RENO ROLDI DE ARAÚJO

Estudo em larga escala dos efeitos da idade sobre os parâmetros reprodutivos e viabilidade de oócitos equinos após injeção intracitoplasmática de espermatozóide (ICSI) usando sêmen sexado

São Paulo
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Tese apresentada ao Programa de Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências

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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que o Projeto intitulado "Estudo em larga escala do efeito da idade sobre os parâmetros reprodutivos e a viabilidade de oócitos equinos após a injeção intracitoplasmática de espermatozóide (ICSI) usando sêmen sexado", protocolado sob o nº 3121/2013, utilizando 22 (vinte e dois) equideos, sob a responsabilidade Prof. Dr. Luciano Andrade Silva Docente da Faculdade de Zootecnia e Engenharia de Alimentos-USP), foi aprovado em reunião de 13/11/2013 e está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo.

We certify that the Research "Large-scale study of age effects on reproductive parameters and viability of equine oocytes after intracytoplasmatic sperm injection (ICSI) using semen", protocol number 3121/2013, utilizing 22 (twenty two) equine, under the responsibility Prof. Dr. Luciano Andrade Silva, was approved in the meeting of day 11/13/2013 and agree with Ethical Principles in Animal Research adopted by Ethic Committee in the Use of Animals of the School of Veterinary Medicine and Animal Science of University of São Paulo.

São Paulo, 28 de novembro de 2013.

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À minha família e amigos dedico este trabalho

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RESUMO

ARAUJO, R. R. **Estudo em larga escala dos efeitos da idade sobre os parâmetros reprodutivos e viabilidade de oócitos equinos após injeção intracitoplasmática de espermatozóide (ICSI) usando sêmen sexado**. [Large-scale study of age effects on reproductive parameters and viability of equine oocytes after intracytoplasmatic sperm injection (ICSI) using sexed semen]. 2015. 106f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015.

O objetivo do presente estudo foi comparar o efeito da idade de doadores de oócitos sobre os parâmetros reprodutivos, a taxa de recuperação e qualidade de oócitos após ICSI utilizando sêmen sexado. Éguas Pôneis de Polo (n = 79) e Puro-Sangue (n = 23) doadoras de oócitos (400-600kg e 3-29 anos) foram utilizados durante as estações reprodutivas de 2009/2010 e 2011 no hemisfério Sul (San Luis, Argentina) e norte (Kentucky, EUA), respectivamente. As éguas foram divididas em três categorias experimentais: Jovens (YM: 3-10 anos), Meia Idade (MA: 11-17a) e Idosas (OM≥18a). Um total de 326 oócitos recuperados *in vivo* a partir de folículos pré-ovulatórios (n=279) e folículos imaturos em crescimento (n=47). Desses, 224 oócitos foram recuperados, classificado e dirigido a um programa comercial ICSI com Pôneis de Polo (primeira estação). Durante a segunda estação, 57 oócitos foram recuperados a partir de folículos pré-ovulatórios e 47 oócitos de folículos em crescimento e congelados em nitrogênio líquido para ser usado posteriormente em outro estudo. Os pontos experimentais avaliados foram: intervalos (dias) entre a aspiração ou a ovulação (AS/OV) ao PGF, PGF ao GnRH (Dia 0=GnRH), AS/OV ao Dia 0, e AS/OV ao Dia+1; edema uterino (UE); tonus cervical (CT); diâmetro máximo do folículo dominante (MdF1) em D0 e D+1, e a taxa de crescimento folicular. O número total de aspirações, o número de folículos aspirados e oócitos por aspiração e/ou folículo, o grau de expansão das células do cúmulos, a presença e qualidade do corpo polar (PB), o volume ooplasma, o intervalo de GnRH a aspiração, a GnRH a ICSI, e a taxa de clivagem (CR) depois da ICSI também foram avaliados. Foram observadas diferenças significativas entre as três categorias experimentais (YM, MA e OM) para: intervalos (dias) entre PGF-GnRH, AS/OV-GnRH, AS/OV-Day 1, edema uterino no Dia 0, CT em Dia 1, MdF1 no D0 e D+1 e volume de ooplasma. A taxa de recuperação *in vivo* (RR) não foi afetada pela idade (média de 102%, 85% e 73,4% de oócitos recuperados por ciclo, por F1 e por folículo, respectivamente, P> 0,05). O CR não diferiu entre as

por ciclo , por F1 e por folículo, respectivamente, P> 0,05). O CR não diferiu entre as categorias experimentais. Em conclusão, um efeito de envelhecimento pode ser observado em vários parâmetros reprodutivos em éguas. Os ovócitos no presente estudo tiveram um menor volume de ooplasma para OM comparados com YM, mas isso não foi eficaz em predizer a viabilidade dos oócitos ou o potencial para o desenvolvimento para a avaliação da taxa de clivagem após ICSI utilizando sêmen sexado.

Palavras-chave: Oócito. Envelhecimento. miRNA. mtDNA. Embriões.

ABSTRACT

ARAUJO, R. R. **Large-scale study of age effects on reproductive parameters and viability of equine oocytes after intracytoplasmatic sperm injection (ICSI) using sexed seme**n. [Estudo em larga escala dos efeitos da idade sobre os parâmetros reprodutivos e viabilidade de oócitos equinos após injeção intracitoplasmática de espermatozóide (ICSI) usando sêmen sexado]. 2015. 106f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia. Universidade de São Paulo, São Paulo, 2015.

The aim of the present study was to compare the effect of oocyte donors` age on reproductive parameters, recovery rate and quality of oocytes after ICSI using sexed semen. Polo Ponies (n=79) and Thoroughbred (n=23) oocyte donor mares (400-600kg and 3-29 years) were used during the 2009/2010 and 2011 reproductive seasons in the Southern (San Luis, Argentina) and in the Northern hemispheres (Kentucky, USA), respectively. Mares were divided into three experimental categories: Young Mares (YM: 3-10y), Middle Age Mares (MA: 11-17y) and Old Mares (OM≥18y). A total of 326 oocytes were recovered *in vivo* from pre-ovulatory follicles (n=279) and immature-growing follicles (n=47). From those, 224 oocytes were recovered, sorted and directed to a commercial ICSI program with Polo Ponies (first season). During the study`s second season, 57 oocytes were recovered from pre-ovulatory follicles and 47 oocytes from growing follicles and frozen in liquid nitrogen to be used in another study. The evaluated experimental end-points were: Intervals (days) between aspiration or ovulation (AS/OV) to PGF, PGF to GnRH (Day 0=GnRH), AS/OV to Day 0, and AS/OV to Day + 1; uterine edema (UE); cervical tone (CT); maximum diameter of dominant follicle (MdF1) on D0 and D+1, and follicular growth rate. Total number of aspirations, number of follicles aspirated and oocytes recovered per aspiration and/or follicle, the degree of cumulus cell expansion, the presence and quality of polar body (PB), the ooplasm volume, the interval from GnRH to aspiration, GnRH to ICSI, and the cleavage rate (CR) after ICSI were also evaluated. Significant differences between the three experimental categories (YM, MA and OM) were observed for: intervals (days) between PGF-GnRH, AS/OV-GnRH, AS/OV-Day +1, uterine edema on Day 0, CT on Day +1, MdF1 on D0 and D+1 and ooplasm volume. The *in vivo* recovery rate (RR) was not affected by age (average of 102%, 85% and 73.4% of oocyte recovered per cycle, per F1 and per follicle, respectively; P>0.05). The CR did not differ among experimental categories. In conclusion, an effect of aging could be observed in

several reproductive parameters in mares. The oocytes in the present study had a smaller ooplasm volume for OM compared to YM, but this was not effective in predicting oocyte viability or the potential to development through evaluation of the cleavage rate after ICSI using sexed semen.

Keywords: Oocyte. Aging. miRNA. mtDNA. Embryos

SUMÁRIO

1 INTRODUCTION AND RATIONALE

Several reproductive technologies, ranging from *in vitro* maturation (IVM), *in vitro* fertilization (IVF), intra-fallopian transfer of oocyte (GIFT), sexed semen and intra-cytoplasmic sperm injection (ICSI), to animal cloning, have been developed and continuously improved through studies in animals and humans (DEVITO et al., 2010; MEIRELLES et al., 2010; WONGTRA-NGAN et al., 2010; BEN-AMI et al., 2011). However, the success of these technologies is strongly dependent on oocyte quality. In this context, female aging has been suggested as a possible factor that negatively influences the results of these technologies (BROEKMANS; SOULES; FAUSER, 2009). The oocyte quality profoundly affects some reproductive events such as oocyte maturation, monospermic fertilization and embryo survival, as well as the maintenance of pregnancy and fetal development (WANG; SUN, 2007). Therefore, the identification of reliable indicators for predicting the quality and oocyte competence and understanding of the physiological factors that regulate these mechanisms are extremely important to the effectiveness of assisted reproduction in domestic species and humans.

Many changes in physiological reproductive patterns are observed during the female aging process. It is known that aging is accompanied by decreased fertility as a result of hormonal, anatomical and histological changes (NAVOT et al., 1994; SAUER et al., 1994; ZAPANTIS; SANTORO, 2002). Additionally, the harmful effect of maternal age on fertility seems to be strongly related to the quality of the oocyte in several species including humans and horses (NAVOT et al., 1994; CARNEVALE; GINTHER, 1995). The loss of quality of the oocyte during aging is a multifactorial process involving different factors at different stages of the estrous cycle. Some of known causes of reduced fertility and oocyte quality related to aging are associated to ultrastructural alterations (mitochondrial dysfunction, spindle/meiotic aberrations leading to aneuploidy or trisomy), depletion of follicle pool, hormonal alteration (DHEA reduction), metabolic disorders (reactive metabolites and oxidative stress, both in oocytes and grannulosa cells, among others (CARNEVALE; BERGFELT; GINTHER, 1993; SHIGENAGA; HAGEN; AMES, 1994; KEEFE et al., 1995; COLLADO; BLASCO; SERRANO, 2007; BROEKMANS; SOULES; FAUSER, 2009; BENTOV et al., 2011; KUSHNIR et al., 2012; LI et al., 2012; SZAFAROWSKA; JERZAK, 2013). Despite the efforts to understand and treat infertility related to female aging, by the use

of *in vivo* or *in vitro* assisted reproductive techniques (BEN-AMI et al., 2011), the complete elucidation of the mechanisms has not been determined.

Experimental models using rats, mice, pigs, cattle and horses have been developed to study oocyte quality and competence during maturation, fertilization and embryo development. In humans, oocyte morphology has been associated with viability (BORINI et al., 2005; WANG; SUN, 2007). The mare has recently been suggested as a promising model for the study of infertility related to human aging because of the similarity between the reproductive cycles of these species and similarities of aging associated with reproductive changes (GINTHER et al., 2004; CARNEVALE, 2008). Fertility in the mare decreases with age, starting around 10 years of age and some mares reach reproductive senescence after 20 years of age (CARNEVALE, 2008). Therefore, the elucidation of the mechanisms related to infertility (specially related to decreased oocyte quality) and its correlation with age, along with advances in assisted reproductive techniques (ART) in the mare, may contribute to obtain pregnancies from older donors, and may also be used as model to understand infertility in women. In this sense, the morphological evaluation of the equine oocyte represents an alternative noninvasive method for assessment of oocyte quality and competence being of utmost importance for the efficiency of assisted reproduction in domestic species.

In Brazil, few laboratories have been devoted to studies involving *in vitro* production of equine embryos (FERNANDES, 2008). Unlike bovine, advances in biotechnologies of reproduction in horses occur more slowly mainly due to the high cost of maintaining animals on farms, the difficulty of obtaining material in slaughterhouses, as well as the difficulty in establishing an efficient routine of *in vitro* production of embryos. However, the need for techniques that improve reproductive performance, and encourage the preservation of genetic material in horses has increased considerably since the equestrian market in Brazil has been growing satisfactorily (LIMA; SHIROTA; BARROS, 2006).

The Brazilian equine herd ranks as the third largest herd in the world, with more than six million heads. In 2006 a report was published by the Center for Advanced Studies in Applied Economy, University of São Paulo (ESALQ) that aimed to increase knowledge of the horse industry, or better the Horse Complex Agribusiness in Brazil (LIMA; SHIROTA; BARROS, 2006). The estimates presented, calculated based on conservative criteria, indicate that the complex moves more than $ 7.5 billion annual economic value, generating approximately 3.2 million direct and indirect jobs. Therefore, research which aims to deepen knowledge in physiological mechanisms to improve the reproductive performance of the equine species are of great importance for the sector in the country.

2 GENERAL PURPOSE

Assess oocyte quality by analyzing the total number of copies of mtDNA, the evaluation of morphological characteristics prior and posterior maturation and consequently select equine oocytes with greater potential for *in vitro* embryo development. In parallel, a model for studying oocyte maturation comparative *in vivo* and *in vitro* will be developed in order to understand the molecular and cellular changes that occur during these maturation processes, emphasizing the effect of female aging on this process.

2.1 SPECIFIC OBJECTIVES AND HYPOTHESES

2.1.1 Experiment 1

Objectives:
Compare the age effect of donor mares on reproductive parameters, the rate of oocyte recovery and the viability of oocytes by ICSI with use of sexed semen.

Hypotheses:
Lower reproductive parameters, lower quality and smaller oocytes associated with lower in vitro development of embryos are observed in older mares.

2.1.2 Experiment 2

Objectives:
Compare different oocyte collection methods on the rate of oocyte recovery, oocyte quality and its relation with the morphology of the cumulus-oocyte complex in a large number of

mares.

Hypotheses:

Higher recovery will be obtained by follicular aspiration of oocytes in vivo matured when compared with oocytes recovered from growing follicles, both in vivo and in vitro; Equine oocytes collected from follicles in vivo matured have better quality and grater layer of cumulus-oocyte complex cells.

2.1.3 Experiment 3

Objectives:

The purpose of this study was to evaluate the levels of miRNAs and mtDNA in follicular fluid and oocytes of young and old mares, and to identify possible sets of differentially expressed miRNAs and different amounts of mtDNA between young and old mares.

Hypotheses:

Old mares have low levels of oocytes mtDNA and different levels of miRNAs in oocyte and follicular fluid that may be related to decreased fertility.

3 LITERATURE REVIEW

3.1 *IN VITRO* PRODUCTION OF EQUINE EMBRYOS

The development of assisted reproductive technologies in horses (eg .: *in vitro* fertilization - IVF, intra-cytoplasmic sperm injection - ICSI and cloning) has been relatively slow compared to other domestic species such as ruminants and pigs (GALLI et al., 2007). Despite the scarce availability of ovaries from the slaughterhouse and the lack of interest of horse breeders and associations, the development of efficient methods for recovery of oocytes from live mares have aroused interest for IVF in horses (HINRICHS, 2005). *In vitro* fertilization, is usually performed from the culture of oocytes with a capacitated sperm, but this procedure is not efficient in the equine species. However, the equine oocyte *in vitro* fertilization is possible using ICSI. The first cultures of equine embryo were realized in vivo, into the oviduct of mares and sheep (as recipients). The objective was to achieve better embryo development rates, around 18-36% of fertilized oocytes (GALLI et al., 2007). Subsequently, parallel advancement of *in vitro* maturation conditions and culture media allowed us to obtain high rates of embryo development and maturation from *in vitro* culture of embryos produced by ICSI, from 5 to 10% in the first studies to 38 % in recent studies (HINRICHS et al., 2005; GALLI et al., 2007). In 2003 it was reported the birth of the first equine cloned by somatic cells nuclear transfer (SCNT, WOODS et al., 2003). From this date, SCNT technology has being improved due to advances in the basic points of the *in vitro* production of embryos, including cryopreservation. The development of an efficient protocol for equine oocyte activation (LAZZARI et al., 2002; LAGUTINA et al., 2005) and the application of piezo-electric support (WESTHUSIN et al., 2003) or manipulation-free area for reconstruction of the embryo during SCNT (BOOTH et al., 2001; OBACK; WELLS, 2002; LAGUTINA et al., 2007) were key steps to success of the procedure of cloning by SCNT in this species. Activation rates of equine oocytes over 70% of cleavage has been reported in embryos fertilized, in partenothes embryos and embryos undergoing nuclear transfer (GALLI et al., 2007, 2008). The use of parthenogenetic activation and embryo culture until the blastocyst stage is a routine widely employed to control *in vitro* production of embryos in several species, including equine species (GALLI; LAZZARI, 2008; GALLI et al., 2008).

Equine embryos produced *in vitro* by ICSI and SCNT and cryopreserved can achieve rates up to 69% of pregnancy with birth rate of 83% (GALLI et al., 2007). In nuclear transfer, pregnancy rates and birth of foals are still estimated based on a small number of embryos produced *in vitro* and transferred to recipients (GALLI et al., 2007; JOHNSON et al., 2010). Rates of blastocyst formation on reconstructed embryos by nuclear transfer are between 5-28%, pregnancy rates between 11-54%, and birth rates between 23-38% (GALLI et al., 2007; JOHNSON et al., 2010).

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3.1.1 Oocyte competence and quality

Oocyte competence is defined as the ability of the oocyte to resume meiosis, cleave after fertilization and develop into a normal embryo, resulting in a healthy individual (WANG; SUN, 2007). The successful application of reproductive technologies is dependent on the competence of the oocyte, which in turn is dependent on the quality. Despite the terminology of oocyte competence be well sedimented, no work in the literature precisely defines the oocyte quality. It is understood by oocyte quality in this study as the evaluation of morphological and biochemical characteristics that determine its competence. For the complete success of the early reproductive events, the oocyte has to go through a series of stages of maturation during folliculogenesis, including: meiotic, cytoplasmic, molecular and epigenetic maturation.

3.1.2 Oocyte maturation

Mammalian oocytes remain quiescent in ovarian follicles at the stage of prophase of meiosis, a period called the germinal vesicle stage (GV; RICHARD, 2007). The resumption of meiosis *in vivo*, indicated by germinal vesicle breakdown (GVBD), happens upon stimulation of luteinizing hormone (LH) or after removal of the oocyte from the follicular environment (inhibitory environment, FAN; SUN, 2004; SIRARD et al., 2006). After any of these stimuli, the maturation promoting factor (MPF) is synthesized or activated and the

oocyte completes the first stage of meiosis releasing the first polar body (SIRARD et al., 2006). At this point, the oocyte is blocked in metaphase II in meiosis under the influence of cytosolic factors (SIRARD et al., 2006).

