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Marilena Rizzo

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Aneuploidy in horse oocytes and embryos; how late is too late? The ‘fertility clock’ doesn’t only tick for career women

Aneuploidie in paarden eicellen en embryos; hoe laat is te laat? De ‘fertiliteitsklok’ tikt niet alleen voor carrière vrouwen

(met een samenvatting in het Nederlands)

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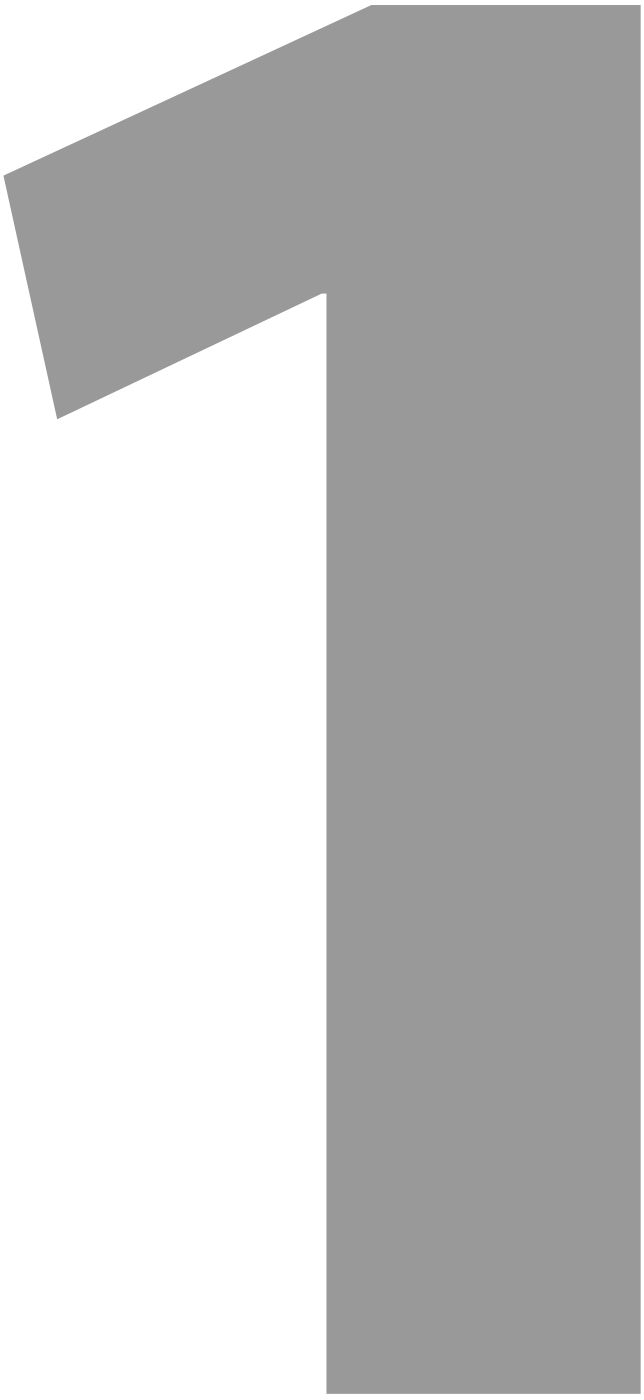

Chapter 1

General introduction

INTRODUCTION

In the last two decades, advanced assisted reproduction techniques have become an integral part of sport horse breeding, with more than 1000 foals born worldwide from *in vitro* produced (IVP) embryos. Although conventional *in vitro* fertilization (IVF) is unsuccessful in the horse, Ovum-Pick-Up (OPU; oocytes recovered by trans-vaginal follicle puncture and aspiration) combined with ICSI (Intracytoplasmic Sperm Injection; injection of a single spermatozoon into an oocyte) or somatic cell nuclear transfer (a form of cloning) can be used to produce equine embryos *in vitro*. Indeed, *in vitro* embryo production has a number of potential advantages for commercial horse breeding, including: a) more efficient use of expensive frozen semen (including the possibility of producing multiple embryos from a single straw) [1]; b) more chances to obtain embryos, and consequently foals, from sub-fertile stallions or mares [1]; c) genetic salvage, the possibility of producing embryos from mares that die suddenly or undergo emergency euthanasia; d) the opportunity to perform OPU sessions throughout the year, as an outpatient procedure (particularly appreciated by owners of actively competing mares) and at locations at considerable distance from an experienced ICSI laboratory [2]; e) the opportunity to use cryopreservation to distribute genetically valuable embryos worldwide [3]. However, despite the many potential benefits and despite becoming increasingly clinically widespread, ART methods continue to suffer from a relatively low efficiency; moreover, the likelihood of success varies markedly from laboratory to laboratory.

The two limiting steps to a successful equine *in vitro* embryo production (IVEP) program are the fact that a relatively small percentage of injected oocytes develop to a blastocyst, and the lower likelihood of pregnancy after the transfer of an *in vitro* produced compared to an *in* vivo derived embryo into a recipient mare. Until approximately 10 years ago, less than 10% of injected oocytes developed into a transferable blastocyst; in the last decade, blastocyst production rates per injected *in vitro*-matured oocyte have reached ~ 40 % in research programs [1, 4, 5], while recent papers on commercial OPU-ICSI programs have reported that approximately 60 % of OPU sessions yield a blastocyst, with a mean of 1.3-1.8 embryos per mare per OPU session [6, 7]. Although the likelihood of pregnancy after transfer of *in vitro* produced embryos also varies between centers, recent papers have reported success rates ranging between 50 % and 80 %. On the other hand, 16 to 25 % of these pregnancies undergo embryonic or fetal death before day 60 of gestation, such that live foals born per embryo transfer rates range between 30 and 70 % [1, 8, 9]. This variability in the success of establishing pregnancy, and in the incidence of early embryonic loss (EEL) between laboratories/clinics,

could be influenced by the ability to accurately and effectively identify and select IVP embryos [5, 10], the intrinsic quality and developmental competence of the embryos [11], and the synchrony and fertility of the recipient mares used [12, 13].

Of course, early pregnancy loss (EPL) is not only an issue after ART, but is one of the major sources of inefficiency and economic loss in all branches of the horse breeding industry. Even after natural mating or standard artificial insemination, the incidence of early pregnancy loss between days 12 and 60 is reported to be around 10% to 15% for young mares, and to rise to 30% or more for mares older than 18 years [14-16].

There are various possible causes of early pregnancy loss; these include abnormalities of the oviductal or uterine environments, especially when the deficits are chronic [7, 17]; failure of oviductal transport [17]; or intrinsic abnormalities of the embryo (e.g. retarded development, a higher incidence of apoptotic cells or reduced developmental competence) [11, 13, 17, 18]. To test the hypothesis that the uterine environment is an important contributor to embryonic loss in aged, sub-fertile mares, Ball *et al*. transferred morphologically normal Day 7 and Day 8 embryos to the uterus of normal young, fertile mares and compared embryo survival to when similar embryos were transferred to the uterus of older, sub-fertile mares [18]. That the pregnancy rate after transfer was similar in the two groups of recipient mares indicated that the uterine environment is not the major cause of EEL; rather, it suggests that intrinsic embryonic defects are more likely to be responsible for the majority of pregnancy losses in aged, sub-fertile mares [18]. In a subsequent study, Ball *et al*. (1989) recorded a high rate of embryonic loss in sub-fertile mares before Day 14 [19]. This was not correlated to a reduced likelihood of fertilization 4 days after ovulation, essentially confirming that EEL is primarily the consequence of reduced developmental potential intrinsic to the ovulated oocyte and resulting zygote, or that it may arise during intra-oviductal development of the early embryo. In this respect, when oocytes from aged sub-fertile donor mares are transferred to the uterus of normal young recipients, pregnancy rates are considerably lower than for oocytes from young fertile donors (31% vs 92%), suggesting that an age-associated decline in oocyte quality is the principal contributor to the age-related decline in fertility in mares [14]. This agrees with the finding that chromosomal and other abnormalities of oocytes do not markedly affect fertilization rates, but do significantly increase the risk of EEL in aged females [14, 15, 20, 21].

Age-associated fertility decline is also an important issue in human reproduction, where advanced maternal age is associated with an increased incidence of embryonic aneuploidies of

meiotic origin (the embryos contain an incorrect number of chromosomes because they develop from an oocyte containing an incorrect number of chromosomes) [22, 23]. Indeed, aneuploidy is the primary cause of developmental arrest, implantation failure, miscarriage and congenital birth defects in human (ART) pregnancy [24, 25], and the incidence rises significantly with advancing maternal age. Although the prevalence of aneuploidy in equine oocytes is unknown, it has previously been reported that *in vivo* matured oocytes from old mares show chromosomal misalignment, which could potentially result in mis-segregation and lead to aneuploidy [26]. Since equine embryos have also been shown to suffer from aneuploidy [27], it seems reasonable to propose that embryonic aneuploidy of meiotic origin may be an important to contributor to the increased incidence of early pregnancy loss in aged mares.

The main objective of this doctoral thesis was therefore to study the effect of advanced mare age on the quality and developmental competence of equine oocytes and embryos. In particular, the goals of the project were: 1) to evaluate the effect of advanced mare age on the incidence of chromosomal and spindle abnormalities in *in vitro* matured equine oocytes; 2) to elucidate the mechanisms that predispose to meiotic spindle and chromosome segregation abnormalities in equine oocytes, by investigating the role of inter-chromosomal cohesion and the spindle assembly checkpoint (SAC) in the dynamics of spindle formation and chromosome (mis)alignment and (mis)segregation; 3) to indirectly estimate the incidence of mitosis-derived chromosomal aneuploidy in IVP and *in vivo* derived equine blastocysts by quantifying micronucleus formation and investigating whether a correlation exists between the morphological characteristics of IVP embryos and the proportion of (ab)normal nuclei. The following paragraphs of this chapter review the mechanisms by which aneuploidy can arise in oocytes and embryos, highlighting the impact of maternal age and *in vitro* embryo production.

Maternal aging and aneuploidy

Embryonic aneuploidy is a condition in which all or some of the embryonic cells show an abnormal number of chromosomes. This abnormal chromosome number originates from one or more mis-segregation events that, assuming one of the parents isn't affected by aneuploidy, can arise during: a) the mitotic divisions within the primordial germ cell pool during fetal life, which creates the gamete progenitors; b) the meiotic divisions within oocytes and/or spermatogonia; or c) the first few mitotic divisions within the embryo following fertilization [25]. Although all of these processes have been shown to contribute to embryonic aneuploidy, more than 90% of mis-segregation errors originate from errors of meiosis within the oocyte [22, 28-30].

In mammals, oocytes initiate the first meiotic division during fetal life. During this period, the duplicated homologous chromosomes pair up and undergo recombination, simultaneously forming a unique "bivalent" structure. Meiosis within the oocytes then arrests at the dictyate stage, and the bivalent configuration of meiotic prophase I is maintained until follicular recruitment and the resumption of meiosis sometime after puberty. In women and horses, this period of oocyte quiescence can last decades . During this extended period of meiotic arrest, oocytes can be exposed to molecular and cellular aging processes that can impair or alter any or all of; mitochondrial function, the strength of the cohesion between homologous chromosomes, the ability of the oocyte to correctly form a spindle and/or to accurately detect when all the chromosomes are correctly aligned on the metaphase plate.

Meiosis and chromosome mis-segregation

The zygote is formed by the fusion of paternal and maternal gametes. To ensure the formation of a diploid conceptus, germ cells must undergo two (reductional) meiotic divisions (Meiosis I and II) (Figure 1) in order to generate haploid oocytes and sperm cells.

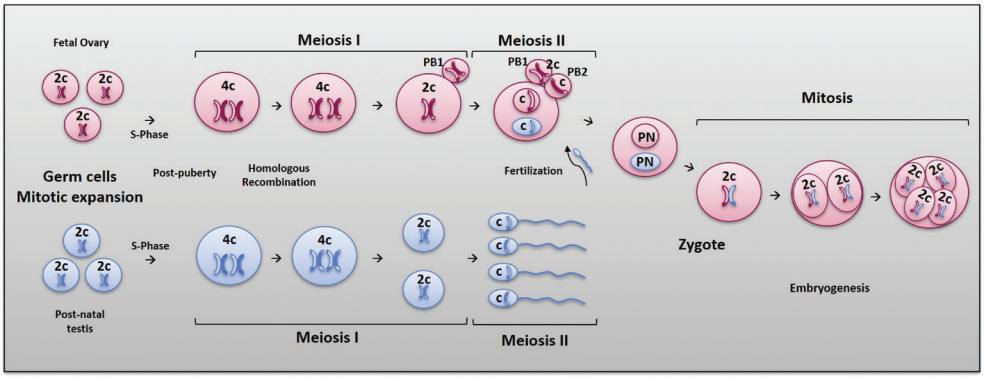

Figure 1. Cell divisions leading to the formation of the zygote. Division occurring in the female (pink) and male (blue) pre-meiotic germ cells, going through the first and second meiotic divisions (Meiosis I and II), followed by zygote formation after fertilization and the early stages of embryogenesis. c, haploid chromosome number; PB, polar body; PN, pronucleus.

While spermatogenesis is a continuous process with recurring waves of meiosis, mammalian eggs remain arrested in prophase one of meiosis until a surge of luteinizing hormone (LH) triggers both ovulation of the dominant follicle and activation of the contained oocyte. The oocyte is then released from meiotic arrest and completes the first meiotic division. Meiosis I

is also called the "reductional division" because it consists of the segregation of the bivalents (homologous chromosomes) into two pairs of sister chromatids; one pair of sister chromatids remains in the oocyte cytoplasm, while the other pair is extruded into the first polar body (PB1). The second meiotic division is only triggered following fertilization by a spermatozoon, and involves the segregation of the dyad into two single chromatids (equational division), one of which is extruded into the second polar body (PB2) while the other remains in the oocyte. The completion of Meiosis II is followed by the formation of two parental pronuclei, which then fuse to initiate entry into the subsequent embryonic mitotic cell cycles. When either the sperm cell or the oocyte carries an incorrect number of chromosomes, an aneuploid zygote will result. Most embryonic aneuploidies arise from segregation errors during one of the two meiotic divisions within the oocyte [22, 29]. Segregation errors occur most frequently during the first meiotic division and consist of 1) non-disjunctions (NDJ), 2) pre-division or "premature separation of sister chromatids" (PSSC) and 3) reverse segregation (RS) [31, 32]. Meiotic non-disjunctions can occur during both the first and second meiotic divisions; an homologous chromosome ("true", "classic" or "MI" non-disjunction) or a single chromatid (MII non-disjunction) is wrongly dispatched to the oocyte or the polar body leading to hyper- or hypoploidy, respectively (Figure 2, B-C). Meiosis I NDJ occurs mainly in oocytes of young women (< 20 years old), involves the larger chromosomes (chr. 1-5) and decreases in frequency with maternal age [32, 33]. Meiotic pre-divisions and reverse segregation are mainly caused by a lack of cohesion between the sister chromatids. Meiotic pre-divisions involve a premature breaking down of the bivalent (MI) or dyad (MII), during the first or second division, respectively, leading to the generation of two separated pairs of sister chromatids (univalents) or two single chromatids, which can act as independent units, increasing the risk of mis-segregation (Figure 2, D-E). Reverse segregation consists of a precocious separation of the sister chromatids of both homologues during meiosis I, the sister kinetochores of which form bipolar attachments generating a mature oocyte containing two non-sister chromatids that may segregate randomly during Anaphase II but can still generate a normal haploid oocyte. Both PSSC and RS are mainly observed in the oocytes of women of advanced maternal age (> 33 years old), and primarily affect the acrocentric chromosomes (chr. 13-15 and 21-22) [32, 33].

While it is known that NDJ occurs more commonly in young women whereas PSSC and RS are more prevalent in older women, the mechanisms and underlying defects leading to these errors are unclear. It is probable that various molecular mechanisms contribute at different levels to generate errors in chromosome segregation during meiosis. In the case of maternal

aging, the mechanisms that seem to play the most important roles are: a) Loss of inter-chromosomal cohesion; b) Abnormalities in Chromosome-Spindle interaction; and c) Lack of stringency of the Spindle Assembly Checkpoint (SAC) mechanism.

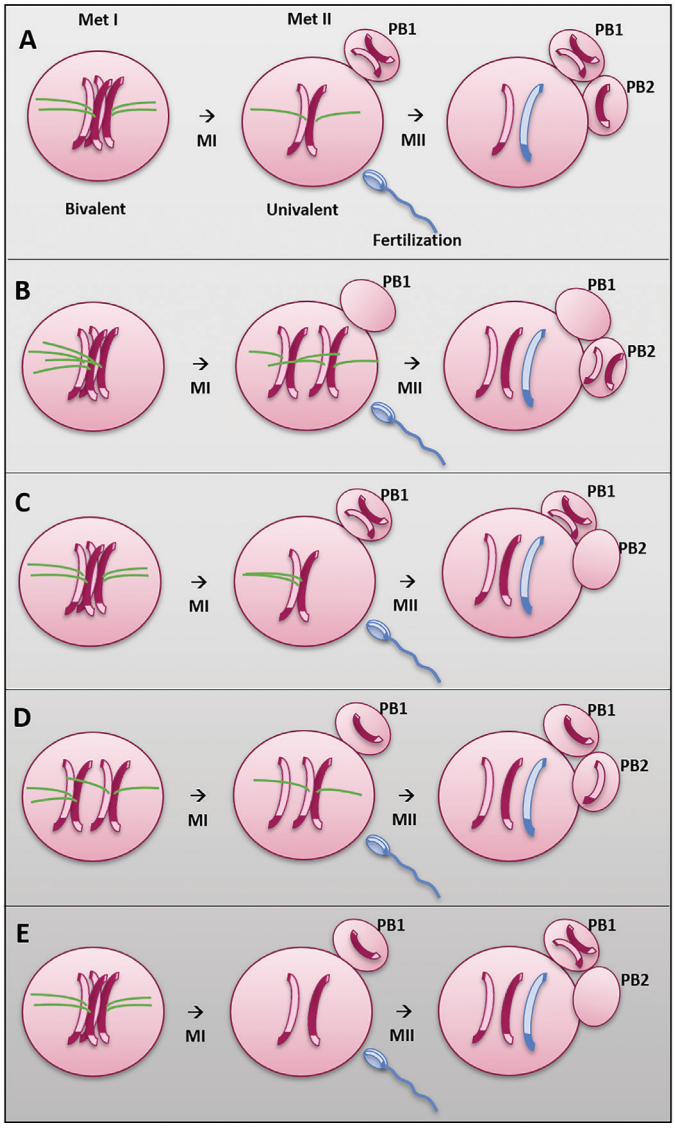

Figure 2. Schematic representation of some of the mechanisms leading to oocyte aneuploidy (e.g. trisomy). (A) normal chromosome segregation leading to a euploid zygote. (B) Meiosis I non-disjunction: homologous chromosomes fail to segregate, remaining in the oocyte following meiosis I. (C) Meiosis II non-disjunction: failure of the two sister chromatids to segregate at meiosis II. (D) Meiosis I pre-division: premature breaking down of the bivalent leading to the generation of two separated pairs of sister chromatids (univalents), which can segregate intact or prematurely divide into chromatids during meiosis I and randomly segregate during meiosis II, resulting in a trisomic zygote. (E) Meiosis II pre-division: premature separation of the sister chromatids (dyad) prior to Anaphase II; the two single chromatids may act as single units and potentially mis-segregate at anaphase II,

Maternal aging and loss of cohesion

During the first meiotic division, the chromosome bivalents must be held together in order to ensure a pairwise alignment and correct orientation prior to the onset of anaphase I. Homologous chromosomes are held together by having undergone recombination events (crossover) during the replication phase, which gives rise to DNA-links at the crossover sites, and by having the sister chromatids locked together by cohesins. The cohesion complex is composed of different subunits important for the maintenance and regulation of chromosomal cohesion. Cohesins were first discovered in mitotically dividing cells and identified as members of the structural maintenance chromosome (SMC1 and SMC3) and kleisin (RAD21) protein families, and the Scc3 (also known as its two isoforms SA1 and SA2). So far, five different proteins have been identified as meiotic-specific cohesion subunits encoded by different genes. Specifically, the meiotic paralogs in mammals for SMC1, RAD21 and SA1/SA2 have been reported and named as SMC1β, REC8 and STAG3, respectively [34]. These proteins form complexes that function as a molecular "glue", acting as a ring-like structure that surrounds and holds together the sister chromatids. By the time of the first meiotic division, the anaphase-promoting complex/cyclosome (APC/C) is activated and ubiquinates its target proteins (Cyclin B and Securin) whose degradation, in turn, activates the enzyme Separase. Separase is responsible for the cleavage of the meiotic-specific cohesin REC8, leading to the separation of the homologous chromosomes [35, 36]. However, to avoid premature separation of the single chromatids, cohesion must be maintained at the centromere region prior to the activation of anaphase II. The protection of this centromeric cohesion is provided by the Shugoshin proteins (Sgo1 and Sgo2) [36]. In particular, the shugoshins are thought to localize on the centromeric and pericentromeric region of the chromatids and play a crucial role in the centromeric localization of protein phosphatase 2A (PP2A), with which they cooperate to counteract the phosphorylation of Rec8 by Separase [37-39].

Any disturbance in the function of the cohesin complex results in weakened cohesion between sister chromatids leading to premature and/or incorrect separation, which can in turn lead to aneuploidy. It is generally thought that, in mammalian oocytes, cohesins are synthesized and established around the time of birth, with minimum turnover during post-natal life [40-43]. Therefore, it is possible that species with a prolonged period of meiotic arrest are more susceptible to a gradual loss and/or reduction in function of the cohesion complex. Many research groups have focused on age-related disfunction of chromosome cohesion in human oocytes, and via mouse models. The results obtained so far have shown that, in case of advanced

maternal age, there is a clear association between aneuploidy and a reduction in expression of cohesins (Rec8, SA3, SMC1b), an increase in the distance between the sister kinetochores (i.e. a greater interkinetochore (iKT) distance), and a decline in the activity of the proteins required to prevent precocious removal of the cohesins. [42, 44-49]. Moreover, *in vitro* maturation and maternal aging are known to influence the sensitivity of the oocytes to mitochondrial damage, and therefore increase the risk of oxidative damage by reactive oxygen species (ROS); this has been reported to lead to chromosome segregation errors by inducing premature loss of cohesion [50-54]. Despite the clear involvement of cohesin disfunction in the generation of meiotic aneuploidies, it is still unclear which molecular mechanisms steer this process.

