its highly compacted structure protects paternal DNA during the transport to the oocyte and after the zygote formation (CARRELL et al., 2016). The high degree of compaction is acquired during spermatogenesis when the nucleosomes composed by histones are replaced by toroids, formed by a exclusively sperm nuclear protein called protamine (ZINI; AGARWAL, 2011). However, part of sperm DNA remains attached to histones, around 5 to 15% in human spermatozoa (CARRELL; HAMMOUD, 2010). Between then, the variants H2A, H2B, H3, H4 and the testicle-specific variant tH2B (GATEWOOD et al., 1990; KIMMINS; SASSONE-CORSI, 2005). The testicle and spermatic-specific variants such as H2AL1, H2AL2, H2BL1 were described in both, spermatids and mature spermatozoa in murine (GOVIN et al., 2007) and it has already been demonstrated that some paternal histones are maintained in zygote chromatin, unlike protamine that after fertilization is immediately replaced (VAN DER HEIJDEN et al., 2008).

So far, studies in mice and human sperm described the presence of nucleosome structures, although their results vary considerably. Some studies found sperm histones enriched next to promoter regions of genes related to embryo development, miRNA, HOX genes, imprinted genes and chromatin binding sites like CCCTC-binding factor (ARPANAHI et al., 2009; BRYKCZYNSKA et al., 2010; CARONE et al., 2014; HAMMOUD et al., 2009). On the opposite side, other studies found nucleosome structures enriched at intergenic regions or gene deserts (SAITOU; KURIMOTO, 2014; SAMANS et al., 2014; YAMAGUCHI et al., 2018). These divergent results may be partially explained due to differences in experimental details and technical process used to map the nucleosome over the sperm DNA. In general, the preferential approach to study nucleosome-binding DNA uses micrococcal nuclease (MNase), which is an enzyme that preferentially digests naked DNA and keeps intact nucleosome-associated DNA (around 150 bp). Studies in different cells types demonstrated that the nucleosome accessibility can differ depending of MNase

concentration used (CARONE et al., 2014; MIECZKOWSKI et al., 2016). Some nucleosomes regions seem to be preferentially accessed at high MNase concentration and some regions at lower. Considering the unique way in which sperm DNA is packaged, differences between the level of DNA compaction, the use and concentration of reduced agents like DTT to break disulfide bonds between protamine and the concentration of MNase used for digestion could be variables to explain the difference between studies and possible variability of nucleosome assembly between species.

While there are many studies describing the nucleosome structures in mice and human sperm, a little is known about remaining histones in other species. Immunohistochemistry analysis of boar testes and epididymis indicated that only somatic cells (spermatogonia, spermatocytes and round spermatids) and not elongated spermatids or mature spermatozoa were imunoreacted to histone H1 (NAKAZAWA et al., 2002). For stallion sperm, Ketchum et al. (2018) described the participation of histone H4 acetylation during chromatin remodeling in the spermatogenesis process. Studies with bull sperm also identified histones with immunostaining analysis (KUTCHY et al., 2017, 2018) and western blotting (DE OLIVEIRA et al., 2013). However, these histones in the nucleus could be vestiges from spermatogenesis, without significant importance in the chromatin remodeling in the mature sperm. Based on that, we decided to investigate the presence of nucleosome-binding DNA in different mammalian species (stallion, boar, ram and monkey ejaculated sperm) with focus in bovine sperm, using mice as model species. This study could be the first step to further standardized protocols like immunoprecipitation and DNA sequencing (ChIP-Seq) of nucleosome-binding DNA and histone modifications for different livestock species to study epigenetic marks in mature spermatozoa.

7.2 MATERIAL AND METHODS

The present study was developed at Department of Animal Science at University of California, Davis (UC Davis). All animals' manipulations were performed by specialized technicians from UC Davis facilities and with procedures already approved by Institutional Animal Care and Use Committee (IACUC) from this University.

7.2.1 Sperm samples collection

For horse, ram and pig we performed the analysis with fresh semen collected by artificial vagina, electroejaculator and gloved-hand technique, respectively. For bull and monkey sperm, we used cryopreserved semen thawed at 37°C for 30 sec. For mice, sperm were isolated from the caudal epididymis of 10-week-old mice by slicing the tissue and put in 1.5 mL of TYH medium (119.4 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM Mg_2SO_4, 25 mM $NaHCO_3$, 1 mM sodium pyruvate, 25 mM sodium lactate, 5.6 mM glucose, 1 U/ml of penicillin G). Then, sperm were separated by swim-up for 1 h on incubator (37°C, 5% CO_2) and the upper phase was removed and centrifuged 2,000G/5 min to recover the cells. As positive control, we used bovine fibroblast, a somatic cell exclusively compacted by nucleosome. All chemical reagents and solutions used in this study were purchased from Sigma-Aldrich (St. Louis, MO, E.U.A.) unless otherwise stated.

7.2.2 Percoll® gradient

To avoid somatic cells contamination, sperm samples from horse, pig, ram, bull and monkey were submitted to Percoll® gradient. For that, 500 µL of semen was added on the top of 4 mL Percoll® gradient (2 mL 45% and 2 mL 90%), centrifuged 700G/15 min and the pellet with sperm was washed in 1 mL of PBS (1,100G/5 min). The concentration was evaluated at a Neubauer chamber.

7.2.3 MNase digestion

We followed the protocol described by Hisano et al. (2013) for mice and human sperm with few modifications. Sperm were pre-treated with DTT, centrifuge 2,000G/5 min, washed in 1 mL of DPBS (2,000G/5 minutes), and diluted in Buffer 1 solution (0.3M sucrose, 15 mM Tris-HCl pH 7.5, 60 mM KCl, 5 mM $MgCl_2$ and 0.1 mM EGTA) with 1% DOC and 0.5% NP-40 and incubated on ice for 10 min. To titrate the ideal MNase concentration, 2 x 10^6 spermatozoa were digested with different MNase concentrations diluted in MNase buffer (0.3M sucrose, 85 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$ and 2 mM $CaCl_2$) during 5 min. MNase treatment was stopped with 5 mM EDTA and placed on ice for 5 min. Samples were centrifuged at 17,000G/10 min and the

supernatant containing the digested DNA was recovered and processed for DNA extraction with proteinase K, phenol:chloroform and precipitated with ethanol. Extracted DNA was quantified in Nanodrop® and visualized in 2.5% agarose gel in TBE stained with ethidium bromide. An aliquot of each sample was also evaluated in the Bioanalyzer® (Agilent Technologies Inc, Santa Clara, CA, USA) using the high-sensitivity DNA chip according to the manufacture instructions.

7.3 RESULTS

7.3.1 Mice and bovine sperm digestion

The identification of nucleosome structure in sperm has been described mainly in mice and human. Based on that, we collected sperm samples from mouse epididymis to test the efficiency of this protocol. In this case, we followed the Hisano et al. (2013) protocol for mice, using 50 mM DTT/2 hours. After the tritation of MNase for mice, we performed a test with mice and bovine sperm samples under the same conditions. For mice sperm samples, we were able to get mononucleosome-sized DNA with 30U of MNase (Figure 1A). After that, we performed the digestion with 3 individual males with 30U and different concentrations of MNase for bovine sperm samples (Figure 1B). However, the same protocol was unable to get nucleosome band for bovine samples.

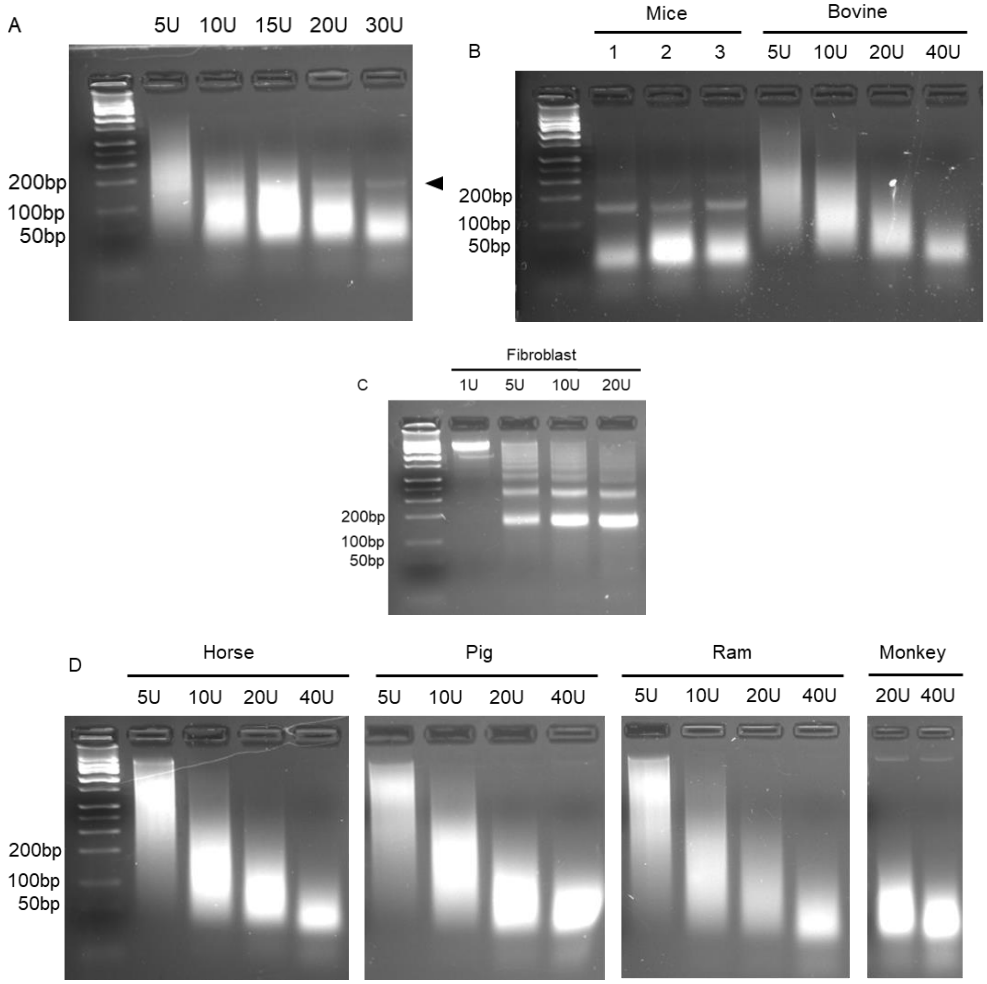
In attempt to check the efficiency of MNase protocol, we tested in fibroblast, a somatic cell exclusively compacted by nucleosome structures. In this experiment, it was evident the presence of different size of nucleosome (Figure 1C), even for low MNase concentrations (5U), and for higher concentrations (10U and 20U), more intense band at mononucleosome-sized DNA (around 150 bp). This result indicated that our protocol was efficient to get mononucleosome-sized DNA.