In horses, as in most mammalian species, meiotic or nuclear maturation is gradually acquired during follicular growth, specifically after the follicle reaches 20 mm in diameter (HINRICHS, 2010). During follicular development the oocyte grows and gains meiotic competence in three stages. First acquires the ability to undergo germinal vesicle breakdown, then is able to progress to metaphase I, and finally gain ability to progress to metaphase II (RODRIGUEZ; FARIN, 2004). The full developmental competence of the oocyte requires synchrony between nuclear and cytoplasmic maturation (SUN; NAGAI, 2003; KRISHER, 2004).

The cytoplasmic maturation involves events that enable the oocyte to complete its development. During this process, occurs an increase in the number and reorganization of intracellular organelles as the mitochondria, lysosomes, endoplasmic reticulum and Golgi complex (WATSON, 2007). In addition, oocytes accumulate calcium into intracytoplasmic vesicles, which is required for the fertilization process (BROMFIELD; MESSAMORE; ALBERTINI, 2008) and also develops sensitivity to inositol 1,4,5-triphosphate (IP3) by increasing the number of receptors in preparation for fertilization (EPPIG et al., 2004).

The first signs of cytoplasmic maturation in oocytes are the condensation of the nucleolus and the depletion of ribosomes. These events represent the end of the maturation phase changes due to modification of the transcriptional and translational machinery (SIRARD et al., 2006). Inefficient cytoplasmic oocyte maturation can lead to the interruption of maternal embryonic development control, and as a result of failure in development (VASSENA et al., 2003). To date, there is no accurate method of measuring the complete cytoplasmic maturation than the rates of fertilization and embryonic development (KRISHER, 2004). In this sense, the importance of oocyte competence in embryonic development has stimulated extensive research aiming to identify indicators for predicting the quality of the oocyte.

3.1.3 Methods for evaluating the quality of the oocyte

Traditional methods of assessing the quality of the oocyte are based on morphological classification of the follicle, the oocyte-cumulus complex and the polar corpuscle and/or meiotic spindle (WANG; SUN, 2007). Despite the prediction of oocyte quality by evaluating morphological characteristics be a subjective method, its use provides valuable information for pre-selection of oocytes with high expertise for developing information, maximizing the results of embryo development (WANG; SUN, 2007).

The cumulus-oocyte complex is usually classified by the degree of compaction of cumulus cells and characteristics of the oocyte cytoplasm. In cattle, for example, groups report better developmental competence in oocytes with compact cumulus and dark ooplasm compared to oocytes with expanded cumulus and dark ooplasm (BLONDIN; SIRARD, 1995; DE WIT; WURTH; KRUIP, 2000; DE WIT; KRUIP, 2001). However, contradictory results have also been reported for this species using this classification (BILODEAU-GOESEELS; PANICH, 2002; COTICCHIO et al., 2004). In horses, a better rate of maturation is observed in oocytes with expanded compared to compact cumulus (approximately 60 vs 51%, respectively; HINRICHS; WILLIAMS, 1997; HINRICHS; SCHMIDT, 2000; GALLI et al., 2007). However the ability of oocytes with expanded cumulus to develop to the blastocyst stage is not different when compared to oocytes with compact cumulus (LAGUTINA et al., 2005; GALLI et al., 2007; HINRICHS, 2010), different from that observed in ruminants and pigs.

3.1.4 Mitochondria

The organization and activity of mitochondria play an important role among several events involved in oocyte quality, cytoplasmic maturation, fertilization and embryonic development (VAN BLERKOM; RUNNER, 1984; MAY-PANLOUP et al., 2007). The pattern of mitochondrial distribution is a highly dynamic process during oocyte maturation and early embryonic development (STOJKOVIC et al., 2001; SUN et al., 2001; BAVISTER; SQUIRRELL, 2000; AMBRUOSI et al., 2009). Consistent results indicate that inadequate distribution of mitochondria in the ooplasm is a marker of cytoplasmic immaturity and is

strongly related to low development capacity (BAVISTER; SQUIRRELL, 2000; SUN et al., 2001; AU et al., 2005; BREVINI et al., 2005). Mitochondria contain only a single copy of DNA (mtDNA). In contrast to somatic cells, each oocyte has a large number of mitochondria in the cytoplasm (PIKÓ; MATSUMOTO, 1976). Notably, studies in human oocytes (SANTOS; EL SHOURBAGY; ST JOHN, 2006), cattle (STOJKOVIC et al., 2001; TAMASSIA et al., 2004) and pigs (EL SHOURBAGY et al., 2006) showed that the average the number of mitochondrial copies was significantly lower in groups of unfertilized oocytes compared to groups of fertilized oocytes. Furthermore, deletions of mtDNA are associated with impaired quality of oocytes, resulting in inefficient embryonic development (HSIEH et al., 2002; GIBSON; KUBISCH; BRENNER, 2005). Recently, a review on transmission of diseases related to mtDNA was published in collaboration with the research group in which this project will be developed (POULTON et al., 2010). The review covers applications of recent advances in genetic techniques for the management of mtDNA-related disorders. In this same group, other studies report the importance of mtDNA on the quality of oocytes and potential of embryonic development in cattle (BURGSTALLER et al., 2010; CHIARATTI et al., 2010). Mitochondria are the main energy producing oocytes. Its use the cycle of oxidative phosphorylation to produce ATP required for cellular activities. In addition, mitochondria are involved in the production of reactive oxygen species (ROS) and apoptosis (STOJKOVIC et al., 2001; RAMALHO-SANTOS et al., 2009; WAI et al., 2010). To date, no study has been conducted in the equine species trying to correlate the amount of copies of mtDNA in oocytes with growth and oocyte quality, follicular development, the aging of the female and the potential of embryonic development.

3.1.5 MicroRNAs (miRNAs)

Another molecules that regulates oocyte development and consequently embryo development is the miRNAs. MicroRNA are small non-coding RNAs (~ 21 nucleotides), that regulates the mRNA translation of protein coding genes in a sequential specific manner, resulting in the cleavage or repression of the correlated mRNA (LEE; FEINBAUM; AMBROS, 1993; LAGOS-QUINTANA et al., 2001; LAU et al., 2001; LEE; AMBROS, 2001). The post-transcriptional mechanism of regulation involves the interaction of

microRNAs with mRNAs encoded in the 3 'UTRs where the complementarity near perfection or the incompatibility inhibits translation by mRNAs degradation or by blocking translation without degrading the target (KIM; NAM, 2006).

MicroRNAs are expressed as capped and transcribed polyadenylated RNA polymerase II (LEE et al., 2004). From primary miRNA transcripts (Pri-miRNA), the formation of precursor miRNAs (pre-miRNAs) precedes functionally mature miRNAs through successive processing events catalyzed by RNase III family, Drosha and Dicer (MURCHISON; HANNON, 2004). Mature miRNAs (~ 20NT) associated with the complex induced RNA silencers (RISC) primarily drive the partial translational repression of target mRNAs by a not very well understood mechanisms (FILIPOWICZ, 2005).

MiRNAs are important in the regulation of different biological processes, including development time, proliferation, differentiation, migration and apoptosis (AMBROS, 2003; KLOOSTERMAN; PLASTERK, 2006; SCHICKEL et al., 2008; BARTEL, 2009). Recently, differential microRNA levels associated with age in human and equine follicular fluid revealed possible paths that can be used to determine fertility and the success of *in vitro* fertilization (SILVEIRA et al., 2012; DIEZ-FRAILE et al., 2014). More studies that aim to elucidate the molecular mechanisms related to the decline of oocyte quality and decreased fertility associated with aging are extremely important, both for horses as for humans.

3.1.6 Oocyte quality and the age effect

An important factor that requires attention when you mention oocyte competence, maturation and embryo development is its relationship with age, or better, with an aging female. Aging is associated with physiological changes and decline of fertility in most mammalian species. In humans, female fertility declines from approximately 35 years (HULL et al., 1996; SPANDORFER et al., 2004). In the mare, this decline begins around age 10 (CARNEVALE, 2008). The quality of the oocyte has been indicated as the primary cause of reduced fertility associated with aging in mares (CARNEVALE; GINTHER, 1995), and in other species (ZAPANTIS; SANTORO, 2002; OTTOLENGHI et al., 2004; BAIRD et al., 2005). Recently the mare has been suggested as a promising model for the study of infertility related to aging in humans due to the similarity in the reproductive cycle and similarities of aging associated with reproductive changes (GINTHER et al., 2004; CARNEVALE, 2008).

Changes in fertility in the mare associated with aging occur early in pregnancy before the embryo into the uterus. Embryos recovered from oviducts of old mares showed delayed cleavage, fewer cells and a larger number of morphological abnormalities, even though fertilization rate did not differ between young and old mares (CARNEVALE; BERGFELT; GINTHER, 1993). In a subsequent study (CARNEVALE; GINTHER, 1995), the oocytes were collected directly from pre-ovulatory follicles of young and old mares and transferred to the oviduct of young mares as order to eliminate a possible confounding effect between age and environment of the reproductive tract. As a result, the smaller amount of pregnancies was detected by transfer of oocytes from old as compared to young mares, suggesting that oocytes from old mares are less capable for fertilization and embryo development. In humans, decreased oocyte quality and development capacity related to maternal age have also been reported (OTTOLENGHI et al., 2004; BAIRD et al., 2005). Thus, the elucidation of mechanisms related to infertility (and/or decreased oocyte quality) and its correlation with aging, along with advances in assisted reproductive techniques (ART) in the mare, has a great contribution to equine reproduction, and can also be used to validate and improve studies in other species, specifically in the human species.

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4 LARGE-SCALE STUDY OF AGE EFFECTS ON REPRODUCTIVE PARAMETERS AND VIABILITY OF EQUINE OOCYTES AFTER INTRACYTOPLASMATIC SPERM INJECTION (ICSI) USING SEXED SEMEN

ABSTRACT

The aim of the present study was to compare the effect of oocyte donors` age on reproductive parameters, recovery rate and quality of oocytes after ICSI using sexed semen. Polo Ponies (n=79) and Thoroughbred (n=23) oocyte donor mares (400-600kg and 3-29 years) were used during the 2009/2010 and 2011 reproductive seasons in the Southern (San Luis, Argentina) and in the Northern hemispheres (Kentucky, USA), respectively. Mares were divided into three experimental categories: Young Mares (YM: 3-10y), Middle Age Mares (MA: 11-17y) and Old Mares (OM≥18y). A total of 326 oocytes were recovered *in vivo* from pre-ovulatory follicles (n=279) and immature-growing follicles (n=47). From those, 224 oocytes were recovered, sorted and directed to a commercial ICSI program with Polo Ponies (first season). During the study`s second season, 57 oocytes were recovered from pre-ovulatory follicles and 47 oocytes from growing follicles and frozen in liquid nitrogen to be used in another study. The evaluated experimental end-points were: Intervals (days) between aspiration or ovulation (AS/OV) to PGF, PGF to GnRH (Day 0=GnRH), AS/OV to Day 0, and AS/OV to Day + 1; uterine edema (UE); cervical tone (CT); maximum diameter of dominant follicle (MdF1) on D0 and D+1, and follicular growth rate. Total number of aspirations, number of follicles aspirated and oocytes recovered per aspiration and/or follicle, the degree of cumulus cell expansion, the presence and quality of polar body (PB), the ooplasm volume, the interval from GnRH to aspiration, GnRH to ICSI, and the cleavage rate (CR) after ICSI were also evaluated. Significant differences between the three experimental categories (YM, MA and OM) were observed for: intervals (days) between PGF-GnRH, AS/OV-GnRH, AS/OV-Day +1, uterine edema on Day 0, CT on Day +1, MdF1 on D0 and D+1 and ooplasm volume. The *in vivo* recovery rate (RR) was not affected by age (average of 102%, 85% and 73.4% of oocyte recovered per cycle, per F1 and per follicle, respectively; P>0.05). The CR did not differ among experimental categories. In conclusion, an effect of aging could be observed in several reproductive parameters in mares. The oocytes in the

present study had a smaller ooplasm volume for OM compared to YM, but this was not effective in predicting oocyte viability or the potential to development through evaluation of the cleavage rate after ICSI using sexed semen.

Keywords: Oocyte. Aging. ICSI. Sexed semen. Embryos.

4.1 INTRODUCTION

Several reproductive technologies, ranging from *in vitro* maturation (IVM), *in vitro* fertilization (IVF), intra-fallopian transfer of oocyte (GIFT), sexed semen and intra-cytoplasmic sperm injection (ICSI), to animal cloning, have been developed and continuously improved through studies in animals and humans (DEVITO et al., 2010; MEIRELLES et al., 2010; WONGTRA-NGAN et al., 2010; BEN-AMI et al., 2011). However, the success of these technologies is strongly dependent on oocyte quality. In this context, female aging has been suggested as a possible factor that negatively influences the results of these technologies (BROEKMANS; SOULES; FAUSER, 2009). The oocyte quality profoundly affects some reproductive events such as oocyte maturation, monospermic fertilization and embryo survival, as well as the maintenance of pregnancy and fetal development (WANG; SUN, 2007).

Many changes in physiological reproductive patterns are observed during the female aging process. It is known that aging is accompanied by decreased fertility as a result of hormonal, anatomical and histological changes (NAVOT et al., 1994; SAUER et al., 1994; ZAPANTIS; SANTORO, 2002). Additionally, the harmful effect of maternal age on fertility seems to be strongly related to the quality of the oocyte in several species including humans and horses (NAVOT et al., 1994; CARNEVALE; GINTHER, 1995). The loss of quality of the oocyte during aging is a multifactorial process involving different factors at different stages of the estrous cycle. Some of known causes of reduced fertility and oocyte quality related to aging are associated to ultrastructural alterations (mitochondrial dysfunction, spindle/meiotic aberrations leading to aneuploidy or trisomy), depletion of follicle pool, hormonal alteration (DHEA reduction), metabolic disorders (reactive metabolites and oxidative stress, both in oocytes and grannulosa cells, among others (CARNEVALE;

BERGFELT; GINTHER, 1993; SHIGENAGA; HAGEN; AMES, 1994; KEEFE et al., 1995; COLLADO; BLASCO; SERRANO, 2007; BROEKMANS; SOULES; FAUSER, 2009; BENTOV et al., 2011; KUSHNIR et al., 2012; LI et al., 2012; SZAFAROWSKA; JERZAK, 2013). Despite the efforts to understand and treat infertility related to female aging, by the use of *in vivo* or *in vitro* assisted reproductive techniques (BEN-AMI et al., 2011), the complete elucidation of the mechanisms has not been determined.

Experimental models using rats, mice, pigs, cattle and horses have been developed to study oocyte quality and competence during maturation, fertilization and embryo development. In humans, oocyte morphology has been associated with viability (BORINI et al., 2005; WANG; SUN, 2007). The mare has recently been suggested as a promising model for the study of infertility related to human aging because of the similarity between the reproductive cycles of these species and similarities of aging associated with reproductive changes (GINTHER et al., 2004; CARNEVALE, 2008). Fertility in the mare decreases with age, starting around 10 years of age and some mares reach reproductive senescence after 20 years of age (CARNEVALE, 2008). Therefore, the elucidation of the mechanisms related to infertility (specially related to decreased oocyte quality) and its correlation with age, along with advances in assisted reproductive techniques (ART) in the mare, may contribute to obtain pregnancies from older donors, and may also be used as model to understand infertility in women. In this sense, the morphological evaluation of the equine oocyte represents an alternative noninvasive method for assessment of oocyte quality and competence being of utmost importance for the efficiency of assisted reproduction in domestic species.

The use of ICSI, as well as others ARTs in horses, has been relatively slowly applied compared to other domestic species such as ruminants and pigs (GALLI et al., 2007). Despite the limited availability of ovaries from the slaughterhouse and low interest from horse breeders and associations, the development of efficient methods for recovering oocytes from live mares have aroused interest for IVF in horses (HINRICHS, 2005). In vitro fertilization is usually performed from *in vitro* cultured oocytes with an *in vitro* capacitated sperm, but it is not efficient in horses. However, fertilization of the equine oocyte *in vitro* is achieved with the use of ICSI. The use of this technique to study the effect of age-related oocyte quality is relevant, since the oocytes can be retrieved *in vivo* by follicular aspiration and fertilized *in vitro.* This eliminates the problematic factors related to the abnormality of the reproductive tract of old mares, as well as factors related to the quality of the cumulus cells and zona pellucidae, for example (CARNEVALE; GINTHER, 1995, ALTERMATT et al., 2009).

Another biotechnology that shows great potential to be applied in the equine species is

sexed semen. Some articles has been published using equine sexed semen and this has generated interest from the equine industry and many breed associations (BUCHANAN et al., 2000; LINDSEY et al., 2002b, 2005; MORRIS, 2004; SUH; SCHENK; SEIDEL, 2005; MARI et al., 2010). A limitation for the use of sexed semen in horses is the amount of semen that can be sex sorted per hour (approximately 5-10x10^6 spermatozoa/h; (MARI et al., 2005) and the minimum inseminating dose required to acquire satisfactory pregnancies rate commercially. The doses $\geq$ 300x10^6 progressively motile spermatozoa per insemination has been recommended to obtain good fertility in mares (PICKETT et al., 1989; SIEME et al., 2004). However, pregnancies of mares with sexed semen have been reported with rates of 38% using deep uterine insemination (LINDSEY et al., 2005), and 25 to 72% with the use of hysteroscopic deposition of semen in the uterine tubal junction (2 to 20x10^6 sptz; (LINDSEY et al., 2002a, 2002b, 2005; MORRIS, 2004) or by surgical insemination directly into the oviduct (150,000 sptz; (BUCHANAN et al., 2000)). There is still limited information available on stallion sperm quality after sex sorting and its use for oocyte fertilization. Satisfactory results after ICSI with sexed semen has been reported in cattle and swine (PROBST; RATH, 2003; JO et al., 2014). Thus, ICSI might be a viable alternative to circumvent the problem related to the low available dose of sexed semen to be used, since only one sperm is needed for fertilization of the oocyte.

Thus, the aim of this study was to compare the oocyte donors' age effect on the reproductive parameters, recovery rate and oocyte quality evaluated by oocyte and grannulosa cells morphology and cleavage rate after ICSI using sexed semen.