Chromosome segregation and spindle assembly

During both mitotic and meiotic chromosome segregation, the spindle plays a central role. The spindle is a transient cytoskeletal structure formed by dynamic microtubules that interact with chromosome-kinetochores (molecular protein complexes located at the centromere of each chromatid) and ensure correct chromosome segregation. Spindle assembly requires the generation of a microtubule network arranged in a barrel-fusiform shape, with the chromosomes distributed along its equator (metaphase plate) and connected by the appropriate microtubules to one of the two opposing spindle poles. Although the spindle serves the same purpose during meiosis and mitosis, meiotic and mitotic spindle assembly differ from each other in a number of aspects. In somatic cells, spindle assembly is mainly organized by the two centrosomes, each of which regulates the formation of one of the two spindle poles. The centrosomes are each composed of two centrioles and are surrounded by a pericentrosomal matrix. These structures act together as the main microtubule organizing centers (MTOCs). Oocytes of many species, including mammals, lose their centrioles during oogenesis, and they therefore need to adopt alternative acentrosomal strategies to assemble the spindle during meiosis [55, 56]. In the absence of centrosomes, chromatin has been shown to actively induce microtubule nucleation [57] using the RanGTP- and CPC (Chromosomal Passenger Complex)-based chromatin-dependent pathways [58]. RanGTP has been proposed to be the dominant mechanism for microtubule formation in human oocytes and has been well described in Xenopus extracts [59]. RanGTP promotes the release of various spindle assembly factors (SAFs) in close proximity to the chromosomes, which are in turn responsible for the nucleation of the microtubules. In contrast to the centrosome-guided spindle formation in somatic cells, the microtubules nucleated at the chromosomes follow an “inside out” direction; they are initially orientated

randomly, but are subsequently organized into a parallel and bipolar form by the activity of various microtubular proteins (motor and kinesin-like proteins), yielding the classical barrel shaped spindle [31, 56, 60]. During acentrosomal spindle assembly, not only the chromatin but also the kinetochores have been shown to nucleate microtubules, most likely by a similar RanGTP-pathway; however, the molecular recruitment and signaling of kinetochore-driven nucleation still needs to be demonstrated and is not fully understood [61, 62]. Mouse oocytes have developed an alternative route for spindle organization involving multiple non-centrosomal MTOCs that each assembles microtubule aster-like structures. These MTOCs accumulate around the oocyte nucleus and, after nuclear envelope breakdown (NEBD), they form a dense microtubule array onto which the chromosomes are arranged. The activity of the antiparallel motor kinesin-5, together with the migration and sorting of the acentrosomal MTOCs to two opposite poles, establishes spindle bipolarity, elongating the initial compact array into a barrel form with large poles [63]. In horses, MTOCs have not been identified and a chromosome-mediated origin of microtubule nucleation, similar to that in human oocytes, has been proposed [64].

Formation of the spindle is not the only process that differs between meiosis and mitosis. Mammalian female gametes are the largest cells in the body, and meiotic divisions within oocytes are asymmetrical in order to produce a single fertilizable egg containing large stores of the mitochondria, RNAs and proteins required for early embryo development [65]. This makes the oocyte an even more unusual environment in which to form a spindle, where the main initial objective is to bi-orientate the homologous chromosomes. During mitotic spindle assembly, the interactions between the microtubule plus-ends emanating from the spindle poles and attaching onto the chromosome kinetochores, enable chromosome movement towards the metaphase plate and the bi-orientation of the two sister chromatids attached to opposing poles. Once anaphase takes place, a canonical shortening of kinetochore-microtubules due to microtubule depolymerization at both the poles and at the kinetochores, generates a poleward pulling-force that segregates the chromosomes, moving them toward the corresponding pole and guaranteeing a correct partitioning of the genetic material to the respective daughter cells [66]. While this process is relatively straightforward and applicable to what happens during the second meiotic division, meiosis I has the important and unique task of pairing and segregating homologous chromosomes, a process that requires that the kinetochores of both sister chromatids remain paired and act as a single unit when attaching to the microtubules deriving from the appropriate spindle pole, forming a so-called amphitelic attachment (Figure 3) [67].

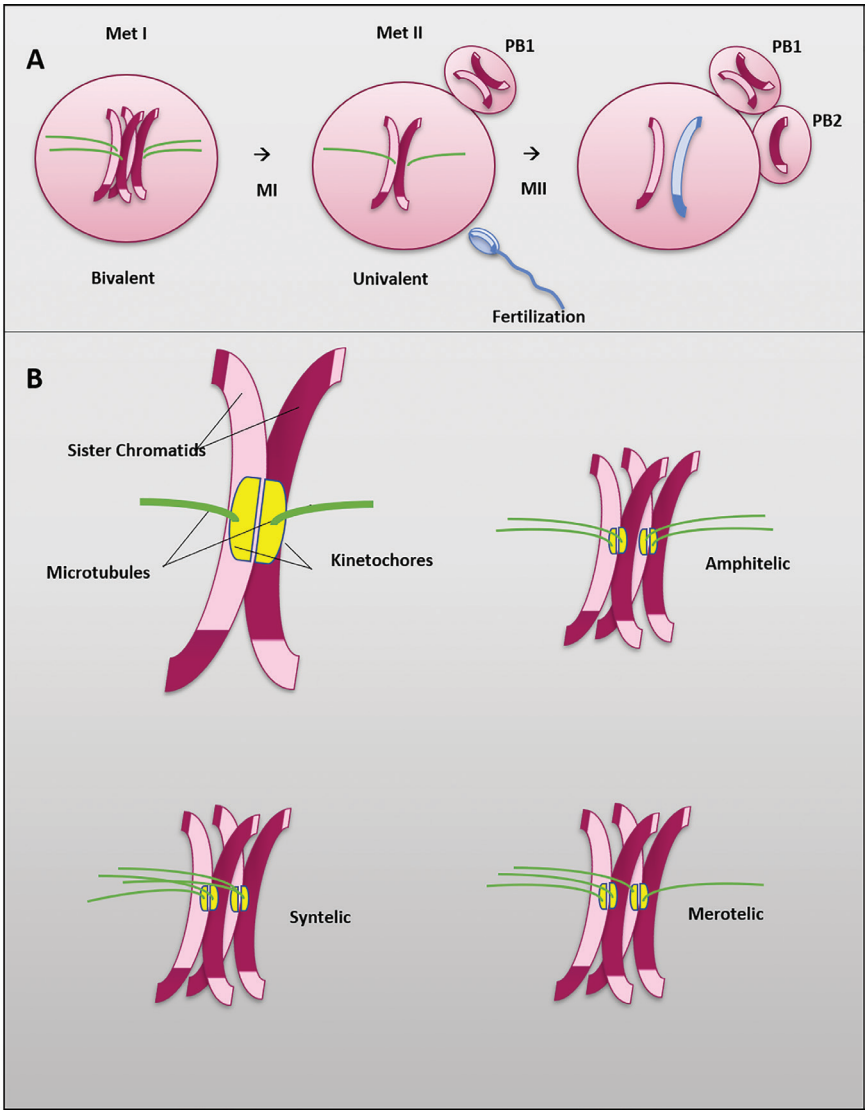

Figure 3. Schematic representation of kinetochore-microtubule attachments. (A) normal chromosome segregation during meiosis I and II. (B) Sister chromatid kinetochore attachment to the opposite spindle poles is supposed to take place during metaphase of the second meiotic division. During Metaphase I, each kinetochore pair within a homologous chromosome bivalent has a monopolar attachment, with the two sister kinetochore pairs directed to opposite spindle poles (amphitelic attachment). Syntelic (both pairs of sister kinetochores directed towards the same spindle pole) and merotelic (one of the kinetochore pairs attaches to both spindle poles simultaneously) attachment lead to chromosome mis-segregation and MII aneuploidy. MI, meiosis I; MII, meiosis II; Met I, metaphase I; Met II, metaphase II; PB, polar body.

The correct interaction between microtubules and kinetochores is a delicate and important step, not only in ensuring the correct alignment of the homologous chromosomes or sister chromatids on the metaphase plate, but also in guiding their separation towards the appropriate spindle pole. More specifically, kinetochore and microtubules (K-MTs) must interact with each other to form a bond between the outer layer of the kinetochore and the MT plus-end fibers. Both the "outer kinetochore" and MTs are highly dynamic and unstable and, before a properly stabilized attachment can be achieved, they go through multiple rounds of "forming and destroying" the K-MT attachment, until all chromosomes are both attached and correctly biorientated [68-70]. Considering the instability of the K-MT attachments, it is not surprising that incorrect attachment of the kinetochore to the microtubules during meiosis I and II is considered a cause of lagging chromosomes and mis-segregation events. Furthermore, maternal aging appears to impact microtubule architecture and dynamics, resulting in the formation of transient multipolar spindles, which although they recongregate into bipolar structures, often fail to generate merotelic attachments, leading to mis-alignment and chromosome mis-segregation [71-73]. Therefore, under these circumstances, correct attachment and biorientation becomes a significant challenge. However, to make this process more efficient, cells have developed a surveillance mechanism to identify improper attachment and allow correction of any mistakes. This control machinery is known as the Spindle Assembly Checkpoint (SAC), or simply the "mitotic checkpoint".

Maternal aging and Spindle Assembly Checkpoint stringency

The Spindle Assembly Checkpoint is a control mechanism studied extensively in somatic and cancer cells, and involved in the surveillance of faithful chromosome segregation and cell cycle progression. The main task of the SAC is to prevent anaphase onset until all of the chromosomes are correctly aligned and biorientated, i.e. each kinetochore is properly attached via the microtubules to the respective pole. In the event of errors in kinetochore-microtubule attachment, such as missing or tensionless attachments, the SAC will inhibit anaphase activation and promote error correction (Figure 4) [68]. Although less stringent and with some differences, the SAC has also been shown to be active during meiosis, sharing many of the general functions and mechanisms described for mitosis [74].

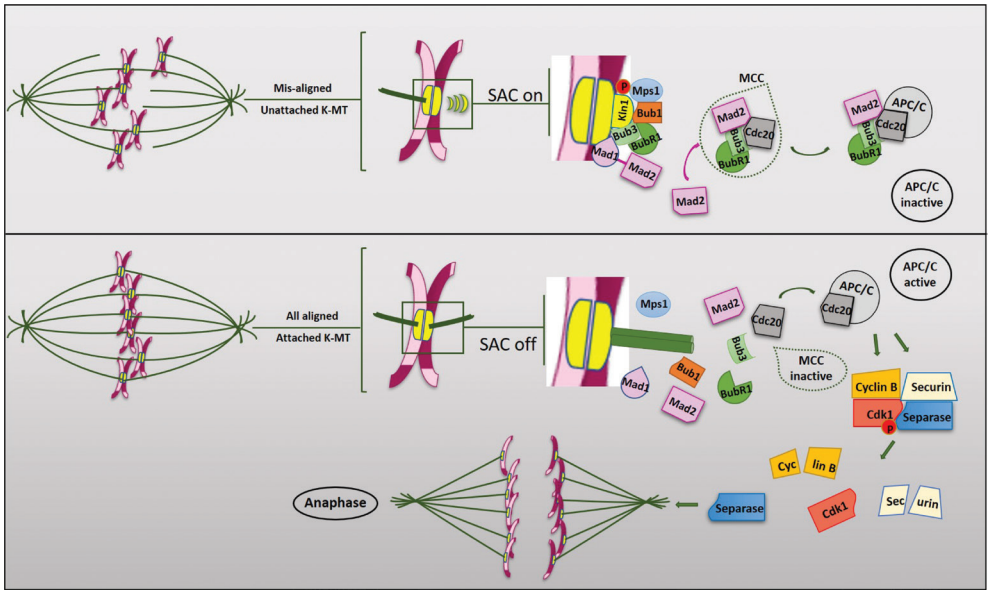

Figure 4. Schematic representation of the Spindle Assembly Checkpoint (SAC) pathway. Mis-aligned and unattached kinetochore-microtubules (K-MT) induce activation of the SAC inhibitory signal. Autophosphorylation of Mps1 triggers the cascade recruitment of Bub3, Bub1 and Mad1. The homodimer Mad1 binds to Mad2, acting as a catalytic unit responsible for the activation and incorporation of further molecules, Mad2 and Ccd20, that together with preformed Bub3 and BubR1, form the Mitotic Checkpoint Complex (MCC) responsible for the inactivation of the anaphase-promoting complex/cyclosome (APC/C). Once proper chromosome alignment is achieved and all the K-MTs are attached, the SAC signal becomes silent. The inactivation of the MCC allows the APC/C-Cdc20 to degrade their targets, Cyclin B and Securin, leading to inactivation of Cdk1 and activation of Separase, thus allowing anaphase to proceed. Mps1, multipolar spindle 1; Bub3, budding uninhibited by benzimidazoles 3 homolog; Bub1, budding uninhibited by benzimidazoles 1 homolog; BubR1, budding uninhibited by benzimidazoles 1 homolog β; Mad1, mitotic arrest deficient 1; Mad2, mitotic arrest deficient-like 1; Cdc20, cell division cycle 20; Cdk1, cyclin-dependent kinase 1; P, phosphorylation.

The SAC-derived block of the transition to anaphase relies on the inhibition of the anaphase promoting complex/cyclosome (APC/C) activator CDC20 (Cell Division Cycle protein 20) by the Mitotic Checkpoint Complex (MCC), which is the final effector of the entire inhibition-signaling pathway. The MCC consists of the proteins MAD2, BUBR1, BUB3 and CDC20, the recruitment and assembly of which depends on BUB1 and MAD1, and is mainly orchestrated by the kinase MPS1. The latter has been described as the master regulator of the SAC [68]. MPS1 accumulates at the kinetochores during early prometaphase, and its activation by autophosphorylation triggers the onset of a cascade mechanism essential for the functioning of

the SAC, in particular through its important interactions with the KMN network (the predominant K-MT binding complex consisting of the three sub-complexes KLN1-C, MIS12-C and NDC80-C). NDC80-C-dependent recruitment of MPS1 at the kinetochore occurs in a MT-binding-dependent manner, and its activated form phosphorylates the outer-kinetochore protein, KLN1, inducing the formation of phospho-docking sites for BUB3 which, in turn, brings along its paralog BUB1 and, by proxy, BUBR1 and MAD1 [51]. The homodimer MAD1 binds to MAD2 yielding a catalytic unit responsible for the activation and incorporation of further molecules, MAD2 and CDC20, to the preformed BUB3 and BUBR1, forming the MCC complex which is able to diffuse throughout the cytosol and inhibit the APC/C. The temporary SAC-dependent block of anaphase guarantees the time required to correct the K-MT attachment errors. In particular, the error-correction machinery is orchestrated by the kinase Aurora B (AURKB) and its meiosis-specific homolog Aurora C (AURKC). Through their tension-sensing function, this family of kinases recognizes incorrect K-MT embeddings, and induces their cleavage by phosphorylating components at the interface of the microtubule-kinetochore interaction, thus promoting correction. For a more extensive review, see references [68], [75] and [76].

From the description above, it is clear that the SAC is critical to ensuring the proper segregation of chromosomes; conversely, any malfunction of one of the SAC components could predispose to chromosome mis-segregation and aneuploidy. For this reason, various studies on human and murine oocytes have focused on the cell-checkpoint functions during the first and second meiosis, and have proven that loss of SAC proteins is strikingly correlated with an increased incidence of mis-segregation, leading to the hypothesis that a weakened SAC is one of the age-related predispositions to aneuploidy [77-80].

Aneuploidy and early mitotic divisions

Chromosomal instability is a well-known issue affecting human IVF embryos. Although embryonic aneuploidy often has a meiotic origin, post-zygotic aneuploidy is also relatively common during early embryo development. Studies based on genome-wide screening of individual blastomeres have revealed that more than 70% of cleavage-stage human IVF embryos contains cells with complex large-scale structural chromosomal imbalances [81]. While aneuploidies of meiotic origin are characterized by all cells of an embryo showing the same abnormal chromosome number, post zygotic aneuploidy is distinguished by chromosomal mosaicism, where a single embryo contains a complement of both normal (euploid) and

abnormal (aneuploid) cells [82], and which arises from chromosome segregation errors during mitosis of one of the first few cleavage divisions after fertilization [83]. Mosaicism may involve the loss or gain of whole chromosomes (such that the resulting cells will be hypo- and hyperploid respectively), sub-chromosomal segmental imbalances (segmental aneuploidy), and complex chromosomal rearrangements characterized by a chaotic pattern of multiple aneuploidies involving several abnormal cell lines. Furthermore, a wide range of chromosomal errors has been detected in human embryos, not only during the first cleavage divisions but at all stages of pre-implantation development. The reason why these first divisions are more error-prone is still obscure and the exact mechanisms that modulate the risk of mitotic chromosome segregation errors, or ensure removal of compromised cells, are poorly understood and still under investigation [84]. However, it is likely that multiple factors, including advanced maternal age may predispose to post-zygotic (mitotic) aneuploidies in human embryos [85].

Different mechanisms have been listed as possible causes of mitotic aneuploidy; abnormal zygotic spindle formation, chromosome fragmentation, endoreplication, failed or asymmetric cytokinesis, blastomere fusion and chromosome breakages. However, anaphase lagging and non-disjunction in mitosis are thought to be the two most common errors leading to mitotic aneuploidies in human embryos (Figure 5) [84].

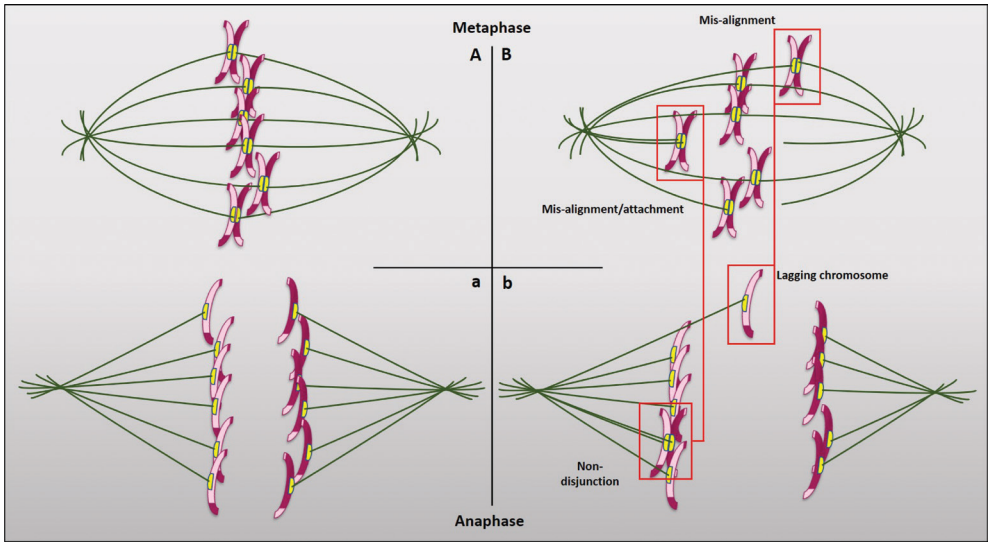

Figure 5. Representative scheme of chromosome misalignment/wrong kinetochore-microtubule attachment during anaphase lagging and non-disjunction of the chromosomes, responsible for mitotic aneuploidies in embryos. (A) correct kinetochore-microtubule attachment and alignment of the chromosomes on the metaphase plate prior to anaphase onset; (a) normal chromosome segregation and partitioning during anaphase; (B) and (b) misaligned chromosomes (not aligned on the metaphase plate) and incorrect K-MT attachments (both kinetochores attached to the same spindle pole) leading to a lagging chromosome or non-disjunction during mitotic anaphase, resulting in an aneuploid embryo.

Anaphase lagging during mitosis has been ascribed to chromatids showing defects in their movement, leading to them falling behind during anaphase probably due to an improper connection to the spindle apparatus. Consequently, the lagging chromatid is not incorporated into the nucleus of one of the daughter cells, resulting in a monosomy in one of the two daughter cells. If the lagging chromosomes remain separate from the main group of chromosomes at the time of nuclear envelope reformation, they result in the formation of small nucleus-like structures known as micronuclei (MN), which are a hallmark of chromosomal instability and DNA damage [86]. Similar to what has already been described for meiosis II, mitotic non-disjunction is the failure of the sister chromatids to separate from each other, resulting in daughter cells with a loss or gain of a whole chromosome.

Proper spindle assembly during mitosis, with all the duplicated chromosomes aligned along the metaphase plate and the respective kinetochores correctly linked through the microtubules to

the opposite poles, guarantees the accurate partitioning of the genomic material over the daughter cells. However, the first zygotic divisions are far from being simple examples of mitosis. It has been suggested that the higher incidence of mitotic errors during the first cleavage divisions may relate to the fact that these divisions are directed by the cellular machinery inherited from the oocyte, since embryonic genome activation (active mRNA transcription and protein translation) only starts (depending on species) at around the 4-8 cell stage [87]. Thus, the first cell cleavage divisions rely on maternally stored cytosolic components which, unlike DNA, are divided together with the cytoplasmic material during the first divisions, making faithful chromosome segregation increasingly challenging. Furthermore, during the first zygotic division, the maternal and paternal genomic material is replicated independently within the two distinct parental pronuclei. After nuclear envelope breakdown, it appears that two separate spindles form, one for each parental pronucleus. The axes of these two spindles then have to align before the onset of anaphase in order to guarantee correct chromosome segregation. Failure of correct congression may lead to a tripolar division, formation of four haploid cells or to the formation of multinucleate two-cell embryos. In addition, spindle formation during the first division does itself not follow the canonical path. As described above, in somatic cells bipolar spindle assembly is driven by two centromeres, each formed of a pair of centrioles surrounded by pericentriolar matrix. Although oocytes lose their centrioles during oogenesis, in man and the horse, the zygote inherits two centrioles from the sperm cell at fertilization. However, although these centrioles duplicate during the pronucleus phase (forming two centrosomes), the ability of these centrosomes to nucleate microtubules, and their contribution to the formation of a first spindle encompassing both maternal and paternal chromosomes is still unclear. The lack of a good animal or cellular model is one of the major limitations to the study of chromosome segregation errors during early mammalian cleavage divisions. In fact, aneuploidy is relatively infrequent in mouse embryos, while the oocyte-zygote transition cannot be studied in embryonic stem cell lines. Recent studies have shown that equine embryos are also affected by mosaicism and aneuploidy [27]. This, together with many other analogies between reproductive physiology in mares and women (both are monovulatory / uniparous, and have similar durations of ovarian follicular waves and reproductive senescence, etc. [88]), has increased the interest in the use of the horse as an alternative, and more natural, animal model for the study of the effect of advanced maternal age on the origin of aneuploidy in oocytes and embryos.

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2

Chapter 2

Advanced mare age impairs the ability of *in vitro*-matured oocytes to correctly align chromosomes on the metaphase plate

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ABSTRACT

Advanced mare age is associated with declining fertility and an increased risk of early pregnancy loss. Compromised oocyte quality is probably the primary reason for reduced fertility, but the defects predisposing to embryonic death are unknown. In women, advanced age predisposes to chromosome segregation errors during meiosis, which lead to embryonic aneuploidy and a heightened risk of miscarriage. The aim of the study was to evaluate the effect of advanced mare age on chromosome alignment and meiotic spindle morphology in *in vitro*-matured (IVM) oocytes.