7.3.2 MNase protocol in livestock species and primate

We tested the protocol of MNase digestion with fresh sperm samples of horse, pig and ram, and thawed sperm from monkey. In all cases, sperm were incubated with 10 mM DTT/30 min and different MNase concentration. We decided to decrease the DTT from 50 mM/2 hours to 10 mM/30 min due to differences of sensibility between

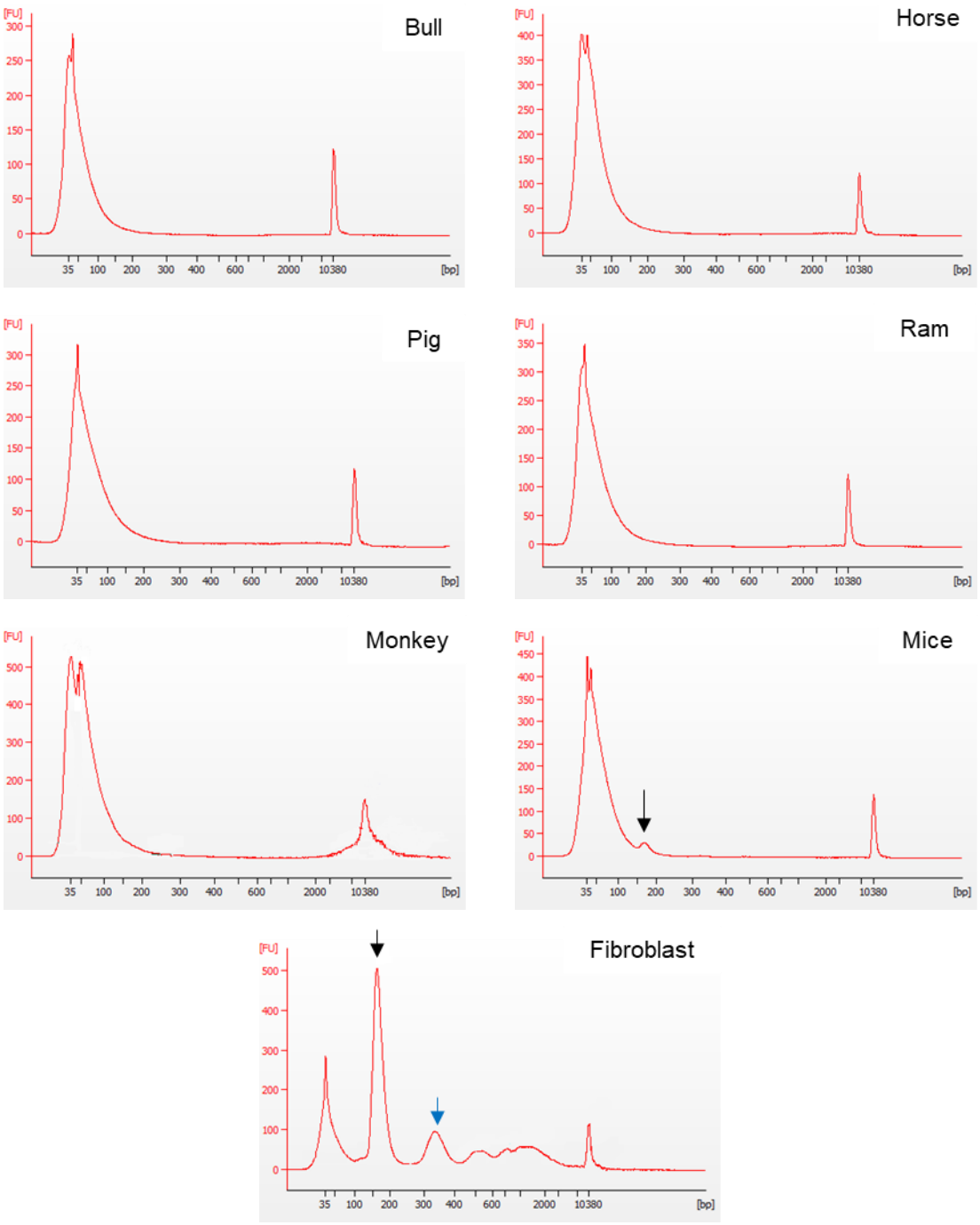
species. Horse and ram sperm presented after treatment head fragmentation or detach that impair the washes after this step (data not shown). For monkey, due to the small amount of sperm recovered after Percoll®, we could test only two MNase concentrations. In this experiment we verified that all species had the same digestion profile with increased doses of MNase, being the 40U the efficient concentration to digest the DNA under 100 bp (Figure 1D). However, we were not able to identify nucleosome band for any of these species. For a more accurate DNA evaluation, we analyzed the 40U digested DNA samples on Bioanalyzer (Figure 2).

Figure 1. MNase digestion of sperm DNA with increasing concentrations in different species and cell types



Legend: A- MNase digestion of mice DNA sperm with nucleosome DNA band around 150 bp at 30U lane (arrowhead point); B- MNase digestion of sperm DNA from 3 different mices (1 to 3) and bovine sperm DNA sample with increasing concentration of MNase; C- Bovine fibroblast MNase digestion with different nucleosome-sized DNA; D- MNase digestion of horse, pig, ram and monkey sperm DNA.

Figure 2. Bioanalyzer profile of sperm samples and fibroblast digested with 40U MNase.



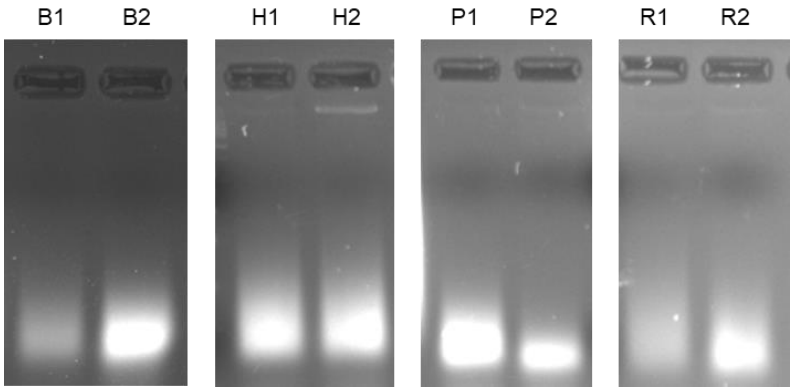
Legend: For all samples the left and right peak are ladders (35 and 10380 bp respectively). Black arrow in the mice and fibroblast samples indicate mononucleosome sized-DNA and blue arrow in the fibroblast sample indicates dinucleosome sized-DNA.

Bioanalyzer results confirmed the previous gel results, no band around 150 bp for bull, horse, pig, ram and monkey sperm samples. A small band of 150 bp for mouse

samples and different band sizes for fibroblast samples, with a more prominent band around 150 bp representing the mononucleosome-sized DNA.

To excluded individual effects, we tested two different bulls, stallions, boars and rams and again, we were not able to get nucleosome band from these samples, confirming our previous results (Figure 3).

Figure 3. MNase digestion (40U) of different bulls, stallions, boars and rams



Legend: MNase digestion of bull (B1 and B2), horse (H1 and H2), pig (P1 and P2) and ram (R1 and R2) sperm DNA samples.

7.4 DISCUSSION

Histones marks were found in human and mouse sperm preferentially located near to developmental genes (BRYKCZYNSKA et al., 2010; CASTILLO et al., 2014; HAMMOUD et al., 2009, 2014; HISANO et al., 2013). These raised the question if histones modifications can be passed to the embryo or if they have an important role on embryo development and epigenetic memory. Therefore, we started to investigate the presence of nucleosome structures using MNase digestion in the sperm of different livestock species, with focus in bovine sperm, in attempt to standardize this method for further studies of nucleosome-binding DNA or histones modification like immunoprecipitation and sequencing analysis (ChIP-Seq). However, for any sperm samples evaluated in the present study, with exception of mice sperm, we were not able to identify mononucleosome DNA.

The chromatin in the spermatocytes has the same group of histones as any other somatic cell in the organism. As the cell starts the meiosis events, new DNA-binding proteins start to bind and organize the chromatin into a new fashion. Somatic histones are replaced by sperm-specific histones in spermatogonia, but the basic

structural subunit of chromatin organization, the nucleosome, is maintained (ZINI; AGARWAL, 2011). After meiosis, histones are replaced by transition proteins (TPs), which help to destabilize nucleosomes by preventing DNA bending (PRADEEPA; RAO, 2007) and stimulating the repair of eventually DNA strand breaks (CARON; VEILLEUX; BOISSONNEAULT, 2001), uniformly condensing the chromatin. In spermatids, TPs are replaced by protamines; a set of positively charged proteins with two mainly families, protamine 1 and 2.

The presence of extra-nuclear histones in bovine sperm was described by Tovich and Oko (2003). In this study, they identified 4 different types of histones (H3, H2B, H2A and H4) in sperm peri-nuclear theca, considering as somatic histones with non-nuclear localization. After that, they studied the developmental localization of somatic-type histones during bovine spermiogenesis (TOVICH; SUTOVSKY; OKO, 2004). In this second study, somatic histones remained associated with the bovine sperm head in the elongation and maturation phases of spermiogenesis. During the elongation phase, nuclear somatic histone leaves the nuclear localization to be associated with manchette microtubes and post-acrosomal sheath (non-nuclear structures) and later degraded. Recently studies identified, by immunostaining, a weak signal of histones modifications H3K27ac and H3K27me3 presented over the whole head and as a crown close to the membrane, respectively (KUTCHY et al., 2018). Therefore, these studies confirm that is possible to find histone in bull sperm, however our results indicate they are not attached to DNA, as a nucleosome structure, once no mononucleosome DNA was identify after MNase digestion. The same is probably happening to the other species evaluated in the present study.

As far as we know, there is no information about nucleosome presence in mature sperm for other livestock species like sheep, horse and pig. Even for chromatin analysis considering the DNA integrity for these species, there only have a few studies (BATISTA; VAN LIER; PETROCELLI, 2016; CORTÉS-GUTIÉRREZ et al., 2009; HAMILTON et al., 2016). An important difference between the species that we were not able to detect nucleosome structure and mice is the origin of the sperm cell. For bull, stallion, monkey, ram and pig samples, we analyzed ejaculated sperm. However, for mice sperm, we needed to collect sperm from epididymis, which is a storage organ for spermatozoa. In this process, it is possible to increase the chances of somatic cells contaminations or immature cells in the sample, which could explain the difference find between species. Recently, ChIP-Seq comparison of mice sperm samples submitted

only to swim-up and swim-up and percoll verified some differences between sequencing data suggesting swim-up sperm samples could contain non-sperm cells contamination, which could have impacted the ChIP-seq results (YAMAGUCHI et al., 2018). Then, the sperm process can also be a bias to different results between experiments.

Studies in mice verified that H3K27me3 and H3K4me3 in sperm are removed in the zygote and reestablished after embryo genome activation (ZHANG et al., 2016; ZHENG et al., 2016). Then, independently of its location, sperm histones can be removed after fertilization during the replacement of protamine by oocytes histones. So far, for bovine sperm, only two studies detected nucleosome structures using a protocol like ours, but with 0.5% Triton-X as a permeabilization agent previous to MNase digestion instead of NP-40 and DOC (SAMANS et al., 2014; SILLASTE et al., 2017). We tried their method (data not shown) and we were not able to get DNA, probably because in our conditions, Triton-X was not enough to permeabilize the sperm and allows MNase digest the DNA. Recently, Yamaguchi and colleagues (2018) used nucleoplasmin (NPM) to improve the solubility of mice sperm chromatin by removing the protamine. The authors believed that using NPM it is possible to examine the genome-wide distribution of histones without a MNase treatment bias. Either in mice or bovine, these divergent results coming from the protocol used to obtain nucleosome-binding DNA leads us to believe that samples processing previous of MNase digestion (detergents used, use or not of cross-linked agent), MNase concentrations and individual effects could interfere in the access of nucleosome band. Nevertheless, both studies in bovine showed that nucleosome structures are related to repetitive genome elements, with no enrichment on embryonic development genes. Then, we can assume that nucleosome structures, when they are present, may not have important function to sperm chromatin epigenetic status in this species.