4.2 MATERIALS AND METHODS

4.2.1 Animals and experimental design

A total of 102 non-lactating Polo Pony (n=79) and Thoroughbred mares (n=23) weighting between 400 and 600 kg were used during the 2009/2010 and 2011 breeding seasons in the southern (San Luis, Argentina) and northern (Kentucky, EUA) hemisphere, respectively. The Polo Pony mares belonged to a Commercial Program of Equine Assisted

Reproduction (First season) and the Thoroughbred mares to the Maxwell H. Gluck Equine Research Center at the University of Kentucky (Lexington, EUA; Second season). All mares had free access to good quality pasture, balanced mineral salt and water. Mares between 3 and 29 years of age were used as oocyte donors for both seasons and grouped based on age: Young mares (YM: 3-10y; n=54); Middle Age mares (MA: 11-17y; n=24) and Old Mares (OM: ≥18 y; n=24). All procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of São Paulo (First season) and the University of Kentucky (Second season).

4.2.2 Ultrasonography, estrus synchronization and oocyte collection

First and second season. Mares were synchronized with administration of a luteolytic agent (PGF, 7.5 mg, im; Lutalyse) 6 days after follicular aspiration (ASP) or ovulation (OV) and monitored for follicular growth by rectal palpation and ultrasonography every 2 to 5 days. From a total of 441 estrous cycles, only those cycles that met the following criteria were used: donor mares with the same reproductive and nutritional management; at least five exams by transrectal palpation and ultrasonography per cycle; absence of hormonal treatment additional to that provided for synchronization and induction of follicular maturation *in vivo* (PGF, GnRH and hCG); absence of any use of anti-inflammatory drugs and cycles with clinical history of normal health. The mares were monitored by transrectal ultrasonography (US) with intervals of 2 to 5 days after OV until the first detection of a follicle ≥28 mm, after which point they were monitored daily. Treatment with 2000 IU hCG + 1.5mg GnRH was performed to induce oocyte maturation *in vivo* when the follicle diameter was ≥32 mm, uterine edema was present (GINTHER, 1992), and the cervix relaxed. **Second season.** Mares were synchronized as performed in the First season. However, mares were daily monitored by transrectal ultrasonography until detection of a pre-ovulatory follicle ≥28 mm. Treatment with 2000 IU hCG + 1.5mg GnRH was performed as the same criteria described for First season.

4.2.3 Oocyte collection and culture

Oocytes were collected from pre-ovulatory or growing follicles using ultrasound-guided transvaginal aspiration as previously described (CARNEVALE et al., 2005; ALTERMATT et al., 2009). During the first season, follicle aspirations were performed 22 to 28 hours after GnRH-hCG administration by one of two veterinary clinicians previously trained to perform this procedure. During the second season, pre-ovulatory follicles were aspirated approximately 30 hours after GnRH-hCG administration by the same veterinary clinician throughout the entire experiment. In addition, immature follicles (15-25mm) were aspirated on the day that the largest follicle from a known new follicular wave had reached a diameter of ≥20 mm. Oocytes were identified using a stereomicroscope and immediately placed in 500 µL of tissue culture medium (TCM-199; Bio Whittaker, Walkersville, MD, USA) with 10% fetal calf serum, 0.2 mM pyruvate, and 25 µg mL-1 of gentamicin. Oocytes were then matured *in vitro* at 38.5^{o}C in an atmosphere of 6% CO_2 in air (first season) or placed and washed in PBS to be frozen in liquid nitrogen at -196^{o}C (second season).

4.2.4 Oocyte morphologic measurements and parameters

Oocytes were subjectively classified according to the degree of cumulus cell expansion (1- very expanded, 2-expanded and 3-compact), the presence and quality of polar body (PB; absent, normal, degenerated, fragmented or degenerated + fragmented), and the ooplasm volume (OoplV). The OoplV were evaluated subjectively by just one experienced technician unaware of the age of the donor. The interval from GnRH-hCG to aspiration, GnRH-hCG to ICSI, and the cleavage rate (CR) after ICSI were also recorded.

4.2.5 Semen collection, staining, flow sorting and cryopreservation

Sperm preparation and staining were based on the method previously described (LINDSEY et al., 2002b; SUH; SCHENK; SEIDEL, 2005). Briefly, ejaculates from 22

stallions were extended to $25x10^6$ sptz/mL in modified high-potassium Tyrode's medium (KMT) and stored during 18h in a water bath at 15°C protected from environment light. Following storage, samples were centrifuged at 600 g for 10 min, the supernatant removed, and the sperm rich pellets extended to $400x10^6$ sptz/mL. Spermatozoa were stained with 47 mM Hoechst 33342 at $100x10^6$ mL^{-1} of KMT for 30 min at 34°C. Then KMT extender was added to dilute spermatozoa to a final concentration of $75x10^6$ sptz/mL plus 0.75 µL/mL 5% food coloring dye (FD&C #40; Warner Jenkinson, St Louis, MO, USA). Stained samples were filtered through a 50 µm nylon mesh filter (CellTricsTM, Partec GmbH, Germany) by gravity to remove debris or clumped masses of spermatozoa. Stained spermatozoa were sorted into X and Y-chromosome-bearing populations based on DNA content using SX MoFlo sperm sorter (Dako-Cytomation Inc., Fort Collins, CO, USA), equipped with a UV argon laser (wave length 351 at 150 mW) and modified for sorting sperm (JOHNSON; PINKEL, 1986; JOHNSON; WELCH, 1999). The instrument sheath pressure was 40 psi and the trigger rate was adjusted to 22.000 cells/s. Real-time sorting was performed using a 70 µm nozzle tip. Spermatozoa were sorted to 15 mL total volume, and incubated at room temperature for 75 min. After sorting, samples were centrifuged at 850x g for 20 min at 22°C, and the supernatant removed. The resulting sperm pellet was re-suspended in KMT extender and frozen as previously described (LINDSEY et al., 2002b) for later use with ICSI procedure.

4.2.6 Intracytoplasmatic sperm injection and embryo culture

All ICSI procedures were performed by only one experienced technician unware of the mare's age, and in accordance to a protocol previously described (ALTERMATT et al., 2009) with minor modifications. X sorted sperm were used for all ICSI procedures. Oocytes without a polar body or oocytes that were classified as damaged (broken zona pellucida or disrupted oolema) either before or during ICSI were not included in the analyses. Prior to ICSI, cumulus oocyte complexes (COC) were denuded by gentle pipetting in medium containing hyaluronidase (200 IU ml^{-1}; Sigma, St. Louis, MO, USA). After removal of cumulus cells and just before sperm injection, oocytes were examined under an inverted microscope (200x). Frozen sperm were obtained from ejaculates of 22 different fertile stallions and processed as previously described (ALTERMATT et al., 2009). Spermatozoa were selected based on

progressive motility and normal morphology and the one selected spermatozoon was injected into each oocytes using a micromanipulator and Piezo-driven injection system (Prime Tech Inc., Ibaraki-ken, Japan). Injected ova were incubated in DMEM/F12 media containing 10% fetal calf serum at 38.5^{o}C in 5% CO_2, 5% O_2 and 90% N_2. Presumptive zygotes were observed at 24, 48 and 72h (±2h) after ICSI (Time 0).

4.3 STATISTICAL ANALYSES

Data were analyzed for overall differences using SAS general linear mixed model (SAS Institute Inc.; Cary, NC, USA). End points that were not normally distributed, according to Shapiro-Wilk tests were transformed to natural logarithms or ranked. ANOVA was performed; differences between two means were evaluated by T-test and differences between ≥2 means were evaluated by Tukey test. RR, quality of the PB and CR were analyzed with chi-square or Fisher's exact test. Nonparametric data were evaluated using the Kruskal-Wallis test. A probability of $P \leq 0.05$ was considered a significant difference. A probability of $P > 0.05$ and ≤ 0.10 indicated that a difference approached significance. Data are reported as mean ± SEM.

4.4 RESULTS

A total of 519 cycles of oocyte donor mares were monitored during two breeding seasons. From 441 cycles monitored for ET and ICSI procedures in a commercial program during the first season, 216 cycles were assigned to follicular aspiration according to the criteria previously established. During the second season, 78 cycles were monitored in Thoroughbred mares. A total of 326 oocytes were recovered *in vivo* from pre-ovulatory follicles (n=279) and immature-growing follicles (n=47) during the two breeding seasons. From those, 224 oocytes from Polo Ponies were recovered, sorted and used for ICSI (first season; Table 1). Fifty seven and 47 oocytes were recovered from pre-ovulatory and growing follicles, respectively during the second season (Table 2 and 3). The oocytes were recovered, evaluated, processed and frozen in liquid nitrogen for further use.

Table 1 - Effect of age of Polo Pony mares on reproductive parameters, collection and viability of equine oocytes recovered from pre-ovulatory follicles assessed by morphology and cleavage rate after intracytoplasmatic sperm injection (ICSI) with sexed semen

End points	Combined Group	Young (3-10y) N=42	Intermediate (11-17y) N=24	Old (≥18y) N=13	P-Value
No of OPU sections	216	101	78	37	
Average age (years)	11.56 ± 0.92	5.83 ± 0.44	14.06 ± 0.47	23.09 ± 1.03	P<0.001
Corrected average age[1] (years)	11.3 ± 0.4	6.0 ± 0.3**a**	14.3 ± 0.3**b**	23.3 ± 0.5**c**	P<0.001
Intervals (d)					
OV/As[2] a PGF	6.7 ± 0.1	6.7 ± 0.1	6.6 ± 0.1	6.6 ± 0.2	P>0.80
PGF to GnRH-HCG (Day 0)	5.5 ± 0.2	4.8 ± 0.2**a**	5.8 ± 0.3**b**	7.2 ± 0.9**c**	P<0.001
OV/As to Day 0	12.2 ± 0.2	11.4 ± 0.2**a**	12.5 ± 0.3**ab**	13.8 ± 1.0**b**	P<0.002
OV/As to Day +1	13.1 ± 0.2	12.4 ± 0.2**a**	13.5 ± 0.3**ab**	14.5 ± 1.0**b**	P<0.004
Uterine edema					
Day 0	2.34 ± 0.06	2.43 ± 0.08**a**	2.37 ± 0.10**ab**	2.01 ± 0.17**b**	P<0.05
Day +1	1.61.± 0.07	1.67 ± 0.10	1.65 ± 0.12	1.36 ± 0.16	P=0.26
Cervical tone					
Day 0	2.53 ± 0.04	2.58 ± 0.05	2.55 ± 0.06	2.34 ± 0.10	P=0.10
Day +1	2.61 ± 0.04	2.66 ± 0.05**a**	2.64 ± 0.06**a**	2.42 ± 0.10**b**	P<0.03
Dominant Follicle (F1)					
Maximum Ɵ on Day 0	35.1 ± 0.2	36.0 ± 0.3**a**	34.8 ± 0.4**a**	32.6 ± 0.7**b**	P<0.001
Maximum Ɵ on Day +1	36.8 ± 0.2	37.7 ± 0.3**a**	36.8 ± 0.3**a**	34.4 ± 0.8**b**	P<0.001
Growth rate					
Day -10 to 0	3.04 ± 0.1	3.19 ± 0.1	2.97 ± 0.1	2.75 ± 0.2	P>0.13
Day 0 to +1	1.73 ± 0.1	1.71 ± 0.2	1.66 ± 0.2	1.94 ± 0.4	P>0.75
Ovum pick-up parameters (Pre-ovulatory follicles) and ICSI results					
No of OPU sections	216	101	78	37	
No of aspirated follicles	313	130	127	56	
N of secondary follicles 2[os]	89	33	40	16	
No of recovered oocytes	224	97	87	40	
Recovery rate % (n)					
Oocytes/cycle	104% (224/216)	96% (97/101)	112% (87/78)	108% (40/37)	P>0.76
Oocytes/follicle	71.6% (224/313)	75% (97/130)	68.5% (87/127)	71.4% (40/56)	P>0.90
F1 follicle	86.1% (186/216)	85.1% (86/101)	85.9% (67/78)	89.2% (33/37)	P>0.98
F2 follicle	39.8% (35/88)	37.9% (11/29)	39.5% (17/43)	41.2% (7/17)	P>0.99
F3 follicle	33.3% (3/9)	0% (0/2)	60% (3/5)	0% (0/2)	P>0.37
Interval GnRH-OPU (h)	24.3 ± 0.24	23.7 ± 0.35	24.8 ± 0.42	24.5 ± 0.56	P>0.14
Oocyte evaluation after GnRH-HCG (h)	37.6 ± 0.15	37.9 ± 0.15	37.4 ± 0.24	37.1 ± 0.56	P>0.89
COCs Expansion	1.8 ± 0.04	1.8 ± 0.05	1.7 ± 0.05	1.7 ± 0.10	P>0.65
N of Polar Bodies (PB) evaluated	188	84	69	35	
Absent BP	3.2% (6)	4.8% (4)	1.4% (1)	2.9% (1)	P>0.51
Normal BP	83.5% (157)	83.3% (70)	85.6% (59)	80% (28)	P>0.77
Degenerated BP	5.3% (10)	4.8% (4)	4.3% (3)	8.6% (3)	P>0.63
Fragmented PB	8.0% (15)	7.1% (6)	8.7% (6)	8.6% (3)	P>0.93
Deg. + fragmented BP	13.3% (25)	11.9% (10)	13% (9)	17.2% (6)	P>0.74
Ooplast volume					
Any degree of reduction	22% (50/224)	17% (16/97)	24% (21/87)	33% (13/40)	P=0.11
Reduction ≥10%	11.6% (26/224)	6% (6/97)**a**	14% (12/87)**ab**	20% (8/40)**b**	P=0.05
Reduction ≥15%	4% (9/224)	0% (0/97)**a**	7% (6/87)**b**	7.5% (3/40)**b**	P<0.03
Interval GnRH-HCG to ICSI (h)	39.6 ± 0.3	40.2 ± 0.2	38.8 ± 0.8	39.5 ± 0.8	P>0.19
Cleavage rate % (n)	44.8% (74/165)	49% (40/81)	39% (21/54)	43% (13/30)	P>0.48

[a,b,c] Different superscripts indicate significant difference (P<0.05) among means within a line.

[1] Corrected average age – weighted average based on mare age and number of cycles aspirated.

[2] OV/AS – Day of beginning of experiment related to ovulation (OV) or follicular aspiration (AS).
[3] Day 0 – Day of GnRH + HCG induction.
Fonte: (ARAUJO, 2014)

Table 2 - Effects of age of Thoroughbred mares on reproductive parameters, collection and viability of equine oocytes recovered from pre-ovulatory follicles assessed by morphology

End Points	Combined Group	Young (3-10y) N=12	Old (≥18y) N=11	P-Value
No of OPU sections	57	28	29	
Average age (years)	14.6 ± 2.1	4.5 ± 0.5	23.0 ± 1.01	
Corrected average age[1] (years)	13.3 ± 1.1	4.5 ± 0.3**a**	22.5 ± 0.6**b**	P<0.001
Intervals (d)				
OV/As[2] a PGF	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0. 0	P>0.99
PGF to GnRH-HCG (Day 0)	7.3 ± 0.5	6.5 ± 0.3**a**	8.2 ± 0.9**b**	P<0.05
OV/As to Day 0	13.3 ± 0.5	12.6 ± 0.3**a**	14.2 ± 0.9**b**	P<0.04
OV/As to Day +1	14.5 ± 0.5	13.6 ± 0.3**a**	15.4 ± 1.0**b**	P<0.04
Uterine edema				
Day 0	1.81 ± 0.16	2.19 ± 0.20**a**	1.45 ± 0.21**b**	P<0.01
Day +1	1.17 ± 0.16	1.49 ± 0.24 **a**	0.89 ± 0.20 **b**	P<0.01
Cervical tone				
Day 0	2.25 ± 0.10	2.33 ± 0.11	2.18 ± 0.14	P>0.20
Day +1	2.46 ± 0.11	2.62 ± 0.12	2.32 ± 0.16	P>0.08
Dominant Follicle (F1)				
Maximum Θ on Day 0	34.3 ± 0.4	35.0 ± 0.6**a**	33.6 ± 0.6**b**	P<0.04
Maximum Θ on Day +1	36.1 ± 0.4	36.9 ± 0.5**a**	35.3 ± 0.7**b**	P<0.04
Growth rate				
Day -10 to 0	2.56 ± 0.10	2.68 ± 0.15	2.40 ± 0.11	P<0.15
Day 0 to +1	1.65 ± 0.27	1.11 ± 0.27	2.25 ± 0.46	P=0.03
Ovum pick-up parameters (Pre-ovulatory follicles)				
No of OPU sections	57	28	29	
No of aspirated follicles	67	33	34	
N of secondary follicles 2os	11	6	5	
No of recovered oocytes	55	28	27	
Recovery rate % (n)				
Oocytes/cycle	96.5% (55/57)	100% (28/28)	93.1% (27/29)	P>0.15
Oocytes/follicle	82.1% (55/67)	84.8% (28/33)	79.4% (27/34)	P>0.56
F1 follicle	82.5% (47/57)	85.7% (24/28)	79.3% (23/29)	P>0.71
F2 follicle	72.7% (8/11)	66.7% (4/6)	80.0% (4/5)	P>0.62
F3 follicle	na	Na	na	
Interval GnrH-OPU (h)	30.7 ± 0.10	30.64 ± 0.18	30.8 ± 0.12	P>0.23
Oocyte evaluation after GnRH-HCG (h)	31.5 ± 0.17	31.2 ± 0.33	31.8 ± 0.12	P>0.23
COCs Expansion	1.9 ± 0.17	2.0 ± 0.10	1.9 ± 0.08	P>0.23
N of Polar Bodies (PB) evaluated	46	24	22	
Absent BP	45.7% (21)	37.5% (9)	12 (54.5% (12)	P>0.25
Normal BP	50% (23)	54.2% (13)	10 (45.5% (10)	P>0.55
Degenerated BP	2.15% (1)	4.2% (1)	0% (0)	P>0.33
Fragmented PB	2.15% (1)	4.2% (1)	0% (0)	P>0.33
Deg. + fragmented BP	4.3% (2)	8.4% (2)	0% (0)	P>0.17
Ooplast volume	93.4 ± 1.4	94.9 ± 1.7	91.7 ± 2.2	P>0.22
Any degree of reduction	38% (21/55)	32% (9/28)	44% (12/27)	P>0.35
Reduction ≥10%	33% (18/55)	25% (7/28)	41% (11/55)	P>0.21
Reduction ≥15%	20% (11/55)	18% (5/55)	22% (6/27)	P>0.69

[a,b,c] Different superscripts indicate significant difference (P<0.05) among means within a line.
[1] Corrected average age – weighted average based on mare age and number of cycles aspirated.
[2] OV/AS – Day of beginning of experiment related to ovulation (OV) or follicular aspiration (AS).
[3] Day 0 – Day of GnRH + HCG induction.
Fonte: (ARAUJO, 2014)

Characteristics of the estrous cycle from the different experimental groups are shown in Tables 1-3. Intervals between events (OV/As), hormonal treatments (PGF, GnRH-hCG [Day 0]) and oocyte aspirations, as well as characteristics of the uterus, cervix, and follicles, oocyte recovery rates, morphologic characteristics of oocytes and cleavage rate after ICSI are summarized.