To investigate differences in spindle organization and chromosome alignment between young and old mares, oocytes collected from slaughtered mares were divided into two groups depending on mare age (young, ≤14 years and old, ≥16 years), IVM and stained to visualize chromatin and alpha-tubulin. Spindle morphology, morphometry and chromosome (mis)alignment were evaluated by confocal microscopy and 3D image analysis. Oocytes from old mares showed a higher incidence of chromosome misalignment (47.4% vs. 4.5%; $P<0.001$) and a thicker metaphase plate (mean ± s.d.: 5.8 ± 1.0 µm vs. 4.9 ± 0.9 µm; $P = 0.04$) than oocytes from young mares. Although no differences in spindle morphometry were detected between old and young mares, an increased major spindle axis length was associated with chromosome misalignment (mean ± s.d.: 25.3 ± 6.1 µm vs. 20.8 ± 3.3 µm; $P = 0.01$) irrespective of age. Advanced mare age predisposes to chromosome misalignment on the metaphase II spindle of IVM oocytes. The compromised ability to correctly align chromosomes presumably predisposes to aneuploidy in resulting embryos and thereby contributes to the age-related decline in fertility and increased incidence of early pregnancy loss.

INTRODUCTION

Advanced mare age (in particular beyond 14 years) is associated with a decline in fertility [1-3]. This manifests as a lower success of fertilization in older (81%) than younger mares (96%) [4], and a trebling of the incidence of pregnancy loss between Day 12 and Day 60 in mares aged 18 years or older (30%) compared with mares younger than 12 years (10%) [5-8]. Estimated pre-pregnancy detection losses (between Day 2 and Day 14 after ovulation) are also significantly higher in old (62%; 19.4 ± 1.0 years) than in young mares (9%; 5.7 ± 0.3 years) [4]. The cause(s) of this decline in fertility and associated increase in the incidence of pregnancy loss are likely to be multifactorial; nevertheless, intrinsic oocyte defects are thought to be a major contributor, because transferring oocytes from old mares into the oviduct of younger recipients does not improve the likelihood of fertilization or reduce the risk of pregnancy loss [5]. During development, oocytes undergo two rounds of chromosome segregation (Meiosis I and Meiosis II) to produce a haploid gamete competent to develop into a diploid zygote and embryo, after fusion with the paternal haploid set of chromosomes introduced by the spermatozoon during fertilization. During oocyte meiosis, the formation of Metaphase I (MI) and Metaphase II (MII) spindles and accurate alignment of chromosome pairs (homologous chromosomes in MI; sister chromatids in MII) on either side of the equatorial plate are required for the correct segregation of the chromosomes between the oocyte and the polar bodies, to ensure genomic integrity. In women, advanced age predisposes to chromosome segregation errors in oocytes during both meiotic divisions, which in turn leads to an
increased incidence of embryonic aneuploidy, early miscarriage and birth defects such as Down syndrome [9, 10]. In particular, advanced maternal age seems to impair the ability of the oocyte to detect and correct the presence of misaligned chromosomes [10]. While *in vitro* maturation has been shown to compromise the process of chromosome segregation in mares, the effect of maternal age on the ability to correctly form a spindle and align the chromosomes is unknown [11]. The purpose of this study was to determine whether advanced mare age affects spindle morphology and interferes with proper alignment of chromosomes in *in vitro*-matured MII oocytes.

MATERIALS AND METHODS

Oocyte collection and culture

Ovaries were recovered from slaughtered mares within 15 min of death and divided into two groups, depending on the age of the mare (young, ≤14 years and old, ≥16 years) as determined by examining the microchip number and associated passport. Ovaries were packaged at 21–25°C as previously described for short shipment [12] and transported to the laboratory within 5 h. Cumulus–oocyte complexes (COCs) were then immediately recovered from the ovaries by scraping the follicle wall with a bone curette and flushing the dislodged cells with embryo flushing medium (Euroflush, IVM Technologies, Leeuwarden, the Netherlands), as described by Hinrichs et al. [13]. Only follicles ≤30 mm were selected for scraping, to avoid the collection of oocytes that had already matured. Recovered COCs were evaluated with a dissecting microscope at 10–60x magnification, and only oocytes with at least one layer of intact cumulus cells were used, irrespective of the morphology of the cumulus (expanded or compact); denuded oocytes were discarded. COCs were then matured in a 50:50 mixture of Dulbecco's minimal essential medium (DMEM) and Ham's F12 (GIBCO BRL Life Technologies, Bleiswijk, the Netherlands) supplemented with 10 % fetal calf serum (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) 0.125 µg/mL epidermal growth factor (Prepotech Inc., Rocky Hill, New jersey, USA), 0.1 IU/mL follicle stimulating hormone (Sigma-Aldrich), 0.6 mmol/L cysteine (Sigma-Aldrich), 0.1 mmol/L cysteamine (Sigma-Aldrich), 0.1% insulin (Coming Life Science, New York, USA), 0.1% transferrin (Coming Life Science) and 0.1% sodium selenite (Coming Life Science) for 26 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. After maturation, cumulus cells were removed by brief exposure to Hepes-buffered synthetic oviduct fluid (H-SOF; Avantea, Cremona, Italy) containing 1 µg/mL of hyaluronidase (Sigma-Aldrich) followed by gentle pipetting through a fine bore pipette. Only oocytes displaying a first polar body were used for further analysis.

Oocyte fixation and immunostaining

Oocytes were fixed in microtubule-stabilizing solution for 1 h at 38°C (medium M [14]), followed by 2% paraformaldehyde (Coming Life Science) for 30 min at room temperature and stored in phosphate-buffered saline (PBS; B. Braun, Hessen, Germany) at 4°C until further processing. Prior to staining, the oocytes were washed three times in PBS containing 3 mg/mL polyvinylpyrrolidone (Sigma-Aldrich) (PBS-PVP) for 5 min. Next, they were incubated

overnight at 4°C in PBS containing a 1:250 dilution of a mouse monoclonal anti-a-tubulin antibody (T5168, Sigma-Aldrich) to label the microtubules in the spindle. The oocytes were then washed twice in 0.1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 5 min and incubated for 1 h at room temperature in a blocking solution containing 0.1 mol/L glycine (Sigma-Aldrich), 1% goat serum (Sigma-Aldrich), 0.01% Triton X-100 (Sigma-Aldrich), 0.5% BSA and 0.02% sodium azide (Sigma-Aldrich) in PBS. Thereafter, the oocytes were incubated for 1 h at 37°C in PBS containing 0.5% Triton X-100, 0.5% BSA and 1:100 goat anti-mouse Alexa Fluor 488 antibody (A11029; Invitrogen Corp., Carlsbad, California, USA). Following two 5 min washing steps in PBS supplemented with 0.1% BSA and 0.1% Triton X-100 and one in PBS alone, the oocytes were incubated for 30 min at room temperature in PBS-PVP containing 1:100 Hoechst 33342 (Sigma-Aldrich) to label the DNA, washed in PBS-PVP for 5 min and mounted on glass slides (Superfrost Plus, Menzel, Braunschweig, Germany) with anti-fade mounting medium (Vectashield; Vector Laboratories, Burlingame, California, USA).

Image acquisition and analysis

Image acquisition was performed using a Leica TCS-SP5 confocal laser scanning microscope equipped with a 639 objective (Leica Microsystem, Wetzlar, Germany). Hoechst 33342 was stimulated with a 351-nm laser, and emission was detected between 414 and 466 nm (blue channel), Alexa Fluor 488 was separately stimulated with a 488-nm laser, and emission was detected in the 511–577 nm range (green channel). The microtubular spindle, labelled with Alexa Fluor 488, and the chromosomes, labelled with Hoechst 33342, were identified using sequential confocal sections (Z-stacks) at 0.42-µm intervals. A three-dimensional (3D) image of the spindle and chromosomes was then created and analyzed using Imaris 8.2 software (Bitplane AG, Zurich, Switzerland). The spindle was assessed for gross morphological parameters (Table 1). The shape of the spindle was classified as ‘normal’ if it was bipolar and fusiform, or ‘abnormal’ if it was tri- or tetrapolar or severely misshapen. Chromosomes were considered to be misaligned when they were displaced by ≥2 µm from the metaphase plate, as described by Coticchio et al. [15]. Chromosome misalignment was only assessed in morphologically normal spindles and was scored as absent if all of the chromosomes were properly aligned along the spindle equator, mild when up to five chromosomes were positioned ≥2 µm from the equator or severe when more than five chromosomes were distant from the equator (Table 1; Supplementary Items 1–3). The Imaris surface tool was used to render solid surfaces best representing both the spindle and the chromosomes, regardless of spindle

orientation (Supplementary Item 4). To create a 3D image of the meiotic spindle, the green channel was selected, a Gaussian smoothing filter was applied (detail level of 0.0853 µm), and a threshold for surface creation was selected on the basis of absolute intensity using 51 and 166 arbitrary units as lower and upper thresholds, respectively. The 3D image for the metaphase plate was created in a similar fashion by selecting the blue channel and using 27.2 and 213 arbitrary units, respectively, as the lower and upper thresholds for absolute fluorescence intensity. If the polar body was in close proximity to the spindle, this was filtered out on the basis of size and or position (x, y, z). Three-dimensional measurements of the spindle (pole-to-pole length, width and volume) were taken from the ellipsoid statistics (Figure 1). DNA dispersion over the metaphase plate was assessed by creating a three-dimensional bounding box (a rectangular prism) around the metaphase plate and by measuring the width of this box (the length of the side parallel to the spindle's main axis) (Figure 1). Misaligned chromosomes were not included in the bounding box (Supplementary Item 4).

Data analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 24.0 software (IBM, Armonk, New York, USA). To analyze differences between groups, Fisher's exact test was used for categorical variables (spindle type and chromosome misalignment), and independent *t* tests were used to compare spindle and metaphase plate morphometric parameters. Significance was set at $P \leq 0.05$.

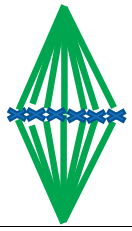
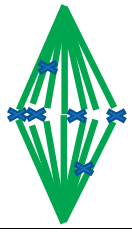
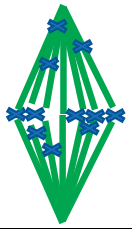
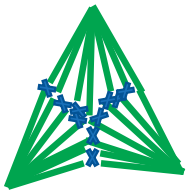
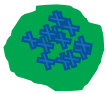
Spindle type	Definition	Diagrammatic representation
Normal without misalignment	**Bipolar fusiform shape with all chromosomes aligned at the equator**	
Normal with mild misalignment	**Bipolar fusiform shape with one to five chromosomes displaced from the equator**	
Normal with severe misalignment	**Bipolar fusiform shape with more than five chromosomes displaced from the equator**	
Tri- or tetra-polar	**Three or four defined poles**	
Severely misshapen	**Poorly defined or missing poles**	

Table 1. Diagram illustrating the classification of *in vitro*-matured mare oocytes into normal and abnormal Metaphase II spindle shapes and by chromosome alignment

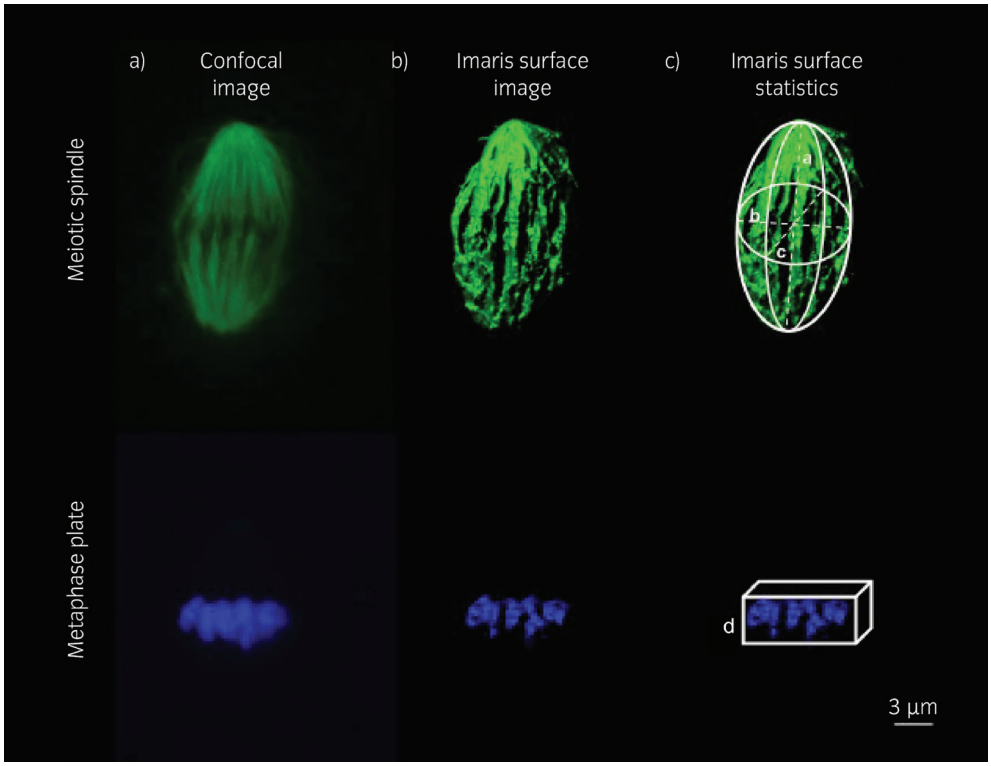


Figure 1. Example of surface rendering of the meiotic spindle and metaphase plate in *in vitro*-matured mare oocytes. a) Confocal image prior to processing; b) image after processing with the Imaris surface tool; c) illustration of the measurements used for the statistic (a, pole-to-pole length; b and c, spindle width; d, metaphase plate thickness). The scale bar is set at 3 µm.

RESULTS

A total of 303 COCs were recovered from 51 mares; 169 COCs from 27 young and 134 COCs from 24 old mares. Mare ages ranged between 2 and 14 years (mean ± s.d.: 7.5 ± 3.5 years) for the young group and between 16 and 27 years (mean ± s.d.: 19 ± 3.9 years) for the old group. No significant difference (P = 0.5) between the two groups was observed for the success of first polar body extrusion after maturation, with 75 of the 169 (44.4%) oocytes from young mares and 54 of the 134 (40.3%) oocytes from old mares reaching MII. Per group, 25 MII oocytes were randomly selected to analyze spindle morphology and chromosome alignment. Of these, three oocytes from the young group and six from the old group had to be excluded from the final analysis as a result of insufficient immunofluorescent staining of the chromatin.

Advanced mare age compromises the ability of *in vitro*-matured MII oocytes to align chromosomes

Representative images of spindles and metaphase plates are shown in Figure 2. MII oocytes from old mares showed a significantly higher incidence of chromosome misalignment (47%) compared with oocytes from young mares (5%) (Figure 3; $P<0.001$). Moreover, while misalignment in the young group was limited to a single oocyte with a single chromosome displaced from the equator, in the old group both mild misalignment (37%) and severe misalignment (11%) were detected.

MII oocytes from old mares have a thickened metaphase plate

DNA dispersion over the metaphase spindle was increased in oocytes from old mares, which showed a significantly ($P = 0.004$) thicker metaphase plate (mean ± s.d.: 5.8 ± 1.0 μm) than young mares (4.9 ± 0.9 μm) (Figure 4).

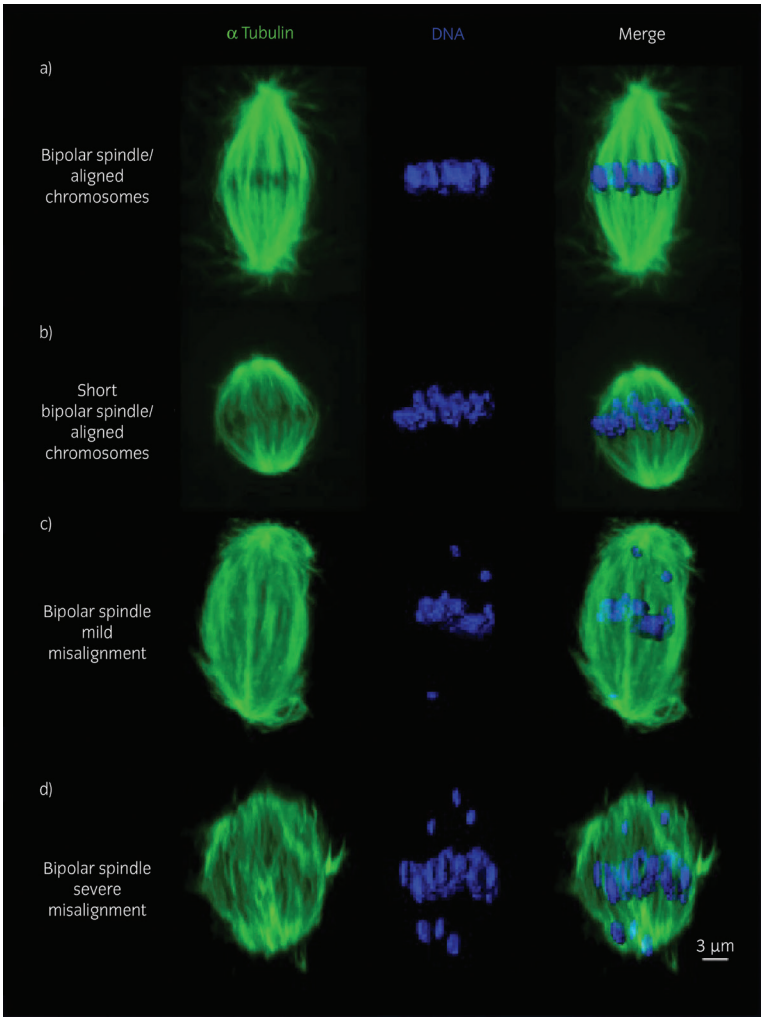


Figure 2. Representative images of a) a bipolar spindle with properly aligned chromosomes on the metaphase plate, from a young mare oocyte; b) bipolar spindle showing a reduced pole-to-pole length but with chromosomes properly aligned on the metaphase plate, from an old mare oocyte; c) bipolar spindle with increased length showing mild chromosome misalignment (≤5 displaced chromosomes), from an old mare oocyte; d) bipolar spindle showing severe chromosome misalignment (>5 displaced chromosomes), from an old mare oocyte. The scale bar is set at 3 µm.

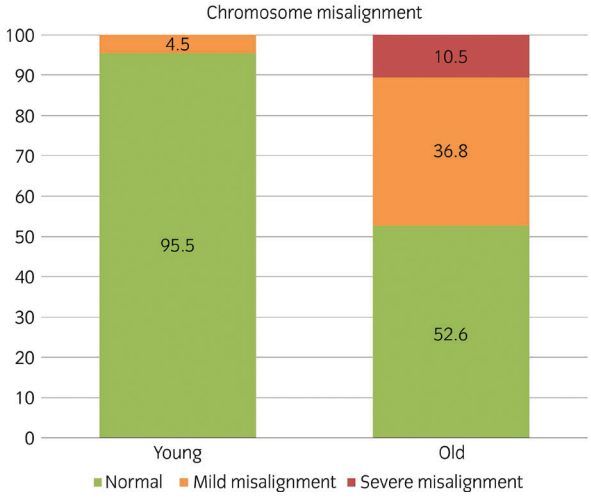


Figure 3. Frequency of chromosome misalignment in MII oocytes from young (≤14 years) and old (≥16 years) mares. Absent: all chromosomes aligned on metaphase plate; mild: 1-5 misaligned chromosomes; severe: >5 misaligned chromosomes.

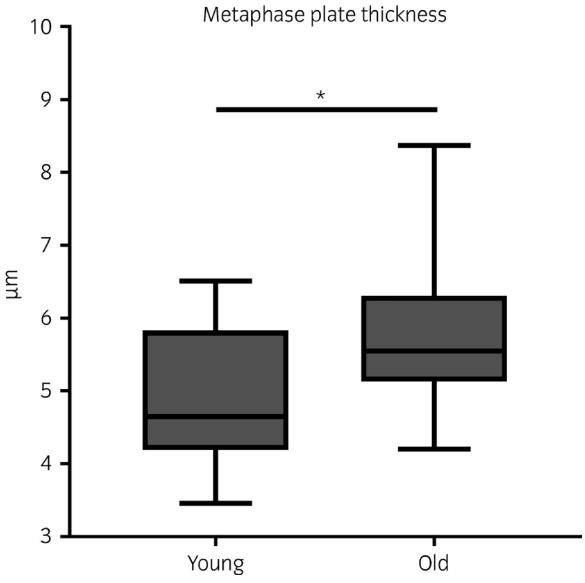


Figure 4. Box plot representation of metaphase plate thickness in the oocytes of mares of different ages (young: ≤14 years and old: ≥16 years). Asterisks indicate significant differences, $P<0.05$.

Spindle length is increased in oocytes displaying chromosome misalignment

All oocytes from young mares and all but one of the oocytes from old mares showed morphologically normal bipolar spindles. The one oocyte from an old mare with a grossly abnormal spindle displayed a tripolar spindle (Supplementary Item 5). Using the 3D image analysis, it was possible to examine spindle morphology methodically in detail, and to record parameters such as spindle major axis dimensions (axes a, b and c; Figure 1; Supplementary Item 4). Although no significant differences in spindle morphometric parameters were detected between oocytes from old and young mares (with a mean ± s.d.: length 21.5 ± 2.5 µm (P = 0.7) and width 12.3 ± 1.9 µm (P = 0.5) in young mare oocytes and 22.2 ± 6.0 µm and 11.8 ± 2.9 µm in old mare oocytes, respectively), the length of the major spindle axis was increased in oocytes displaying chromosome misalignment (mean ± s.d.: 25.3 ± 6.1 µm vs. 20.8 ± 3.3 µm; P = 0.05) irrespective of mare age (Figure 5). Consequently, spindle length was highly variable within the old mare group (range 11- 35 µm), while it was more consistent in the young mare group (range 17-28 µm). Moreover, spindles without misalignment were smaller in old mare than young mare oocytes. In fact, when oocytes with misaligned chromosomes were not considered, both spindle length (mean ± s.d.: 18.3 ± 4.2 µm vs. 26.6 ± 6.4 µm; P = 0.01) and width (mean ± s.d.: 10.4 ± 2.1 µm vs. 13.4 ± 3.2 µm; P = 0.01) in oocytes from old mares were significantly reduced compared with young mares (Figure 5; Supplementary Items 1 and 6).