The presence of histones in sperm nucleus can be indirect evaluated using fluorescent probes as aniline blue test (ANBL) and chromomycin A3 (CMA3). The first one attaches to nucleus-histones and generates a blue color in the sperm head, and the second one competes for protamine binding sites, being an indirect visualization of protamine deficiency. However, both stains present low percentage of positive cells for bull samples (CASTRO et al., 2018; DE OLIVEIRA et al., 2013). We could speculate that for species pre-selected based on reproductive features, which is the case of all livestock species evaluated in the present study, remained nucleosome structures are

not desirable or do not have essential function to pass paternal DNA information to the next generation. For human, a non-selected species, protamine deficiency is considered one of the causes of fertility problems, with a significant variation among individuals (BIANCHI et al., 1996; DE IULIIS et al., 2009; LOLIS et al., 1996; TAVALAEE; RAZAVI; NASR-ESFAHANI, 2009). We cannot affirm that the sperm DNA from species analyzed in our study is completely or exclusively compacted by protamine. Histones can be located at sites of DNA loop domain attachment to nuclear matrix that helps the three-dimensional organization of sperm nucleus (WYKES; KRAWETZ, 2003). However, in the conditions of our experiments, the absence of nucleosome-bind DNA for all the males analyzed lead us to conclude that histones are not attached to DNA for livestock species.

REFERENCES

ARPANAHI, A. et al. Endonuclease-Sensitive Regions of Human Spermatozoal Chromatin Are Highly Enriched in Promoter and CTCF Binding Sequences. **Genome research**, v. 19, n. 8, p. 1338–1349, ago. 2009.

BATISTA, C.; VAN LIER, E.; PETROCELLI, H. Dynamics of sperm DNA fragmentation in raw boar semen and fertility. **Reproduction in domestic animals = Zuchthygiene**, v. 51, n. 5, p. 774–780, 2016.

BIANCHI, P. G. et al. Chromatin Packaging and Morphology in Ejaculated Human Spermatozoa: Evidence of Hidden Anomalies in Normal Spermatozoa. **Molecular human reproduction**, v. 2, n. 3, p. 139–144, mar. 1996.

BRYKCZYNSKA, U. et al. Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. **Nature structural & molecular biology**, v. 17, n. 6, p. 679–87, jun. 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20473313>. Acesso em: 29 nov. 2014.

CARON, N.; VEILLEUX, S.; BOISSONNEAULT, G. Stimulation of DNA Repair by the Spermatidal TP1 Protein. **Molecular reproduction and development**, v. 58, n. 4, p. 437–443, abr. 2001.

CARONE, B. R. et al. High-resolution mapping of chromatin packaging in mouse embryonic stem cells and sperm. **Developmental Cell**, v. 30, n. 1, p. 11–22, 2014. Disponível em: <http://dx.doi.org/10.1016/j.devcel.2014.05.024>.

CARRELL, D. T. et al. The “omics” of human male infertility: integrating big data in a systems biology approach. **Cell and Tissue Research**, v. 363, n. 1, p. 295–312, 2016.

CASTILLO, J. et al. Genomic and proteomic dissection and characterization of the human sperm chromatin. **Molecular human reproduction**, v. 20, n. 11, p. 1041–53, 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/25193639>.

CASTRO, L. S. et al. Effect of bovine sperm chromatin integrity evaluated using three different methods on in vitro fertility. **Theriogenology**, v. 107, p. 142–148, 2018. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0093691X1730540X>.

CORTÉS-GUTIÉRREZ, E. I. et al. Assessment of sperm DNA fragmentation in stallion (Equus caballus) and donkey (Equus asinus) using the sperm chromatin dispersion test. **Reproduction in Domestic Animals**, v. 44, n. 5, p. 823–828, 2009.

DE IULIIS, G. N. et al. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. **Biology of Reproduction**, v. 81, n. 3, p. 517–524, set. 2009. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19494251>. Acesso em: 19 ago. 2014.

DE OLIVEIRA, R. V et al. Molecular morphology and function of bull spermatozoa linked to histones and associated with fertility. **Reproduction (Cambridge, England)**, v. 146, n. 3, p. 263–72, set. 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23904564>. Acesso em: 24 set. 2014.

GATEWOOD, J. M. et al. Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. **Journal of Biological Chemistry**, v. 265, p. 20662–20666, 1990.

GOVIN, J. et al. Pericentric heterochromatin reprogramming by new histone variants during mouse spermiogenesis. **Journal of Cell Biology**, v. 176, n. 3, p. 283–294, 2007.

HAMILTON, T. R. dos S. et al. Induced lipid peroxidation in ram sperm: semen profile, DNA fragmentation and antioxidant status. **Reproduction**, v. 151, n. 4, p. 379–390, abr. 2016. Disponível em: <http://www.reproduction-online.org/content/early/2016/01/25/REP-15-0403.abstract>.

HAMMOUD, S. S. et al. Distinctive chromatin in human sperm packages genes for embryo development. **Nature**, v. 460, n. 7254, p. 473–478, 23 jul. 2009. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2858064&tool=pmcentrez&rendertype=abstract>. Acesso em: 20 jan. 2014.

HAMMOUD, S. S. et al. Chromatin and transcription transitions of mammalian adult germline stem cells and spermatogenesis. **Cell Stem Cell**, v. 15, n. 2, p. 239–253, 2014. Disponível em: <http://dx.doi.org/10.1016/j.stem.2014.04.006>.

HISANO, M. et al. Genome-wide chromatin analysis in mature mouse and human spermatozoa. **Nature Protocols**, v. 8, n. 12, p. 2449–2470, 2013. Disponível em: <http://www.nature.com/doifinder/10.1038/nprot.2013.145>.

KETCHUM, C. C. et al. Early Histone H4 Acetylation during Chromatin Remodeling in Equine Spermatogenesis. **Biology of reproduction**, v. 98, n. 1, p. 115–129, jan. 2018.

KIMMINS, S.; SASSONE-CORSI, P. Chromatin remodelling and epigenetic features of germ cells. **Nature**, v. 434, p. 583–589, 2005.

KUTCHY, N. A. et al. Testis specific histone 2B is associated with sperm chromatin dynamics and bull fertility-a pilot study. **Reproductive Biology and Endocrinology**,

v. 15, n. 1, p. 1–14, 2017.

KUTCHY, N. A. et al. Acetylation and methylation of sperm histone 3 lysine 27 (H3K27ac and H3K27me3) are associated with bull fertility. **Andrologia**, v. 50, n. 3, p. 1–11, 2018.

LOLIS, D. et al. Chromomycin A3-staining as an indicator of protamine deficiency and fertilization. **International journal of andrology**, v. 19, n. 1, p. 23–7, 1996. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/8698534>.

MIECZKOWSKI, J. et al. MNase titration reveals differences between nucleosome occupancy and chromatin accessibility. **Nature Communications**, v. 7, n. May, p. 1–11, 2016. Disponível em: <http://dx.doi.org/10.1038/ncomms11485>.

NAKAZAWA, Y. et al. Replacement of nuclear protein by histone in pig sperm nuclei during in vitro fertilization. **Reproduction**, v. 124, n. 4, p. 565–572, 2002.

PRADEEPA, M. M.; RAO, M. R. S. Chromatin Remodeling during Mammalian Spermatogenesis: Role of Testis Specific Histone Variants and Transition Proteins. **Society of Reproduction and Fertility supplement**, v. 63, p. 1–10, 2007.

SAITOU, M.; KURIMOTO, K. Paternal nucleosomes: Are they retained in developmental promoters or gene deserts? **Developmental Cell**, v. 30, n. 1, p. 6–8, 2014. Disponível em: <http://dx.doi.org/10.1016/j.devcel.2014.06.025>.

SAMANS, B. et al. Uniformity of nucleosome preservation pattern in mammalian sperm and Its connection to repetitive DNA elements. **Developmental Cell**, v. 30, n. 1, p. 23–35, 2014. Disponível em: <http://dx.doi.org/10.1016/j.devcel.2014.05.023>.

SILLASTE, G. et al. A novel hypothesis for histone-to-protamine transition in Bos taurus spermatozoa. **Reproduction**, v. 153, n. 3, p. 241–251, 2017.

TAVALAEE, M.; RAZAVI, S.; NASR-ESFAHANI, M. H. Influence of Sperm Chromatin Anomalies on Assisted Reproductive Technology Outcome. **Fertility and sterility**, v. 91, n. 4, p. 1119–1126, abr. 2009.

TOVICH, P. R.; OKO, R. J. Somatic histones are components of the perinuclear theca in bovine spermatozoa. **Journal of Biological Chemistry**, v. 278, n. 34, p. 32431–32438, 2003.

TOVICH, P. R.; SUTOVSKY, P.; OKO, R. J. Novel Aspect of Perinuclear Theca Assembly Revealed by Immunolocalization of Non-Nuclear Somatic Histones During Bovine Spermiogenesis1. **Biology of Reproduction**, v. 71, n. 4, p. 1182–1194, 2004. Disponível em: <https://academic.oup.com/biolreprod/article-lookup/doi/10.1095/biolreprod.104.030445>.

VAN DER HEIJDEN, G. W. et al. Sperm-derived histones contribute to zygotic chromatin in humans. **BMC developmental biology**, v. 8, p. 34, 2008.

WYKES, S. M.; KRAWETZ, S. A. The structural organization of sperm chromatin. **Journal of Biological Chemistry**, v. 278, n. 32, p. 29471–29477, 2003.

YAMAGUCHI, K. et al. Re-evaluating the Localization of Sperm-Retained Histones Revealed the Modification-Dependent Accumulation in Specific Genome Regions. **Cell**

Reports, v. 23, n. 13, p. 3920–3932, 2018. Disponível em: <https://doi.org/10.1016/j.celrep.2018.05.094>.

ZHANG, B. et al. Allelic Reprogramming of the Histone Modification H3K4me3 in Early Mammalian Development. **Nature**, v. 537, n. 7621, p. 553–557, set. 2016.

ZHENG, H. et al. Resetting Epigenetic Memory by Reprogramming of Histone Modifications in Mammals. **Molecular cell**, v. 63, n. 6, p. 1066–1079, set. 2016.

ZINI, A.; AGARWAL, A. **Sperm Chromatin**. New York, NY: Springer New York, 2011.

8 CHAPTER 4: SPERM DNA METHYLATION PROFILE BETWEEN HIGH AND LOW *IN VITRO* FERTILITY BULLS

8.1 INTRODUCTION

Brazil is the first country in the ranking of bovine *in vitro* embryo production (IVP). It contributes with 61% of the *in vitro* embryos produced in the world (PERRY, 2017). The growth of IVP and its practical application in commercial herds made the researchers, including private companies, start the running to optimize this biotechnology. Initially, studies purposed to improve *in vitro* oocyte maturation rates decreasing the difference between breeds for the number of aspirated follicles and the oocyte quality, and to improve the *in vitro* embryo culture using media with lower fetal serum percentages and increasing embryo survival rates after cryopreservation (LONERGAN et al., 2006; HANSEN et al., 2010; WRENZYCKI; STINSHOFF, 2013; MOUSSA et al., 2015). Once the sperm cryopreservation process has been well established since 1950, the male effect in most studies was left disregarded. However, in the last years, especially due to the widespread use of sex-sorted semen in IVP procedures, the focus of some researchers has turned to understand the “bull effect” and the *in vitro* fertility difference between them, that still leads to inconstancy of IVP results.

In cattle, it became clear that due to reproductive selection pressure over the years excluding subfertile and infertile animals, some sperm attributes like DNA integrity has no longer significant impact on fertility status (BOE-HANSEN; FORTES; SATAKE, 2018; CASTRO et al., 2018; SOUZA et al., 2018). Then, the “bull effect” seen on IVP system could be related to other features, not yet explored, like epigenetic marks on DNA. The main two are histones modifications and DNA methylation. The presence and localization of histones attached to DNA in spermatozoa is controversial and the protocol used can create a bias to access nucleosome-binding DNA (SAITOU; KURIMOTO, 2014). Therefore, the DNA methylation would be a good candidate to be studied since it is considered the most stable epigenetic modification of the chromatin structure.