Table 3 - Effects of age of Thoroughbred mares on reproductive parameters, collection and viability of equine oocytes recovered from immature follicles assessed by morphology

End Points	**Combined Group**	**Young (3-10y) N=12**	**Old (≥18y) N=11**	**P-Value**
No of OPU sections	54	26	28	
Average age (years)	14.6 ± 2.1	4.5 ± 0.5	23.0 ± 1.01	
Corrected average age[1] (years)	13.3 ± 1.1	4.5 ± 0.3**a**	22.5 ± 0.6**b**	P<0.001
No of follicles aspirated	111	59	52	*
No of recovered oocytes	47	23	24	*
No of follicles aspirated/procedure	2.06	2.27	1.86	P>0.54
No of recovered oocytes/procedure	0.87	0.88	0.86	P>0.93
Recovery rate % (n)				
Oocytes/procedure	87.0% (47/54)	88.5% (23/26)	85.7% (24/28)	P>0.94
Oocytes/follicle	42.3% (47/111)	39.0% (23/59)	46.0% (24/52)	P>0.63
Maximum Θ at aspiration				
Largest follicle (F1)	23.08 ± 0.25	23.12 ± 0.32	23.02 ± 0.39	P>0.86
Average Θ of aspirated follicles	20.38 ± 0.37	20.24 ± 0.49	20.62 ± 0.59	P>0.62
COCs expansion	3.00 ± 0.0	3.00 ± 0.0	3.00 ± 0.0	P>0.99
No of Polar bodies evaluated	43	21	22	
Absent BP	97.7% (42)	100% (21)	95.5% (21)	P>0.32
Normal BP	2.3% (1)	0% (0)	4.5% (1)	P>0.32
Degenerated BP	0% (0)	0% (0)	0% (0)	P=1.0
Fragmented PB	0% (0)	0% (0)	0% (0)	P=1.0
Deg. + fragmented BP	0% (0)	0% (0)	0% (0)	P=1.0
Ooplast volume				
Any degree of reduction	23% (11/47)	13% (3/23)	33% (8/24)	P=0.10
Reduction ≥10%	13% (6/47)	4% (1/23)	21% (5/24)	P=0.09
Reduction ≥15%	8.5% (4/47)	0% (0/47)**a**	17% (4/24)**b**	P=0.04

a,b,c Different superscripts indicate significant difference (P<0.05) among means within a line.
[1] Corrected average age – weighted average based on mare age and number of aspiration procedures.
Fonte: (ARAUJO, 2014)

4.4.1 Estrous cycle parameters

Old mares had significantly longer intervals from PGF to Day 0; OV/As to Day 0 and Day +1 than young mares ($P \leq 0.05$; Tables 1 and 2). Middle Age mares had significantly longer interval from PGF to Day 0 than Young mares, but shorter when compared to OM ($P \leq 0.05$; Table 1). No significant difference in cervical tone was observed either for Polo or Thoroughbred (first and second season) mares on Day 0. Old mares had lower uterine edema score on Day 0 than YM in first season ($P \leq 0.05$). However, this was not observed during the second season (Table 2). A decrease in cervical tone was observed for OM on Day +1 during the first season ($P \leq 0.05$). The decrease approached significance for OM during the second season ($P > 0.08$). Maximum diameter of the pre-ovulatory follicle also showed difference between groups ($P \leq 0.05$). Old mares received GnRH-hCG treatment with smaller follicles than YM (Table 1 and 2) and MA (Table 1). Follicular growth rate was higher for OM compared to young Thoroughbred mares ($P \leq 0.05$; second season) from Day 0 to Day +1, but not from Day -10 to 0. This was consistent for both seasons.

4.4.2 Oocyte recovery rates and morphology

A total of 313 and 67 pre-ovulatory follicles were aspirated during the first and second season respectively (Tables 1 and 2). Overall, oocyte recovery rate per cycle and per follicle were between 96.5 to 104 % and 71.6 to 82.1%, respectively. Oocyte recovery rate of F1 and F2 follicles were between 82.5 and 86.1%, and 39.8 and 72.7 %, respectively by season. F3 oocyte recovery reached 33.3% (Table 1). A total of 111 growing follicles and 54 aspirations procedures were performed in Thoroughbred during the second season (Table 3). Forty seven oocytes were recovered, yielding an overall oocyte recovery rate of 87 and 42.3 % per procedure and per growing follicles (follicles ≥15mm and ≤ than 25mm follicles), respectively. The mean diameter of largest and the average diameter of follicles aspirated between groups were not different between groups ($P \geq 0.05$; Table 3). Additionally, there was no difference in oocyte recovery rate from pre-ovulatory or growing follicles among groups during both seasons.

In the first season, the aspiration procedures were performed approximately 24 hours

after GnRH-hCG induction of pre-ovulatory follicular and oocyte maturation. The microscopic morphology evaluation of the oocyte was performed after COCs were submitted to 13.3 hours of *in vitro* maturation, yielding an interval of 37.6 hours after GnRH-hCG treatment. Most oocytes (over 96%) had already resumed meiosis (determined by the presence of first polar body) at the time of evaluation (Table 1). During the second season, the aspiration procedure was performed approximately 30.7 hours after GnRH-hCG induction of pre-ovulatory follicles (Table 2). The evaluation was performed about one hour later. Over 54% of oocytes resumed meiosis. The first polar body was not present in 98% of oocytes recovered from growing follicles, (Table 3). There was no significant difference detected for COCs expansion or PB evaluation among the experimental groups.

Ooplasm volume in oocytes from pre-ovulatory follicles was smaller for OM compared to YM ($P \leq 0.05$; Table 1) or growing follicles ($P \leq 0.05$; Table 3). The MA (Table 1) also had a higher proportion of oocytes with ≥15% ooplasm reduction from pre-ovulatory follicle than YM ($P \leq 0.05$). The smaller ooplasm volume observed in OM and MA had no effect on CR of oocytes subjected to ICSI with sexed semen as shown in Table 1. There was no effect of stallion on CR or group effect.

4.5 DISCUSSION

An effect of age could be observed in several reproductive parameters evaluated in this study. The reproductive status of mares associated with oocyte collection and use of oocyte in ICSI with sexed semen, such as: longer interval between administration of a luteolytic agent and day of gonadotropin-induction or aspiration, differences in uterine edema and cervical tone in the days close to oocyte collection, difference in diameter of the pre-ovulatory follicle at the day of induction and one day after, as well as reduction of ooplasm volume in OM was monitored. However, no difference was found regarding the CR of oocytes from young or old mares subjected to ICSI with sexed semen. It is worth to note that this is the first report of a large IVP of equine embryos using sex sorted semen.

It is generally agreed that the lack of standardization for establishing age groups in mares makes it difficult to compare results between studies in the species (GINTHER et al., 2008). In the present study, grouping of animals by age was intended to cover the young groups (CARNEVALE; BERGFELT; GINTHER, 1993; GINTHER et al., 2008, 2009;

ALTERMATT et al., 2009); and middle age, fully (10-14y; (GINTHER et al., 2008, 2009)) or partially (15-19y; (CARNEVALE et al., 2003)) and the group of old mares (≥18y; (GINTHER et al., 2008, 2009)) or ≥ 20y (CARNEVALE; BERGFELT; GINTHER, 1993; ALTERMATT et al., 2009) of previously studies reported. The present study used Polo Ponies (first season) and Thoroughbred mares (second season) as oocytes donors, whereas other studies used large Ponies -mares and Pony-horse crosses (GINTHER et al., 2008), small Ponies (CARNEVALE; BERGFELT; GINTHER, 1993) or light horse mares (ALTERMATT et al., 2009).

Information related to the effect of age on follicular dynamics during induced estrous cycle in mares used for collection, evaluation and submission of oocytes for ICSI with sexed semen in a single study has not been reported before. A longer interval between treatment with PGF (or beginning of the experiment) and the day of follicular and oocyte maturation (Day 0), and a longer interval between the day of PGF (or beginning of the experiment) and the day of oocyte recovery for approximately two days was observed for the group of OM compared to YM in the present study. Similar results were reported by Ginther et al (2008) that showed an increase of one day in the length of inter ovulatory interval comparing old (≥18 years) and young mares (5-6 years). Difference in the interval between ablation to ovulation for young and old mares (± 1 day) in estrous cycles with induced follicular waves were also reported by the same group (GINTHER et al., 2009).

As reported (GINTHER et al., 2009), the diameter of the pre-ovulatory follicle on Day 0 and Day+1 was smaller for old mares when compared to the other groups. However, the growth rate did not differ probably due to it being retrospectively-calculated to the day of induction for 10 days instead of using a smaller range as reported by (GINTHER et al., 2009). The mare age had a negative effect on the mean diameter of the dominant follicle (MOREL; NEWCOMBE; HAYWARD, 2010). This study demonstrated that follicle size decreases by 0.935 mm for every age period (3 years decrease in age). A smaller ovulatory diameter has also been observed in older women (SANTORO et al., 2003) and mares (MOREL; NEWCOMBE; HAYWARD, 2010).

A greater degree of uterine edema was observed in YM on Day 0 (Table 1 and 2) and on Day+1 (Table 2) when compared to OM. Although the hormonal levels were not measured in the present study, it has been reported that estradiol levels were increased during the pre-ovulatory period in YM (GINTHER et al., 2008), consistent with the ultrasonography findings of the uterine edema and size of the pre-ovulatory follicle in the present study. The

cervical tone was also evaluated in the present study. A difference in the cervical tone on Day +1 was observed among groups during the first season. The lower cervical tone observed for old mares might be result of lower levels of endogen estradiol evidenced in a previous study (GINTHER et al., 2009), however this observation could not be confirmed in the present experiment, since endocrinology was beyond the scope of the study.

The recovery rate of oocytes after *in vivo* maturation or from immature follicles did not differ among young, middle age and old mares (Table 1-3). Usually, recovery of oocytes by aspiration of the gonadotropin-stimulated dominant pre-ovulatory follicle is performed to obtain oocytes for oocyte transfer (HINRICHS, 2010) or ICSI. The high recovery rate of oocytes from pre-ovulatory follicles in the present study (71.6-82.1%, table 1 and 2, respectively) are consistent with other authors' reports (HINRICHS; KENNEY; KENNEY, 1990; CARNEVALE; GINTHER, 1995; HINRICHS et al., 1998; CARNEVALE et al., 2004). In the present study, oocytes were aspirated on average 24 (first season) and 30 hours (second season) after induction of follicular and *in vivo* oocyte maturation. According to Katrin Hinrichs (2010), 24 hours are sufficient to allow the expansion of the oocyte cumulus cells in response to gonadotropin, facilitating oocyte recovery, and 35 h after treatment is the maximal time limit that can be assumed that the follicle will not ovulate before aspiration. The degree of cumulus cells expansion did not differ among groups (Table 1 and 2), consistent with the similar recovery rate of oocytes among mares with different ages. For the immature follicles, the recovery rate (42.3%) was lower or higher than other studies previously reported (≤25%; (BRÜCK et al., 1992; COOK; SQUIRES; JASKO, 1993; DUCHAMP; BÉZARD; PALMER, 1995; KANITZ et al., 1995; MARI et al., 2005) and 50-60%; (BØGH et al., 2002; COLLEONI et al., 2007; JACOBSON et al., 2010), with no difference detected among groups.

Age-associated changes in oocyte morphology were identified in the present study. A smaller ooplasm volume was observed in oocytes from middle age and old mares when compared to young mares This was observed in oocytes from both pre-ovulatory (table 1) and growing follicles (table 3). In another study (FRANK-GUEST; STOKES; CARNEVALE, 2010), a larger ooplasm volume was found in oocytes of 14-19 and 20-23 years mares when compared to 24-28 or 3-13 years mares. However, these findings were not consistent with a previous report from the same group (ALTERMATT et al., 2009) a year before, in which only the thickness of zona pellucidae , the perivitelline space, and the inner ZP volume (central cavity within the ZP) were different between young and old mares. According to Altermatt et al. (2009), the age of oocyte donors did not affect the cleavage rate, blastocyst development

and pregnancy rate after ICSI. The decrease in the zona pellucidae thickness in old mares and the increase in perivitelline space and inner ZP volume was associated with oocytes that failed to develop. In the present study, age did not affect the cleavage rate of oocytes subjected to ICSI with sexed semen despite the lower ooplasm volume observed in middle age and old mares, (Table 1). Frank-Guest et al. (2010) concluded that the age of oocyte donors affect the potential production of pregnancies and embryo loss after ICSI. In a retrospective study, the same authors evaluated several morphological parameters (diameter of the oocyte, ZP thickness with matrix, inner ZP diameter, ooplasm diameter, ooplasm volume and perivitelline space volume) of oocytes and concluded that the morphological measurements are affected by age, but no measure was consistently predictive of potential oocyte development. In contrast, a correlation between decreased fertility and changes in morphology of human oocytes associated with aging has been reported (XIA, 1997). Studies in humans relate the quality and/or oocyte morphology and their association with aging and/or viability or developmental potential of the oocyte (OTTOLENGHI et al., 2004; BAIRD et al., 2005; BORINI et al., 2005; SUN et al., 2005; WANG; SUN, 2007). In horses, invasive methods of evaluation using confocal microscopy assessing the equine oocyte for meiotic stage, spindle quality, spindle orientation, thickness of the F-actin band and the percentage of oocytes image with foci of microfilaments showed no difference between the different age groups (GINTHER et al., 2009). Even though potentially efficient, invasive methods disqualify the use of oocyte for subsequent use in ART. Therefore, there is still a need to identify reliable indicators for predicting oocyte quality and competence, and the understanding of the physiological factors that regulate these mechanisms are extremely important for the efficiency of assisted reproduction in domestic species and humans.

The possibility of influencing the sex of an offspring through semen sexing has brought great interest of the equine industry after the first report of the birth of a foal resulting from this technique (BUCHANAN et al., 2000). The major limitation of this technology is the amount of semen that can be sex sorted per hour and the minimum inseminating dose required to acquire commercially satisfactory pregnancies rate in horses. The use of ICSI in equines is a viable alternative to circumvent the problem related to the low available dose of sexed semen to be used, since only one sperm is needed for fertilization of the oocyte (ICSI). In vitro production of embryos using sexed semen has been reported in pigs, buffalo, cattle, among others (PROBST; RATH, 2003; WILSON et al., 2006; BATHGATE et al., 2007; LIANG et al., 2008). To our knowledge, this is the first reported use of sexed semen for IVP

horse embryos. Although an initial study, where only cleavage rate of embryos was reported, further studies investigating embryonic development and pregnancy rates are needed.

In conclusion, the parameters used to evaluate the oocyte viability and age affects did not show difference in fertility between young and old mares after ICSI. Studies evaluating a more advanced stage of embryo development (such as blastocyst and pregnancy) after ICSI should be performed to better infer this issue.

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5 LARGE-SCALE STUDY ON EQUINE OOCYTE RECOVERY AND MORPHOLOGICAL EVALUATION AFTER *IN VIVO* OR POST-MORTEM (*IN VITRO*) COLLECTION

ABSTRACT

The aim of the present study was to compare different methods of oocyte collection and the morphology of the cumulus-oocyte-complex in a large number of mares. A total of 913 oocytes were recovered. Of these, 279 were recovered *in vivo* from pre-ovulatory follicles, 47 from growing follicles and 587 oocytes from *in vitro* follicular aspiration from slaughterhouse ovaries. Ovaries (n=484) were collected in a slaughterhouse and transported to the laboratory. The total number of aspirations, number of follicles aspirated and oocytes recovered per aspiration and/or follicle, the degree of cumulus cell expansion, the presence and quality of polar body (PB), and the interval from GnRH to aspiration (*in vivo*) were evaluated. The recovery rate (RR) of oocytes from growing follicles did not differ between *in vivo* and *in vitro* aspirations (42.3% vs. 47%, respectively, P=0.53). However, oocytes recovered *in vitro* had a higher proportion of degeneration and lower layer of cumulus cells compared to oocytes recovered *in vivo*. In conclusion, higher recovery rates were obtained by ultrasound-guided *in vivo* transvaginal aspiration of pre-ovulatory follicles, when compared to growing follicles, both by transvaginal aspiration (TVA) or recovery of oocytes from ovaries from slaughterhouse. The method of oocyte collection to be chosen will depend of the availability of animals or equine tissues by the laboratory.

Keywords: Oocyte recovery. OPU. Aging. Oocyte quality.

5.1 INTRODUCTION

Many reproductive technologies, ranging from *in vitro* maturation (IVM), *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) to animal cloning, has been

developed and continuously improved in recent decades through studies in animals and humans (DEVITO et al., 2010; MEIRELLES et al., 2010; WONGTRA-NGAN et al., 2010; BEN-AMI et al., 2011). In the horse, the *in vitro* embryo production (IVP) has presented notable progress in recent years and its clinical use has recently being reported (GALLI et al., 2007; HINRICHS et al., 2012). IVF is not efficient in the equine specie (HINRICHS, 2010a), and fertilization of the equine oocyte *in vitro* is usually achieved with the use of ICSI. Rates ranging from 10% to 40% of *in vitro* equine blastocyst production have been reported among laboratories (HINRICHS et al., 2005; CHOI et al., 2007; GALLI et al., 2007; RIBEIRO et al., 2008).