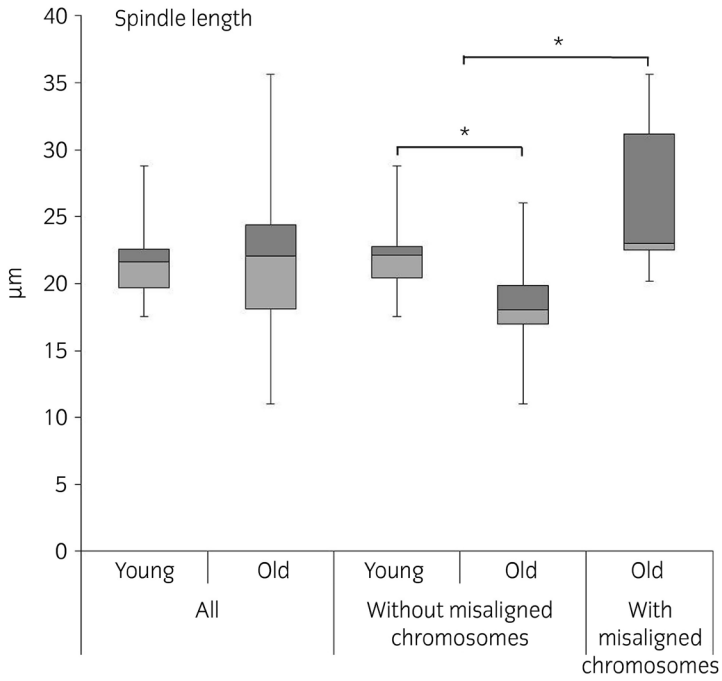


Figure 5. Box plot representation of spindle length (pole-to-pole) in the oocytes of mares of different ages (young: ≤14 years and old: ≥16 years) and morphological (aligned vs. misaligned chromosomes) classes. Asterisks indicate significant differences, $P<0.05$.

DISCUSSION

In vitro-matured MII oocytes from old mares showed a higher incidence of chromosome misalignment than oocytes from young mares [16]. Similarly in women, Battaglia et al. [9] reported that 79% of the MII spindles in oocytes from 40- to 45-year-old women showed clear displacement of chromosomes from the metaphase plate, whereas only 17% of MII spindles in oocytes from 20- to 25-year-old women showed chromosome misalignment; they proposed that age may alter spindle components and/or the timing of the meiotic phases and thereby predispose to irregularities of the microtubular spindle and chromosome misalignment. As defects in chromosome alignment have been shown to be predictive of aneuploidy in oocytes [9], it is probable that the increased incidence in misalignment detected in MII oocytes in mares >16 years old will be reflected by an increased prevalence of aneuploidy in fertilized oocytes, zygotes and embryos from older mares. The mechanism underlying the increased incidence of

chromosome misalignment in oocytes from aged females is unknown; however, Yun et al. [17] suggested that dysfunction of the spindle assembly checkpoint (SAC), a mechanism involved in regulating and correcting kinetochore microtubule attachment prior to anaphase, might play an important role in this process. Yun et al. [17] found that the SAC in oocytes from aged mice was more 'permissive', that is allowed anaphase to progress before chromosome alignment at the metaphase plate was complete, and unable to correct 'faulty' attachment between the kinetochores and the microtubules of the spindle; in particular, some SAC components such as mitotic arrest deficient 2 (MAD2) and aurora kinase C (AURKC) showed reduced function, especially when the oocytes were matured and handled *in vitro* [17]. We propose that compromised function of the SAC may also contribute to impaired chromosome alignment and segregation in aged mares and increase the risk of aneuploidy in mature oocytes. Morphometric analysis revealed that the meiotic spindle in horse oocytes has axial dimensions of approximately 21 x 12 μm. These measurements differ from those previously reported by Franciosi et al. [11] (19.89 ± 1.15 μm vs. 17.28 ± 0.81 μm); however, the latter authors measured the spindle axes on the basis of a projected image rather than using confocal microscopic Z-stacks and 3D image reconstruction and analysis, as in the present study. Our results show that the spindle in horse oocytes is larger than that in human oocytes (12 x 9 μm) [15] but smaller than in mice oocytes, where the major axis ranges from 20 to 30 μm [18]. In addition, the length of the MII spindle in horse oocytes seems to be influenced by both maternal age and chromosome misalignment. In fact, when spindles with misaligned chromosomes were not considered, advanced mare age was associated with a significant reduction in pole-to-pole spindle length. This is similar to what has previously been reported for aged, inbred (CBA) mice [19] and may result from compromised activity of microtubule motor proteins in oocytes from aged females. There was

also a relationship between chromosome alignment and spindle length in horse oocytes, with spindle length being significantly longer in oocytes with misaligned chromosomes. Bromfield et al. [20] previously reported a relationship between the number of displaced chromosomes and the overall length of the meiotic spindle in frozen-thawed human oocytes. The percentage of slaughterhouse-derived oocytes that successfully matured to the MII stage in the present study is similar to that reported in previous studies (20-85%) [21, 22], and the absence of a significant difference in the percentage of oocytes reaching MII between young and old mares mirrors the findings of Brinsko et al. [23] who reported similar development to MII for young (≤15 years; 28/168) and old mares (>15 years; 11/66). However, meiotic spindles require considerable energy (e.g. GTP and ATP) for assembly and to ensure maintenance of bipolarity

and correct interaction between the kinetochores and the microtubules, which in turn ensure correct alignment of the chromosomes onto the metaphase plate. It has previously been shown that oocytes from aged mares are more susceptible to mitochondrial damage during *in vitro* maturation [24, 25], and it is therefore possible that, during *in vitro* maturation, oocytes from aged mares are unable to satisfy the energy requirements to ensure spindle integrity and correct microtubule-kinetochore interaction, thereby increasing the risk of improper spindle assembly and chromosome misalignment.

In conclusion, the present study demonstrates that advanced mare age compromises the ability of *in vitro*-matured equine oocytes to correctly align their chromosomes along the metaphase plate. This is likely to predispose to aneuploidy in the oocytes after fertilization-induced completion of the second meiotic division and in resulting embryos. This may in part explain the age-related reduction in oocyte developmental competence and the increase in the incidence of early pregnancy loss observed in aged mares.

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AUTHOR CONTRIBUTIONS

M. de Ruijter-Villani and T.A.E. Stout conceived the study. M. Rizzo and K.D. Ducheyne developed the experimental design with advice from M.S. Cristarella, M. Quartuccio, T.A.E. Stout and M. de Ruijter-Villani. M. Rizzo and K.D. Ducheyne performed the experiments with the support of C. Deelen and M. Beitsma. K.D. Ducheyne, M. Rizzo and M. de Ruijter-Villani performed interpretation of the data. T.A.E. Stout and M. de Ruijter-Villani supervised the project. M. de Ruijter-Villani, K.D. Ducheyne and M. Rizzo wrote the manuscript, which was edited by T.A.E. Stout. All authors have approved the final version of the manuscript.

CONFLICT OF INTERESTS

No competing interests have been declared.

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3

Chapter 3

Advanced mare age predisposes to aneuploidy and weakening of centromeric cohesion in *in vitro* matured oocytes

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Submitted

ABSTRACT

Aneuploidy of meiotic origin is a major contributor to age-related subfertility and an increased risk of miscarriage in women. Although age-related aneuploidy has been studied in rodents, the mare may be a more appropriate animal model to study reproductive aging. Similar to women, aged mares show reduced fertility and an increased incidence of early pregnancy loss; however, it is not known whether aging predisposes to aneuploidy in equine oocytes. We evaluated the effect of advanced mare age on (1) gene expression for cohesin components, (2) incidence of aneuploidy and (3) chromosome centromere cohesion (measured as the distance between sister kinetochores) in oocytes matured *in vitro*. Oocytes from aged mares showed reduced gene expression for the centromere cohesion stabilizing protein, Shugoshin 1. Moreover, *in vitro* matured oocytes from aged mares showed a higher incidence of aneuploidy and premature sister chromatid separation, and weakened centromeric cohesion. We therefore propose the mare as a valid model for studying effects of aging on centromeric cohesion; cohesion loss predisposes to disintegration of bivalents and premature separation of sister chromatids during the first meiotic division, leading to embryonic aneuploidy; this probably contributes to the reduced fertility and increased incidence of pregnancy loss observed in aged mares.

INTRODUCTION

Advanced age in women predisposes to chromosome segregation errors during the meiotic divisions in oocytes. When this happens, the resulting oocyte will have an abnormal number of chromosomes (aneuploidy) and, once fertilized, will give rise to an aneuploid embryo. Embryonic aneuploidy is known to be the major cause of developmental arrest, implantation failure, miscarriage and congenital birth defects in human reproduction [1, 2]. While 20% of mature oocytes in women between the ages of 25 and 30 years exhibit aneuploidy (i.e. an abnormal number of chromosomes), in women older than 35 years the proportion of aneuploid oocytes increases to 50%; then rises again to more than 60% for women above 40 years old [3]. However, studies on human oocytes and embryos are limited by availability, practicality and, above all, ethical concerns; and although non-human primates might appear to represent the closest animal model [4], some of the aforementioned limitations also apply. However, women are not the only mammals in which fertility is susceptible to the effects of aging. Age-related aneuploidy and variations in fertility have been investigated in various animal models, with the majority of targeted studies carried out on rodents. Despite the considerable advantages of rodent models, there are also important differences in reproductive physiology and lifespan between mice and women. By contrast, in horses just as in women, advanced female age is associated with decreased fertility and an increased risk of early pregnancy loss [5-7]. The likelihood of an equine embryo developing successfully to day 4 decreases in mares above 14 years of age (81% vs 96%) [8] while mares older than 18 years are three times more likely to suffer pregnancy loss between Days 16 and 60 than mares below 12 years of age (30% vs 10%) [9-11]. Analogies between mares and women are however not limited to the decrease in fertility with increasing age, but can be extended to other aspects of their reproductive biology: women and mares are both (1) mono-ovulatory, with a long follicular phase and a similar time course to ovulation; (2) show comparable age-related changes in cycle length and hormone concentrations; (3) have a long time interval (decades) to reproductive senescence; (4) exhibit meiotic oocyte arrest for decades; (5) show reduced fertility as a result of intrinsic oocyte defects [5, 12-14]. We recently showed that advanced maternal age impairs the ability of horse oocytes to correctly align their chromosomes on the metaphase plate [15], presumably predisposing them to aneuploidy. Even though it has previously been argued that the mare represents a potentially valuable model for reproductive aging in women [5], the incidence and the origin of aneuploidy in horse oocytes is unknown. Although various factors may contribute to the genesis of chromosome segregation errors and consequently to aneuploidy, in human

oocytes weakened cohesion between the centromeres is proposed to play a pivotal role [16]. During the first meiotic division, the homologous chromosomes are held together by both recombination sites and cohesin complexes. In mammalian oocytes, the cohesin complex is a ring-like protein structure that wraps around the chromosome arms and centromeres and is composed of two maintenance proteins (Sms1β and Smc3), a kleisin (Rec8) and Stag3 [17]. Other accessory proteins, such as Pds5B, Sororin and Wapl are weakly associated with the cohesin complex and regulate the dynamic interaction between cohesin and the chromatin. In this respect, Sororin and Pds5B interact to stabilize cohesin loading onto the chromatin, whereas Wapl facilitates dissociation of cohesin by competing with Sororin for binding to Pds5B [17].
During anaphase of the first meiotic division (anaphase I), Separase cleaves the cohesin along the chromosome arms, allowing bivalent resolution and the consequent release and separation of the homologous chromosomes [18]. The centromeric cohesin is protected against Separase by two Shugoshin isoforms (Sgo1 and Sgo2) and continues to hold the sister chromatids together during the initial stages of the second meiotic division. At the onset of anaphase II, Shugoshin disappears allowing the residual centromeric cohesin to be cleaved by Separase, which in turn allows segregation of the sister chromatids [17].
Although components of the cohesin complex are loaded onto the chromosomes during the pre-meiotic S phase of fetal development, in adult women a gradual loss of cohesin has been shown to occur in oocytes as a result of aging [16]. This can lead to the premature separation of the two sister chromatids of a bivalent, generating two univalents or even four separate single chromatids. In the present study, we investigate the suitability of the mare as an animal model for future studies of the effect of advanced maternal age on the stability of centromeric cohesion and the risk of oocyte aneuploidy.

MATERIALS AND METHODS

Oocyte collection and culture

Ovaries were recovered from slaughtered mares within 15 min after death, divided into two groups depending on the age of the mare (young, ≤ 14 years; old, ≥ 16 years) and maintained at 21-28 ºC for 2-4 hours during transport, as described previously [15]. Mare age was determined by reading the microchip and consulting the corresponding passport. Cumulus-oocyte complexes (COCs) were collected by scraping the wall of incised follicles with a bone curette and flushing out the dislodged cells with embryo flushing medium (Euroflush; IMV

Technologies, Leeuwarden, The Netherlands) supplemented with 0.4 % Heparin (Heparin sodium 5000 IU/mL; LEO Pharma BV, Denmark) as described previously [15]. To prevent the collection of *in vivo* matured oocytes, only follicles ≤30 mm were scraped. The collected COCs were identified using a dissecting microscope and only COCs with at least one layer of intact cumulus cells were used in the following studies. Collected COCs were first held at 21-22 ºC for 12 hours in HEPES-buffered synthetic oviduct fluid (H-SOF; Avantea, Italy) and subsequently matured *in vitro* for 26 hours at 38.5°C in 5% CO_2-in-air in a 50:50 mixture of Dulbecco's minimal essential medium (DMEM) and Ham's F12 (GIBCO BRL Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% fetal calf serum (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA), 0.125 µg/mL epidermal growth factor (Peprotech Inc., Rocky Hill, New Jersey, USA), 0.1 IU/mL follicle-stimulating hormone, 0.6 mmol/L cysteine and 0.1 mmol/L cysteamine (Sigma-Aldrich Chemical Co.), 0.1% insulin, 0.1% transferrin and 0.1% sodium selenite (VWR International BV, Amsterdam, The Netherlands). After oocyte maturation, cumulus cells were removed by exposing the COCs briefly to H-SOF supplemented with 1 µg/mL hyaluronidase (Sigma-Aldrich Chemical Co.) before gentle pipetting through 131 and 55 µm pipettes (EZ-strip, Research Instruments Ltd, Falmouth, UK). For the gene expression study, the oocytes were further divided on the base of the appearance of the COCs (expanded or compact) before maturation and on the successful extrusion of the first polar body after maturation. Only oocytes showing first polar body extrusion were used for producing chromosome spreads.

RNA extraction and cDNA synthesis

Total RNA was extracted from pools of 10 oocytes using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. The quantity and quality of total RNA were determined, respectively, by spectrophotometry and using an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, California, USA) with an RNA 6000 Nano Labchip kit (Agilent) in accordance with the manufacturer's instructions. Only samples with an RNA integrity number (RIN) of 7.5 or greater were used for analysis. Reverse transcription was performed as described previously [19] using Superscript III (Invitrogen Corporation, Carlsbad, California, USA), in a total volume of 20 µl made up of 10 µl of sample containing 1000 ng of RNA which had been treated with DNAse I (30 min at 37♁C followed by 10 min at 65♁C; 1 IU/mg of RNA; RNAse-Free DNase set, Qiagen).

Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously [19]. The primers used in the present study (Table 1) were produced at Eurogentec (Seraing, Belgium), with specificity tested by DNA sequencing (ABI PRISM 310 Genetic analyzer; Applied Bio-system, Foster City, California, USA). Real-time PCR was carried out in 15 µl of reaction mix including 7.5 µl of IQ SYBR® Green Supermix (BioRad, Veenendaal, The Netherlands), 0.5 µM of primer, and 1 µl of cDNA, on an IQ5 Real-Time PCR detection System (BioRad). Cycle conditions included a 3 min denaturation step at 95°C, followed by 40 cycles of amplification (15 s at 95°C, 30 s at the primer specific annealing temperature and 30 s at 72°C). A melting curve and standard curve were performed to verify product specificity and enable quantification of expression for each gene. Relative gene expression was expressed as the ratio of target gene expression to the geometric mean of expression for two housekeeping genes (PGK1 and SRP14), selected after stability evaluation using GeNorm (Biogazelle, Zwijnaarde, Belgium).

Gene	Sequence	T_a (°C)	Amplicon size (bp)	GenBank Accession no.
REC8	F: 5'-GGTCTACTTTCAACAATGCCAG-3' R: 5'-GCTCCACCATATCAATGCGG-3'	58°	213	XM_00150319
SGO1	F: 5'-CAGGGATTTATTTGTGACGG-3' R: 5'-TGTCTTGATTAGGAATGGTAGG-3'	60°	209	XM_00559982
SGO2	F: 5'-GATATACTTCCCGAAGAAAGCC-3' R: 5'-TTTGATTCCCGAGATGATACAC-3'	62°	125	XM_00560217
STAG3	F: 5'-CTATGACACTAATGACCTCCCT-3' R: 5'-CATCCAACACCCAATCTCCT-3'	58°	251	XM_00560315
WAPL	F: 5'-AAATCAAGAGTTCACTGACGAC-3' R: 5'-ACAAAGGGACAAATTCTGATGG -3'	58°	215	XM_00149932
PGK1	F: 5'-CTGTGGGTGTATTTGAATGG-3' R: 5'-GACTTTATCCTCCGTGTTCC-3'	54°	151	XM_00561428
SRP14	F: 5'-CTGAAGAAGTATGACGGTCG-3' R: 5'-CCATCAGTAGCTCTCAACAG-3'	55°	101	XM_00150358

Table 1. Details of primer pairs used in the present study.

Chromosome spreads and immunostaining

The zona pellucida was removed by exposing the oocytes briefly to 0.1% pronase (Sigma-Aldrich Chemical Co.) in a phosphate-buffered saline-polyvinylpyrrolidone (PBS-PVP) solution (B. Braun, Hessen, Germany - Sigma-Aldrich Chemical Co.). After inducing cell swelling by incubating the oocytes in a hypotonic solution consisting of 1% sodium citrate (VWR International BV) in ultrapure water (Milli-Q®, MQ) for 8 minutes, the oocytes were

lysed as described previously [20], using a chromosome spread solution consisting of 0.15% Triton X-100 and 3 mM dithiothreitol (Sigma-Aldrich Chemical Co.), and 0.2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) on a cover slip. After lysis, air was gently blown on the slide using a plastic Pasteur pipet (Sigma-Aldrich Chemical Co.) to evenly spread the chromosomes, after which the spreads were air-dried overnight in a humidified chamber at 37°C. Prior to staining of chromosome spreads, non-specific staining was blocked by incubation for one hour in 3% bovine serum albumin (BSA, Sigma-Aldrich Chemical Co.) in PBS at room temperature. The chromosome spreads were then incubated overnight at 4 °C in PBS containing a 1:250 dilution of a purified human anti-centromere CREST antibody (Cat. No. 15-235; Antibodies Incorporated, Davis, California, USA) and 0.5% BSA. The chromosome spreads were then washed three times in PBS with 5% BSA (PBS-BSA) before being incubated in PBS-BSA containing a 1:100 dilution of goat anti-human Alexa Fluor 488 (A11013; Life Technologies, Eugene, Oregon, USA) for 3 hours at room temperature. After three washes with 0.1% Triton X-100 in PBS-BSA, the chromosome spreads were incubated in MQ containing a 1:1000 dilution of Hoechst 33342 (Sigma-Aldrich Chemical Co.) for 10 minutes at room temperature to stain the chromatin. After an additional washing step with MQ, the coverslips were mounted on glass slides with Vectashield H-1000 (Vector Laboratories Inc., Peterborough, UK).

Image acquisition and analysis

Image acquisition was performed using a confocal laser scanning microscope (Leica TCS-SPE-II; Leica Microsystems, Wetzlar, Germany) equipped with a 63x objective. Hoechst 33342 was stimulated with a 405 nm laser and the emission was detected between 414 and 466 nm (blue channel), Alexa Fluor 488 was separately stimulated with a 488 nm laser and emission was detected in the 511-577 nm range (green channel). A 3-dimensional image of the chromosomes and the kinetochores was acquired using sequential confocal sections (Z-stacks) at 0.17 μm intervals and then analyzed using Imaris 8.2 software (Bitplane AG, Zurich, Switzerland). Euploid MII horse oocytes should contain a total of 32 dyads, composed of 64 sister chromatids, with two sister kinetochores on each sister chromatid pair. Aneuploidy results in deviation from this number. The Imaris spot tool was used to segment the kinetochores, by selecting the green channel, applying a Gaussian smoothing filter (detail level of 0.0853 μm) and a threshold for surface creation on the basis of absolute intensity (using 51 and 166 arbitrary units as lower and upper thresholds, respectively). The distances between sister chromatids (interkinetochore

distance: iKD) was measured using the MatLab (Math Works, Natick, Massachusetts, USA) plug-in function "spots-to-spots closest distance". This automatic sister kinetochore recognition was checked for accuracy and manually adjusted when kinetochores were falsely linked.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, California, USA). Normality was analyzed using the D'Agostino-Pearson and Shapiro-Wilk normality tests. Comparison of the iKD between groups was performed using an unpaired T-test. Dichotomous data, such as the percentage of aneuploid oocytes, was analyzed using Fisher's exact test.

RESULTS

Six hundred and sixty-nine COCs were collected from the ovaries of 221 mares (106 young and 115 old mares). Mare ages ranged between 2 and 14 years (mean ± SD: 9.4 ± 3.0 years) for the young group, and between 16 and 27 years (mean ± SD: 20.5 ± 3.6 years) for the old group. No significant difference between the young and old groups was observed for the success of first polar body extrusion after *in vitro* maturation (49.2 % vs 46.7 %; P = 0,5).

For the gene expression study, three hundred and twenty oocytes were divided into 8 groups on the basis of mare age (young versus old), cumulus appearance before maturation (compact versus expanded) and extrusion of the first polar body after maturation (MII and non-MII). For each of the 8 groups, 4 pools of 10 oocytes were used for mRNA extraction.

Three hundred and forty-nine oocytes that showed first polar body extrusion after *in vitro* maturation were used to make chromosome spreads. However, a usable chromosome spread that could be imaged resulted in only 199 cases; moreover, 131 of these spreads could not be analyzed fully due to either insufficient separation of the chromosomes or a poor fluorescent signal for the kinetochore stain. Only spreads where all of the sister chromatids and kinetochores were distinguishable were used for the analysis. Single or unpaired sister chromatids were excluded from iKD analysis.

Figure 1. Box plot showing mRNA expression for SGO1 in oocytes from young (≤14 years) and old (≥16 years) mares. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range. *P < 0.05.

MII oocytes from old mares show reduced expression of *Shugoshin 1* mRNA

Quantitative RT-PCR revealed measurable mRNA expression for all of the target genes in all samples, while amplification of the -RT blanks did not result in measurable amounts of product. Gene expression for Rec8, Stag3, Sgo2 and Wapl was similar in oocytes from young and old mares, whereas Sgo1 expression was significantly lower in oocytes from old compared to those from young mares, irrespective of the success of maturation or initial cumulus appearance (Figure 1).