DNA methylation occurs by the addition of methyl group (CH_3) in cytosine residues forming 5-methyl cytosine (5mC). Usually genes have cytosine and guanidine

(CG) rich regions close to codification regions called CpG island that offers possible methylation sites. Genes with high frequency of CpG island close to protomer regions shows low transcription activity and the gene expression is silenced (AHLUWALIA, 2009). The DNA methylation is established in two specific moments: in the zygote after fertilization and in primordial germ cells (PGCs) before the migration to the gonadal tissues. In the zygote, the paternal DNA suffers active DNA demethylation at the same time of protamine-histone exchange, while maternal DNA due to specific marks on its chromatin remains unaffected to be passive demethylated later (NAKAMURA et al., 2012). In PGCs, 90% of their methylation patterns is lost when genome-wide leads to erasure parental imprints and reactivation of X chromosome in females (LEE; HORE; REIK, 2014). Both events are determinant to established pluripotent features to lineage of cells derived from zygotes and PGCs. In mice, the *de novo* methylation is established at embryonic day E13.5 in pro-spermatogonia and the methylome is complete prior to birth, and sustained though all spermatogenesis stages until mature spermatozoa (SEISENBERGER et al., 2012). Recently, the whole genome DNA methylation analysis in cattle showed that sperm presents a specific methylation pattern when compared to somatic cells (ZHOU et al., 2018a).

Studies in human described differences for methylation profile between fertile and subfertile men (ASTON et al., 2015; LAQQAN; HAMMADEH, 2018; NASRI et al., 2017). The methylation profile on CpGs sites within imprinted control regions like H19 gene may serve as epigenomic biomarker of infertility in men (PENG et al., 2018). Also, Benchaib et al. (2005) verified that global methylation levels on sperm DNA in humans could not affect fertilization rates, but interfere on embryo development. In buffalos, differences on DNA methylation of genes related to embryo development were described among bulls with high fertility and subfertiles (VERMA et al., 2014). In the comparison between sex-sorted and conventional sperm samples, no difference was found for global methylation pattern, however individual differences were found for IGF2 and IGF2R, among bulls (CARVALHO et al., 2012).

Several technologies have been described to analyze genome-wide DNA methylation and they can be classified in two types: region-base and single-base resolution techniques. The first one is based on immunoprecipitation approaches that uses methyl (MeDIP) or methyl-CpGs binding domain MBD2 protein (MBD-seq) antibodies to pull down genomic regions that are methylated and then sequence or identify genes using microarrays. The single-base resolution approaches use bisulfite

treatment to convert unmethylated cytosine (C) to thymine (T) while methylated cytosine remains unchanged. Bisulfite treated DNA can be evaluated by next-generation sequencing or microarrays. For single-base resolution approaches it is possible to analyze the whole-genome sequencing (WGBS) or target to CpGs rich regions, using a methodology called reduced representation bisulfite sequencing (RRBS). In this analysis before the bisulfite conversion, the genomic DNA is digested with MspI restriction enzyme which recognized CCGG sites. These regions are more enriched in CpG islands, promoter or genic regions of the genome. Also, this enrichment step reduces the number of reads required to a good coverage, decreasing the costs of sequencing when compared to the WGBS. The studies of sperm methylation profile in bovine sperm are relatively new (JIANG et al., 2018; PERRIER et al., 2018; ZHOU et al., 2018a). Overall, they agree that bull sperm is hypomethylated, unlike what is seen in humans, primates and mice (KOBAYASHI et al., 2012; MOLARO et al., 2011). Considering the windows of reprogramming methylation are crucial, how the paternal DNA is marked could impair on further embryo development. Then, in the present study we decided to investigate the DNA methylation, using RRBS approach, between bulls with different *in vitro* fertility to verify if their methylation pattern could be interfering on *in vitro* embryo production.

8.2 MATERIAL AND METHODS

8.2.1 Experimental design: Ranking and selection of the bulls with high and low *in vitro* fertility

The present study was based on a retrospective database from the reproduction biotechnology company In Vitro Brazil between the years of 2012 to 2015 (IVB, Mogi Mirim, São Paulo, Brazil). In this period, IVB yielded around 5,000 IVP manipulations of 500 bulls. The database contained information such as the number of aspirated and cultured oocytes, cleavage structures and embryos produced from each manipulation. Using this information, we calculated *in vitro* rates: cleavage rate (cleavage structures/oocytes cultured), blastocyst rate (embryos produced/oocytes cultured) and embryo development rate (embryos produced/cleavage structures) for all IVP manipulations. In order to represent IVP rates for each bull, we selected only animals with more than 5 recorded manipulations and each of them with more than 50 oocytes

cultured/manipulated. In addition, IVP protocol used was homogenized to select only manipulations using Percoll® gradient and X sex-sorted semen. Based on that, 140 bulls from different breeds fitted the criteria and were included to the ranking.

Bulls were ranked based on embryo development rate (embryos produced/cleavage structures). From this ranking, we selected the top 5 and the bottom 5 bulls (n=5 per group) that had commercially available semen straws. The *in vitro* rates from selected bulls were confirmed in other study from our group (ALMEIDA, 2018). They presented similar cleavage rate (HF: 86.66±1.15 *vs* LF: 84.94±0.95; p=0.25) and different blastocyst (HF: 29.41±1.56 *vs* LF: 16.02±0.92; p<0.0001) and embryo development rates (HF: 33.96±1.67 *vs* LF: 18.91±1.09; p<0.0001).

8.2.2 Reagents and solutions

All chemical reagents and solutions used in this study were purchased from Sigma-Aldrich (St. Louis, MO, E.U.A.) unless otherwise stated.

8.2.3 Sperm DNA extraction

Sperm DNA extraction was performed according to Anvar and colleagues (2015) protocol, with a few modifications. One straw of each bull was thawed at 37 °C for 30 seconds and washed twice (400 x g/5 min) in 1 mL of DPBS (Sigma Aldrich®) to remove the extender. The spermatozoa pellet was recovered, and cells were incubated overnight at 55°C with lysis buffer (10 mM Tris-HCl pH 8, 5 mM EDTA, 200 mM NaCl, 0.5% SDS and 0.5% Triton-X 100), proteinase K (20 mg/mL) and 50 mM DTT. The DNA was extracted with the same sample volume of phenol:chloroform and precipitated with 1/10 of sample volume of 3M sodium acetate and 2x of 100% ethanol. Precipitated DNA was washed in 500 µL of 70% ethanol, resuspended in water and evaluated for quality and quantification with Nanodrop® and Qubit®, respectively.

8.2.4 Library preparation and sequencing

For RRBS analysis, we used the Premium RRBS kit (Diagenode, Denville, NJ, United States). Briefly, the protocol started with 100 ng of genomic DNA digested with

MspI, followed by adapter ligation, end repair, pooling samples, bisulfite conversion and PCR amplification to enrichment and produce the final sequencing library. The pooled library was sequenced with Illumina platform (HiSeq 4000, single-read 100bp length).

8.2.5 Data analysis

The reads were trimmed to remove low quality bases and the adapters using Trim_galore with default parameters (Babraham Bioinformatics, Babraham Institute). To avoid methylation bias caused by introduction of unmethylated Cs during sample processing, two additional bases were removed from the 3' end. Trimmed reads were mapped to reference genome (*Bos taurus* ARS-UCD1.2) using Bismark platform with default mode (KRUEGER; ANDREWS, 2011). Conversion efficiency was estimated using unmethylated spike-in control sequence provided by the kit. Methylation data were generated using the Bismark_methylation_extractor tool and hierarchical clustering, principal components analysis (PCA) and comparison of methylation level between the two groups were analyzed by methylKit R package (AKALIN et al., 2012). The comparison between HF and LF was made using logistic regression test and it was included in comparison different methylation cytosines (DMC) with minimum 5 reads coverage, ≥25% of difference and q-value <0.01. All analyses were conducted using the NCBI *Bos taurus* Annotation Release 106 annotation. Since duplicate removal by coordinates is not recommended for RRBS data, PCR bias was addressed by removing bases with unusually high coverage (>99.9%). Genes containing DMCs were subjected to DAVID Functional Annotation Tool to extract functional annotation clustering.

8.3 RESULTS

8.3.1 Descriptive analysis

The summary sequencing data is presented in Table 1. Samples 1 to 5 represent HF group and 6 to 10, LF group. For most of the samples, total reads produced after sequencing was around 30 million and a few reads were removed after trimming (sequences analyzed). The average percentage of mapped reads was

89.41% and the mapping efficiency was 31.03%. The bisulfite conversion calculated using the spike-in controls provided by the kit indicates a media efficiency of 99.2%.

Table 1. Number of reads produced after sequencing and included in the analysis.

Sample	Total reads number	Number of sequences analyzed	Mapped reads (%)	Mapping efficiency (%)	Bisulfite conversion efficiency (%)
1H	40,785,371	40,623,107	90.9	29.10	99.5
2H	30,662,646	30,593,172	91.6	30	98.9
3H	52,040,293	52,029,126	84.1	28.4	99.6
4H	40,905,477	40,867,961	90.4	33.5	99.4
5H	28,679,231	28,568,189	86.4	29.2	99.2
6L	33,886,558	33,692,986	90.5	28.3	99.4
7L	34,719,685	34,706,614	86.9	39.6	99.6
8L	31,500,828	31,451,193	91.1	32	98.2
9L	16,014,611	15,946,677	91.1	27.7	99.3
10L	15,638,072	15,630,579	90.9	32.5	99.4

Legend: Sequences analyzed = number of reads included in the analysis after trimming; Mapped reads = number of reads mapped to the reference genome/sequences analyzed; Mapping efficiency = number of uniquely mapped reads/sequences analyzed

Regarding the cytosine context, Table 2 presents the total number of cytosines included in the analysis (>5 reads coverage and after removed unusually high coverage), and the percentage of methylated cytosine. Most cytosine is localized in CpG regions, and around 1% is localized at CHG or CHH regions, respectively, being H an adenine or timine. On average, 1,155,605 cytosines per sample were included in the analysis of group comparison.

Table 2. Cytosines (Cs) context after Bismark alignment.