The success of the assisted reproductive technologies (ART) in horses is strongly dependent on the oocyte competence. Oocytes may be collected from ovaries of slaughtered mares (CHOI et al., 2009; CREMONESI; ANDERSON; LANGE-CONSIGLIO, 2010), from recently dead mares (HINRICHS, 2010a; HINRICHS et al., 2012), or from live mares via transvaginally ultrasound-guided follicle aspiration (TVA; (ALTERMATT et al., 2009; JACOBSON et al., 2010; HINRICHS et al., 2012; VELEZ et al., 2012)). In the laboratory, the collection of oocytes from ovaries usually is performed trough aspirations of the follicular contents from visible follicles after removing the excess of ovarian tissues using scissors (CREMONESI; ANDERSON; LANGE-CONSIGLIO, 2010). The aspiration is done using needles connected or not to vacuum pumps (HINRICHS, 1991; DELL'AQUILA et al., 2001; CREMONESI; ANDERSON; LANGE-CONSIGLIO, 2010), or trough scrapping the follicle wall (CHOI et al., 2009; HINRICHS, 2010a). The recovery of oocytes using bone curettes to scrap the follicle has been reported to recover oocytes with major layer of cumulus cells (HINRICHS, 1991) and might be related to better oocyte competence after IMV and ICSI (DELL'AQUILA et al., 2001). However, this procedures is time consuming compared with aspiration solely, requiring more labor, and trained personnel (HINRICHS, 2010a). Recovering oocytes from recently dead mares is a promising procedure, since valuable mares can reproduce after death. On the other side, recovery of oocytes from live mares allows the use of the animal for competition or training while its oocytes can be used for further application in ART. It is however, worth to keep in mind that exercise might decrease oocyte quality and success of the ARTs potential (MORTENSEN et al., 2009, 2010; SMITH et al., 2012).

Oocyte competence seems to be related to its morphology (XIA, 1997). In humans, a correlation between decreased fertility and changes in morphology of human oocytes associated with aging was reported (XIA, 1997). In contrast, Frank-Guest et al. (2010)

evaluating several oocytes morphological parameters in a retrospective study (diameter of the oocyte, ZP thickness with matrix, inner ZP diameter, ooplasm diameter, ooplasm volume and perivitelline space volume), concluded that the morphological measurements are not consistent predictive factors of potential oocyte development. In contrast, a few studies in humans have related the quality and/or oocyte morphology and their association with viability or developmental potential of the oocyte (OTTOLENGHI et al., 2004; BAIRD et al., 2005; BORINI et al., 2005; SUN et al., 2005; WANG; SUN, 2007). Differences in oocytes morphology in horses affecting *in vitro* maturation rates has been reported (HINRICHS, 2010b). Oocytes with expanded zona pellucidae have shown to have higher maturation rate compared to oocytes showing compact zona pellucidae.

Unlike bovid, advances in biotechnologies of reproduction in horses have developed more slowly mainly due to the high cost of keeping animals on farms, the difficulty of obtaining material in slaughterhouses, as well as the difficulty in establishing an efficient routine of *in vitro* production of embryos. However, the demand for techniques that could improve the reproductive performance leading the preservation of genetic material in horses has increased considerably (VIEIRA et al., 2013; FERNANDES et al., 2014). Thus, the present study aims to compare different methods of oocyte collection and to compare the collection method with the morphology of the cumulus-oocyte-complex.

5.2 MATERIALS AND METHODS

Oocyte collection and evaluation was performed *in vitro* (experiment 1) and *in vivo* (experiment 2).

5.2.1 Experiment 1 - *in vitro* oocyte collection and morphological evaluation

Oocytes were collected from ovaries of mares slaughtered at the abattoir of Araguari-MG (Granja POMAR). Ovaries (approximately 30 per shipment, usually once a week) were packed in plastic bags immersed in saline (0.9%) containing antibiotic (amicacin), wrapped in

a thermal container (~24-35°C), and immediately transported to the laboratory of Molecular Morphophysiology and Development (FZEA, USP-Pirassununga, SP, Brazil). In the laboratory, excess tissue was removed from the ovaries with scissors to facilitate visualization of the follicles. To recover the oocytes, all visible follicles were punctured with 12G needle connected in a 10 mL syringe. Then the follicles were opened with a scalpel blade, and the granular layer was scrapped using 0.5 cm bone curette to guarantee the recovery of a possible remaining oocyte-cumulus complex. The contents of the curette were washed in petri dishes with Hepes-buffered TCM 199 with Hank's salts (Gibco Life Technologies, Inc., Grand Island, NY) supplemented with gentamicin (25 mg / mL, Gibco). The contents of the petri dishes were examined using a dissecting microscope at 10-20x. Cumulus-oocyte-complexes were rinsed in holding medium and were classified as compact, expanded, very expanded or degenerated depending upon granulosa and cumulus expansion. The time elapsed since the slaughter of mares and removal of the ovaries until recovery of oocytes for maturation in the laboratory was recorded, as well as ovaries transport time and variation of temperature. After recovery, oocytes were classified according to presence and the degree of cumulus cell expansion (1-very expanded, 2-expanded and 3-compact).

5.2.2 *In Vitro* maturation

After washing in holding medium and classification, oocytes were washed three times in maturation medium (TCM 199 with Earle's salts, 5mU/mL FSH Sioux Biochemicals, Sioux Center, IA 10% FBS and 25 / mL gentamycin) and cultured in droplets with a ratio of 10 µL of medium / oocyte under mineral oil (Sigma Chemical Co., St. Louis, MO) at 38.2°C in 5% CO_2 in air for 24-26h as described (CHOI et al., 2009) with a maximum of 10 oocytes per drop. After maturation, oocytes were stripped by pipetting in 0.05% hyaluronidase solution (CHOI et al., 2003).

5.2.3 Experiment 2 – *In vivo* oocyte collection

5.2.3.1 Animals and experimental design

A total of 102 non-lactating Polo Pony mares (n=79) and Thoroughbred (n=23) weighing between 400 and 600 kg were used in 2009/2010 and 2011 breeding season in the southern (San Luis, Argentina) and northern (Kentucky, EUA) hemisphere, respectively. The Polo Pony mares belonged to a Commercial Program of Equine Assisted Reproduction (First season) and the Thoroughbred mares to the Maxwell Gluck Equine Research Center at University of Kentucky (Lexington, EUA; Second season). The mares had free access to good quality pasture, balanced mineral salt and water during the first and second seasons. Mares 3 to 29 years of age were used as oocyte donors during both seasons. All procedures were performed in accordance with the Institutional Animal Care and Use Committee at São Paulo University (First season) and University of Kentucky (Second season).

5.2.3.2 Ultrasonography, follicular synchronization and oocyte collection

First season. Mares were synchronized with the administration of a luteolytic agent (PGF, 7.5 mg, im; Lutalyse) 6 days after aspiration or ovulation and monitored by rectal palpation and ultrasonography every 2 to 5 days for follicular growth control. From a total of 441 monitored estrous cycles, only those cycles that met the following criteria were used for the present study: donor mares with the same reproductive and nutritional management; at least five exams by transrectal palpation and ultrasonography per cycle; absence of hormonal treatment additional to that provided for synchronization and induction of follicular maturation *in vivo* (PGF, GnRH and hCG); and absence of any use of anti-inflammatory drugs and cycles with clinical history of normal health. Mares were monitored by transrectal ultrasonography (US) with intervals of 2 to 5 days after ovulation (D0) until the first detection of a follicle ≥30 mm, then the mares were daily monitored. Treatment with 2000 IU hCG + 1.5mg GnRH analog was performed to induce oocyte maturation *in vivo* when the follicle diameter was ≥35 mm, uterine edema was present (GINTHER, 1992), and the cervix presented relaxed. **Second season.** Mares were synchronized as performed in the First season. Day 0 was the day of ovulation and the mares were daily monitored by transrectal

ultrasonography until detection of a pre-ovulatory follicle ≥35 mm. Treatment with 2000 IU hCG + 1.5mg GnRH was performed following the same criteria described for First season.

5.2.3.3 Oocyte collection and culture

Oocytes were collected from pre-ovulatory or growing follicles using ultrasound-guided transvaginal aspiration as previously described (CARNEVALE et al., 2005; ALTERMATT et al., 2009). During the first season, follicle aspirations were performed 22 to 28 hours after GnRH-hCG administration by one of two veterinary clinicians previously trained to perform this procedure. During the second season, pre-ovulatory follicles were aspirated approximately 30 hours after GnRH-hCG administration. All follicle aspirations were conducted by one veterinary clinician. Aspiration of immature follicles was performed on the day that the largest follicle from a known new follicular wave had reached a diameter of ≥20 mm. Growing follicles with diameters between 15 and 25 mm were aspirated. Oocytes were identified using a stereomicroscope and immediately placed in 500 µL of tissue culture medium (TCM-199; Bio Whittaker, Walkersville, MD, USA) with additions of 10% fetal calf serum, 0.2 mM pyruvate, and 25 µg mL-1 of gentamicin. Oocytes were then submitted to maturation (first season) or placed and washed in PBS to be frozen (second season). The maturation step was done at 38.5^{o}C in an atmosphere of 6% CO_2 in air.

5.2.3.4 Oocyte morphologic measurements and parameters

Oocytes from both experiments were classified according to the degree of cumulus cell expansion (1-very expanded, 2-expanded and 3-compact; as in experiment 1) and the presence and quality of polar body (PB; absent, normal, degenerated, fragmented or degenerated + fragmented).

5.3 STATISTICAL ANALYSIS

Data were analyzed for overall differences using SAS general linear mixed model (SAS Institute Inc.; Cary, NC, USA). End points that were not normally distributed, according to Shapiro-Wilk tests, were transformed to natural logarithms or ranked. ANOVA was performed; differences between two means were evaluated by t test and differences between ≥2 means were evaluated by Tukey test. RR, quality of the PB and CR were analyzed with chi-square or Fisher's exact test. Nonparametric data were evaluated using the Kruskal-Wallis test. P values <0.05 were used to indicate significance. Data are reported as mean ± SEM.

5.4 RESULTS

5.4.1 Experiment 1 - *In Vitro* oocyte collection

A total of nine collections of oocytes (repetitions) were performed. One repetition containing 76 oocytes showed contamination after maturation. Only data from collection, oocyte recovery and classification from this repetition were used for the analysis. The ovaries left the slaughterhouse with an average temperature of 32ºC and arrived at 30ºC (Table 1). The average time of transport was 4 hours. After collection and identification, oocytes were classified based on the morphology of cumulus cells (Table 2). From a total of 511 oocytes collected (8 repetitions), 491 were submitted to IVM. Some oocytes had the zona pellucidae broken or were lost during handling, leaving 423 oocytes evaluated after IVM. The rate of maturation after IVM (based on the presence of the first polar body) was 43.7% (185/423).

5.4.2 Experiment 2 – *In Vivo* oocyte collection

A total of 519 cycles of oocyte donor mares were monitored during two breeding

seasons. From 441 cycles monitored for ET and ICSI procedures in a commercial program during the first season, 216 cycles assigned to follicular aspiration were selected according to criteria previously established. During the second season, 78 cycles were monitored in Thoroughbred mares. A total of 326 oocytes were recovered *in vivo* from pre-ovulatory follicle (n=279) and immature-growing follicles (n=47) for the two breeding seasons. From those, 224 oocytes were recovered, sorted and submitted for maturation (first season; Table 3). Fifty seven and 47 oocytes were recovered from pre-ovulatory and growing follicle, respectively, during the second season (Table 3). The oocytes were recovered, evaluated, processed and frozen for a related study.

Table 1- Ovary collection and oocyte recovery (09 repetitions)

End points	**Mean**	**SEM**	**Total**
Slaughter duration (min)	200.00	14.12	.
Number of animals slaughtered	91.00	9.98	819
Number of females	30.00	4.48	270
Number of ovaries collected	53.78	8.64	484
Outlet temperature (^{o}C)	32.17	0.17	.
Shipping time (min)	245.63	11.93	.
Arrival temperature (^{o}C)	30.11	0.59	.
Δ Temperature	2.50	0.67	.
Δ T/h	0.76	0.20	.
Total number of follicles	137.44	18.96	1237
Number of follicle/ovary	2.56	0.17	.
Number of oocytes recovered	65.22	7.19	587
Number of oocytes/ovary	1.21	0.12	.
Recovery rate (N oocyte/follicle)	0.47	0.03	.

Fonte: (ARAUJO, 2014)

Table 2 – Cumulus-Oocyte-Complex classification (Experiment 1; *in vitro* collection)

End points	**Total**
Number of oocytes classified	401
Number of compact (%)	189 (47.1%)
Number of Expanded (%)	79 (19.7%)
Number of denuded (%)	105 (26.2%)
Number of degenerated (%)	28 (7%)

Fonte: (ARAUJO, 2014)

5.4.2.1 Oocyte recovery rates and morphology

A total of 313 and 67 pre-ovulatory follicles were aspirated during the first and second season respectively (Table 3). Overall, oocyte recovery rate per cycle and per follicle were between 96.5 to 104 % and 71.6 to 82.1%, respectively. Oocyte recovery rate per F1 and F2 follicles were between 82.5 to 86.1% and 39.8 to 72.7 %, respectively. F3 oocyte recovery reached 33% (Table 3). A total of 111 growing follicles and 54 aspirations procedures were performed in Thoroughbred during the second season. Forty seven oocytes were recovered, yielding an overall oocyte recovery rate of 87 and 42.3 % per procedure and per growing follicles (≥15mm and <≤ than 25mm follicles), respectively.

Table 3 - Recovery rate parameters and oocyte morphology after *in vivo* or *in vitro* collection methods

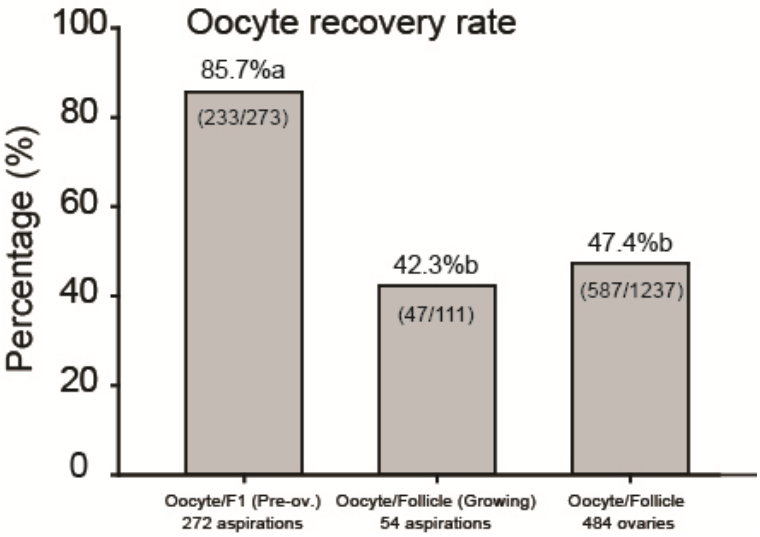
	First season	**Second season**		**Second season**
End points	**(Pre-ov. folls.)**	**(Pre-ov. folls.)**	**Combined seasons (Pre-ov. Folls.)**	**(Immature folls.)**
No of aspirations	216	57	273	54
No of aspirated follicles	313	67	380	111
N of secondary follicles 2os	89	11	100	*
No of recovered oocytes	224	55	279	47
Recovery rate % (n)				
Oocytes/cycle	104% (224/216)	96.5% (55/57)	102% (279/273)	87.0% (47/54)
Oocytes/follicle	71.6% (224/313)	82.1% (55/67)	73% (279/380)**x**	42.3% (47/111)**y**
F1 follicle	86.1% (186/216)	82.5% (47/57)	85% (233/273)	*
F2 follicle	39.8% (35/88)	72.7% (8/11)	*	*
F3 follicle	33.3% (3/9)	*	*	*
Interval GnrH-OPU (h)	24.3 ± 0.24**a**	30.7 ± 0.10**b**	*	*
COCs Expansion	1.76 ± 0.04 **a**	1.96 ± 0.07**b**	*	3.00 ± 0.0
N of oocytes evaluated for PB	188	46	234	43
Absent BP	3.2% (6)**a**	45.7% (21)**b**	12.0% (27)**x**	97.7% (42)**y**
Normal BP	86% (157/182)	92% (23/25)	87% (180/207)	100% (1/1)
Degenerated BP	5.0% (10/182)	4.0% (1/25)	5.0% (11/207)	0% (0/1)
Fragmented PB	8.0% (15/182)	4.0% (1/25)	8% (16/207)	0% (0/1)
Deg. + fragmented BP	14% (25/182)	8% (2/25)	13.0% (27/207)	0% (0/1)

a,b Different superscripts indicate significant difference (P<0.05) among means within a line between first and second season (Pre-on folls).
x,y Different superscripts indicate significant difference (P<0.05) among means within a line between Combined seasons and Second season (Immature follicle).
Fonte: (ARAUJO, 2014)

In the first season, the aspiration procedures were performed approximately 24 hours after GnRH-hCG induction of pre-ovulatory follicular and oocyte maturation. The microscopic morphology evaluation of the oocyte was performed after COCs were submitted

to 13.3 hours of *in vitro* maturation, yielding an interval of 37.6 hours after GnRH-hCG treatment. Most oocytes (> 96%) had already resumed meiosis (determined by the presence of first polar body) at the time of evaluation (Table 3). During the second season, the aspiration procedure was performed approximately 30.7 hours after GnRH-hCG induction of pre-ovulatory follicles. The evaluation was performed about one hour later. Over 54% of oocytes resumed meiosis. The first polar body was absent in 98% of oocytes recovered from growing follicles. The recovery rate did differ ($P<0.05$) from pre-ovulatory follicles and/or growing follicles (Table 3; Figure 1). However, the number of hours after GnRH-hCG to pre-ovulatory oocyte collection in vivo affects the degree of COCs expansion. The percentage of oocytes with absence of PB differed significantly between season and between follicular status.

Figure 1 – *In vivo* and *in vitro* recovery rate of oocytes from pre-ovulatory or immature follicles



Fonte: (ARAUJO, 2014)

5.5 DISCUSSION

Different methods to collect equine oocytes and different stages of follicular development during aspiration and oocyte recovery were demonstrated in the present study. Higher recovery rates were obtained by ultrasound-guided transvaginal aspiration of pre-

ovulatory follicles (Figure 1), when compared to growing follicles, both by TVA or recovery of oocytes from ovaries from slaughterhouse. This is important, since collection of oocytes is of utmost importance, being a fundamental step for subsequent use in oocyte transfer, ICSI and/or *in vitro* embryo production.