MII oocytes from old mares show an increased incidence of aneuploidy

Representative images of chromosome spreads for euploid and aneuploid oocytes from young and old mares are shown in Figure 2. MII oocytes from old mares showed a significantly higher incidence of aneuploidy (20/36 = 54.6 %) than oocytes from young mares (5/32 = 15.2 %) (Figure 3A; $p < 0.05$). Of the total 25 aneuploid oocytes from young (5) and old (20) mares, 20 (80%; 5 and 15 from young and old mares respectively) showed hypoploidy (<64 sister chromatids). In the aged mare group, 14 of the 20 aneuploid oocytes (70%) showed an uneven number of sister chromatids (Figure 3b), consistent with unbalanced premature separation of sister chromatids (PSSC) during meiosis I. In the young group, the incidence of even (3/5) and

uneven (2/5) numbers of sister chromatids in aneuploid oocytes was similar. In addition, one euploid and one aneuploid oocyte from the old mare group and one aneuploid oocyte from the young mare group showed 2 unpaired sister chromatids.

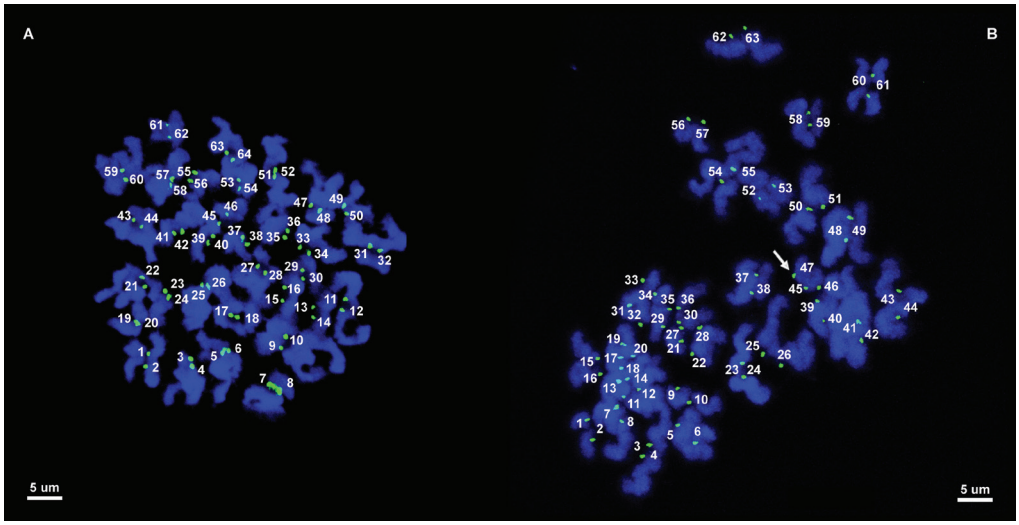

Figure 2. Representative maximum projection and individual z stacks of chromosome spreads for euploid and aneuploid MII oocytes from young and old mares. Green, kinetochores (CREST); blue, chromatin (Hoechst). (A) A total of 64 CREST positive foci (32 pairs of sister chromatid kinetochores) are seen in an euploid MII oocyte from a young mare. (B) A total of 63 CREST positive foci are counted in an aneuploid MII oocyte from an old mare; the white arrow indicated the uneven and unpaired kinetochore. Bar, 5µm.

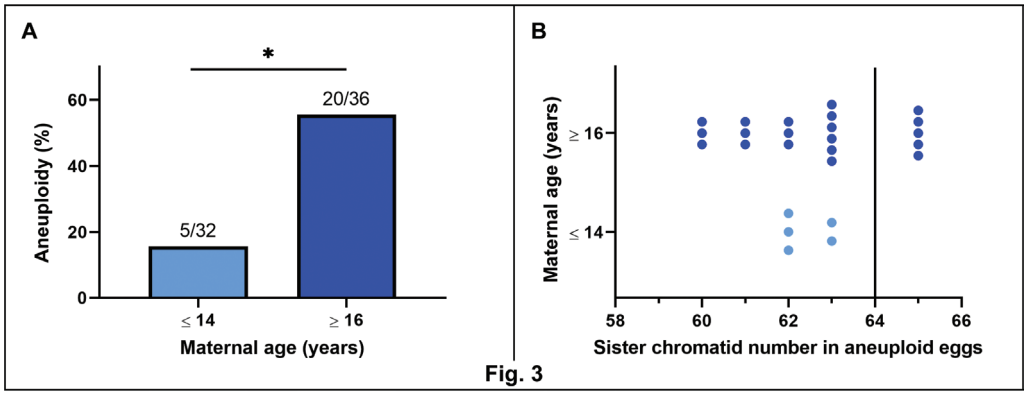

Figure 3. (A) Incidence of aneuploidy in *in vitro* matured MII horse oocytes from mares of different ages (*, $p < 0.05$). Numbers above bars indicate the number of aneuploid oocytes as a proportion of oocytes analyzed. (B) Frequency distribution of sister chromatid counts for the oocytes identified as aneuploid. The vertical line indicates the euploid number.

MII oocytes from old mares show weakened centromeric cohesion

The iKD between sister chromatids was greater in MII oocytes from old mares (mean ± SD, 1.96 ± 0.74 µm) than in those from young mares (1.33 ± 0.40 µm; $p < 0.0001$) (Figure 4A-C). When oocytes were divided on the basis of ploidy, the iKD was similar between euploid and aneuploid oocytes within young (mean ± SD, 1.32 ± 0.32 vs. 1.14 ± 0.18 µm) or within old mares (mean ± SD, 1.91 ± 0.44 vs. 2.03 ± 0.57 µm), respectively (Figure 4D).

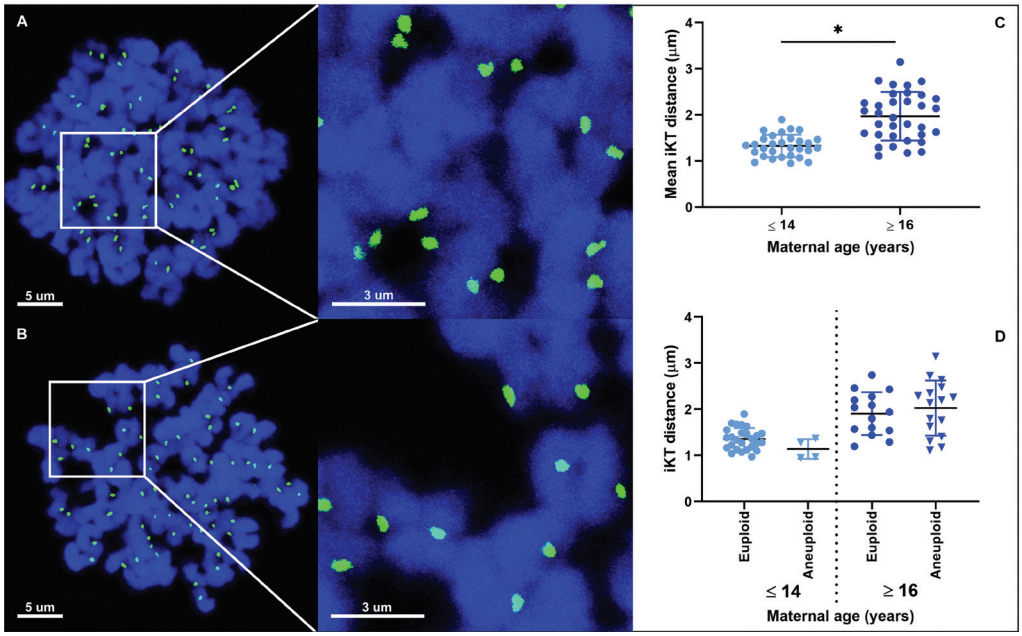

Figure 4. Representative maximum projection images of chromosome spreads for *in vitro* matured MII oocytes from young (A) and aged (B) mares. Green, kinetochores (CREST); blue, chromatin (Hoechst 33342). Note the increased iKD (i.e. separation of the CREST signals) in oocytes from old mares. Bar, 5 and 3 µm. Scatterplots of iKD categorized by mare age (C) (*, $p < 0.0001$) and by mare age and aneuploidy (D).

DISCUSSION

The results of this study demonstrate that advanced age predisposes to aneuploidy in mare oocytes, as has previously been reported for women. Not only was the incidence of aneuploidy in MII oocytes from mares (15% in young and 55% in old mares) comparable to that reported for women (20% in women younger than 35 years; 60% in women older than 35 years) [3], but

the mechanics of mis-segregation also appear to be analogous. It has been reported that 80% of aneuploidies seen in the oocytes of reproductively aged women are the result of premature separation of the sister chromatids [3, 21, 22]. Whereas in mice, chromatid count in aneuploid oocytes is biased to even numbers [23] and only 31% are consistent with age-related PSSC [24, 25], our results show that 75% of aneuploid oocytes from aged mares have an uneven chromatid number. This is most likely the result of premature separation of the bivalents followed by random segregation of the sister chromatids during the first meiotic division. This is consistent with weakening of centromeric cohesion as further evidenced by the increased distance between sister kinetochores in MII oocytes from aged mares. It is interesting to note that the age-related increase in iKD observed in mare MII oocytes (1.33 ± 0.40 μm in young compared to 1.96 ± 0.74 μm in aged mares) is of a similar magnitude to that reported in women (0.82 ± 0.03 μm in women <35 years, compared to 1.1 ± 0.03 μm in women > 35 years [16]). Unlike in mice, where the iKD is increased not only in oocytes from aged females but also in aneuploid oocytes from young females [25], the iKD did not differ between euploid and aneuploid MII equine oocytes within a given mare age group, suggesting that mare oocytes are a better model for studying the effect of maternal aging on centromeric cohesion.

The similarities in the incidence of age-related oocyte aneuploidy and degree of centromeric cohesion loss between mares and women identified in our study, together with what has been previously described about similarities between these two species in reproductive biological characteristics and reproductive senescence [5], indicate that the mare is a useful model for studying the age-dependent mechanisms that predispose to oocyte and embryo aneuploidy.

The molecular origin of the observed increase in iKD remains to be elucidated. It may involve the loss of a particular component of the cohesion complex. The maintenance and regulation of centromeric cohesion is important to avoid premature breakdown of the bivalent into two separate sister chromatids (univalents). It is generally accepted that the protection of cohesion by the Shugoshin protein family is a mechanism conserved for mitosis and meiosis, and both members of the Shugoshin protein family have been reported in mammals [26-29]. However, the specific role and functions of the two proteins (Sgo1 and Sgo2) during the first or second meiotic division are not yet clear and sometimes differ between species. Recently it was demonstrated that Shugoshin 1 plays a pivotal role in mammalian meiosis. Shugoshin 1 depletion induces premature dissociation of cohesin at the centromere and along the chromosome arms, allowing premature separation of the sister chromatids in cows [30]. It is therefore possible that the reduced expression of *Shugoshin 1* mRNA observed in aged mare

oocytes reflects a reduction of Shugoshin 1 function during meiosis, which would predispose to deterioration of centromeric cohesion in oocytes.

Another possible cause of centromeric cohesion loss in oocytes from older females is increased oxidative damage [31, 32]. During *in vitro* maturation, oocytes are subject to various oxidative insults; since oocytes from aged mares appear to be more sensitive to mitochondrial damage [33, 34] and therefore, oxidative stress, it is possible that the higher incidence of aneuploidy and weakened centromere cohesion observed in the present study are higher than they may be for oocytes of aged mares allowed to mature *in vivo*.

In conclusion, we propose that the weakening of centromeric cohesion observed in *in vitro* matured oocytes from aged mares predisposes to disintegration of bivalents into univalents and premature separation of the sister chromatids during the first meiotic division. Therefore, sister chromatids, instead of just the homologous chromosomes, might segregate during the first meiotic division, either in a balanced or unbalanced fashion (Figure 5), predisposing to embryonic aneuploidy of meiotic origin. This may partly explain the reduced fertility and the increased incidence of early pregnancy loss in aged mares, and suggests that the horse could be a valuable animal model for studying the molecular mechanisms underlying the effects of maternal aging on oocyte chromosome mis-segregation and aneuploidy.

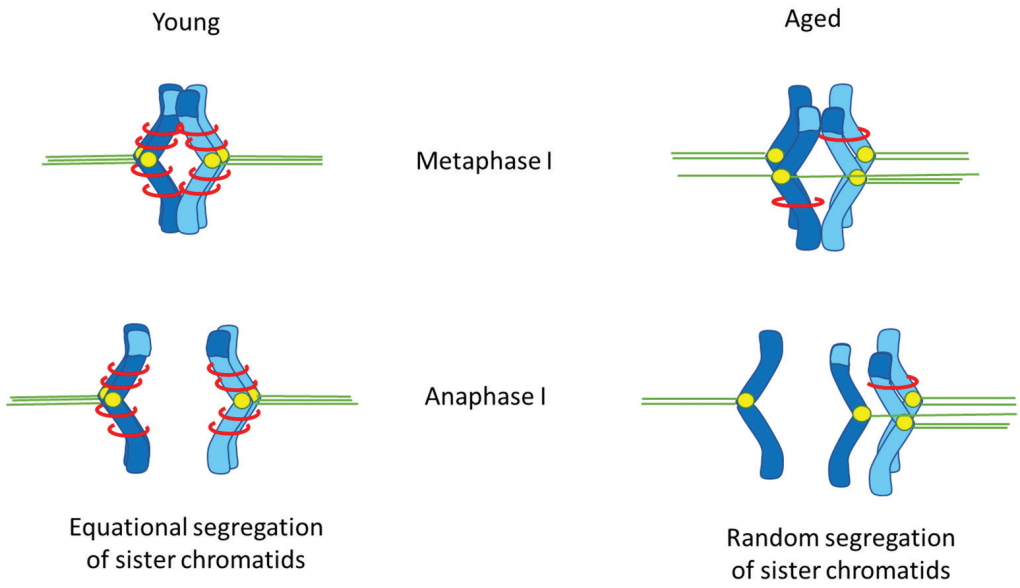

Figure 5. Schematic representation for loss of chromosome cohesion and the generation of single chromatids in MII oocytes as a result of maternal aging. The effect of advanced maternal age is depicted on a bivalent (dark and light blue) during meiosis I. Aging is associated with weakened cohesion (red rings). The increased distance between sister chromatid kinetochores prevents them from working as a single unit. They are therefore subject to abnormal attachment to microtubules and random segregation, as opposed to equal segregation.

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AUTHOR CONTRIBUTIONS

Marilena Rizzo performed part of the experiments, analysed the data and wrote the paper. Nikole du Preez performed some of the experiments. Marta de Ruijter conceived the study, analysed the data and wrote the paper. Claudia Deelen and Mabel Beitsma helped collecting

the COCs and deliver technical support for the experiments. Kaatje Ducheyne and Tom Stout edited the manuscript.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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4

Chapter 4

Reduced Mps1 activity leads to meiotic spindle instability in *in vitro* matured oocytes from aged mares

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To be submitted

ABSTRACT

Aneuploidy originating during meiosis in oocytes is the major contributor to reduced fertility, implantation failure and miscarriage in women of advanced age. It has been postulated that the error-prone nature of chromosome segregation in oocytes of older women may be attributable to a less stringent Spindle Assembly Checkpoint (SAC), however the underlying molecular deficits are not known. In the present study, we used horse oocytes to investigate the expression and function of selected SAC components. Oocytes from old mares (≥ 16 y.o.) showed lower mRNA expression for Mps1, Spc25 and AurkC than oocytes from young mares (≤ 14 y.o.). Moreover, MII oocytes from aged mares showed a higher incidence of spindle abnormalities, but not of chromosome misalignment, when exposed to the Mps1 inhibitor Cpd5 after nocodazole-washout. In contrast, Aurora kinase inhibition after nocodazole-washout severely impaired microtubule organization and spindle formation in all oocytes, irrespective of mare age. In conclusion, gene expression of the SAC kinase Mps1 is reduced in oocytes from aged mares. Moreover, spindles of aged mare's oocytes are more prone to destabilization when Mps1 activity is inhibited. Together, this suggests that compromised Mps1 activity predisposes to faulty chromosome-cytoskeleton interaction and errors of chromosome segregation in aged mares.

INTRODUCTION

Oocytes from women of advanced age have an elevated risk of chromosome mis-segregation during the meiotic divisions; this yields an aneuploid oocyte which, after fertilization, will give rise to an aneuploid embryo. Embryonic aneuploidy of meiotic origin is the primary cause of the increased incidence of arrested embryonic development, implantation failure and early miscarriage observed in women of advanced reproductive age [1, 2]. Meiosis is initiated during fetal development, but arrests at the prophase of the first meiotic division before birth and does not resume until follicle recruitment and initiation of oocyte maturation during adulthood, up to decades later. This long period of meiotic arrest and metabolic quiescence is associated with exposure to molecular and cellular aging processes, such as mitochondrial dysfunction, and loss of molecular components required for chromosome cohesion and adequate spindle formation and function [1, 2]. These deficits compromise chromosome-cytoskeleton interactions during meiosis and predispose to oocyte aneuploidy. While some of these deficits, such as loss of cohesion between sister chromatids, have been studied extensively in human oocytes, others including defects in spindle formation have received little attention. Correct alignment of the chromosomes, accompanied by proper aggregation and organization of the cytoplasmic microtubules, is essential for the creation of a normal bipolar spindle and for equal partitioning of chromosomes during anaphase [1, 3, 4].

During both mitosis and meiosis, potential errors in chromosome segregation are supposed to be prevented by a complex surveillance mechanism known as the Spindle Assembly Checkpoint (SAC). The SAC is composed of a number of molecular components, which collectively monitor kinetochore-microtubule (K-MT) attachment and prevent precocious anaphase onset before homologous chromosomes or sister chromatids have achieved attachment to their opposite spindle poles, during meiosis I and II respectively [5, 6]. The SAC-derived block of the transition into anaphase relies on the final effector of the inhibition-signaling pathway, the Mitotic Checkpoint Complex (MCC). The MCC consists of the proteins Mad2, BubR1, Bub3 and Cdc20. The recruitment and assembly of the MCC at the kinetochores depends on Bub1 and Mad1, and is mainly orchestrated by the kinase Mps1, which has been described as the master regulator of the SAC [7]. Once assembled, the MCC is able to diffuse throughout the cytosol and inhibit the anaphase promoting complex/cyclosome (APC/C), ensuring that there is time to correct the faulty K-MT attachment via the error-correction machinery, which is coordinated primarily by the kinase, Aurora B (AurkB) and/or its meiosis-specific homolog Aurora C (AurkC). Studies on human and murine oocytes indicate that loss

of SAC function is closely correlated with an increased incidence of chromosome mis-segregation [8-11]. In addition, maternal aging has been associated with reduced gene expression of key SAC components in human oocytes, suggesting that a weakened SAC could be an important contributor to age-related aneuploidy [12].

Similar to women, mares of advanced age suffer from reduced fertility. Although aged mares appear to have a similar ability to conceive as young mares (91% fertilization rate for mares less than 7 years vs 85% for mares older than 17 years), they suffer an increased incidence of early pregnancy loss (<10% in young vs 30-70% in mares older than 18 years) [for review see 13]. A decline in oocyte quality during the long period of meiotic arrest (4-25 years), has been proposed to be the principal contributor to the age-related decrease in fertility in mares [14-17]. The similarities between women and mares with regard to the duration of oocyte meiotic arrest (years to decades) and of the time interval to reproductive senescence, suggest that the mare could represent a valuable 'natural' model for studying the processes underlying reproductive aging. Recent studies on oocytes from mares of advanced age show that their ability to correctly align the chromosomes on the metaphase plate is compromised both after *in vitro* [18] and *in vivo* [19] maturation. We hypothesize that the observed inability to correctly align the chromosomes on the meiotic spindle may arise from a defective spindle assembly checkpoint. The aim of this study was therefore to investigate the effects of advanced mare age on gene expression and function of selected SAC components in equine oocytes.

MATERIALS AND METHODS

Oocyte collection and *in vitro* maturation

Ovaries were collected from slaughtered mares within 15 min of death, divided into two groups depending on the age of the mare (young, ≤ 14 years; old, ≥ 16 years) and transported to the laboratory within 4 hours at 21-25 °C as described previously [18]. Cumulus-Oocyte Complexes (COCs) were recovered from the ovaries by scraping the follicle wall and flushing the dislodged COCs using embryo flushing medium (Euroflush, IMV Technologies, Leeuwarden, the Netherland) as described previously [18]. A dissecting microscope with 10-60x magnification was used for evaluating the recovered COCs. Only oocytes with at least one layer of intact cumulus cells were used, denuded oocytes were discarded. For the PCR experiment, COCs were further subdivided depending on the cumulus morphology (Compact or Expanded) at recovery. The oocytes were then washed in Hepes-buffered synthetic oviduct

fluid (H-SOF; Avantea, Cremona, Italy) and matured *in vitro* for 26h at 38°C in a humidified atmosphere containing 5% CO2 as described previously [18]. After maturation, oocytes were denuded from the surrounding cumulus cells by gentle pipetting through a fine bore pipette after a brief exposure to a 1 µg/mL solution of hyaluronidase (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) in H-SOF. For the PCR experiments, oocytes were then divided into two groups depending on the presence or absence of the first polar body. For the SAC component inhibition experiments, only oocytes showing polar body extrusion were used.

Ovaries from 41 young (mean age ± SD: 8.9 ± 4.1 years; range: 2-14 years) and 77 old (mean age ± SD: 20.3 ± 3.7 years; range: 16-28 years) were used in the present study. A total of 320 oocytes (160 COCs from each age group), selected for cumulus appearance at recovery (compact and expanded), and which showed or failed to show polar body extrusion after maturation (matured and not-matured) were randomly selected for qRT-PCR analysis. A further 320 oocytes (160 from young and 160 from old mares), not selected for cumulus appearance at recovery, and showing a polar body after *in vitro* maturation were randomly selected for SAC component inhibition studies and immunofluorescent analysis. Of the immunostained oocytes, 68 (24 and 44 from young and old mares, respectively) had to be excluded due to inadequate immunofluorescent staining for tubulin or chromatin.