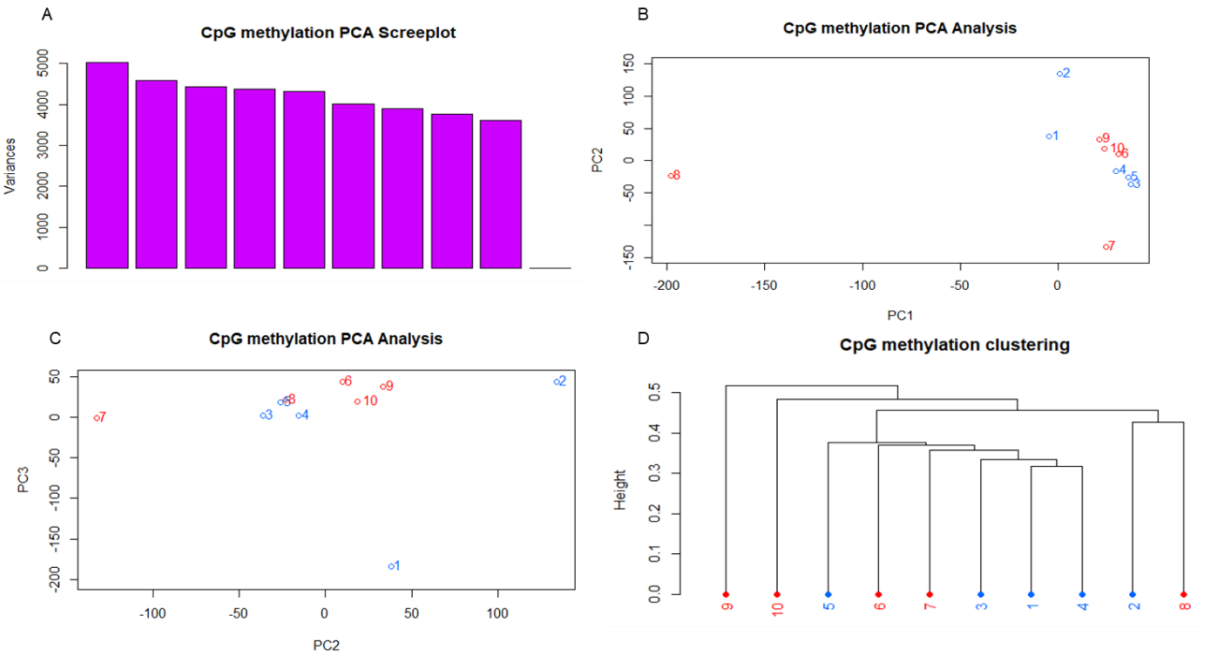
Sample	Total number of Cs analyzed	CpG methylated (%)	CHG methylated (%)	CHH methylated (%)	Unknown (CN or CHN) Methylated (%)
1H	1,359,342	18.1	1.0	0.8	0.9
2H	1,149,524	15.1	0.9	0.8	0.8
3H	1,513,324	13.4	1.0	0.8	1.1
4H	1,470,192	20.7	1.0	0.8	1.2
5H	1,007,143	15.8	1.0	0.8	1.3
6L	1,214,302	12.4	0.9	0.7	1.0
7L	1,389,823	33.5	1.2	0.9	1.8
8L	1,136,312	25.7	1.0	0.8	1.2
9L	640,994	13.7	0.9	0.7	0.7
10L	675,092	19.9	1.0	0.8	1.0

Legend: Total number of Cs analyzed are cytosines with more than 5 reads coverage and after removed unusually high coverage.The percentage is calculated individually for each context following the equation: %C methylated context = C methylated/(C methylated + C unmethylated) * 100

Samples were cluster based on the similarity of their methylation profiles (Figure 1A-C). For principal components analysis (PCA), nine principal components were

identified, and they were similar from each other (Figure 1A). The relation between PCA1 x PCA2 and PCA2 x PCA3 are presented in the Figure 1B and 1C respectively. Overall, samples from HF and LF were close to each other, without clustering between samples from the same group. The hierarchical clustering indicated similar results (Figure 1D).

Figure 1. Principal components analysis (PCA) and hierarchical clustering of CpG methylation from 10 individual bull samples.

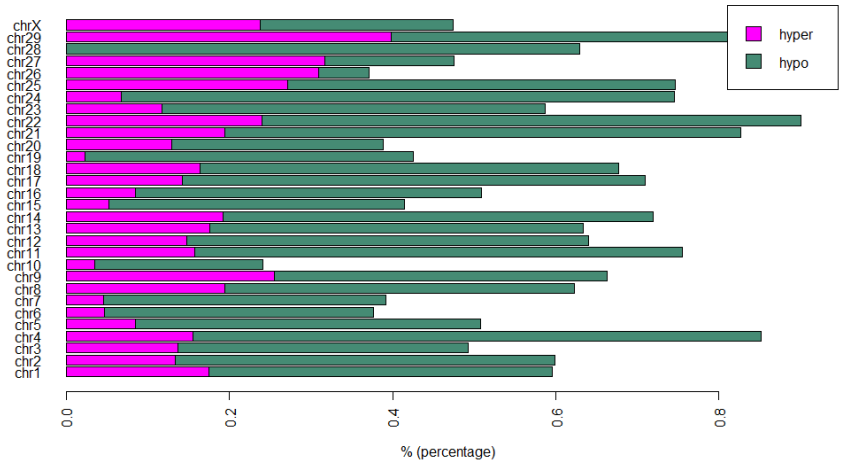


Legend: Blue samples (1 to 5) are the high *in vitro* fertility group and red samples (6 to 10) are the low *in vitro* fertility group. A- Nine principal components (PC) identified from 10 samples of sperm methylation profile; B – PC1 x PC2 for each sample; C – PC2 x PC3 for each sample. Samples closer to each other in principal component space are similar in their methylation profiles. D – hierarchical clustering of 10 samples of sperm methylation profile using Pearson's correlation distance and Ward clustering method.

8.3.2 Differentially methylation cytosines (DMC) between HF x LF bulls

In the comparison between HF and LF bulls, we identified 440 DMC with ≥25% of difference (q-value<0.01), being 327 hypermethylated (74%) and 113 hypomethylated (26%) in the HF group. The frequency of DMC covered CpG indicated they were distributed over all chromosomes and the HF group presented a higher proportion of hypomethylation regions. DMC were hyper- and hypo- methylated over all the chromosomes, with exception of chr28, presenting only hypomethylated regions (Figure 3). Chr 26 and 27 presented more hyper- then hypomethylated regions.

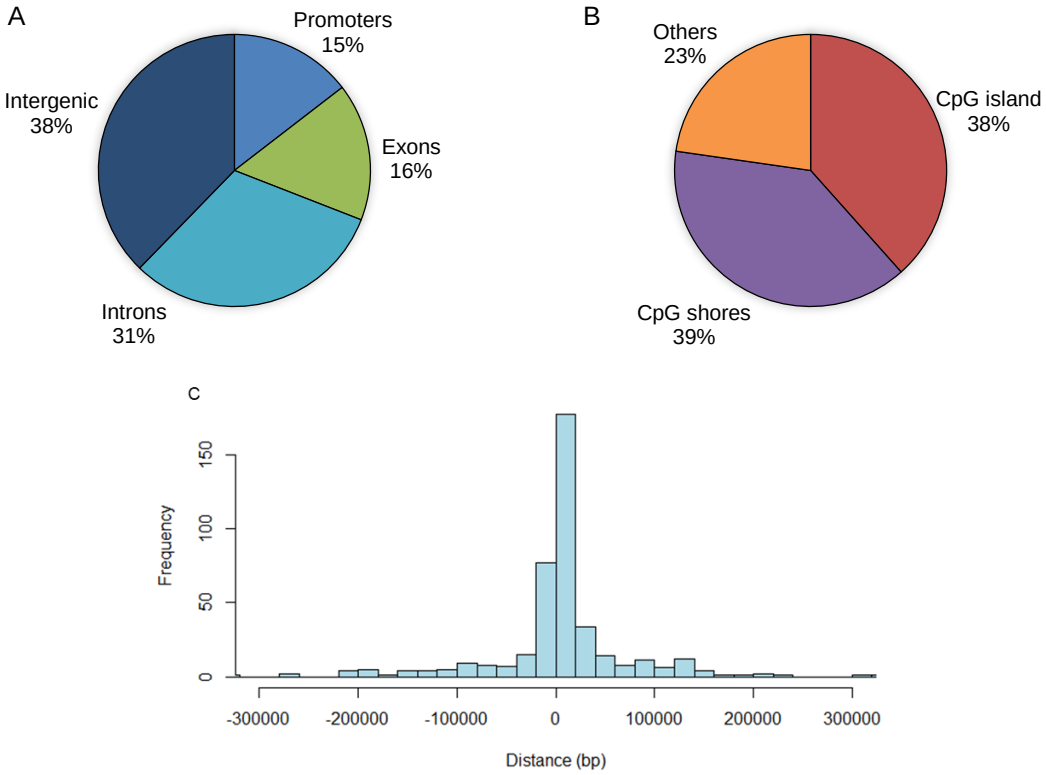
Figure 3. Percentage of hyper- or hypomethylated regions per chromosome in the comparison of sperm DNA methylation of high/low *in vitro* fertility groups.



Legend: Bar plots of frequency of DMC over all covered CpG (percentage in the x axis) per chromosome (y axis: chromosome 1 to 29 and sexual chromosome X). DMC with hypermethylation in HF group compares to LF group are pink and DMC with hypomethylation in HF group compares to LF group are green (q value<0.01 and methylation difference ≥25%).

Regard the DNA features, most part of DMC are localized on intergenic regions (38%), followed by introns (31%), promoters (15%) and exons (16%) (Figure 4A). Similar proportion of DMC was identified in CpG island (38%) and CpG shores (39%). Most part of the DMC are localized around the transcription start site (TSS), up or downstream and the distance of these DMC to the nearest TSS is presented in the Figure 4C. Of these 440 DMC, 184 genes were identified. However, after DAVID function annotation, no enriched pathway was defined by these genes once all of them presented enrichment score < 1. Table 3 shows the genes ID, names and distance to the gene.

Figure 4. Comparison of sperm DNA methylation of high/low *in vitro* fertility groups and the DNA feature of different methylation cytosines (DMC) overlapping genome annotation.



Legend: A- Pie chart indicating percentages of differentially methylated CpGs on promoters, exons, introns and intergenic regions; B- Pie chart indicating percentages of differentially methylated CpGs on CpG islands, CpG island shores (defined as 2kb flanks of CpG islands) and other regions outside of shores and CpG islands; C- Distance to transcription start site (TSS; 0bp in the x axis) for differentially methylated CpGs are plotted from HF versus LF analysis. Each bar represents a window of bp (±20kb).

Table 3. Genes identified from different methylation cytosines (DMC) in the comparison of sperm DNA methylation of high/low *in vitro* fertility groups.

Gene ID	Gene name	Distance to TSS
LOC112444653	5.8S ribosomal RNA	-700; 368; 1434; 2124
ACAD9	Acyl-coa dehydrogenase family member 9(ACAD9)	-17899
ACOXL	Acyl-coa oxidase-like(ACOXL)	16151; 16173
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif 2(ADAMTS2)	-88369
ALDOA	Aldolase, fructose-bisphosphate A(ALDOA)	924
AMDHD2	Amidohydrolase domain containing 2(AMDHD2)	523
ANK3	Ankyrin 3, node of Ranvier (ankyrin G)(ANK3)	-43
AMH	Anti-Mullerian hormone(AMH)	2928; 3000
ASPHD1	Aspartate beta-hydroxylase domain containing 1(ASPHD1)	25
AGA	Aspartylglucosaminidase(AGA)	13632
ATP1A3	Atpase Na+/K+ transporting subunit alpha 3(ATP1A3)	-8740
ATG9B	Autophagy related 9B(ATG9B)	-5810