In this study, a large number of ovaries were collected and transported to the laboratory for recovery of oocytes (n = 484 oocytes, Table 1). Approximately 60 ovaries were lost in the slaughter line during collection, either by presenting of pathology (ex: abscess, atresia, etc), or other impediment on the slaughter line. Ovaries arrived at the laboratory with an average time of 4h at 30^{o}C temperature. The transporting time allowed the vast majority of oocytes to be collected within 6-8h postmortem, a critical period in which the probability of producing embryos and successful pregnancies to term is higher (CARNEVALE et al., 2004; RIBEIRO et al., 2008). For oocyte transfer or ICSI, it is recommended that the ovaries be removed from the mare as soon as possible after mare's death (HINRICHS, 2010a); however, delays in recovery from mares up to 5 h post-mortem have been associated with production of viable foals from shipped ovaries (CARNEVALE et al., 2003). The temperature during transport of the ovaries in the present study was within the recommended range (HINRICHS, 2010a), being 37^{o}C for transport with a maximum time of 2 h or at room temperature when prolonged transport is needed.

A total of 587 oocytes were recovered from 1237 follicles of the ovaries obtained at a slaughterhouse, resulting in a recovery rate of 47% (Table 1). Similar rates ranging from 46-48% were obtained in previous reports (HINRICHS, 1991; DELL'AQUILA et al., 2001). Recovery of oocytes from immature follicles in the mare is complicated by the closer and stronger attachment of the equine oocyte-cumulus complex to the follicle wall (HAWLEY; ENDERS; HINRICHS, 1995). Higher recovery rate has been reported using scrapping of the follicles instead of aspirations only (DELL'AQUILA et al., 2001). In the present study, all follicles were opened after aspiration and scrapped to recover a possible remained cumulus-oocyte-complex. The same method was attempted by (DELL'AQUILA et al., 2001), but the authors abandoned the idea since an unexpected low number of oocytes was recovered (~19% of total oocytes recovered). A similar number of aspirated follicles per ovary were reported for both studies: 2.64 (DELL'AQUILA et al., 2001) and 2.56 in the present study, yielding an oocyte recovery rate/aspirated ovary of 1.27 and 1.21, respectively. A higher recovery rate of oocytes/ovary (2.03; 414 oocytes/204 ovaries) was reported recently (GOMES et al., 2012), however, the authors did not state how many follicles were aspirated in each ovary. It is

important to remember that nutrition have a significant effect on the number of follicles in the ovary (VAN NIEKERK; VAN NIEKERK, 1997). Additionally, it is vital to evoke that the laboratories will not always have access to materials from slaughterhouses to obtain equine oocytes. For example, the supply of horse tissues from slaughterhouses in the United Sates has been limited since 2007 with federal regulations resulting in the closing of slaughterhouses (HINRICHS, 2010b). In some cases, the recovery of oocytes from live mares is therefore, necessary.

In the present study, oocytes were recovered either from pre-ovulatory follicles or from growing immature follicles (table 3). A recovery rate of 85% was observed for pre-ovulatory F1 follicles. Usually, recovery of oocytes by aspiration of the gonadotropin-stimulated dominant pre-ovulatory follicle is performed to obtain oocytes for oocyte transfer (HINRICHS, 2010a) or ICSI. The high recovery rate of oocytes from pre-ovulatory follicles in the present study are consistent with other reports (65-80%; (HINRICHS; KENNEY; KENNEY, 1990; CARNEVALE; GINTHER, 1995; HINRICHS et al., 1998; CARNEVALE et al., 2004)). In the present study, oocytes were aspirated on average 24 (first season) and 30 hours (second season) after induction of follicular and oocyte maturation *in vivo*. Twenty four hours allows the cumulus cells to expand in response to gonadotropin (HINRICHS, 2010b), facilitating oocyte recovery, and 35 h is the limit that can be assumed that the follicle will not ovulate before aspiration. The degree of cumulus cells expansion appeared to differ between seasons for pre-ovulatory follicle, however it was not enough to affect significantly the recovery rate between seasons. A significant difference in the degree of cumulus cells expansion between oocytes recovered from pre-ovulatory follicle and immature follicles were observed, as expected. Only compact cumulus-oocyte-complex cells were observed for oocytes recovered from growing follicles. When considering oocytes recovered from excised ovaries, higher percentage of compact (47%) was observed, compared to expanded, denuded or degenerated. A difference in in the oocyte morphology in oocytes recovered from excised ovaries have been reported to be related to the method of oocyte collection (ZHANG et al., 1989; DELL'AQUILA et al., 2001; HINRICHS, 1991). A greater layer of cumulus cells was obtained when follicles were scrapped compared to aspiration (DELL'AQUILA et al., 2001).

For immature follicles, the recovery rate in the present study (42.3%) was in between other studies previously reported (≤25% (BRÜCK et al., 1992; COOK; SQUIRES; JASKO, 1993; DUCHAMP; BÉZARD; PALMER, 1995; KANITZ et al., 1995; MARI et al., 2005) and 50-60% (BØGH et al., 2002; COLLEONI et al., 2007; JACOBSON et al., 2010). A significantly higher proportion of oocytes without a polar body were observed for these

groups of oocytes after denudation by pipetting with hyaluronidase solution than for oocytes recovered from pre-ovulatory follicles. When evaluating season, a higher proportion of oocytes without PB was observed for the second season (aspirations ~30h after gonadotropin treatment) compared to the first season (~24 h after gonadotropin treatment). It is worth to mention that oocytes in the first season were evaluated for PB presence after 14h of IVM (about of 38h after GnRH treatment of pre-ovulatory follicle; data not shown), while in the second season the oocytes were evaluated immediately after recover (~31h after GnRH treatment), which shows this difference in the maturation rate.

In conclusion, different methodologies for collecting equine oocytes from different stages of follicular aspiration, and oocyte recovery rate were presented in the present study. Higher recovery rates were obtained by ultrasound-guided transvaginal aspiration of pre-ovulatory follicles, when compared to growing follicles, both by TVA or recovery of oocytes from ovaries from slaughterhouse. The method of oocyte collection to be chosen will depend of the availability of animals or equine tissues by the laboratory.

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6 AGE-ASSOCIATED MIRNA AND MTDNA LEVELS IN EQUINE OOCYTE AND FOLLICULAR FLUID INDICATE POSSIBLE NEW MARKERS FOR THE EFFECTS OF AGING ON FERTILITY

ABSTRACT

Aging is known to be associated with decreased fertility in females. The aim of the present study was to identify follicular fluid (FF) and oocyte miRNAs and to quantify oocyte mtDNA levels in young and old mares. Non-lactating Thoroughbred mares (n=23) aging from 3 to 29 years and weighting between 400 and 600 kg were used as FF and oocyte donors. Mares were assigned in two experimental groups: Young Mares (YM: 3-10y; n=12) and Old Mares (OM: ≥18 y; n=11). FF was recovered from pre-ovulatory follicles and oocytes were recovered from pre-ovulatory or growing follicles (≥15mm and ≤25mm) using ultrasound-guided transvaginal follicular aspiration. Seventy seven oocytes were used to determine the relative amount of mtDNA (Young: n=35 and Old: n=42). For evaluation of age-associated differential micro-RNAs levels, eight samples of FF and oocytes (young n=4; old n=4) were used. The relative amount of mtDNA in equine oocytes from young and old mares before and after *in vivo* maturation was not significantly different among groups. Neither the effects of follicle status, age or interaction were significant ($P<0.05$). Twenty one and 32 cell-secreted vesicles containing miRNAs were present only in FF of young and old mares, respectively. Increased levels of eca-mir-3615 and decreased levels of eca-mir-873 were detected in FF from young mares compared to old mares. Nineteen and four miRNAs were detected only in oocytes from young and old mares, respectively. Higher levels of eca-mir-299 and lower levels of eca-mir-342-3p were detected in oocytes from young compared to old mares. In conclusion, age-associated intracellular molecules in equine FF and oocytes and its potential effects on fertility was demonstrated and discussed in the present study. The results suggest that the mare can serve as an good animal model to study female infertility during the aging process.

Keywords: Oocyte. Aging. miRNA. mtDNA. Embryos.

6.1 INTRODUCTION

Sexual reproduction is an evolutionary and complex process capable of creating a new individual from two others, by the fusion of the female and male gametes. The release of the mature oocyte is the result of a process known as folliculogenesis in which primordial follicles develop inside the ovaries into pre-ovulatory follicles, leading to ovulation (BAERWALD; ADAMS; PIERSON, 2012). In the male side, one single capacitated spermatozoa is able to fertilize the oocyte resulting in a viable embryo. However, the success of fertilization and embryo development is strongly dependent on oocyte quality (OTTOLENGHI et al., 2004; MAY-PANLOUP et al., 2007; FAIR, 2010; YAMAMOTO et al., 2010), which can affect important reproductive events such as oocyte maturation, monospermic fertilization and embryo survival, as well as the maintenance of pregnancy and fetal development (WANG; SUN, 2007). In most mammalian species, aging negatively influences the developmental potential of oocytes and embryos (CARNEVALE; GINTHER, 1995; ZAPANTIS; SANTORO, 2002; OTTOLENGHI et al., 2004; BAIRD et al., 2005; BROEKMANS; SOULES; FAUSER, 2009). In humans, female fertility declines at approximately 35 years (HULL et al., 1996; SPANDORFER et al., 2004). In the horse, there are few studies aimed at elucidating the relationship of oocyte competence and the aging process (CARNEVALE; GINTHER, 1995; GINTHER et al., 2004; CARNEVALE, 2008; RAMBAGS et al., 2014). Nevertheless it is known that this cause of decrease in fertility begins at around 10 years of age in the mare (CARNEVALE, 2008). The mare has been suggested as a promising model for the study of age related infertility in women, as many similarities between the reproductive physiology of these species and similarities of aging associated problems have been described (GINTHER et al., 2004; CARNEVALE, 2008).

Recently, age-associated differential microRNA levels in horse and human follicular fluid revealed potential pathways that can be used to determine fertility and success of *in vitro* fertilization (SILVEIRA et al., 2012; DIEZ-FRAILE et al., 2014). MicroRNA (miRNA) are endogenous, small noncoding RNAs (~21 nucleotides) that regulates mRNA translation of protein-coding genes in a sequence-specific manner, resulting in cleavage or translational repression of the corresponding mRNA (LEE; FEINBAUM; AMBROS, 1993; LAGOS-QUINTANA et al., 2001; LAU et al., 2001; LEE; AMBROS, 2001). miRNAs are important in the regulation of different biological processes, including developmental timing, proliferation, differentiation, migration, and apoptosis (AMBROS, 2003; KLOOSTERMAN;

PLASTERK, 2006; SCHICKEL et al., 2008; BARTEL, 2009).

In addition to miRNAs, mitochondrial DNA (DNA) is also known to be involved in developmental potential and quality of the oocyte (CHIARATTI; MEIRELLES, 2010; CHIARATTI et al., 2010; POULTON et al., 2010). The organization and activity of mitochondria play an important role in several events related to the quality of the oocyte, oocyte cytoplasmic maturation, fertilization, and embryonic development (VAN BLERKOM; RUNNER, 1984; MAY-PANLOUP et al., 2007). The pattern of mitochondrial distribution is determined by a highly dynamic process during oocyte maturation and early embryonic development (BAVISTER; SQUIRRELL, 2000; STOJKOVIC et al., 2001; SUN et al., 2001; AMBRUOSI et al., 2009). Consistent results showed that inadequate distribution of mitochondria in the ooplasm is a cytoplasmic marker indicating immaturity and is related to low capacity of embryo development. Unlike somatic cells, each oocyte has a large amount of mitochondria each containing only one copy of mtDNA (PIKÓ; MATSUMOTO, 1976). Notably, studies with oocytes from women (SANTOS; EL SHOURBAGY; ST JOHN, 2006), cows (STOJKOVIC et al., 2001; TAMASSIA et al., 2004) and sows (EL SHOURBAGY et al., 2006) showed that the average number of mtDNA was significantly smaller in groups of unfertilized oocytes when compared to groups of fertilized oocytes. Furthermore, mtDNA deletions are associated with oocyte impaired quality, resulting in inefficient embryonic development (HSIEH et al., 2002; GIBSON; KUBISCH; BRENNER, 2005). Different studies show conflicting results when trying to clarify the relationship of the decline in the amount of mtDNA with aging (CHEN et al., 1995; KEEFE et al., 1995; MÜLLER-HÖCKER et al., 1996; BLOK et al., 1997; BRENNER et al., 1998; BARRITT et al., 1999; BARRITT; COHEN; BRENNER, 2000). Recently, this relationship has been demonstrated in mice (KUSHNIR et al., 2012). In 2014, a study (RAMBAGS et al., 2014) using slaughterhouse horse ovaries as a source of oocytes showed a significant decrease in the copy number of mtDNA in oocytes from older mares (≥ 12 years old) after *in vitro* maturation (IVM). To date, no study correlating mtDNA copy number in oocytes with follicular development, aging and potential for embryonic development has been conducted in the equine.

Therefore, the aim of the present study was to evaluate follicular fluid and oocyte miRNAs and mtDNA oocyte levels in young and old mares, and to identify a possible set of miRNAs differentially expressed and differential amount of mtDNA between these two groups. It is hypothesized that old mares have low levels of oocytes mtDNA and different levels of miRNAs in oocyte and follicular fluid that may be related to decreased fertility.

6.2 MATERIALS AND METHODS

6.2.1 Animals and experimental design

Non-lactating Thoroughbred mares (n=23) aged 3 to 29 years and weighing between 400 and 600 kg were used as oocyte and follicular fluid donors during the 2011 breeding season in at the Maxwell H. Gluck Equine Research Center at the University of Kentucky (Lexington, USA). The mares had free access to pasture, trace-mineral salt and water. Mares were assigned in two experimental groups: Young Mares (YM: 3-10y; n=12) and Old Mares (OM: ≥18 y; n=11). All procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of Kentucky and with the Ethical Committee for Animal Experimentation Care at the University of Sao Paulo.

6.2.2 Oocyte and follicular fluid collection

In vivo follicular maturation was induced by administration of 2000 IU hCG + 1.5 mg GnRH when the largest follicle reached a size ≥ 32 mm of diameter. Follicular aspiration was performed 30h later by transvaginal ultrasound-guided procedure as previously described (FRANK-GUEST; STOKES; CARNEVALE, 2010). The collected follicular fluid (>10 mL) was then centrifuged at 1500 x g for 15 min, and stored at -80^{o}C until processing for cell-secreted vesicles RNA isolation.

Oocytes were recovered from pre-ovulatory or growing follicles using ultrasound-guided transvaginal aspiration as previously described (CARNEVALE et al., 2005; ALTERMATT et al., 2009). Pre-ovulatory follicles were aspirated approximately 30 hours after GnRH-hCG administration and all procedures performed by only one operator. The recovery of immature follicles was performed on the day in which the largest follicle from a known new follicular wave had reached a diameter of ≥20 mm. For that, each follicle was daily tracked by ultrasonography after total ovary follicular ablation to identify the growing

follicles to be submitted to aspiration. Growing follicles presenting diameters between 15 and 25 mm were then aspirated.

Oocytes were identified using a stereomicroscope and classified according to the degree of cumulus cells expansion, as previously described (ALTERMATT et al., 2009). Then, the cumulus cells were removed by pipetting each oocyte in a 0.05% hyaluronidase solution (CHOI et al., 2003). The oocytes were then classified according to polar body presence, size and homogeneity of the cytoplasm. After this step, the oocytes were washed in ten drops of PBS + 0.1% PVA and frozen for later analysis in 10 µL of PBS + 0.1% PVA into 1.5 ml Eppendorf tubes by immediate deposition of the tube in liquid nitrogen and subsequent storage at -80° C. Additionally, RNAaseOUT^{TM} (Recombinant Ribonuclease Inhibitor, InvitrogenTM, Life Technologies Corporation) was added to the PBS + 0.1% PVA freezing solution.

6.2.3 Determination of mitochondrial DNA copy number

The amount of mtDNA was determined as previously described (CHIARATTI; MEIRELLES, 2010). Briefly, the relative amount of mtDNA was estimated using thermocycler ABI PRISM SDS 7500 HT Real-time PCR System (Applied Biosystems) and TaqMan assay to amplify part of the MT-RNR2 gene. The relative amount of mtDNA was calculated by the standard curve method as proposed by the equipment manufacturer and is expressed relatively to the amount of mtDNA in immature oocytes collected from young mares. Data were processed with the aid of SDS v.2.3 (Applied Biosystems) software.

6.2.4 Cell-secreted vesicles isolation

Cell-secreted vesicles were isolated from ovarian follicular fluid through a series of differential ultracentrifugation steps as previously described (THÉRY et al., 2006). Samples were spun at 300 X g for 10 min and 2000 X g for 10 min to remove residual cells and debris, at 10,000 X g for 30 min to remove microparticles, and two times at 100,000 X g for 70 min (33000 rpm, rotor SW55) to pellet the microvesicles. The pellets were suspended in 50 µl of

PBS (pH 7.4) and used for RNA isolation with Tri-reagent BD.

6.2.5 MicroRNA isolation and reverse transcription

Total RNA and miRNA were isolated from microvesicles preparations isolated from follicular fluid and oocyte using TRI-REAGENT BD (Molecular Research) according to the manufacturer's instructions. RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer. Samples were stored at -80^{o}C. Quantifiable reverse transcribed miRNAs were generated using the miScript PCR System (Qiagen #218193, Venlo, Limburg, Netherlands) according to the manufacturer's instructions. Briefly, the reverse transcription reaction was carried out with approximately 100 ng of total RNA for oocytes and 300 ng for microvesicles. Total RNA, including the small RNA fraction, was incubated with 5X miScript HiFlex Buffer, 10X miScript Nucleic Mix, RNase-free water, and miScript Reverse Transcriptase at 37°C for 60 min followed by 5 min at 95°C.