RNA extraction, cDNA synthesis and Quantitative Real Time-PCR (qRT-PCR)

Oocytes used for qRT-PCR were divided into 8 groups depending on the mare's age (young or old), cumulus appearance before *in vitro* maturation (compact or expanded) and on the presence or absence of a polar body after *in vitro* maturation (MII or non-MII). Oocytes were briefly washed in PBS (B. Braun, Hessen, Germany), and then transferred with a minimum amount of medium into pre-labeled cryovials, snap frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted from pools of 10 oocytes (n=4 per group) using the Invisorb® Spin Cell RNA Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. Each pool of oocytes was lysed in 600 ml lysis buffer, and total RNA was eluted with 20 µl RNase-free water, after which RNA concentration and integrity were measured as described previously [20]. The RNA was then treated with DNAse I (30 min at 37 °C followed by 10 min at 65 °C; 1 IU/mg of RNA; RNAse-Free DNase set, Qiagen Valencia, California, USA) and reverse transcribed into cDNA in a total volume of 20 µl containing 10 µl of sample using Superscript III (Invitrogen Corporation, Carlsbad, California, USA) as described previously [20]. Minus RT blanks were prepared from 5 µl of sample under the same conditions, but in the absence of

reverse transcriptase. Primer pairs (Table 1) for target genes (*Mad2, Bub1, Bub3, Bub1b, Mps1, Ndc80, Spc25, AurkB, AurkC*) and housekeeping genes (*Pgk1* and *Srp14*) were designed using PerlPrimer [21] and produced at Eurogentec (Seraing, Belgium). Primer specificity was tested by DNA sequencing (ABI PRISM 310 Genetic analyzer; Applied Bio-system, Foster City, California, USA). Real-time PCR was performed as described previously [20] in 15 µl of reaction mix that included 7.5 µl of IQ SYBR® Green Supermix (BioRad, Veenendaal, The Netherlands), 0.5 mM of primer, and 1 µl of cDNA, on an IQ5 Real-Time PCR detection System (BioRad). The cycle conditions were composed of an initial denaturation at 95 °C for 3 min, followed by 40 amplification cycles consisting of 95°C for 3min, the primer-specific annealing temperature (see Table 1) for 30sec and 72 °C for 45sec. A melting curve and standard curve were performed for each gene to verify product specificity and enable expression quantification. The stability of the housekeeping genes was determined using GeNorm [22] and the geometric mean of the expression of SRP14 and PGK1 was used for normalizing the starting quantities of the target genes.

Gene	Sequence	T_a (°C)	Amplicon size (bp)	GenBank Accession no.
MAD2L1	F: 5'-GCAGTTTGATATTGAGTGTGAC-3' R: 5'-TCCTGATTCCTCCCATTTCTC-3'	58°	213	XM_001503199.3
BUB1	F: 5'-GACTCAAATACGGAACAAAGG-3' R: 5'-TCTGTCTTCATTTACCCACTG-3'	60°	209	XM_005599825.1
BUB3	F: 5'-GAAGTACGCCTTCAAGTGTC-3' R: 5'-TTTACGAATCCATCAGAACCAC-3'	62°	125	XM_005602172.1
BUB1B	F: 5'-GCAGATATGATATTTCAGGAAGGG-3' R: 5'-CTGGTTTGAAGCCTTGAGAG-3'	58°	251	XM_005603154.1
MPS1	F: 5'-GGTTCAAGTAAGGTATTTCAGG-3' R: 5'-ATTTCCACACTCCATTACCA-3'	58°	215	XM_001499324.3
NDC80	F: 5'-AGTTGAATATAAGCAGACCCAC-3' R: 5'-GCTTGTAGGGACTTCATGGA-3'	62°	231	XM_001492535.3
SPC25	F: 5'-GCAGACTTGTATAAAGATCGAC-3' R: 5'-CTATCTGACACTTCATAGTCCC-3'	60°	157	XM_005601567.1
AURKB	F: 5'-GAAGAAGAGCCATTTCATCGT-3' R: 5'-ACTCCAGAATCAAGTAGATCCT-3'	62°	178	XM_001504814.3
AURKC	F: 5'-GCATCTACAACACCCAAATATCC-3' R: 5'-GTTCTCGGGCTTAATATCCCT-3'	60°	225	XM_005596580.1
PGK1	F: 5'-CTGTGGGTGTATTTGAATGG-3' R: 5'-GACTTTATCCTCCGTGTTCC-3'	54°	151	XM_005614287.1
SRP14	F: 5'-CTGAAGAAGTATGACGGTCG-3' R: 5'-CCATCAGTAGCTCTCAACAG-3'	55°	101	XM_001503583.3

Table 1. Details of the primer pairs for target and housekeeping genes used in the present study.

Nocodazole, Cpd5 and ZM447439 treatment of oocytes

On the basis of the mRNA expression results, the function of two of the selected SAC components (Mps1 and AurkC) was further analyzed using a specific inhibition approach. In order to evaluate the function of Mps1 and AurkC on spindle assembly and chromosome alignment, matured oocytes displaying a polar body were first incubated for 10 min at 38°C with 5% CO2 in IVM medium containing 20μM Nocodazole (M1404, Sigma-Aldrich Chemical Co.), to depolymerize the MII-spindles. Following Nocodazole washout, the oocytes were left to re-form their spindles for 120 min in clean IVM with different concentrations of either CPD5 (0, 200 or 500nM) or ZM447439 (0, 5 or 10μM; S1103, Selleck Chemicals, Houston, USA) in order to inhibit MPS1 or Aurora Kinase. The Mps1 inhibitor Cpd5 was kindly supplied by Prof. Geert Kops (Kops Lab - Cell division and chromosome segregation, Oncode Institute, Utrecht University). The oocytes in the control group were not treated with nocodazole or inhibitors.

Oocyte fixation

Before fixing, all oocytes were exposed to cold shock by placing on ice for 9 min to depolymerize unstable microtubules. The oocytes were then treated with a glycerol-based microtubule-stabilizing solution (Medium M) for 1 h at 37 °C [23], (25% (v/v) glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 50 mM imidazole, 4% Triton-X-100, and 25 mM phenylmethylsulphonylfuoride; pH 6.7: all from Sigma-Aldrich Chemical Co.), and subsequently fixed with a 2% solution of paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) in PBS.

Immunostaining, confocal imaging and 3-dimensional image analysis

Fixed oocytes were immunostained for α-tubulin and chromatin as described previously [18]. Oocytes were washed in PBS with 3mg/ml PVP (Sigma-Aldrich Chemical Co.) (PBS-PVP) three times for 5 min before being incubated over-night in PBS containing 1:250 mouse monoclonal anti-α-tubulin antibody (T5168, Sigma-Aldrich Chemical Co.) at 4 °C. Oocytes where then washed twice for 5min in a 0.1 % solution of BSA (Sigma-Aldrich Chemical Co.) in PBS and incubated for 1 h at Room Temperature (RT) in blocking solution containing 0.1 M glycine, 1% goat serum, 0.01 % Triton X-100, 0.5% BSA and 0.02% sodium azide (all from Sigma-Aldrich Chemical Co.), followed by incubation in the dark at 37 °C in PBS containing 0.5% Triton X-100, 0.5% BSA and a 1:100 Goat Anti-Mouse Alexa Fluor® 488 second

antibody (A11029, Invitrogen Corporation). After two washing steps of 5 min in PBS containing 0.1% BSA and 0.1% Triton X-100, and two washing steps of 5 min in PBS alone, the oocytes were incubated in the dark for 30 min at RT in PBS-PVP containing 5µg/ml of Hoechst (Hoechst 33342, Sigma-Aldrich Chemical Co.). After a brief wash in PBS-PVP, the oocytes were mounted on glass slides (SuperfrostPlus; Menzel, Braunschweig, Germany) with Vectashield (Vector Laboratories, Burlingame, California, USA) to avoid photobleaching. Slides were imaged using a confocal laser scanning microscope (Leica TCS-SPE-II, Leica Microsystems, Wetzlar, Germany) at a magnification of x630. Metaphase II spindles were identified, and sequential sections were taken throughout the whole spindle at 0.42-µm intervals (z-step size). Image-acquisition and analysis of spindle morphology and chromosome alignment were performed using 3D analysis software (Imaris 8.1; Bitplane AG, Zurich, Switzerland) as described previously by Rizzo [18]. Gross morphology of MII spindles and chromosome misalignment were analyzed and classified as described previously [18]. Spindles were classified as normal if bipolar, and abnormal if tri- or tetra-polar or severely mis-shapen. Chromosome misalignment was classified as absent when all chromosomes were on the metaphase plate, mild if up to 5 chromosomes were displaced from the spindle equator and severe if more than 5 chromosomes were displaced from the spindle equator.

Statistical analysis

Spindle morphology and chromosome misalignment between age and treatment groups were compared by Fisher's exact and Cochran-Mantel-Haenszel tests, using a Chi-squared analysis performed using IBM SPSS Statistics for Windows (Version 24.0, Armonk, New Your, USA). Results were considered statistically significant when $P \leq 0.05$.

RESULTS

Gexpression for *Mps1*, *Spc25* and *AurkC* is reduced in oocytes from aged mares

Quantitative RT-PCR revealed measurable mRNA expression for all target genes in all samples, while the amplification of the – RT blanks did not result in measurable amounts of product. Gene expression for most of the SAC components (*Mad2L2*, *Bub1*, *Bub3*, *Bub1B*, *Ndc80* and *AurkB*) was similar in oocytes from young (≤ 14 years) and aged mares (≥ 16 years) (Suppl. Figure S1). However, expression of *Mps1*, *Spc25* and *AurkC* was significantly reduced in

oocytes from aged compared to young mares, irrespective of maturation stage (germinal vesicle versus MII) or initial cumulus appearance (compact versus expanded) ($P<0.05$) (Figure 1).

To investigate the ability of oocytes to correctly form a bipolar spindle and to align their chromosomes on the metaphase plate, *in vitro* matured MII oocytes were allowed to re-form a spindle after nocodazole-induced microtubule depolymerization. To investigate the importance of Mps1 and AurkB/C function in these oocytes, two different concentrations of an Mps1 inhibitor (Cpd5; [24]) or an AurkB/C inhibitor (ZM447439; [25]) were added after nocodazole washout.

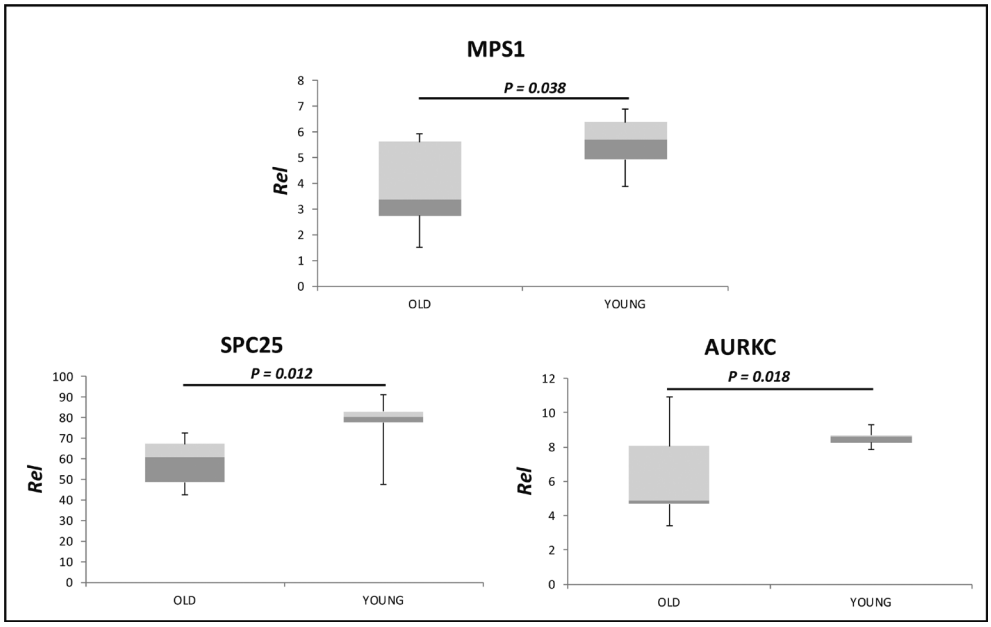


Figure 1. mRNA expression for *Mps1*, *Spc25* and *AurkC* in oocytes from young (≤14 years) and old (≥16 years) mares. The box plot shows the interquartile range, with the median value indicated by the horizontal line; whiskers show the range.

Reduced Mps1 function compromises the ability of oocytes from aged mares to correctly form a bipolar spindle

Oocytes from aged mares were sensitive to the lower dose of Cpd5 exposure (200nM), which induced spindle abnormalities, whereas oocytes from young mares only showed spindle abnormalities when exposed to the higher dose of the inhibitor (500nM: Figures 2 and 3). In

fact, no abnormal spindles were observed in young mare oocytes not exposed to any treatment, exposed to Nocodazole washout only or treated with 200nM Cpd5 after Nocodazole washout (0/60). Only after treatment with 500nM Cpd5 following Nocodazole washout was a significant increase in the proportion of abnormal spindles observed (9/40) (23% vs 0%, P=0.021). Similarly, no significant difference in the incidence of spindle abnormalities was observed in oocytes from aged mares that were not treated (5%, 1/19) compared to those treated with Nocodazole alone (16%, 3/19). However, unlike the young mare group, aged mares' oocytes were susceptible to both concentrations of Cpd5, showing a significantly increased incidence of tri- and tetrapolar spindles compared to the control group (7/21 [33%; P=0.046] and 8/22 [36%; P=0.024] for 200nM and 500nM Cpd5 respectively) (Figure 2). When comparing the two age groups for a given treatment, the incidence of spindle abnormalities was significantly higher only in aged mares' oocytes treated with 200nM of Cpd5 after Nocodazole-washout (33% aged vs 0% young; P=0.01).

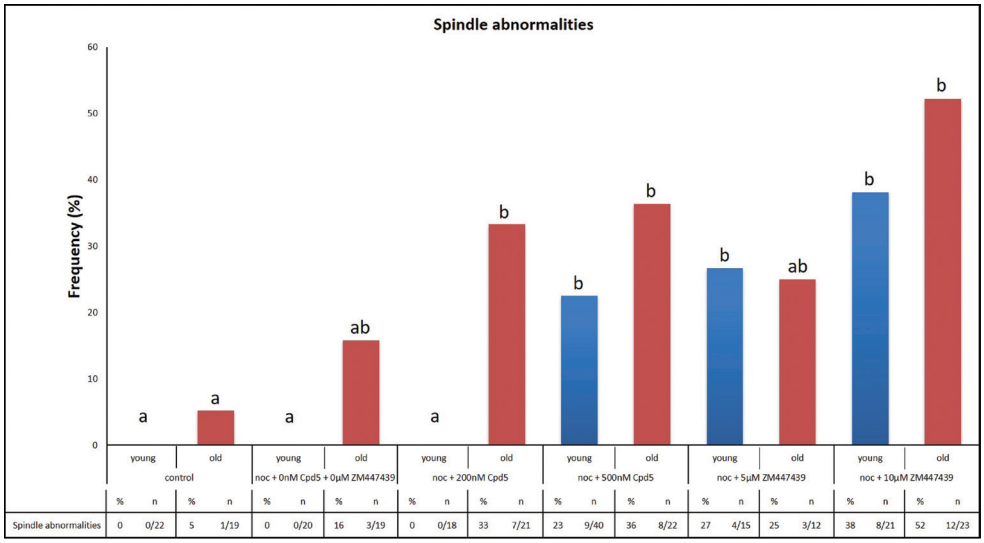


Figure 2. Frequency of spindle abnormalities in MII oocytes from young (≤ 14 years) and old (≥ 16 years) mares, treated with the Mps1 inhibitor Cpd5 (0, 200 or 500nM) or the AurkB/C inhibitor ZM447439 (0, 5 or 10µM) after Nocodazole washout. Oocytes in the control groups were not treated with nocodazole or inhibitor. Bars that share the same superscript do not differ significantly; $P < 0.05$.

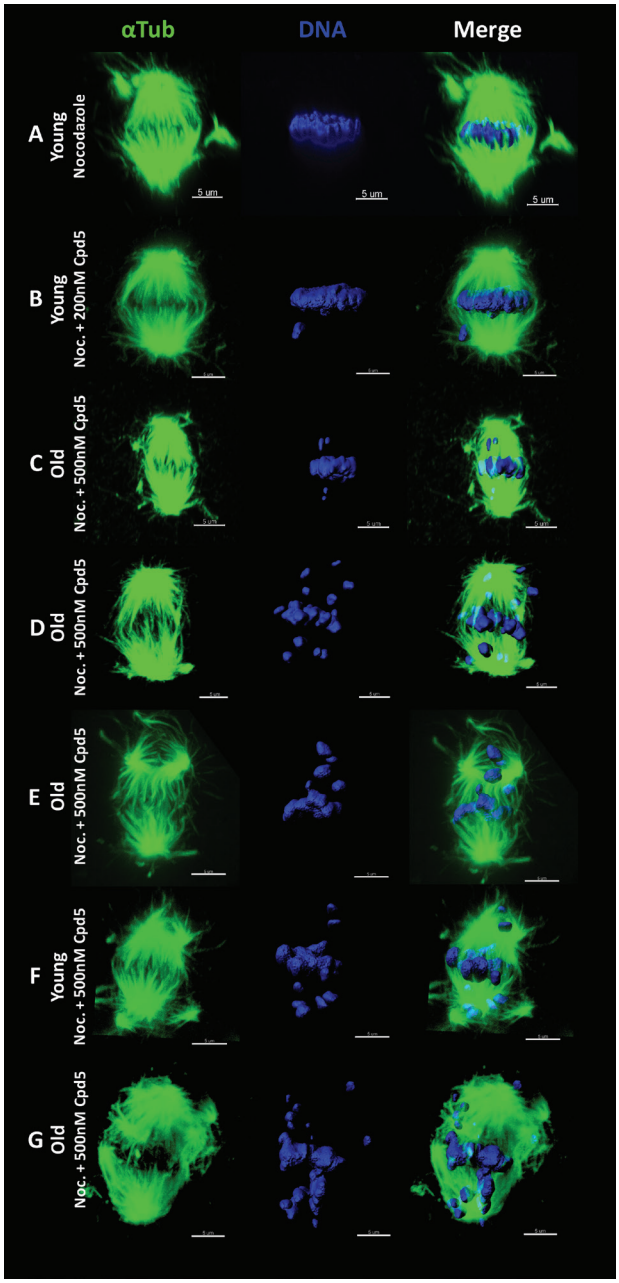
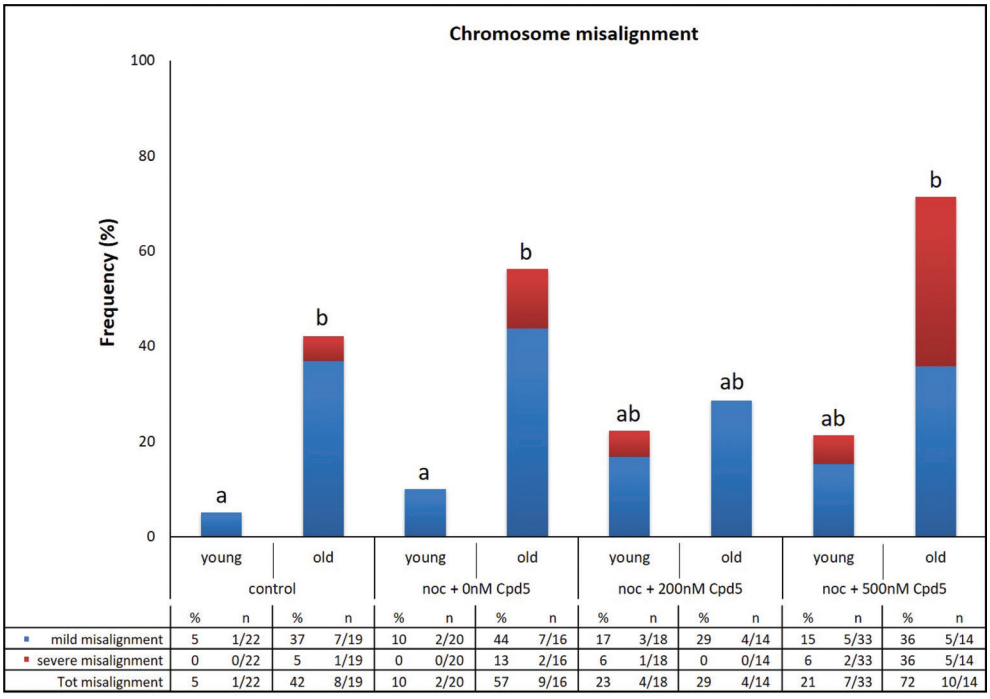


Figure 3. Representative images of normal and abnormal spindles of MII oocytes treated with Mps1 inhibitor Cpd5 after Nocodazole washout. Green, α-tubulin; blue, chromatin. (A) bipolar spindle from a young mare's oocyte after Nocodazole washout only; all chromosomes are properly aligned on the metaphase plate; (B) bipolar spindle from a young mare's oocyte after Nocodazole washout, with mild chromosome misalignment; (C) and (D) bipolar spindles from old mare's oocytes treated with 500nM of Cpd5, with mild (C) and severe (D) chromosome misalignment; (E) tripolar spindle from an old mare's oocyte treated with 500nM of Cpd5; (F) tetrapolar spindle from a young mare's oocyte treated with 500nM Cpd5; and (G) severely mis-shapen spindle from an old mare's oocyte treated with 500nM Cpd5. Scale bar, 5 µm.

Advanced maternal age predisposes to chromosome misalignment in MII oocytes

Oocytes showing meiotic spindle abnormalities were excluded from the analysis of chromosome misalignment. Oocytes from aged mares showed a higher incidence of chromosome misalignment than those from young mares (Figure 4). This age-related difference was visible in every treatment group with the exception of the 200nM Cpd5. Specifically, aged mare oocytes showed a total incidence of chromosome misalignment of 42% (8/19), 57% (9/16), 29% (4/14) and 72% (10/14) in the non-treated Control, Nocodazole, 200nM and 500nM Cpd5 groups, respectively, compared to 5 % (1/22; P=0.006), 10 % (2/20; P=0.004), 23 % (4/18; P=0.7) and 21 % (7/33; P=0.002) for young mare oocytes (Figure 4).



	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n
mild misalignment	5	1/22	37	7/19	10	2/20	44	7/16	17	3/18	29	4/14	15	5/33	36	5/14
severe misalignment	0	0/22	5	1/19	0	0/20	13	2/16	6	1/18	0	0/14	6	2/33	36	5/14
Tot misalignment	5	1/22	42	8/19	10	2/20	57	9/16	23	4/18	29	4/14	21	7/33	72	10/14

Figure 4. Frequency of mild and severe chromosome misalignment in bipolar spindles of MII oocytes from young (≤ 14 years) and old (≥ 16 years) mares treated with the Mps1 inhibitor Cpd5 (0, 200 or 500nM) after nocodazole washout. Oocytes in the control groups were not treated with nocodazole or inhibitor. Bars that share the same superscript do not differ significantly; $P < 0.05$.

Within each age group, there was no significant effect of treatment on the incidence of chromosome misalignment. However, oocytes from aged mares showed a higher percentage of severe misalignment (≥5 chromosomes) after treatment with 500nM Cpd5 (36%, 5/14) when compared to Control (5%, 1/19; P=0.06) or 200nM Cpd5 (0%, 0/14; P=0.04) treatments, although this was not statistically different to the Nocodazole group (13%, 2/16; P=0.2).