Gene ID	Gene name	Distance to TSS
BARX2	BARX homeobox 2(BARX2)	96746
DEFB13	Beta-defensin 13(DEFB13)	114
BCAT2	Branched chain amino acid transaminase 2(BCAT2)	6253
CACNG7	Calcium voltage-gated channel auxiliary subunit gamma 7(CACNG7)	-8377
CLSTN2	Calsyntenin 2(CLSTN2)	86000; 86007
CD34	CD34 molecule(CD34)	-53913
CD48	CD48 molecule(CD48)	9195
C23H6orf15	Chromosome 23 open reading frame, human c6orf15(c23h6orf15)	12963
C6H4orf48	Chromosome 6 c4orf48 homolog(c6h4orf48)	-1522
CFAP100	Cilia and flagella associated protein 100(CFAP100)	1449
COL6A2	Collagen type VI alpha 2 chain(COL6A2)	24126
CTBP2	C-terminal binding protein 2(CTBP2)	469; 501; 506; 508
CLEC3B	C-type lectin domain family 3 member B(CLEC3B)	6103
CBS	Cystathionine-beta-synthase(CBS)	6008
DCN	Decorin(DCN)	-367291
DNASE2	Deoxyribonuclease 2, lysosomal(DNASE2)	5666
DAAM2	Dishevelled associated activator of morphogenesis 2(DAAM2)	58225
POLD1	DNA polymerase delta 1, catalytic subunit(POLD1)	10126
DUSP14	Dual specificity phosphatase 14(DUSP14)	-26412
EBF3	EBF transcription factor 3	-1499
EFHB	EF-hand domain family member B(EFHB)	11881; 397920
ELOB	Elongin B	-76
ENDOU	Endonuclease, poly(U) specific(ENDOU)	11030
EOMES	Eomesodermin(EOMES)	2227
EPS8L1	EPS8 like 1(EPS8L1)	13687
ERCC4	ERCC excision repair 4, endonuclease catalytic subunit(ERCC4)	-439178
ESRRB	Estrogen related receptor beta(ESRRB)	47499
FAM178B	Family with sequence similarity 178, member B(FAM178B)	29029
FEZ2	Fasciculation and elongation protein zeta 2(FEZ2)	71478
FRMPD2	FERM and PDZ domain containing 2(FRMPD2)	-6936
FERMT2	Fermitin family member 2(FERMT2)	-177
FKBP5	FK506 binding protein 5(FKBP5)	-2116
GPER1	G protein-coupled estrogen receptor 1(GPER1)	-3314
GIPR	Gastric inhibitory polypeptide receptor(GIPR)	6794; 6859
GRM4	Glutamate metabotropic receptor 4(GRM4)	101139
GET4	Golgi to ER traffic protein 4 homolog (S. Cerevisiae)(GET4)	3585
GON4L	Gon-4-like (C. Elegans)(GON4L)	-21902
HAUS8	HAUS augmin like complex subunit 8(HAUS8)	26090
LOC112446802	Histone H2B type 2-E-like	-367784
LOC104969236	Histone H4-like(LOC104969236)	1555
HOXC10	Homeobox C10(HOXC10)	-1280
HOXC4	Homeobox C4(HOXC4)	23605; 23641; 23646; 23767
HYAL2	Hyaluronoglucosaminidase 2(HYAL2)	133
IGSF10	Immunoglobulin superfamily member 10(IGSF10)	-133
IPO9	Importin 9(IPO9)	-77911; -14457
INTS1	Integrator complex subunit 1(INTS1)	4386

Gene ID	Gene name	Distance to TSS
IFI16	Interferon gamma inducible protein 16(IFI16)	-5058
IL17F	Interleukin 17F(IL17F)	6428; 6605
IL21R	Interleukin 21 receptor(IL21R)	1666; 1668
IL5RA	Interleukin 5 receptor subunit alpha(IL5RA)	-8263
LOC100126230	Jy-1(loc100126230)	-199935
KLHDC7A	Kelch domain containing 7A(KLHDC7A)	-119296
KLHDC9	Kelch domain containing 9(KLHDC9)	-709; -683
KIAA1211L	KIAA1211 like(KIAA1211L)	19966; 19979; 43938
LMAN1	Lectin, mannose binding 1(LMAN1)	-82194
LGSN	Lengsin, lens protein with glutamine synthetase domain(LGSN)	-146158
LAIR1	Leukocyte associated immunoglobulin like receptor 1(LAIR1)	14124
LMF1	Lipase maturation factor 1(LMF1)	17841
LYAR	Ly1 antibody reactive(LYAR)	795
MST1R	Macrophage stimulating 1 receptor(MST1R)	-5244
MRGPRX2	MAS-related GPR, member X2(MRGPRX2)	38377
MMP25	Matrix metallopeptidase 25(MMP25)	-304
MARS	Methionyl-trna synthetase(MARS)	-1729
MEX3C	Mex-3 RNA binding family member C(MEX3C)	-396489
MICALL2	MICAL like 2(MICALL2)	9747
MIR2368	MicroRNA 2368(MIR2368)	41141
MIR2402	MicroRNA 2402(MIR2402)	25646; 27712
MIR2452	MicroRNA 2452(MIR2452)	39150
MIR2453	MicroRNA 2453(MIR2453)	-261
MIR2887-2	MicroRNA 2887-2(MIR2887-2)	-2576
MIR2900	MicroRNA 2900(MIR2900)	-4270
MIR378D	MicroRNA 378d(MIR378D)	75986
MIR6532	MicroRNA 6532(MIR6532)	63072
MINDY4B	MINDY family member 4B	-23012
NDUFB7	NADH:ubiquinone oxidoreductase subunit B7(NDUFB7)	6394
NPHP3	Nephrocystin 3(NPHP3)	-136911
NEK11	NIMA related kinase 11(NEK11)	1305
NLRP13	NLR family pyrin domain containing 13(NLRP13)	1220
NR5A2	Nuclear receptor subfamily 5 group A member 2(NR5A2)	-202440
NOM1	Nucleolar protein with MIF4G domain 1(NOM1)	1290
LOC514002	Olfactory receptor 18(LOC514002)	2170
LOC112444476	Olfactory receptor 5AL1-like	-69452
LOC529511	Olfactory receptor 5I1(LOC529511)	16429
LOC100295735	Olfactory receptor 7A17-like(LOC100295735)	-491
LOC100138271	Oviduct-specific glycoprotein-like(LOC100138271)	21850; 21852
OSBPL10	Oxysterol binding protein like 10(OSBPL10)	138084; 138116
LOC100297779	P antigen family member 5(LOC100297779)	112187
PGLYRP1	Peptidoglycan recognition protein 1(PGLYRP1)	618
PLEKHB2	Pleckstrin homology domain containing B2(PLEKHB2)	579054; 595134
PNKP	Polynucleotide kinase 3'-phosphatase(PNKP)	4495
GALNT11	Polypeptide N-acetylgalactosaminyltransferase 11(GALNT11)	48300
KCNN3	Potassium calcium-activated channel subfamily N member 3(KCNN3)	-1066

Gene ID	Gene name	Distance to TSS
KCTD11	Potassium channel tetramerization domain containing 11(KCTD11)	401
KCNK10	Potassium two pore domain channel subfamily K member 10(KCNK10)	153917
KCNJ15	Potassium voltage-gated channel subfamily J member 15(KCNJ15)	57100
PRPF31	Pre-mRNA processing factor 31(PRPF31)	-1038
PSMG4	Proteasome assembly chaperone 4(PSMG4)	7809; 7825
PDIA2	Protein disulfide isomerase family A member 2(PDIA2)	18108
PRKCG	Protein kinase C gamma(PRKCG)	7220; 7229; 7738
PPP1R13L	Protein phosphatase 1 regulatory subunit 13 like(PPP1R13L)	5935
PPP1R12B	Protein phosphatase 1, regulatory subunit 12B(PPP1R12B)	777
PTPN18	Protein tyrosine phosphatase, non-receptor type 18 (brain-derived)(PTPN18)	430
PTPN5	Protein tyrosine phosphatase, non-receptor type 5(PTPN5)	-46441
PTPRN2	Protein tyrosine phosphatase, receptor type N2(PTPRN2)	125787; 125789; 125793; 125796; 125826; 125845
RAB11FIP5	RAB11 family interacting protein 5(RAB11FIP5)	-30200
RGL3	Ral guanine nucleotide dissociation stimulator like 3(RGL3)	-96; -75
RALBP1	Rala binding protein 1(RALBP1)	20160
RAPGEF2	Rap guanine nucleotide exchange factor 2(RAPGEF2)	1141630
RBBP8	RB binding protein 8, endonuclease(RBBP8)	-265346
RIPK4	Receptor interacting serine/threonine kinase 4(RIPK4)	-47097
RMI2	Recq mediated genome instability 2(RMI2)	9475
LOC100296255	Regulator of G-protein signaling 16(LOC100296255)	-214741
ARHGAP22	Rho gtpase activating protein 22(ARHGAP22)	-3159
RPP40	Ribonuclease P/MRP subunit p40(RPP40)	8672; 8691
RPN1	Ribophorin I(RPN1)	-9617
RBFOX1	RNA binding protein, fox-1 homolog 1(RBFOX1)	94590
ROGDI	Rogdi homolog(ROGDI)	944; 949
LOC112443721	S-antigen protein-like	-189245
SCGB1D	Secretoglobin, family 1D, member 2(SCGB1D)	-9175
SEMA3B	Semaphorin 3B(SEMA3B)	6065
SEMA6B	Semaphorin 6B(SEMA6B)	2729
SRSF4	Serine and arginine rich splicing factor 4(SRSF4)	8333; 8371
SERPINB10	Serpin peptidase inhibitor, clade B (ovalbumin), member 10(SERPINB10)	45231
SPCS3	Signal peptidase complex subunit 3(SPCS3)	78737
SSR1	Signal sequence receptor subunit 1(SSR1)	31090
SKOR2	SKI family transcriptional corepressor 2(SKOR2)	-90339
SRGAP3	SLIT-ROBO Rho gtpase activating protein 3(SRGAP3)	145
SLC5A5	Solute carrier family 5 member 5(SLC5A5)	7214
SBNO2	Strawberry notch homolog 2(SBNO2)	6856
SAE1	SUMO1 activating enzyme subunit 1(SAE1)	939
SYT5	Synaptotagmin 5(SYT5)	-4476
TBX2	T-box 2(TBX2)	2670
TEX44	Testis expressed 44	34855
THADA	THADA, armadillo repeat containing(THADA)	-1238; -1179
TRAF4	TNF receptor associated factor 4(TRAF4)	6039
TNFRSF13B	TNF receptor superfamily member 13B(TNFRSF13B)	22557
TRNAC-ACA	Transfer RNA cysteine (anticodon ACA)(TRNAC-ACA)	-3213; 62445
TRNAE-CUC	Transfer RNA glutamic acid (anticodon CUC)(TRNAE-CUC)	136

Gene ID	Gene name	Distance to TSS
TRNAE-UUC	Transfer RNA glutamic acid (anticodon UUC)(TRNAE-UUC)	-2041; -2027
TRNAG-GCC	Transfer RNA glycine (anticodon GCC)(TRNAG-GCC)	197427
LOC509184	Transmembrane emp24 domain-containing protein 9(LOC509184)	1257
TMEM192	Transmembrane protein 192(TMEM192)	44183
TMEM220	Transmembrane protein 220(TMEM220)	-4409; -4404
TMEM223	Transmembrane protein 223(TMEM223)	461
TMEM45B	Transmembrane protein 45B(TMEM45B)	-75
TAP2	Transporter 2, ATP binding cassette subfamily B member(TAP2)	-9695
LOC104970173	Tropomyosin alpha-3 chain-like(LOC104970173)	-270821; -209978; -153312
TULP1	Tubby like protein 1(TULP1)	-32433
TP53	Tumor protein p53(TP53)	9104
TP53	Tumor protein p53(TP53)	9121
UQCR11	Ubiquinol-cytochrome c reductase, complex III subunit XI(UQCR11)	-5353
USP42	Ubiquitin specific peptidase 42(USP42)	31892
LOC101906408	Uncharacterized LOC101906408(LOC101906408)	1736
LOC107132214	Uncharacterized LOC107132214(LOC107132214)	1414
VGLL4	Vestigial like family member 4(VGLL4)	1102
XYLT1	Xylosyltransferase 1(XYLT1)	1266
ZADH2	Zinc binding alcohol dehydrogenase domain containing 2(ZADH2)	8120; 8122
ZBTB21	Zinc finger and BTB domain containing 21(ZBTB21)	14599; 14693; 14701
ZBTB3	Zinc finger and BTB domain containing 3(ZBTB3)	872
ZSCAN4	Zinc finger and SCAN domain containing 4	-11723
ZCCHC2	Zinc finger CCHC-type containing 2(ZCCHC2)	53851
ZDHHC3	Zinc finger DHHC-type containing 3(ZDHHC3)	20248
LOC789960	Zinc finger protein 256(LOC789960)	16029
ZNF316	Zinc finger protein 316(ZNF316)	9581
ZNF350	Zinc finger protein 350(ZNF350)	798
LOC104968479	Zinc finger protein 525-like(LOC104968479)	11107
LOC510913	Zinc finger protein 664(LOC510613)	-8769; -3881
LOC100299712	Zinc finger protein 665(LOC100299712)	16228
LOC508131	Zinc finger protein 665(LOC508131)	23196
ZNF8	Zinc finger protein 8(ZNF8)	3636
ZNF827	Zinc finger protein 827(ZNF827)	-386
ZMYND15	Zinc finger, MYND-type containing 15(ZMYND15)	431