6.2.6 Real-Time PCR expression analysis of miRNAs

Expression of mature miRNAs was examined in cell-secreted vesicles preparations isolated from the follicular fluid of four different animals in each group (i.e., young and old mares) using primers designed based on the mature equine sequences available on mirBase (www.mirBase.org). In addition, expression of mature miRNAs also was examined in oocytes for both groups. The profiler plate contains 347 mature miRNA sequences (used as forward primers) and three endogenous controls: U6 snRNA, RNU43 snoRNA, and U1 snRNA (Apêndice A). Each analysis was performed in 6-µl reactions containing 2 X SYBR Green I master mix (Roche Applied Sciences), 10 µM Universal reverse primer and miRNA specific forward primer (SBI), and 0.1 µl cDNA. Real-time PCR was conducted using the LightCycler480 PCR system (Roche Applied Sciences) with 384-well plates. The PCR cycle conditions were as follows: 95^{o}C for 5 min, 45 cycles of 95^{o}C for 10 sec, 55^{o}C for 15 sec, and 72^{o}C for 15 sec followed by a melt curve analysis to confirm amplification of single cDNA products, as described (SILVEIRA et al., 2012). To identify differences in the presence of

microvesicle miRNAs isolated from follicular fluid and oocyte of young and old mares, raw Ct values were normalized to the geometric mean of at least three stable miRNAs between groups, and statistical differences were assessed at $P<0.05$ using a Student t-test.

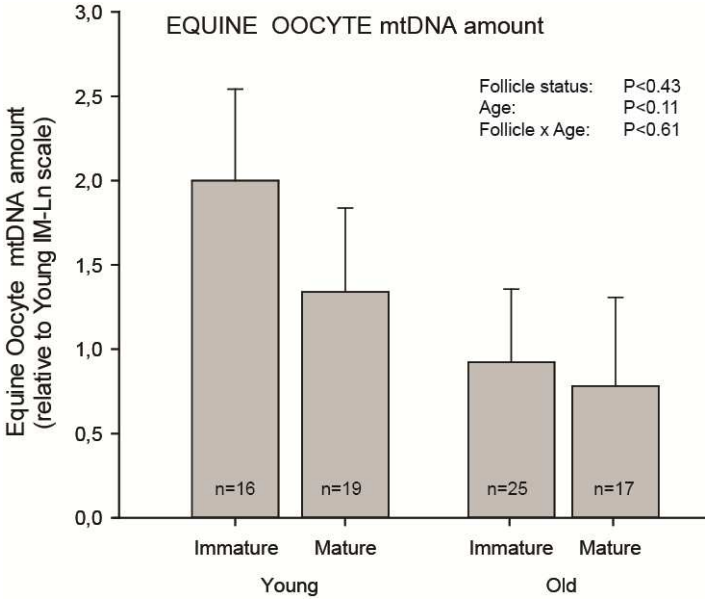
6.3 RESULTS

Fifty seven oocytes from pre-ovulatory follicle and forty seven oocytes from growing follicles (n=104) were recovered. Seventy seven oocytes were used to determine the relative amount of mtDNA (Young: n=35 and Old: n=42), while eight oocytes (young n=4; old n=4) were used to evaluate age-associated differential micro-RNA levels. Remainder oocytes were used for another study, which will be published elsewhere.

6.3.1 MtDNA

Relative amount of mtDNA in equine oocytes from young and old mares before and after *in vivo* maturation was not significantly different among groups (Figure 1; $P > 0.05$). However, young mares appeared to have numerically higher amount of mtDNA than older mares.

Figura 1 – Relative amount of mtDNA in equine oocytes from young and old mares before and after *in vivo* maturation.



Fonte: (ARAUJO, 2014)

6.3.2 MiRNAs

The presence of miRNAs in cell-secreted vesicles present in follicular fluid and miRNAs in oocytes was examined by RT qPCR. MiRNA was considered to be present when the Ct value was ≤37. Twenty one miRNAs were present only in cell-secreted vesicles from follicular fluid of young mares (Table 1).

These 21 miRNAs are predicted to target several signaling pathways (DIANA-mirPath; http://diana.imis.athena-innovation.gr/), with the Ubiquitin mediated proteolysis signaling pathway being the most significantly of them (Table 2).

The miRNAs versus pathways heat map is shown (Figure 2). A total of 32 miRNAs were presented only in microvesicles from follicular fluid of old mares (Table 3). The most significantly targeted pathway was the Neutrophin signaling pathway (table not shown).

Table 1 - miRNAs present only in cell-secreted vesicles from follicular fluid of young mares

MiRNA	Averag. Ct	miRNA	Averag. Ct
let-7d	35.48	ecamiR-495	35.59
ecamiR-26a	34.02	ecamiR-500	36.53
ecamiR-283-3p	35.51	ecamiR-501	35.34
ecamiR-98	35.77	ecamiR-503	35.60
ecamiR-125-b	34.75	ecamiR-508-5p	36.05
ecamiR-205	35.81	ecamiR-767-5p	36.22
ecamiR-371-3p	35.49	ecamiR-769-3p	35.70
ecamiR-380	35.92	ecamiR-876-5p	36.90
ecamiR-381	35.11	ecamiR-1244	34.65
ecamiR-382	35.83	ecamiR-1282	35.09
ecamiR-491-5p	35.70		

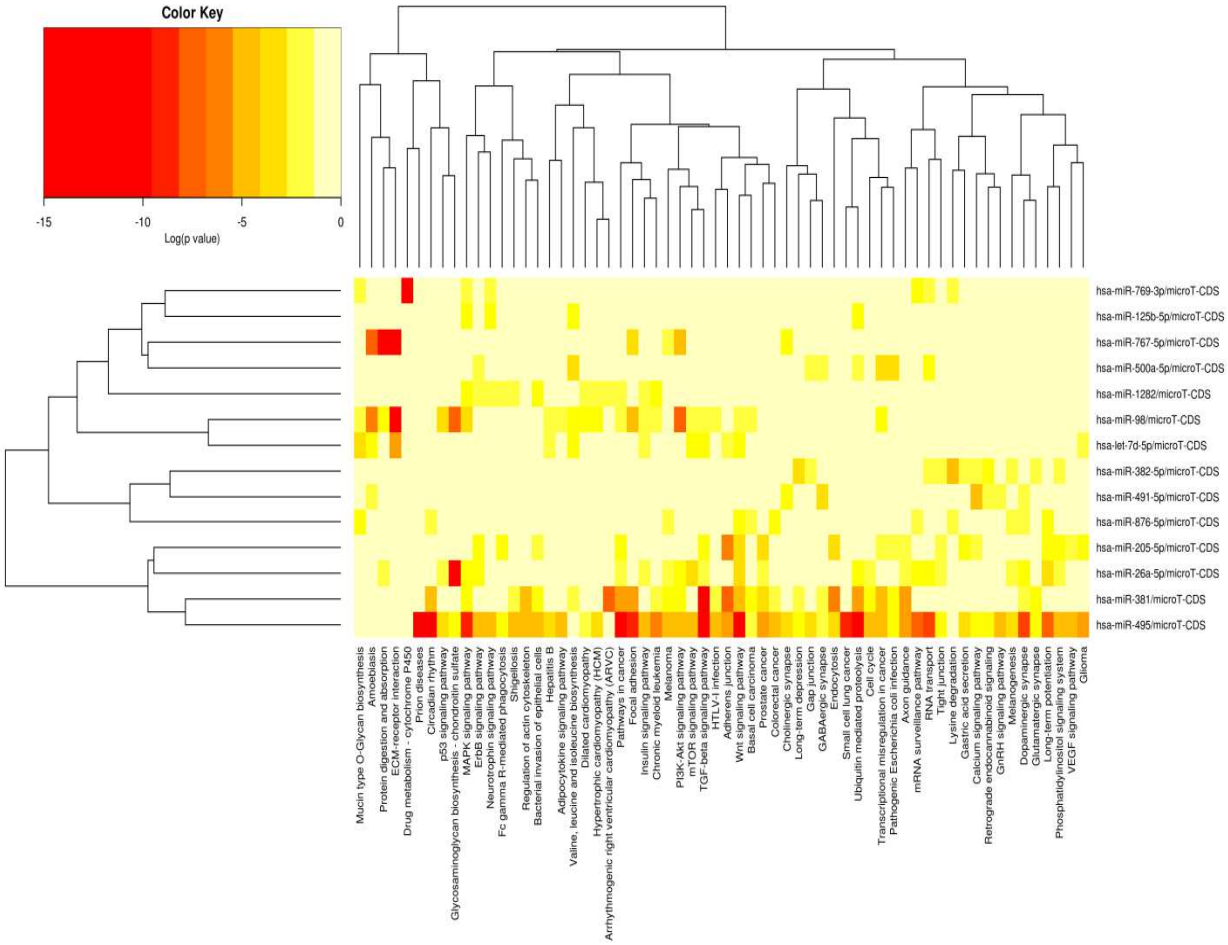
The RNA input was ~300 ng total per reaction, and only Ct values <37 cycles were considered.
Average Ct values are reported + standard deviation.
Fonte: (ARAUJO, 2014)

Table 2 - The most "important" Predicted targeted signaling pathways, p-value number of genes targeted and number of miRNAs involved for miRNAs present only in Follicular Fluid of Young mares are seen in this table. The complete table can be seen in Appendix A

	KEGG pathway	p-value	#genes	#miRNAs
1.	Ubiquitin mediated proteolysis (hsa04120)	5,88E-27	69	12
2.	Focal adhesion (hsa04510)	8,92E-16	89	14
3.	TGF-beta signaling pathway (hsa04350)	3,25E-15	43	10
4.	PI3K-Akt signaling pathway (hsa04151)	5,13E-15	136	13
5.	MAPK signaling pathway (hsa04010)	2,43E-14	110	14
6.	Long-term potentiation (hsa04720)	2,08E-13	38	13
7.	Wnt signaling pathway (hsa04310)	3,03E-13	72	13
8.	Melanoma (hsa05218)	1,83E-10	36	11
9.	Dopaminergic synapse (hsa04728)	1,26E-09	60	13
10.	Neurotrophin signaling pathway (hsa04722)	1,63E-08	55	13

Fonte: (ARAUJO, 2014)

Figure 2 – miRNAs present in cell-secreted vesicles versus pathways heat map for follicular fluid in Young mares (clustering based on significance levels). Darker colors represent lower significance values. The attached dendograms on both axes depict hierarchical clustering results for miRNAs and pathways, respectively.



Fonte: (ARAUJO, 2014)

Table 3 - miRNAs present only in microvesicles from follicular fluid of old mares

MiRNA	Averag. Ct	miRNA	Averag. Ct
ecamiR-10b	33,99	ecamiR-200b	35,11
ecamiR-29a	33,91	ecamiR-206	35,13
ecamiR-30b	33,24	ecamiR-215	34,29
ecamiR-30c	34,5	ecamiR-221	35,51
ecamiR-31	34,28	ecamiR-338-5p	35,01
ecamiR-99b	34,27	ecamiR-410	35,53
ecamiR-133a	34,6	ecamiR-423-3p	33,07
ecamiR-134	32,25	ecamiR-490-5p	35,02
ecamiR-140-3p	33,84	ecamiR-497	35,05
ecamiR-146b-3p	34,48	ecamiR-509-5p	35,32
ecamiR-146b-5p	34,76	ecamiR-543	34,29
ecamiR-150	34,94	ecamiR-598	35,11
ecamiR-151-5p	34,87	ecamiR-767-3p	34,53
ecamiR-181a	34,58	ecamiR-769b	35,4
ecamiR-181b	34,93	ecamiR-872	34,67
ecamiR-196b	36,02	ecamiR-1468	35,54
ecamiR-873	0.21 (±0.002)	0.35 (±0.03)	0,01

The RNA input was ~300 ng total per reaction, and only Ct values <37 cycles were considered.
Average Ct values are reported + standard deviation.
Fonte: (ARAUJO, 2014)

Using normalized expression values, the amount of two miRNAs (eca-mir-3615 and eca-mir-873) were significantly different when the follicular fluid of young and old mares were compared (table 4). Higher levels of eca-mir-3615 and lower levels of eca-mir-873 were detected in follicular fluid from young compared to old mares.

Table 4 - miRNAs significantly different in microvesicles from follicular fluid of old and young mares

MiRNA	Old ΔCt (±SEM)	Young ΔCt (±SEM)	T-test
ecamiR-3615	0.31 (±0.02)	0.19 (±0.02)	0,01
ecamiR-873	0.21 (±0.002)	0.35 (±0.03)	0,01

The RNA input was ~300 ng total per reaction, and only Ct values <37 cycles were considered.
Average Ct values are reported + standard deviation.
Fonte: (ARAUJO, 2014)

Presence of miRNAs was also examined in oocytes from young and old mares (n=4/group). From 350 miRNAs examined, 19 were detected only in oocytes from young

mares and 4 were detected only in oocytes from old mares (Table 5).

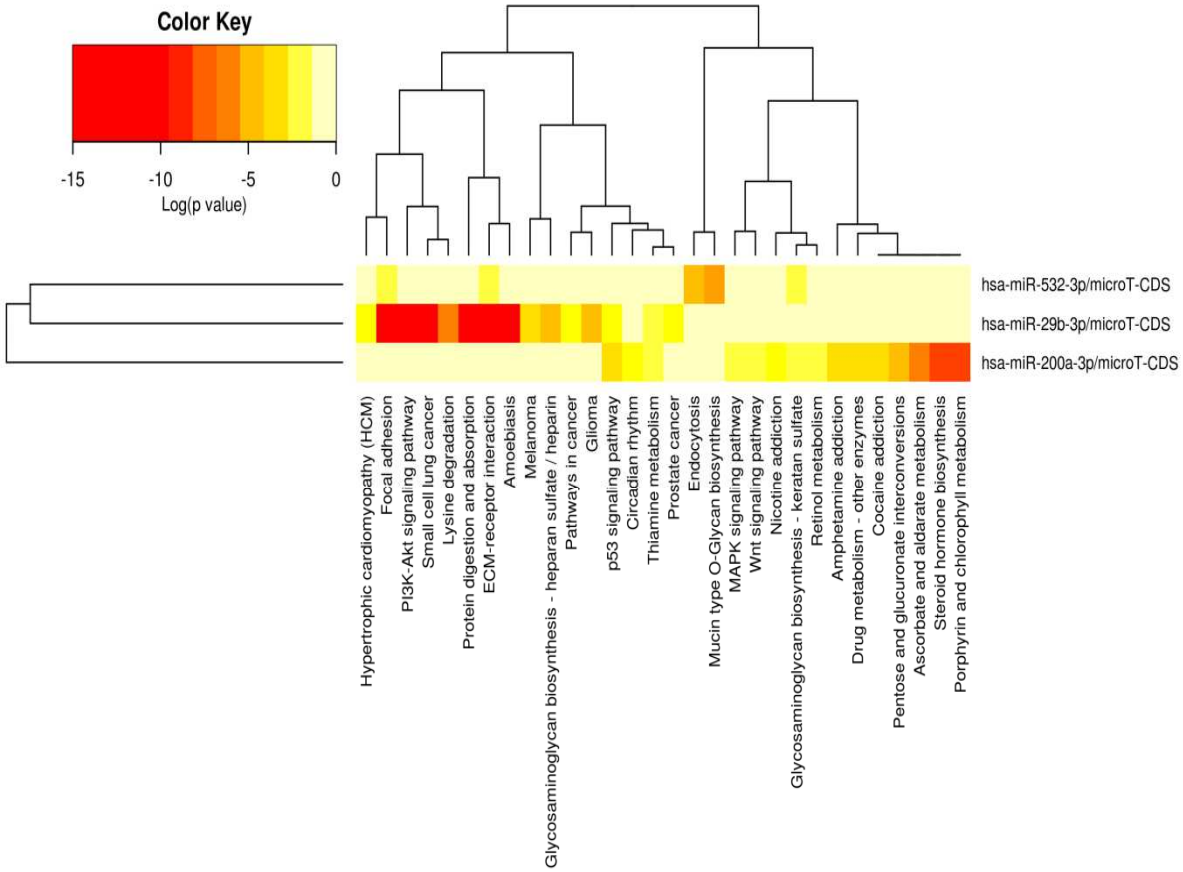
Table 5 - miRNAs present only in oocytes from young mares

MiRNA	Averag. Ct	miRNA	Averag. Ct
ecamiR-22	37,39	ecamiR-324-3p	31,99
ecamiR-27b	43,42	ecamiR-326	41,49
ecamiR-33a	40,86	ecamiR-328	39,98
ecamiR-127	34,57	ecamiR-374b	40,08
ecamiR-138	39,52	ecamiR-384	42,71
ecamiR-139-5p	40,72	ecamiR-410	39,04
ecamiR-154	39,53	ecamiR-491-5p	37,76
ecamiR-187	40,18	ecamiR-503	35,97
ecamiR-199-5p	41,99	ecamiR-504	43,39
ecamiR-218	40,85		
miRNAs present only in oocytes from old mares			
MiRNA	**Averag. Ct**	**miRNA**	**Averag. Ct**
ecamiR-29b	41,28	ecamiR-376b	41,62
ecamiR-200a	42,08	ecamiR-532-3p	40,42
miRNAs significantly different in oocytes from old and young mares			
MiRNA	**Old ΔCt (±SEM)**	**Young ΔCt (±SEM)**	**T-test**
ecamiR-299	0.35 (±0.08)	0.12 (±0.01)	0,02
ecamiR-342-3p	0.12 (±0.003)	0.14 (±0.003)	0,03

The RNA input was ~100 ng total per reaction, and only Ct values <37 cycles were considered.
Average Ct values are reported + standard deviation.
Fonte: (ARAUJO, 2014)

The 19 miRNAs detected only in oocytes from young mares were mostly related to the PI3K-Akt signaling pathway, while the 4 miRNAs detected in old mares were mostly related to the ECM-receptor interaction signaling pathways (Figure 3, table 6). Two miRNAs (eca-mir-299 and eca-mir-342-3p) were significantly different between young and old mares oocytes. Higher levels of eca-mir-299 and lower levels of eca-mir-342-3p were detected in oocytes from young compared to old mares ($P<0.05$; Table 5).

Figure 3 – miRNAs versus pathways heat map for Oocyte in Old mares (clustering based on significance levels). Darker colors represent lower significance values. The attached dendograms on both axes depict hierarchical clustering results for miRNAs and pathways, respectively.



Fonte: (ARAUJO, 2014)

Table 6 - Predicted targeted signaling pathways, p-value number of genes targeted and number of miRNAs involved for miRNAs present only in oocytes of Old Mares.