The aurora kinase inhibitor ZM447439 severely impairs microtubule organization and spindle formation in *in vitro* matured oocytes

Treatment with ZM447439 after Nocodazole washout increased the incidence of spindle abnormalities in oocytes from both young (27% (4/15; P=0.021) and 38% (8/21; P=0.001) for the 5µM and 10µM ZM447439 groups respectively) and aged mares (52% (12/23) for the 10µM ZM447439 group; P=0.005) compared to the Control and Nocodazole treatment groups (Figure 2). However, this was not statistically significant for oocytes from aged mares treated with 5µM ZM447439 (25%, 3/12; P=0.27). The most common spindle abnormalities observed in oocytes treated with 5µM ZM447439 were monopolar, tripolar or mis-shapen spindles, while the most prominent abnormalities after treatment with 10µM ZM447439 were the complete absence of microtubule organization, absence of chromosome congression and monopolar spindles. Representative images are shown in Figures 5 and 6. On the other hand, oocytes treated with ZM447439 which did re-form a bipolar spindle, did not show an increased incidence of chromosome mis-alignment compared to the control or the Nocodazole treatment groups (Figure 7), they did however show a very short spindle major axis compared to control oocytes (Figure 5B).

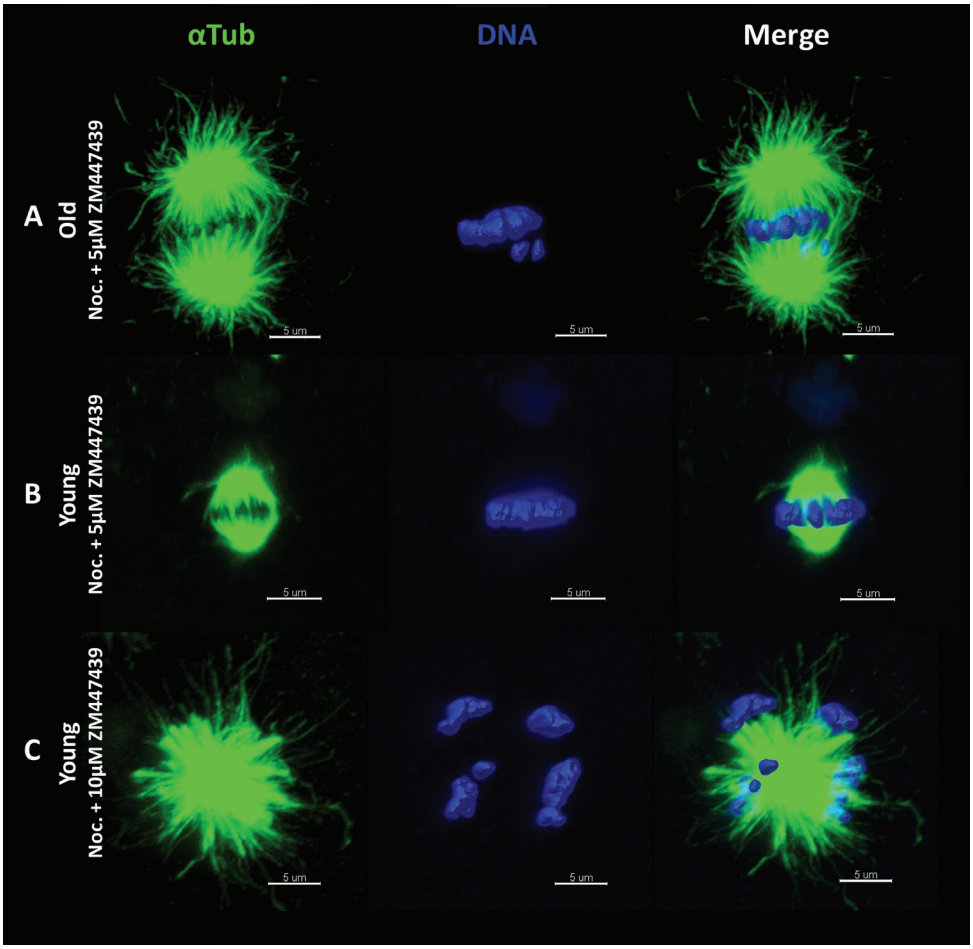


Figure 5. Representative images of spindles of MII oocytes treated with the AurkB/C inhibitor ZM447439 after Nocodazole washout. Green, α-tubulin; blue, chromatin. (A) bipolar spindle showing mild chromosome misalignment from an old mare's oocyte treated with 5μM ZM447439; (B) bipolar spindle showing normal chromosome alignment but with very short pole-to-pole length, from a young mare's oocyte treated with 10μM of ZM447439; (C) monopolar spindle from a young mare's oocyte treated with 10μM of ZM447439. Scale bar, 5 μm.

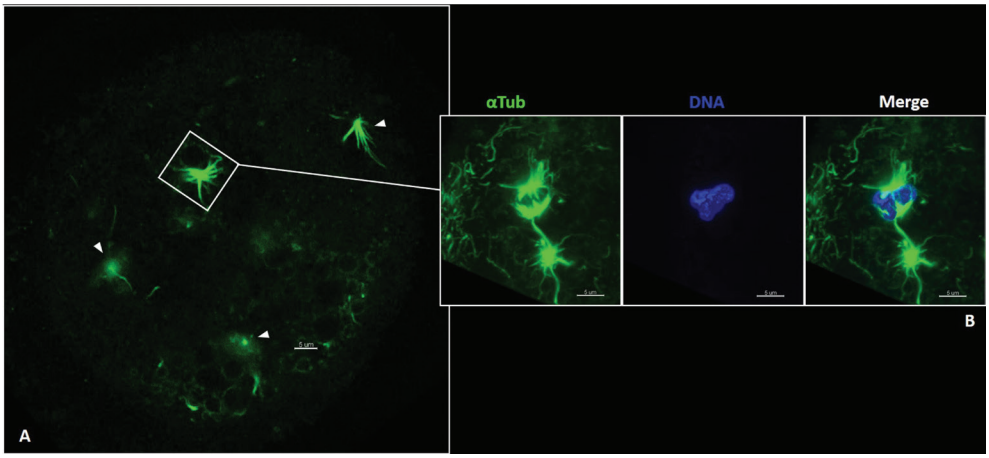
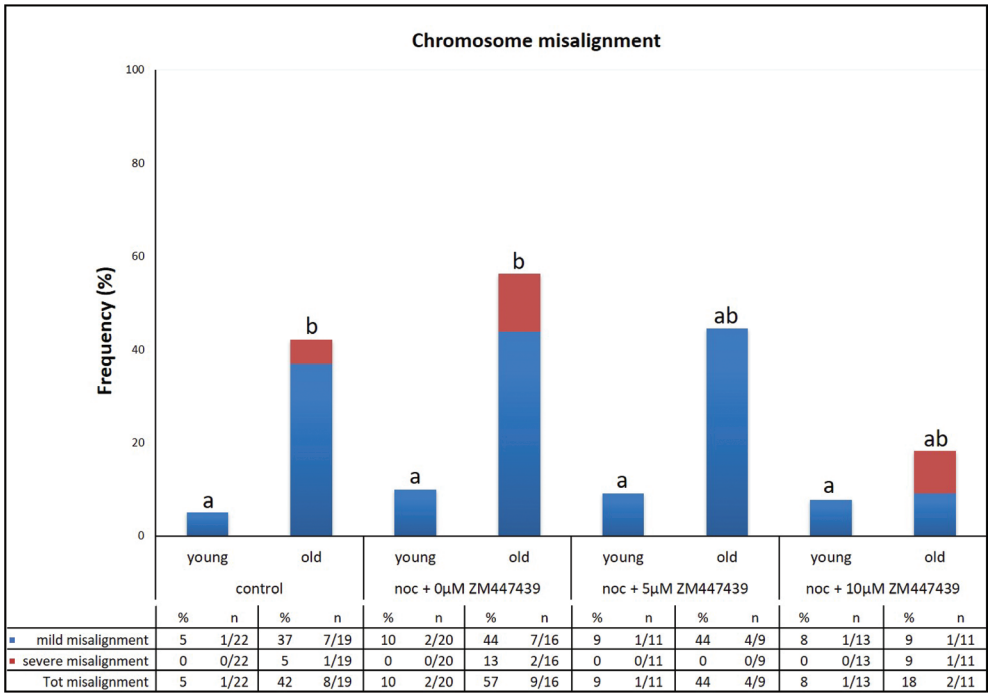


Figure 6. Representative image of MII oocyte from an old mare after treatment with 10μM ZM447439 with complete absence of microtubule organization. Green, α-tubulin; blue, chromatin. (A) Arrowheads indicate tubulin dispersed within the cytosol; (B) maximum projection of the failed attempt to re-form a spindle. Scale bar, 5 μm.



	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n
mild misalignment	5	1/22	37	7/19	10	2/20	44	7/16	9	1/11	44	4/9	8	1/13	9	1/11
severe misalignment	0	0/22	5	1/19	0	0/20	13	2/16	0	0/11	0	0/9	0	0/13	9	1/11
Tot misalignment	5	1/22	42	8/19	10	2/20	57	9/16	9	1/11	44	4/9	8	1/13	18	2/11

Figure 7. . Frequency of mild and severe chromosome misalignment in MII oocytes from young (≤ 14 years) and old (≥ 16 years) mares treated with the AurkB/C inhibitor ZM447439 (0, 5 or 10μM) after Nocodazole washout. Oocytes in the control groups were not treated with nocodazole or inhibitor. Bars that share the same superscript do not differ significantly; $P < 0.05$.

DISCUSSION

The present study demonstrates that *in vitro* matured MII oocytes from aged mares have a reduced mRNA expression for three key components of the SAC: *Mps1*, *AurkC* and *Spc25*. This is similar to what has previously been observed in murine and human oocytes from aged females, where a down regulation of genes encoding for various SAC components (such as Mad2 and Bub1) was associated with an increased incidence of aneuploidy [12, 26, 27], suggesting that an age-related compromise of the SAC could be an important contributor to meiotic aneuploidy.

We hypothesized that the reduced mRNA expression for *Mps1*, *AurkC* and *Spc25* could result in a similar reduction in abundance of their respective proteins leading to impaired SAC function. Although we attempted to assess Mps1 and AurkC protein abundance in oocytes using immunofluorescence, the lack of specific antibodies prevented us from being able to explore their expression. Therefore, to further test the hypothesis that compromised Mps1 or AurkC activity may contribute to aneuploidy in oocytes from aged mares , we evaluated the ability of oocytes from both young and old mares to re-assemble a metaphase II spindle after nocodazole-induced microtubule de-polymerization in the presence of different concentrations of an inhibitor for either Mps1 (Cpd5) or AurkB/C (ZM447439). While treatment with 500nM Cpd5 resulted in an increase in the incidence of tri- and tetrapolar spindles in both age groups, treatment with 200nM Cpd5 induced spindle abnormalities only in oocytes from old mares. Although the incidence of chromosome misalignment after Cpd5 inhibition did not differ statistically when compared to the Nocodazole group, a numerical increase in severe misalignment was observed in the bipolar spindles of oocytes from aged mares treated with 500nM Cpd5. Furthermore, with the exception of the 200nM Cpd5 treatment, oocytes from aged mares showed a higher incidence of chromosome misalignment than oocytes from young mares subjected to the same treatment. We propose that the increased sensitivity of aged mares' oocytes to Mps1 inhibition is due to reduced Mps1 abundance and/or function. Mps1 has several functions during meiosis; it phosphorylates a number of substrates that simultaneously promote error correction and play a crucial role in the SAC dependent inhibition of the anaphase promoting complex/cyclosome. By phosphorylating multiple residues on Knl1, Bub1 and Mad1, Mps1 stimulates sequential recruitment of SAC proteins to unattached kinetochores [28] [29]. Moreover, Mps1 promotes correct chromosome orientation and alignment by regulating the function and localization of AurkC, and regulates the stability of the kinetochore-microtubule attachments by phosphorylating the Ska complex [30, 31]. A previous study on

mouse oocytes [10] demonstrated that disruption of Mps1 leads to accelerated anaphase I progression and an increase in the incidence of aneuploidy, probably due to insufficient recruitment of SAC proteins to unattached kinetochores. In the same study, Mps1-depleted oocytes failed to localize AurkC either on the chromosome or on the spindle middle-zone, suggesting that Mps1 is an important regulator of the chromosomal passenger complex (CPC; composed of AurkC, INCENP, survivin and borealin) in oocytes [10, 32]. It has recently been shown that the CPC regulates centrosome clustering in cancer cells that express supernumerary centromeres [33], and that Mps1 disruption inhibits this clustering leading to the formation of multipolar spindles [24]. Horse oocytes, like human oocytes, assemble their spindle via a process that does not involve centrosomes or microtubule organizing centers (MTOCS) [34, 35]. After nuclear envelope break down (NEBD), the microtubules start nucleation from the chromosomes, and γ-tubulin is only detectable on the minus end of the microtubules as a diffuse staining at the spindle poles. As recently shown in human oocytes, the absence of MTOCs predisposes to spindle instability, with the spindle frequently fragmenting into multipolar spindle intermediates. The multiple poles then re-cluster to form a bipolar spindle structure. However, this spindle reorganization often results in incorrect attachment of the chromosomes to the spindle poles, leading to mis-alignment and mis-segregation of the chromosomes [34]. It is possible that, similar to cells expressing supernumerary centrosomes, the clustering of the multiple acentrosomal spindle poles in oocytes lacking MTOCs is also regulated by CPC. We deem it likely that the observed increased incidence of multi-polar spindle formation after Mps1 inhibition in horse oocytes is caused by impaired CPC function. It is therefore possible that in aged mares' oocytes the impaired expression of Mps1 causes spindle instability, which in turns results in a higher frequency of chromosome misalignment and mis-segregation.

In the present study, inhibiting AurkB/C using ZM447439 induced spindle abnormalities with a similar frequency in oocytes from young and aged mares. The most frequent abnormalities observed were the formation of a monopolar spindle or the formation of short bipolar spindles; when the concentration of ZM447439 was increased to 10μM a proportion of oocytes was unable to re-form a spindle and instead had their chromosomes dispersed through the cytoplasm. These findings are similar to what has previously been observed in xenopus and mouse oocytes. The overexpression of a dominant-negative Aurk-B mutant (Aur-B122R) in Xenopus oocytes (which lack AurkC) induced the formation of short bipolar spindles or monopolar spindles [36]. AurkB stabilizes spindle microtubules by inhibiting the activity of the catastrophe kinesin MCAK through a phospho-conformational switch that reduces microtubule

association [36, 37]. Therefore, AurkB inhibition results in an increased instability of the spindle microtubules. Similarly to Xenopus, inhibition of AurkB/C in murine oocytes, by treatment with 5-10μM ZM447439, decreased MCAK phosphorylation and induced the formation of multipolar, monopolar and apolar spindles with concomitant severe mis-alignment of the chromosomes [38, 39]. Therefore, it is reasonable to speculate that the spindle abnormalities observed in horse oocytes after AurkB/C inhibition are a consequence of decreased phosphorylation of the catastrophe kinesin MCAK, which in turn induces instability of the spindle microtubules.

CONCLUSIONS

Oocytes from mares of advanced age show a reduced expression of genes encoding for three key components of the Spindle Assembly Checkpoint (SAC), *Mps1*, *AurkC* and *Spc25*. Moreover, oocytes from aged mares show an increased sensitivity to Mps1 inhibition, but not to AurkB/C inhibition, when compared to oocytes from young mares. We therefore propose that compromised SAC, in particular Mps1, activity in oocytes from aged mares contributes to spindle instability. In turn, spindle instability could result in improper kinetochore-microtubule attachment and predispose to chromosomal misalignment, mis-segregation and, ultimately, aneuploidy.

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CONFLICTS OF INTEREST

None of the authors have competing interests to declare.

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5

Chapter 5

Reduced Mps1 activity leads to meiotic spindle instability in *in vitro* matured oocytes from aged mares

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ABSTRACT

In vitro embryo production (IVEP) is an increasingly popular means of breeding horses. However, ultimate success is limited by a high incidence of early embryonic loss. While there are various possible causes of pregnancy failure, chromosomal abnormalities such as aneuploidy are important potential contributors. This study evaluated the frequency of micronucleus formation as a proxy for the incidence of aneuploidy in *in vitro* produced (IVP) and *in vivo* derived horse blastocysts. In addition, associations between IVP embryo morphology, the incidence of nuclear abnormalities and the likelihood of pregnancy were investigated. IVP blastocysts exhibited a higher frequency of cells with micronuclei than *in vivo* derived embryos (10 % vs. 1 %; P = 0.05). This indicator of chromosomal instability may explain the higher incidence of pregnancy failure after transfer of IVP-embryos. Frequency of micronuclei was not, however, reliably correlated with bright field microscopic morphological characteristics. Nevertheless, IVP embryos reaching the blastocyst stage after Day 9 of *in vitro* culture were less likely to yield a pregnancy (27% vs 69%), while embryos that had expanded prior to transfer were more likely to undergo embryonic death (44% vs 10%). These findings indicate that current embryo culture conditions are sub-optimal, and that speed of embryo development is correlated with pregnancy survival.

INTRODUCTION

Over the last decade, *in vitro* embryo production (IVEP) has become an increasingly common clinical procedure in equine reproduction. IVEP is used to overcome both female and male sub-fertility, and allows optimal use of limited stocks of semen. Moreover, IVEP combined with embryo cryopreservation facilitates embryo production outside the breeding season, allows optimal use and easy scheduling of recipient mares, and can be used to produce embryos from competing mares without risk of interfering with their competition schedule. IVP also makes it possible to salvage the genetic material from mares that die suddenly. In order to produce embryos *in vitro*, immature oocytes are harvested either by transvaginal aspiration (ovum pick up: OPU) or by follicle scraping (*post mortem*) and then matured *in vitro* [1]. Since conventional *in vitro* fertilisation with equine gametes is not reliably successful, oocytes that reach the metaphase II (MII) stage are fertilised by intracytoplasmic sperm injection (ICSI) [2], and subsequently cultured *in vitro* until they reach the blastocyst stage, at which point they are transferred into a suitable recipient mare or are cryopreserved. Although the success of equine OPU-ICSI has improved progressively over the last 10-15 years, equine IVP results are still variable between laboratories and mares. Recent reports suggest that between 7.2 % and 44 % of injected oocytes develop into a blastocyst [3, 4] and, although the likelihood of pregnancy after transfer ranges between 40% and 80 %, there is a relatively high incidence of pregnancy loss (16 % - 20 %) before Day 60 of gestation [5, 6]. While various factors may contribute to IVEP failure, in human IVF programs embryonic aneuploidy is recognised as the primary contributor to developmental arrest, early embryonic death and miscarriage [7-12]. Although it is known that IVEP increases the number of apoptotic cells in horse embryos [13, 14], and cellular aneuploidy is detectable in equine embryos [15], the incidence and severity of aneuploidy in equine IVP-embryos is unknown, and it is unclear whether *in vitro* embryo production increases the risk of aneuploidies arising during the early mitotic divisions in horse embryos.

A recent study on human IVP embryos examined the correlation between the presence of nuclear abnormalities detected by confocal microscopy and aneuploidy detected by cytogenetic analysis, and demonstrated that micronuclei (MN, i.e. a small 'nucleus' separate from the main nucleus) are a reliable indicator of chromosomal aneuploidy [16]. This was confirmed by Vazquez-Diez, Yamagata [17] who demonstrated in a time-lapse study of mouse embryo development that MN were the result of severely lagging chromosomes. In particular, Kort *et al.* (2016) found that, in blastocysts, the incidence of MN was higher in embryos showing

developmental arrest, and they suggested that a threshold percentage of cells undergoing normal mitosis is required during the early cell divisions if embryonic development is to proceed. If the fidelity of chromosome segregation exceeds this threshold, normal embryonic development can continue.

Current techniques for selecting IVP embryos both in animal [18, 19] and human [20, 21] ART rely on morphological assessment. In addition to morphology, timing of blastocyst formation *in vitro* is a good indicator of quality for human [22, 23], bovine [24] and porcine [25] embryos. Blastocyst formation at an earlier age has been associated with a higher cell number, a higher ratio of inner cell mass to trophectoderm cells and fewer apoptotic cells. Recent time-lapse studies in human 1-8 cell embryos have revealed that chromosome mis-segregation induces a delay in the completion of the first cleavage divisions [26, 27]; it is therefore possible that slowly developing embryos have a higher incidence of aneuploid cells.

Although several attempts have been made to develop a scoring system for equine IVP embryos (Tremoleda et al. 2003; Lewis et al. 2016), a morphological classification that takes account of the timing of embryo development and is associated with embryo survival post-transfer is lacking. Since the morphology of IVP equine embryos differs substantially both from their *in vivo* derived equivalents and from IVP-embryos from other species, the extrapolation of scoring systems is challenging. In fact, *in vivo* derived equine embryos are, at any given time post-fertilisation, developmentally more advanced than IVP embryos [13-15]; they are larger, contain more cells and develop a visible blastocoel earlier than IVP embryos. IVP embryos from other species are more transparent, which makes their morphological quality easier to assess than that of equine IVP embryos.

The aims of this study were therefore to; 1) indirectly quantify chromosomal aneuploidy in IVP and *in vivo* derived equine blastocysts by evaluating their nuclear morphology; 2) investigate whether a correlation exists between bright field microscopic morphological characteristics of IVP embryos, their speed of development and the proportion of normal nuclei; 3) evaluate whether bright field microscopic morphological characteristics and speed of development could be used to predict pregnancy establishment and survival following embryo transfer.

MATERIAL AND METHODS

Experimental design

Experiment 1: indirect quantification of chromosomal aneuploidy in *in vivo* and *in vitro* derived equine blastocysts by evaluating morphology of individual nuclei

This experiment was designed to investigate the impact of IVEP on post-zygotic embryonic aneuploidy. Fourteen *in vivo* derived Day 6-7 blastocysts and 34 IVP Day 6-13 blastocysts were fixed and stained to image the DNA and the actin cytoskeleton. The total number of nuclei and the incidences of micronuclei (MN), cells showing nuclear fragmentation (NF) and mitotic cells (MC) were recorded and calculated as proportions of total cell number (MN%, NF% and MC%) (Figure 1). The incidences were compared between the *in vivo* derived and the IVP embryos.