Legend: Distance to the nearest transcription start site (TSS) – positive number = upstream and negative number = downstream

8.4 DISCUSSION

After fertilization, the paternal DNA suffers active demethylation process and this step is considered an erase of sperm epigenetic marks to stablish the feature of pluripotency in the embryo cells. However the DNA demethylation is not complete, retaining the methylation pattern in some regions (GUO et al., 2014) and the disturb of

these regions can cause embryonic death (SAMPATH KUMAR et al., 2017). Then, in the present study we investigated the sperm DNA methylation profile between high (HF) and low (LF) *in vitro* fertility bulls with expectation that this epigenetic signal could explain the differences for blastocyst production. To analyze the genome-wide DNA methylation we performed the reduced representation of bisulfite sequence technique (RRBS). Although this approach does not coverage the whole genome, is a single-base resolution technique, cost-effective, enriched to CpG regions and then with a good feature to start investigating different methylation regions related to *in vitro* fertility. Based on our library characterization, the average for mapped reads and mapping efficiency were good and similar to describe in the literature for bovine samples using RRBS technique (ZHOU et al., 2016), indicating the efficiency of our library preparation and good coverage of our sequencing data.

In a comprehensive analysis of bovine sperm DNA methylation, Perrier and colleagues (2018) compared sperm to other cell types and bovine to other species by different methylation approaches. They verified that global DNA methylation level in bull sperm is lower than other sperm species and, for the cell type comparison, sperm was less methylated than somatic cells. This hypomethylated profile of bovine sperm cell was also described in whole-genome bisulfite conversion analysis (ZHOU et al., 2018b). Overall, the percentage of methylation in our study, either in CpG context or CHG and CHH was low, in agree with other studies.

The PCA and hierarchical clustering did not indicate a grouping pattern, normally expected for this descriptive analysis, among samples of the same group. We got nine principal components and all of them explain somewhat the variation. Here we only presented the three higher (PC1 x PC2 and PC2 x PC3). This result indicated that the variance is more related to the bull sample than to the group, pointing to a similarity for methylation context in all samples analyzed. In our case, we worked with a higher number of samples per group (n=5) when compared to other studies performing RRBS data. Inside our groups, we have bulls from different breeds and the breed effect on global methylation was described previously (PERRIER et al., 2018). However, our groups were balanced to have the same breeds in both groups, since our goal was not to evaluate the breed effect but the fertility phenotype. Nevertheless, this could explain the heterogenicity inside each group and part of the non-grouping pattern normally expected for comparison studies.

In the comparison between HF and LF group, we verified 440 DMC, being 327 hypo- (74%) and 113 hyper- methylated (26%) in the HF group compared to LF group. Previous studies that performed RRBS analysis in bovine sperm identified a higher number of DMC. However, the comparisons were made between sperm versus somatic cells (PERRIER et al., 2018) or with oocyte and different embryo stages (JIANG et al., 2018). In a context similar to ours, Kropp and colleagues (2017) compared the global methylation status of bulls with different non-return rates and identified 76 different methylation regions (DMR) with 78% of DMRs had enriched methylation levels in high fertility bulls and 22% had enriched methylation levels in low fertility bulls. This suggests that in a context of same cell type, the differences between groups could be small, but depending on the genes they are related, with significant biological relevance.

Then, the next question is where these DMCs are distributed over the genome. The DNA feature indicated that a good proportion of the regions are overlapping promoters (15%) and exons regions (16%), CpG island (38%) and close to TSS (Figure 4C). The CpG island are 400-500 bp length, normally unmethylated and often located in promoters and the first exon of several genes, including housekeeping genes (BIRD, 1986; DEATON; BIRD, 2011). The methylation status of regulatory DNA sequences can influence on transcriptional activity. When promoters regions are methylated, it is related to gene repression, and low methylation in promoters and high methylation in gene-body can enhance gene expression level (JONES, 1999; BALL et al., 2009). Analysis of genome-wide promoter DNA methylation of embryonic stem cells, embryonic germ cells and sperm revealed similarity pattern between them, suggesting that although sperm is a specialized cell type, its promoters epigenome is already programmed for a pluripotent state (FARTHING et al., 2008). If we consider that the mature sperm is a transcriptional inactivated cell, its methylation profile could be an indicator of past events that occurred during spermatogenesis or established in order to control important genes for the next generation.

Of the 440 DMCs, we could to identify 184 genes and the analysis of function annotation performed by DAVID did not show any strong pathway between these genes. Based on that, we can affirm that DMC between HF and LF groups identified in our study have no clear function either in spermatogenesis process or embryo development, which was our first hypothesis. One possible explanation for this low difference for methylation profile could be the difference of *in vitro* embryo production

between the groups. Although the groups were statistically different for embryo development and blastocyst rate, this increase of 15% in the HF group compared to LF group might not be strong enough to relate the fertility phenotype to a specific methylation profile that could be expressed later as an embryo development difference. In our data analysis, we were criterions regard the parameters used to established different methylation regions because we wanted to identify a methylation pattern in the sperm samples that could really affect the biological process - the embryo production. The mature sperm has a DNA methylation established at embryo stage, in pro-spermatogonia and its sustained though all spermatogenesis stages after birth (SEISENBERGER et al., 2012). Therefore, the sperm DNA methylation is highly conserved among germ cells stages. In addition, DNA methylation is the first step of the central dogma of molecular biology – the control of DNA transcription to RNA synthesis. After that, many molecules and pathways could control and interfere until the protein expression, reinforcing the fact that bull fertility could be a multifactorial since sperm production. Probably, to see a significant alteration cause by sperm DNA methylome, more genes need to be involved and this would culminate in a higher impair on spermatogenesis or a complete embryo development block. In these cases, we could see bulls with altered spermatozoa or lower blastocyst production. We cannot exclude the fact that sperm methylation could have an impact on embryo development, once in our study we only evaluated a low percentage of the whole-genome through RRBS technique. In conclusion, our methylation profile between high and low *in vitro* fertility bulls identified 440 different methylation regions and 184 genes, however we could not stablish a strong pathway between these genes that could explain the distinct *in vitro* embryo production for these bulls.

REFERENCES

ALMEIDA, T. G. Cinética e desenvolvimento embrionário *in vitro* de embriões bovinos de touros de alta e baixa fertilidade. Dissertação de mestrado em Ciência, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2018.

AHLUWALIA, K. B. **Genetics**. Second Edi ed. [s.l.] New Age International Limited, 2009.

AKALIN, A. et al. MethylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. **Genome Biology**, v. 13, n. 10, 2012.

ANVAR, Z. et al. Genomic DNA Extraction from Sperm. **Journal of Advanced Medical**

Sciences and Applied Technologies, v. 1, n. 2, p. 120, 2015. Disponível em: <http://jamsat.sums.ac.ir/index.php/JAMSAT/article/view/16>.

ASTON, K. I. et al. Aberrant sperm DNA methylation predicts male fertility status and embryo quality. **Fertility and Sterility**, v. 104, n. 6, p. 1388–1397.e5, 2015. Disponível em: <http://dx.doi.org/10.1016/j.fertnstert.2015.08.019>.

BALL, M. P. et al. Targeted and Genome-Scale Strategies Reveal Gene-Body Methylation Signatures in Human Cells. **Nature biotechnology**, v. 27, n. 4, p. 361–368, abr. 2009.

BENCHAIB, M. et al. Influence of global sperm DNA methylation on IVF results. **Human reproduction (Oxford, England)**, v. 20, n. 3, p. 768–773, mar. 2005. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15640258>. Acesso em: 24 set. 2014.

BIRD, A. P. CpG-rich islands and the function of DNA methylation. **Nature**, v. 321, n. 6067, p. 209–213, 15 maio 1986. Disponível em: <http://www.nature.com/articles/321209a0>.

BOE-HANSEN, G. B.; FORTES, M. R. S.; SATAKE, N. Morphological Defects, Sperm DNA Integrity, and Protamination of Bovine Spermatozoa. **Andrology**, abr. 2018.

CARVALHO, J. O. et al. The methylation patterns of the IGF2 and IGF2R genes in bovine spermatozoa are not affected by flow-cytometric sex sorting. **Molecular reproduction and development**, v. 79, n. 2, p. 77–84, fev. 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22128039>. Acesso em: 26 jan. 2015.

CASTRO, L. S. et al. Effect of bovine sperm chromatin integrity evaluated using three different methods on in vitro fertility. **Theriogenology**, v. 107, p. 142–148, 2018. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0093691X1730540X>.

DEATON, A. M.; BIRD, A. CpG islands and the regulation of transcription. **Genes and Development**, v. 25, n. 10, p. 1010–1022, 2011.

FARTHING, C. R. et al. Global mapping of DNA methylation in mouse promoters reveals epigenetic reprogramming of pluripotency genes. **PLoS Genetics**, v. 4, n. 6, 2008.

GUO, F. et al. Active and passive demethylation of male and female pronuclear DNA in the mammalian zygote. **Cell Stem Cell**, v. 15, n. 4, p. 447–458, 2014.

HANSEN, P. J. et al. Effects of Gamete Source and Culture Conditions on the Competence of in Vitro-Produced Embryos for Post-Transfer Survival in Cattle. **Reproduction, fertility, and development**, v. 22, n. 1, p. 59–66, 2010.

JIANG, Z. et al. DNA methylomes of bovine gametes and in vivo produced preimplantation embryos. **Biology of Reproduction**, v. 0, n. July, p. 1–11, 2018. Disponível em: <https://academic.oup.com/biolreprod/advance-article/doi/10.1093/biolre/ioy138/5037913>.

JONES, P. A. The DNA methylation paradox. **Trends in Genetics**, v. 15, n. 1, p. 34–37, 1999.

KOBAYASHI, H. et al. Contribution of intragenic DNA methylation in mouse gametic DNA methylomes to establish Oocyte-specific heritable marks. **PLoS Genetics**, v. 8, n. 1, 2012.

KROPP, J. et al. Male fertility status is associated with DNA methylation signatures in sperm and transcriptomic profiles of bovine preimplantation embryos. **BMC Genomics**, v. 18, n. 1, p. 280, 2017. Disponível em: <http://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-017-3673-y>.

KRUEGER, F.; ANDREWS, S. R. Bismark: A Flexible Aligner and Methylation Caller for Bisulfite-Seq Applications. **Bioinformatics (Oxford, England)**, v. 27, n. 11, p. 1571–1572, jun. 2011.

LAQQAN, M.; HAMMADEH, M. E. Alterations in DNA Methylation Patterns and Gene Expression in Spermatozoa of Subfertile Males. **Andrologia**, v. 50, n. 3, abr. 2018.

LEE, H. J.; HORE, T. A.; REIK, W. Reprogramming the methylome: Erasing memory and creating diversity. **Cell Stem Cell**, v. 14, n. 6, p. 710–719, 2014. Disponível em: <http://dx.doi.org/10.1016/j.stem.2014.05.008>.