	KEGG pathway	P-value	#genes	#miRNAs
1.	ECM-receptor interaction (hsa04512)	8,06E-43	24	3
2.	Amoebiasis (hsa05146)	5,94E-11	25	3
3.	Focal adhesion (hsa04510)	2,13E-08	41	3
4.	Protein digestion and absorption (hsa04974)	2,26E-09	24	3
5.	PI3K-Akt signaling pathway (hsa04151)	8,38E-05	54	3
6.	Small cell lung cancer (hsa05222)	8,38E-05	21	3
7.	Lysine degradation (hsa00310)	7,46E-01	10	3
8.	p53 signaling pathway (hsa04115)	4,11E+00	15	3
9.	Hypertrophic cardiomyopathy (HCM) (hsa05410)	6,98E+00	17	3
10.	Nicotine addiction (hsa05033)	1,66E+01	9	3
11.	Porphyrin and chlorophyll metabolism (hsa00860)	0.000161852	10	1
12.	Ascorbate and aldarate metabolism (hsa00053)	0.0003995516	7	1
13.	MAPK signaling pathway (hsa04010)	0.0009037489	34	3
14.	Wnt signaling pathway (hsa04310)	0.001073151	23	3
15.	Dilated cardiomyopathy (hsa05414)	0.001958633	15	3
16.	Transcriptional misregulation in cancer(hsa05202)	0.002936221	26	3
17.	Glycosaminoglycan biosynthesis - keratan sulfate(hsa00533)	0.004984184	3	2
18.	HTLV-I infection (hsa05166)	0.004984184	32	3
19.	Drug metabolism - other enzymes (hsa00983)	0.006216487	10	2
20.	Pathways in cancer (hsa05200)	0.006889805	43	3
21.	Pentose and glucuronate interconversions(hsa00040)	0.009680679	7	1
22.	Glycosaminoglycan biosynthesis - heparan sulfate / heparin (hsa00534)	0.009680679	5	2
23.	Circadian rhythm (hsa04710)	0.01269288	7	2
24.	Steroid hormone biosynthesis (hsa00140)	0.01731559	8	2
25.	Prostate cancer (hsa05215)	0.02348688	13	2
26.	Amphetamine addiction (hsa05031)	0.03253155	12	3
27.	Glioma (hsa05214)	0.03288671	11	2
28.	Dopaminergic synapse (hsa04728)	0.04718523	17	3

Fonte: (ARAUJO, 2014)

6.4 DISCUSSION

Little is known about the physiological role of miRNA and mtDNA in ovarian follicular development and maturation of oocytes and the effects of age on these processes, both in humans and domestic species like the mare. In the present study, a set of differentially expressed microRNAs were detected in FF and oocytes from young and old mares. Similarities with human FF were observed (DIEZ-FRAILE et al., 2014) and strongly

encourages the use of the mare as a human model to study female infertility during the aging process.

Among the predicted pathways targeted by the miRNAs found in this study, the p53 pathway was evidenced in FF and oocyte. The p53 pathway is known to play a crucial role in the process of aging (COLLADO; BLASCO; SERRANO, 2007). In this context, miR-134 is pointed as down-regulator of inhibitors of apoptosis genes (BCL2 and IKBKG) in human's grannulosa cells (DIEZ-FRAILE et al., 2014). In the present study, miR-134 was detected only in the FF of old mares. Similarly, increased expression of miR-134 was noted in human FF (DIEZ-FRAILE et al., 2014), which is in agreement with the increase of apoptosis in grannulosa cells in older women associated with down-regulation of miR-21-5p (DIEZ-FRAILE et al., 2014) combined with high level of miR-134 (DIEZ-FRAILE et al., 2014; KRYSKO et al., 2008). MiR-21-5p acts as anti-apoptotic factor in cultured grannulosa cells and in ovarian tissue (CARLETTI; FIEDLER; CHRISTENSON, 2010).

Differential miRNA expression levels between ovulatory and anovulatory follicles have been observed in FF of mares (DONADEU; SCHAUER, 2013). Among the studied miRNAs, only miR-503 was detected in FF in this work. It has been reported that MiR-503 was not differentially expressed between ovulatory and anovulatory follicles (DONADEU; SCHAUER, 2013). However, in the present study, it was detected only in FF and in oocyte of young mares but not in old mares. In the study by Donadeau et al. (DONADEU; SCHAUER, 2013), only mares less than 14 years were used, which agrees with the finding of mir-503 in FF of young mares in this study. It is known that microRNA-503 targets FGF2 and VEGFA and inhibits tumor angiogenesis and growth (ZHOU et al., 2013). In addition, the combined presence of miR-503 and miR-138 has been used to distinguish type 2 diabetes from obesity-related diabetes (PESCADOR et al., 2013). Additionaly, miR-503 mediates anti-proliferative and anti-invasive actions of the thyroid hormone receptor beta (RUIZ-LLORENTE et al., 2014). In the mare, the role of mir-503 is not known, and its relationship with aging needs to be further elucidated.

Increased expression of miRNA-200c seems to be associated with the signs of oocyte immaturity (TESFAYE et al., 2009). Higher expression of miR-200c (1.90 fold change) was observed in immature bovine oocytes compared to oocytes matured *in vitro* (TESFAYE et al., 2009). Signs of immaturity were also observed through the increased expression of mir-299 and mir-376 in human oocytes in germinal vesicle (GV) stage compared with MII (XU et al., 2011). In this study, miR-200a, miR-200b and miR-376 were found only in oocytes and FF of

old mares. Greater amounts of miR-299 (P<0,02) were also observed in this group of mares. The material was collected from pre-ovulatory follicles after induction of follicular maturation *in vivo*, so this finding may be associated with a possible failure of follicular and oocyte maturation in old mares. In addition, family members of mir-200 (miR-200a, miR-200b and miR-200c) are related to the occurrence of epithelial ovarian cancer (CAO et al., 2014; VILMING ELGAAEN et al., 2014) and its overexpression may determine the aggressiveness of the tumor progression and is recognized as a reliable marker to predict the survival in patients with epithelial ovarian cancer (CAO et al., 2014). Moreover, expression of miR-200c and other miR-200 family members has been shown to be induced or up-regulated by ROS. These miR-200 members are known to stimulate endothelial cell apoptosis and senescence via ZEB1 inhibition (MAGENTA et al., 2011). To date, miRNAs 299 and 376 have been linked to breast cancer and glioma tumors, among others (LOWERY et al., 2009; CHOUDHURY et al., 2012). More studies are desired to identify the relationship of these miRNAs with fertility and aging in humans and other domestic species.

From more than 31,000 articles on microRNAs in several species in the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/), only 40 were found until the time of preparation of this manuscript mentioning at least one of the four miRNAs differentially expressed detected in the present study (mir-299, mir -342-3p, mir-873 and mir-3615). From the total, only one article described the mir-3615 (ZHENG et al., 2013), three articles the mir-299 (DIXON-MCIVER et al., 2008; LOWERY et al., 2009; JIN et al., 2013), six articles the mir-873 (XU et al., 2009; MORGAN; BALE, 2011; SKALSKY; CULLEN, 2011; LI et al., 2012; RAHMAN et al., 2012; NING et al., 2014; SONTAKKE et al., 2014;), and 30 articles the mir-342-3p. Multiple studies relating the diagnosis and involvement of miRNAs to pathological conditions, especially to the most varied types of cancer, among others have been published. To the authors' knowledge, only one of these studies reported a relationship of mir-873 with follicular development (SONTAKKE et al., 2014). Furthermore, to the authors' knowledge, this is the first report illustrating a relationship between differentially expressed miRNAs levels with follicular development (FF and oocytes) and the aging process in any species. Because miRNAs have immense potential as a diagnostic tool for diseases, they can be used to elucidate the mechanisms related to human diseases. However, further studies relating the mechanisms of reproduction and aging are warranted.

Despite differences regarding the expression of miRNAs in FF and oocytes of young and old mares in the present study, the comparisons between the amounts of mitochondrial DNA were not statistically different between groups. The number of mtDNA copies has been

used to indirectly measure the cell metabolism. Greater amounts of mtDNA have been found in competent oocytes compared to infertile oocytes in pigs (EL SHOURBAGY et al., 2006), cattle (STOJKOVIC et al., 2001; TAMASSIA et al., 2004), and in humans (TEMPLETON; MORRIS; PARSLOW, 1996; SANTOS; EL SHOURBAGY; ST JOHN, 2006). It is expected that a more accurate information in this regard (competence x mitochondria in the cell) would be obtained through the functional analysis of mitochondrial activity in oocytes in young and old mares. However, for this study, this assessment was not feasible since this analysis needs to be carried out preferably with fresh oocytes.

The amount of mtDNA did not seem to differ in oocytes of young mares ((RAMBAGS et al., 2014) and present study), human species (JANSEN; DE BOER, 1998) and in the older mares before ((RAMBAGS et al., 2014) and present study) or after (present study) *in vivo* maturation. It suggests that mitochondrial replication in the female germ cell line is completed before the oocyte entering the final stage of maturation (NING et al., 2014). Additionally, the increase in the number of mtDNA copies or mitochondrial activity does not seem to be necessary for oocyte final maturation (VAN BLERKOM; DAVIS; LEE, 1995), but may be required for early embryonic development until the time of embryonic genome activation.

Unlike the findings of this study, oocytes from old mares collected from slaughterhouse ovaries (follicles 5-30 mm) subjected to *in vitro* maturation showed a decrease in the amount of mtDNA copy numbers (RAMBAGS et al., 2014). The amount of mtDNA quantified (RAMBAGS et al., 2014) disregarded selection and dominance of the pre-ovulatory follicle, which would naturally be selected to ovulate and release the oocyte for fertilization. This fact masks the actual ratio of the amount of mtDNA with the fertilization potential and viability of oocytes since the pool of oocytes remain quiescent (arrest) after entering meiosis for a long period of maternal life (EICHENLAUB-RITTER et al., 2011), accumulating mutations, deletions, and duplications in mtDNA over time (CORTOPASSI; ARNHEIM, 1990; SHIGENAGA; HAGEN; AMES, 1994; MELOV et al., 1995; JAZIN et al., 1996; NAGLEY; WEI, 1998). Additionally, a decrease in the amount of mtDNA in oocytes from atretic follicles may occur and might be a cause for decreased mtDNA number in oocytes collected from slaughterhouse ovaries of old mares (RAMBAGS et al., 2014).

As previously suggested (GINTHER et al., 2004; CARNEVALE, 2008; RAMBAGS et al., 2014) the mare is an excellent animal model for the study of infertility related to aging in humans, since fertility decreases significantly with advancing age (TEMPLETON; MORRIS; PARSLOW, 1996; MORRIS; ALLEN, 2002). Similarly to human, the mare is a

monovulatory species and oocytes can be collected ante-mortem from specific follicles or post-mortem from slaughterhouses ovaries enabling data collection for research. Moreover, the interval for reproductive senescence is more similar when compared with women than with other laboratory species such as mice.

In conclusion, age-associated intracellular molecules in equine FF and oocytes and their potential effects on fertility was demonstrated and discussed in this study. Differential expression of miRNAs was found in FF and oocyte of young and old mares. However, its relationship with follicular development and oocyte maturation remains to be elucidated. Additionally, quantification of mtDNA in oocytes of mares showed no significant difference for both follicular growth and the relationship with aging, requiring further studies, since conflicting results have also been reported in other species. The present study strongly encourages the use of the mare as a model to study human female infertility during the aging process.

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APPENDIX A

Supl. Table 1 - The most "important" Predicted targeted signaling pathways, p-value number of genes targeted and number of miRNAs involved for miRNAs present only in Follicular Fluid of Young mares are seen in this table.

	KEGG pathway	p-value	#genes	#miRNAs
1.	Ubiquitin mediated proteolysis (hsa04120)	5,88E-27	69	12
2.	Focal adhesion (hsa04510)	8,92E-16	89	14
3.	TGF-beta signaling pathway (hsa04350)	3,25E-15	43	10
4.	PI3K-Akt signaling pathway (hsa04151)	5,13E-15	136	13
5.	MAPK signaling pathway (hsa04010)	2,43E-14	110	14
6.	Long-term potentiation (hsa04720)	2,08E-13	38	13
7.	Wnt signaling pathway (hsa04310)	3,03E-13	72	13
8.	Melanoma (hsa05218)	1,83E-10	36	11
9.	Dopaminergic synapse (hsa04728)	1,26E-09	60	13
10.	Neurotrophin signaling pathway (hsa04722)	1,63E-08	55	13
11.	mRNA surveillance pathway (hsa03015)	1,84E-07	44	10
12.	RNA transport (hsa03013)	7,70E-07	65	13
13.	Axon guidance (hsa04360)	7,99E-07	60	13
14.	Endocytosis (hsa04144)	6,86E-06	83	13
15.	Progesterone-mediated oocyte maturation(hsa04914)	1,33E-05	39	13
16.	Circadian rhythm (hsa04710)	1,39E-05	20	6
17.	Retrograde endocannabinoid signaling(hsa04723)	1,97E-06	52	13
18.	Pathways in cancer (hsa05200)	9,23E-05	132	14
19.	Notch signaling pathway (hsa04330)	1,37E-04	23	8
20.	p53 signaling pathway (hsa04115)	1,37E-04	32	9
21.	Prostate cancer (hsa05215)	2,02E-04	39	11
22.	Insulin signaling pathway (hsa04910)	3,26E-04	56	13
23.	Adherens junction (hsa04520)	3,28E-04	37	12
24.	Small cell lung cancer (hsa05222)	5,49E-04	37	12
25.	Regulation of actin cytoskeleton (hsa04810)	1,72E-03	84	14
26.	Gap junction (hsa04540)	4,49E-03	38	12
27.	Vibrio cholerae infection (hsa05110)	5,97E-03	25	11
28.	mTOR signaling pathway (hsa04150)	3,73E-02	29	13
29.	Colorectal cancer (hsa05210)	3,96E-02	29	10
30.	GnRH signaling pathway (hsa04912)	4,51E-03	38	13
31.	Shigellosis (hsa05131)	1,50E-01	28	10
32.	Gastric acid secretion (hsa04971)	3,86E-01	32	11
33.	Chronic myeloid leukemia (hsa05220)	4,23E-01	31	12
34.	Amoebiasis (hsa05146)	4,69E-01	43	12
35.	Fc gamma R-mediated phagocytosis (hsa04666)	5,88E-01	38	13
36.	Prion diseases (hsa05020)	7,18E-02	12	4
37.	Pathogenic Escherichia coli infection (hsa05130)	1,01E+00	24	9
38.	Aldosterone-regulated sodium reabsorption(hsa04960)	1,46E-02	18	10

39.	Cell cycle (hsa04110)	1,67E+00	52	12
40.	ErbB signaling pathway (hsa04012)	1,78E+00	38	14
41.	Type II diabetes mellitus (hsa04930)	2,40E+00	21	11
42.	Glutamatergic synapse (hsa04724)	2,51E+00	48	13
43.	Bacterial invasion of epithelial cells (hsa05100)	3,31E+00	30	13
44.	Nicotine addiction (hsa05033)	3,32E+00	22	12
45.	Long-term depression (hsa04730)	3,32E+00	29	13
46.	Hypertrophic cardiomyopathy (HCM) (hsa05410)	4,56E-01	33	12
47.	Calcium signaling pathway (hsa04020)	6,63E+00	66	13
48.	Dilated cardiomyopathy (hsa05414)	9,49E+00	35	12
49.	Epithelial cell signaling in Helicobacter pylori infection (hsa05120)	1,15E+01	28	12
50.	VEGF signaling pathway (hsa04370)	2,57E+00	27	13
51.	ABC transporters (hsa02010)	2,64E+01	19	8
52.	Adipocytokine signaling pathway (hsa04920)	2,69E+01	28	11
53.	Tight junction (hsa04530)	3,71E+01	52	13
54.	Apoptosis (hsa04210)	5,19E+01	36	13
55.	Tuberculosis (hsa05152)	7,25E+01	64	13
56.	Protein processing in endoplasmic reticulum(hsa04141)	7,27E+01	63	13
57.	Oocyte meiosis (hsa04114)	0.0001418391	49	10
58.	Melanogenesis (hsa04916)	0.0001498756	38	12
59.	Amyotrophic lateral sclerosis (ALS) (hsa05014)	0.0002328825	23	11
60.	Glioma (hsa05214)	0.0002686863	31	10
61.	Protein digestion and absorption (hsa04974)	0.000314142	33	11
62.	Dorso-ventral axis formation (hsa04320)	0.0003297035	11	7
63.	GABAergic synapse (hsa04727)	0.0003687468	36	13
64.	HIF-1 signaling pathway (hsa04066)	0.0009942658	40	12
65.	Transcriptional misregulation in cancer(hsa05202)	0.001042677	68	13
66.	Fc epsilon RI signaling pathway (hsa04664)	0.001389476	27	11
67.	Osteoclast differentiation (hsa04380)	0.001479272	46	11
68.	Cholinergic synapse (hsa04725)	0.001600696	44	12
69.	Non-small cell lung cancer (hsa05223)	0.002730583	22	11
70.	Pancreatic cancer (hsa05212)	0.002739565	28	10
71.	Arrhythmogenic right ventricular cardiomyopathy (ARVC) (hsa05412)	0.002876741	29	12
72.	Chemokine signaling pathway (hsa04062)	0.002883268	62	14
73.	Endometrial cancer (hsa05213)	0.003405794	21	10
74.	Serotonergic synapse (hsa04726)	0.003617432	41	13
75.	Inositol phosphate metabolism (hsa00562)	0.009618185	23	9
76.	Hepatitis C (hsa05160)	0.009662439	45	12
77.	B cell receptor signaling pathway (hsa04662)	0.01112414	27	10
78.	Hepatitis B (hsa05161)	0.01349701	52	13
79.	Endocrine and other factor-regulated calcium reabsorption (hsa04961)	0.01476788	22	10
80.	HTLV-I infection (hsa05166)	0.01518114	86	13
81.	Pertussis (hsa05133)	0.02684287	26	12
82.	Phosphatidylinositol signaling system (hsa04070)	0.02780315	30	10
83.	Lysine degradation (hsa00310)	0.03483971	19	13
84.	Toxoplasmosis (hsa05145)	0.03551583	39	11

85. Jak-STAT signaling pathway (hsa04630)	0.04195641	49	13
86. Acute myeloid leukemia (hsa05221)	0.04409976	20	10

Fonte: (ARAUJO, 2014)