LEE, I. et al. New control of mitochondrial membrane potential and ROS formation - A Hypothesis. **Biological chemistry**, v. 382, n. 12, p. 1629–1636, dez. 2001. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/11843176>. Acesso em: 1 set. 2014.

LONERGAN, P. et al. Effect of Culture Environment on Gene Expression and Developmental Characteristics in IVF-Derived Embryos. **Theriogenology**, v. 65, n. 1, p. 137–152, jan. 2006.

MOLARO, A. et al. Sperm methylation profiles reveal features of epigenetic inheritance and evolution in primates. **Cell**, v. 146, n. 6, p. 1029–1041, 2011. Disponível em: <http://dx.doi.org/10.1016/j.cell.2011.08.016>.

MOUSSA, M. et al. Maternal Control of Oocyte Quality in Cattle “a Review”. **Animal reproduction science**, v. 155, p. 11–27, abr. 2015.

NAKAMURA, T. et al. PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. **Nature**, p. 2–7, 2012. Disponível em: <http://www.nature.com/doifinder/10.1038/nature11093>.

NASRI, F. et al. Sperm DNA Methylation of H19 Imprinted Gene and Male Infertility. **Andrologia**, v. 49, n. 10, dez. 2017.

PENG, H. et al. Novel Epigenomic Biomarkers of Male Infertility Identified by Methylation Patterns of CpG Sites Within Imprinting Control Regions of H19 and SNRPN Genes. **Omics : a journal of integrative biology**, abr. 2018.

PERRIER, J. et al. A multi-scale analysis of bull sperm methylome revealed both species peculiarities and conserved tissue-specific features. **BMC Genomics**, v. 19, n. 1, p. 404, 2018. Disponível em: <https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-018-4764-0>.

PERRY, G. 2016 Statistics of Embryo Collection and Transfer in Domestic Farm Animals. **Embryo Transfer Newletter**, p. 1–16, 2017.

SAITOU, M.; KURIMOTO, K. Paternal nucleosomes: Are they retained in developmental promoters or gene deserts? **Developmental Cell**, v. 30, n. 1, p. 6–8, 2014. Disponível em: <http://dx.doi.org/10.1016/j.devcel.2014.06.025>.

SAMPATH KUMAR, A. et al. Loss of Maternal Trim28 Causes Male-Predominant Early Embryonic Lethality. **Genes & development**, v. 31, n. 1, p. 12–17, jan. 2017.

SEISENBERGER, S. et al. The Dynamics of Genome-Wide DNA Methylation Reprogramming in Mouse Primordial Germ Cells. **Molecular cell**, v. 48, n. 6, p. 849–862, dez. 2012.

SOUZA, E. et al. Sperm chromatin alterations in fertile and subfertile bulls. **Reproductive Biology**, n. April, p. 0–1, 2018. Disponível em: <https://doi.org/10.1016/j.repbio.2018.04.001>.

VERMA, A. et al. Genome-wide profiling of sperm DNA methylation in relation to buffalo (Bubalus bubalis) bull fertility. **Theriogenology**, v. 82, n. 5, p. 750–9.e1, 15 set. 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/25023295>. Acesso em: 11 jan. 2015.

WRENZYCKI, C.; STINSHOFF, H. Maturation Environment and Impact on Subsequent Developmental Competence of Bovine Oocytes. **Reproduction in domestic animals**, v. 48 Suppl 1, p. 38–43, set. 2013.

ZHOU, Y. et al. Reduced representation bisulphite sequencing of ten bovine somatic tissues reveals DNA methylation patterns and their impacts on gene expression. **BMC Genomics**, v. 17, n. 1, p. 1–11, 2016. Disponível em: <http://dx.doi.org/10.1186/s12864-016-3116-1>.

ZHOU, Y. et al. Comparative whole genome DNA methylation profiling of cattle sperm and somatic tissues reveals striking hypomethylated patterns in sperm. **GigaScience**, n. April 2018, 2018a. Disponível em: <http://doi.org/10.1093/gigascience/giy039%0Ahttps://academic.oup.com/gigascience/advance-article/doi/10.1093/gigascience/giy039/4965117>.

ZHOU, Y. et al. Comparative whole genome DNA methylation profiling of cattle sperm and somatic tissues reveals striking hypomethylated patterns in sperm. **GigaScience**, n. April, p. 1–13, 2018b. Disponível em: <http://doi.org/10.1093/gigascience/giy039%0Ahttps://academic.oup.com/gigascience/advance-article/doi/10.1093/gigascience/giy039/4965117>.

9 FINAL CONSIDERATIONS

The present study can be considered as one more brick in the wall raised to bull fertility understanding. Here we try to characterize the differences between high and low *in vitro* fertility using global approaches that can point it out to possible pathways, but also raise new questions. Our results go to the same direction of some authors about bull fertility would be a symphony, that molecules orchestrate to produce a “sounds good” spermatozoa (SELLEM et al., 2015; UTT, 2016; YANIZ et al., 2017). When we analyze sperm attributes like acrosome, mitochondria, motility, plasma membrane and DNA, we are looking to the final process of different pathways that happen earlier during spermatogenesis. If we analyze an attribute isolated, like DNA integrity, it might not have an effect on fertility status. However, global approaches can indicate pathways that are modified and/or well established between a distinct group of bulls.

The results of Chapter 1 of this thesis agree with other studies in bovine that sperm DNA damage have not been found as the main cause of decrease of embryo production, on the opposite that what is seen in humans (D’OCCHIO et al., 2013; KIPPER et al., 2016). These findings raise the question about how far the sperm chromatin tests are applicable to bovine samples the way they are performed and if these assays are representing the real bull chromatin status. Future studies comparing sperm samples from different species like human, mice and livestock animals exposing to chemical and physical DNA damage (reactive oxygen species, UV irradiation, acid solutions etc.) could be helpful to understand the behavior of the sperm DNA of each species.

For one of our global approaches performed, we described in the Chapter 2 the differential expressed proteins between high and low *in vitro* fertility bulls. Although our focus was the nuclear proteins, most of differentiated proteins were related to spermatogenesis, fertilization and motility process. They cannot be directly associated to embryo development, but they could be indirect indicators of pass events that happened and culminated to the sperm that will produce embryos with higher development competence. Again, the orchestrated work of different sperm proteins results in a functional sperm ables to fertilize and sustain the embryo development, reinforcing the hypothesis that fertility could be multifactorial.

Following the same direction as Chapter 1, Chapter 3 presented some particularities of bovine sperm and other species with the absence of nucleosome-binding DNA. This study was conducted to standardize the protocol to evaluate nucleosome-binding DNA and consequently DNA regions attached to specific histones modifications of gene activation and silencing that could act as a paternal inheritance to the embryo. Unfortunately, using MNase digestion, we were not able to get nucleosome structures for bull or any other species analyzed, with exception of mice sperm. This study raises the question about how similar is the sperm chromatin of livestock animals to human and mice sperm? Considering the different proportion of protamine 1 and probably absence or not function of protamine 2, how retained histones interact with chromatin from these species? Our results together to other studies (CARONE et al., 2014; SAMANS et al., 2014) indicated that retained histones in bovine sperm are residual from spermatogenesis, with possible no effect on further embryo development, once it seems that histones are not presented as nucleosome structures, involving the DNA and contributing to nuclear compaction or gene signalization.

Finally, in the Chapter 4 we used the reduced representation of bisulfite sequencing (RRBS) as a minimized global approach to evaluate the methylation profile between high and low *in vitro* fertility bulls. In this study we identified different methylation regions, most of them hypomethylated in the high *in vitro* fertility group but unfortunately the genes containing these regions were not enriched for any pathway that could explain the relationship between methylation profile and differences for embryo production. Although high and low *in vitro* fertility groups presented some differences for DNA methylation, they are probably not enough to be related to blastocyst production. Then, our next question is how much the sperm methylation can impact on embryo destiny to turn to a blastocyst?

Although we focus our study to the sperm nucleus, hypothesizing that the difference for *in vitro* embryo production could be related to nuclear protein composition and/or epigenetic modifications because these aspects seem to be directly related to paternal DNA effects expressed after embryo genome activation and then, impacting on the embryo development, we cannot ignore the fact that sperm is acting over the embryo development by the moment it starts the contact with oocyte in the IVF environment. By that time, all the enzymes, metabolites and reactions that happen during IVF can determinate the success or not to reach blastocyst stage. And this

continues when the sperm penetrates the oocyte and how this oocyte will interact with paternal DNA. This window between sperm penetration and paternal DNA modification by the oocyte before the first cleavage is still not well understood for bovine and could be determinant to decide the future of the embryo.

In conclusion, this study indicated that *in vitro* fertility characterization can be multifactorial where small changes in chromatin integrity cannot directly affect, but proteins related to spermatogenesis, motility and fertilization can be related to the fertility phenotype studied. Among the epigenetic marks studied, nucleosome-binding DNA was absent in bovine spermatozoa in the conditions of the present study, and DNA methylation did not have a significant impact on the fertility profile.

REFERENCES

CARONE, B. R. et al. High-resolution mapping of chromatin packaging in mouse embryonic stem cells and sperm. **Developmental Cell**, v. 30, n. 1, p. 11–22, 2014. Disponível em: <http://dx.doi.org/10.1016/j.devcel.2014.05.024>.

DE ZEN, S.; SANTOS, M. C. Demanda por carne está ao alcance do Brasil! Revista DBO, junho de 2015.

D'OCCHIO, M. J. et al. Sperm chromatin in beef bulls in tropical environments. **Theriogenology**, v. 79, n. 6, p. 946–52, 1 abr. 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23434358>. Acesso em: 14 jan. 2014.

FAO. Global agriculture towards 2050. **High-level Expert Forum**, 2009. Disponível em:<http://www.fao.org/fileadmin/templates/wsfs/docs/Issues_papers/HLEF2050_Global_Agriculture.pdf>.

GALLI, C. et al. Ovum Pick up, Intracytoplasmic Sperm Injection and Somatic Cell Nuclear Transfer in Cattle, Buffalo and Horses: From the Research Laboratory to Clinical Practice. **Theriogenology**, v. 81, n. 1, p. 138–151, jan. 2014.

KIPPER, B. et al. Sperm morphometry and chromatin condensation in Nelore bulls of different ages and their effects on in vitro fertilization. **Theriogenology**, v. 1, n. 7, p. 1-7, 2016. Disponível em: <https://doi.org/10.1016/j.theriogenology.2016.08.017>

LONERGAN, P. et al. Embryo development in dairy cattle. **Theriogenology**, v. 86, n. 1, p. 270–277, 2016. Disponível em: <http://dx.doi.org/10.1016/j.theriogenology.2016.04.040>.

PERRY, G. 2016 Statistics of Embryo Collection and Transfer in Domestic Farm Animals. **Embryo Transfer Newletter**, p. 1–16, 2017.

SAMANS, B. et al. Uniformity of nucleosome preservation pattern in mammalian sperm and Its connection to repetitive DNA elements. **Developmental Cell**, v. 30, n. 1, p. 23–35, 2014. Disponível em: <http://dx.doi.org/10.1016/j.devcel.2014.05.023>.

SELLEM, E. et al. Use of combinations of in vitro quality assessments to predict fertility of bovine semen. **Theriogenology**, v. 84, n. 9, p. 1447–1454.e5, 2015.

UTT, M. D. Prediction of bull fertility. **Animal Reproduction Science**, v. 169, p. 37–44, 2016. Disponível em: <http://dx.doi.org/10.1016/j.anireprosci.2015.12.011>.

YANIZ, J. L. et al. Toward an Integrative and Predictive Sperm Quality Analysis in Bos Taurus. **Animal reproduction science**, v. 181, p. 108–114, jun. 2017.