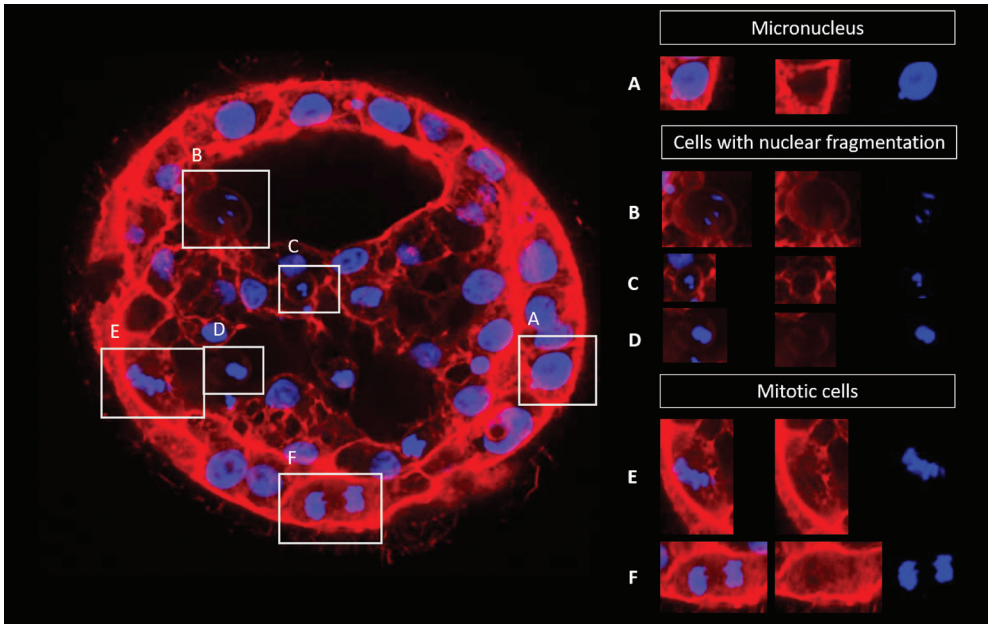

Figure 1. Confocal micrographs of an equine *in vitro*-produced blastocyst (DNA, blue; F-actin, red) illustrating a nucleus with a micronucleus in the 7 o'clock position (A), cells showing different degrees of nuclear fragmentation (B-D) and mitotic cells in metaphase (E) and telophase (F).

Experiment 2: Correlations between speed of blastocyst formation, bright field microscopic appearance and incidence of MN in IVP embryos

This experiment investigated the possibility of a correlation between speed of development, morphological parameters and the proportion of abnormal nuclei in IVP embryos. Embryos were divided into two groups based on the speed at which they reached the blastocyst stage (6-9 days after ICSI or beyond day 9 after ICSI). Each embryo was evaluated using a bright field microscope at a magnification of x200, prior to fixation and immunostaining. The morphological parameters evaluated were selected on the basis of previous reports [4, 14], and were: 1) appearance of the trophectoderm (organisation of a presumptive trophectodermal layer); 2) presence of extruded cells (presence of dark cells extruded from the blastocyst) and 3) obvious expansion (increase in blastocyst diameter with concomitant thinning of the zona pellucida) (Figure 2). The morphological parameters were subsequently analysed with respect to the number of nuclei, MN%, NF% and MC%.

Experiment 3: Correlation between bright field morphology and pregnancy survival after embryo transfer

To determine whether specific morphological phenotypes were associated with a higher chance of developing into a viable pregnancy, IVP embryos that reached the blastocyst stage at day 6-9, or beyond day 9, after ICSI were assessed for the above-mentioned morphological parameters under the same conditions as Experiment 2, and subsequently transferred into a suitable recipient mare (Day 3-5 after ovulation). The likelihood of establishing (Day 7 after transfer) or maintaining pregnancy (Day 35 after transfer) was then correlated with embryonic phenotype and speed of development. Retrospective data on establishment and maintenance of pregnancy after transfer of *in vivo* derived (i.e. flushed) embryos was used for comparative purposes.

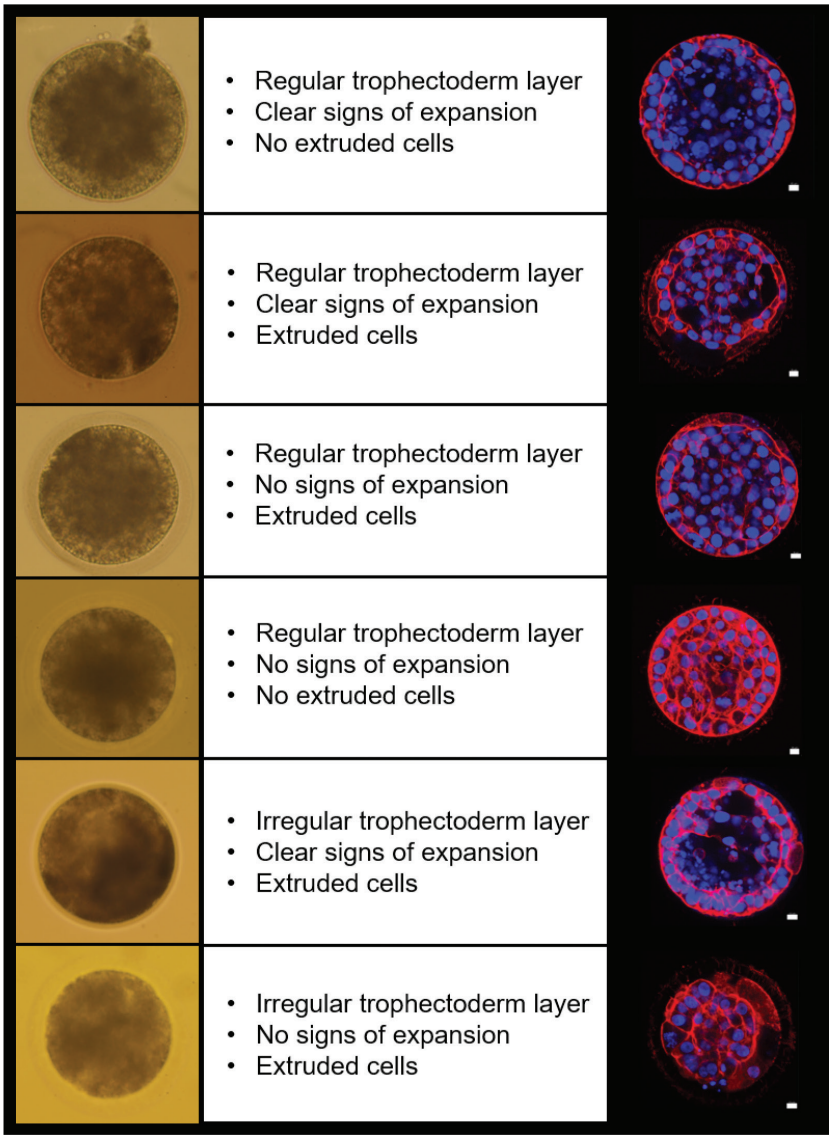

Figure 2. Examples of brightfield morphological classification of equine *in vitro*-produced (IVP) embryos. Photomicrographs show embryos before fixation using brightfield microscopy (left) and after immunofluorescent staining under confocal laser scanning microscopy (right; blue, chromatin; red, F-actin) with their respective classification (centre column). All IVP blastocysts were scored on the basis of three morphological parameters: (1) the presence of a regular trophectoderm layer; (2) signs of expansion (increase in blastocyst diameter with concomitant thinning of the zona pellucida); and (3) the presence of extruded cells. Scale bars = 10 µm.

Animals

All animal procedures were approved by Utrecht University's Animal Experimentation Committee (permission number 2012.III.03.020).

Experiments 1 and 2

A total of 14 *in vivo* derived and 34 IVP embryos were used for the immunofluorescent study. A group of 9 donor mares, aged between 3 and 11 years, was used to provide the *in vivo* derived embryos. Fourteen of the IVP embryos were obtained from 6 donor mares, aged between 4 and 16 years (mean age ± S.D.; 10.2 ± 4.0), subjected to Ovum Pick-Up (OPU); the remaining 20 IVP embryos were produced from oocytes collected *post mortem* from ovaries recovered from 2 different slaughterhouses on 15 different collection days. Five embryos originated from ovaries recovered from young mares (10 ± 1.7; 9-12 y.o.), 8 from old mares (21.4 ± 4.8; 16-33 y.o.) and 7 embryos were derived from mares of unknown age.

Experiment 3

Thirty-seven donor mares were used to produce 66 IVP embryos: 19 IVP embryos were derived from 11 old mares aged between 16 and 26 years (mean ± s.d.:18 ± 3.2), and 47 were derived from 26 young mares aged between 2 and 13 years (mean ± s.d.: 8 ± 3.2). Finally, 54 mares aged between 3 and 15 years (9 ± 2,9) and on Days 3 to 5 after ovulation were used as recipients for ICSI-embryo transfer. Retrospective data on pregnancy establishment and maintenance within our commercial embryo transfer program (from 211 transfers of Day 8 *in vivo* derived embryos performed by the same practitioners) were used for comparative purposes.

***In vivo* embryo production**

The oestrous cycle of donor mares was monitored by transrectal palpation and ultrasonography using a scanner equipped with a 7.5 MHz linear transducer (Mylab Gamma, Esaote, Maastricht, The Netherlands). During early oestrus, mares were examined every other day and, once the dominant follicle exceeded 30 mm, examinations were performed daily. When the follicle exceeded 35 mm in diameter and the mare showed clear uterine oedema indicating oestrus, 1.500 IU of human chorionic gonadotropin (Chorulon, Intervet Nederland BV, Boxmeer, The Netherlands) was administered to induce ovulation and the mare was inseminated with fresh

semen from a single stallion of known good fertility (immunofluorescent staining study) or with chilled- transported or frozen semen from a stallion selected by the mare owner (commercial programme). Embryos were recovered at either Day 6.5 or 7 post ovulation for the immunofluorescent staining study, and at Day 8 for embryo transfer, by standard uterine flushing with lactated Ringer's solution (Ringer's Lactate Solution for irrigation, Baxter, Deerfield, IL, USA) supplemented with 0.5 % v.v. fetal calf serum (FCS, GIBCO BRL Life Technologies, Bleiswijk, The Netherlands) as previously described by Stout [28]. The embryos were then washed 10 times with holding medium (SYNGRO, Vetoquinol, Magny-Vernois, France).

In vitro embryo production

Oocyte collection

Equine cumulus oocyte complexes (COCs) were recovered either by trans-vaginal ultrasound-guided aspiration (ovum pick up: OPU) or by scraping the follicles of ovaries from slaughtered mares.

Recovery of COCs by OPU

The OPU procedure was performed as described previously by Claes, Cuervo-Arango [6]. In short, all follicles larger than 5 mm were punctured via a transvaginal ultrasound guided approach using a 12 G double-lumen needle attached to a vacuum pump (Cook Medical, Bloomington, IN, USA). After aspiration of the follicular fluid, the follicle wall was scraped by rotating the needle and the follicle was flushed 8-10 times with embryo flushing medium (Euroflush, IMV Technologies, Leeuwarden, The Netherlands) supplemented with 0.4 % heparin (5000 IU/mL; Leo Pharma, Balleru, Denmark) at 38.5 °C. The aspirated fluid was recovered in 50 ml tubes (Greiner, Sigma-Aldrich) held in a heated block at 38.5 °C (Gewiss, Cenate Sotto Bergamo, Italy), and subsequently poured through a 70 µm filter (70 µm Falcon™ Cell Strainer, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The content of the filter was rinsed into a petri dish (Greiner Bio-One, Kremsmünster, Austria) and COCs were then identified using a dissecting stereomicroscope (Olympus SZ-60, Olympus, Shinkjuku, Tokyo, Japan) equipped with a warming plate (K-System, Origio, Målov, Denmark) set at 37 °C. The COCs were washed 4 times in Hepes-buffered synthetic oviduct fluid (H-SOF,

Avantea, Cremona, Italy) and held in H-SOF overnight in 2 ml tubes (Nunc™, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Recovery of COCs from abattoir-derived ovaries

Ovaries were collected at the abattoir within 15 min after the mare's death and transported at 21 °C – 25 °C to the laboratory within 5 hours, as described previously [29]. At the laboratory, COCs were harvested by scraping the follicle wall with a bone curette and then flushing the lumen with phosphate buffered saline at 37 °C (PBS, B.Braun, Hessen, Germany) supplemented with 0.5 % (v/v) penicillin/streptomycin (Gibco BRL Life Technologies, Bleiswijk, The Netherlands) as described previously [30]. The COCs were identified, and held overnight, as described for the COCs collected by OPU.

***In vitro* maturation**

After overnight holding in H-SOF, COCs were matured *in vitro* in a 50:50 mixture of Dulbecco's minimal medium (DMEM, Gibco) and Hams F-12 (Gibco) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich), 0.125 µg/mL epidermal growth factor (Peptrotech, London, United Kingdom), 0.1 IU/ml follicle stimulating hormone (Sigma-Aldrich), 0.6 mM cysteine (Sigma-Aldrich), 0.1 mM cysteamine (Sigma-Aldrich), and 0.1 % insulin, transferrin, sodium selenite (ITS; Corning Life Science, New York, USA) under mineral oil for 24h at 38.5 °C in a humidified atmosphere of 5 % CO_2-in-air. After maturation, cumulus cells and corona radiata were removed by exposing COCs to 0.01 % hyaluronidase (Sigma-Aldrich) in H-SOF and gently pipetting through a 155 µm and then a 131 µm capillary plastic pipette (EZ-strip, Origio, Målov, Denmark). Cumulus-denuded oocytes were then washed twice in H-SOF medium supplemented with 10% FCS and maintained in H-SOF until ICSI.

Intra-cytoplasmic sperm injection (ICSI) and embryo culture

Frozen-thawed spermatozoa from stallions with known good fertility via ICSI were selected by a swim-up procedure using a commercial oocyte handling medium (G-MOPS, Vitrolife, Englewood, CO, USA) supplemented with 10 % FCS, as described previously [31]. Oocytes were placed in a 5 µl microdrop of H-SOF under mineral oil at 38°C on a heated stage (Tokai-Hit, Olympus, Shinkjuku, Tokyo, Japan) mounted on an inverted microscope (Olympus IX71, Olympus, Shinkjuku, Tokyo, Japan) equipped with a micromanipulator (Transfer Man NK2,

Eppendorf, Hamburg, Germany). Oocytes with an intact oolemma and an extruded first polar body were injected, as described by Tremoleda, Stout [14]. Just before injection, 2 µl of the motile sperm suspension was mixed with 5 µl of clinical grade polyvinyl pyrrolidone (PVP) (10 % PVP) in Hepes-buffered salt solution (Lucron Bioproducts B.V., Gennep, the Netherlands) to slow sperm cell movement and aid capture. A motile spermatozoon was then immobilised by swiping the injection needle (MIC-50-Angled 30°; Origio, Måløv, Denmark) across its tail and against the bottom of the dish. The spermatozoon was then aspirated tail first into the needle and injected into the ooplasm with a minimal volume of accompanying medium. For sperm injection, the oocyte was held stationary by suction via the holding pipette (MPH-LG-Angled 30°; Origio, Måløv, Denmark) with the polar body positioned at 12 or 6 o'clock and the injection needle advanced through the zona pellucida and plasma membrane at the 3 o'clock position. Presumptive zygotes were cultured in 15 µl microdroplets of a commercial human embryo culture medium (Global medium, LifeGlobal, Guilford, CT, USA) with 10 % FCS under mineral oil (SAGE oil for tissue culture, Origio, Målov, Denmark) in a humidified atmosphere containing 5 % CO_2 and 5 % O_2 at 38.5 °C. Five days after ICSI, cleavage was assessed and cleaved embryos were transferred to DMEM/F-12 with 10 % FBS and cultured in a humidified atmosphere containing 5 % CO_2 and 5 % O_2 at 38.5 °C. Embryo development was evaluated daily using an inverted microscope equipped with a 20x objective (Olympus IMT-2, Olympus, Shinkjuku, Tokio, Japan) from Days 6–13 to identify the day of blastocyst formation.

Morphological assessment of *in vitro* produced blastocysts

All IVP embryos were evaluated using an inverted microscope equipped with a 20x objective (Olympus IMT-2, Olympus, Shinkjuku, Tokyo, Japan), and photomicrographs (Olympus E-330, Olympus, Shinkjuku, Tokyo, Japan) were taken. Subsequently, various parameters were evaluated (Figure 2). First, the developmental rate (time to reach the blastocyst stage) was recorded. Then, the trophectoderm organisation (clearly visible presumptive trophectoderm layer or not), the presence of extruded cells, and obvious expansion of the blastocyst were evaluated.

Immunofluorescent study

Fixation and fluorescent labelling of embryos for nuclear evaluation

Blastocysts were fixed in 2 % paraformaldehyde for 30 minutes at room temperature (RT) and then stored at 4 °C until immunostaining. Fixed embryos were washed 3 times in PBS containing 3 mg/ml polyvinylpyrrolidone (Sigma-Aldrich) (PBS-PVP) for 5 min and permeabilised in 0.1 % saponin (Sigma-Aldrich) in PBS for 30 min at RT. Next, the embryos were incubated in a 0.165 µM solution of Alexa Fluor® 568 phalloidin (Molecular Probes Europe BV, Leiden, The Netherlands) in PBS-PVP for 30 min at RT to label F-Actin. After three 5 min washing steps in PBS-PVP, embryos were incubated for 30 min at RT in a 1:500 dilution of Hoechst 33342 (Sigma-Aldrich) in PBS-PVP to label the DNA. Embryos were mounted in 10 µl droplets of antifade mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA) on glass microscope slides (Superfrost Plus, Menzel, Braunschweig, Germany) using 0.12 mm thick spacer wells (Secure-Seal^{TM} Spacer, Thermofisher Scientific, Waltham, MA, USA), and sealed under a coverslip using nail polish.

Confocal imaging

The immunostained embryos were analysed using a Leica SPE-II – DMI4000 confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with a 65x objective. The images produced by the lasers were merged using the Leica Application Suite X® software (LAS, Leica). 561 nm and 405 nm lasers were used to excite the Alexa Fluor® 568 phalloidin and the Hoechst 33342 respectively. The whole embryo was imaged by acquiring optical slices at 1 µm intervals, and Z-stacks were oversampled at 0.467 µm to ensure that all MN were captured.

Analysis of confocal images

The number of nuclei, MN, NF and MC were counted manually using ImageJ [32]. The percentage of MN (MN%), NF (NF%) and MC (MC%) were calculated as the number of MN, NF and MC respectively, divided by the total number of nuclei. The grid tool was used to place a grid over the image and the cell counter plugin was used to count the number of nuclei, MN, NF and MC. The counting tool allowed tracking of the nuclei, MN, NF and MC throughout the Z-stack, thereby avoiding double counting. A MN was defined as a small (1/6 to 1/3 of nuclear diameter) extranuclear DNA body (i.e. Hoechst 33342 positive) within the same cell as an

obvious nucleus [33], a MC was defined as a cell with a mitotic figure (metaphase, anaphase or telophase) and a NF was defined as a cell with a fragmented nucleus (Figure 1).

Embryo transfer and diagnosis of pregnancy

In vivo derived (n = 211) and IVP-embryos (n = 66) were transferred into recipient mares by 4 experienced practitioners using the technique described by Wilsher and Allen [34, 35]. Embryo-recipient synchrony ranged from +1 to -5 days (recipient ovulated from one day before to 5 days after the donor mare) for *in vivo* derived embryos. IVP-embryos were transferred into recipient mares that ovulated 3 to 5 days previously, as described by Claes, Cuervo-Arango [6]. The pregnancy status of recipient mares was examined by ultrasonography of the genital tract at 1 and 5 weeks after transfer.

Statistical analysis

Data were analysed using SPSS Statistics for Mac (version 24.0: IBM Corp., Armonk, NY, USA). Independent samples T-tests were conducted to compare the number of nuclei, MN, MC, NF and MN%, MC% and NF% respectively between *in vivo* derived and IVP blastocysts. A general linear model was conducted to investigate interactions between the developmental rate, trophectoderm organisation, presence of extruded cells and embryonic expansion with the number of nuclei, MN, MC, NF and MN%, MC% and NF%. Since no interactions were found, independent samples T-tests were used to explore the impact of delayed development, trophectoderm organisation, presence of extruded cells and obvious expansion on the number of nuclei, MN, MC, NF, MN%, MC% and NF% of IVP-embryos. Initial and ongoing pregnancy results for IVP- and *in vivo* derived embryos were compared using a Chi-squared test. Chi squared tests (or independent samples T-tests if expected cell values < 5) were conducted to explore the impact of delayed development, trophectoderm organization, extruded cells and embryonic expansion on initial and ongoing pregnancy results for IVP-embryos. Significance was set at $P < 0.05$.

RESULTS

A total of 100 IVP-embryos were produced for this study. Thirty-four were immunostained and 66 were transferred into recipient mares. Of the 34 stained presumptive blastocysts, 6 of the 20 IVP embryos derived from slaughtered mares were found to be degenerate (2 in each group, respectively of young, old and of unknown age), and 1 of the 14 IVP embryos derived from the OPU program was composed of an unusually low number of cells (60 nuclei); all 7 were excluded from further analysis. The pregnancy results for 211 *in vivo* derived embryos in the commercial embryo transfer program of our clinic were used as a comparison for the IVP-ET pregnancy results.

IVP blastocysts show a higher incidence of micronuclei than *in vivo* derived embryos

In experiment 1, the total number of nuclei and the percentage with MN (MN%), cells showing nuclear fragmentation (NF%) and mitotic cells (MC%) were evaluated in 14 *in vivo* derived (6 early blastocysts and 8 expanded blastocysts) and 27 IVP blastocysts (Table 1).

Embryo production method	Developmental stage	No. of embryos	No. of nuclei/embryo	MN%	NF%	MC%
In vivo	Early blastocyst	6	480.2 ± 103.4[a]	0.7 ± 0.6[a]	1.6 ±	3.4 ± 0.4[a]
	Expanded blastocyst	8	1490.0 ± 193.1[b]	0.3 ± 0.1[a]	0.3 ± 0.1[a]	2.0 ± 0.4[a]
In vitro	Blastocyst	27	261.0 ± 21.4[a]	9.6 ± 1.2[b]	15.9 ±	3.4 ± 0.3[a]

Table 1. Total number of nuclei and relative percentage of micronuclei (MN%), cells showing nuclear fragmentation (NF%) and mitotic cells (MC%) in equine blastocysts produced *in vivo* and *in vitro*. Unless indicated otherwise, data are given as a mean ± s.e.m. Within columns, values with different superscript letters differ significantly ($P<0.05$).

IVP embryos showed higher incidences of cells with MN (19 times) and cells showing nuclear fragmentation (16 times) than *in vivo* embryos (Table 1). Expanded *in vivo* derived blastocysts were composed of more cells and contained a lower proportion of mitotic cells (MC%) than early *in vivo* derived blastocysts and IVP embryos. IVP embryos were composed of a similar number of cells with a MC% similar to the *in vivo* derived early blastocysts.

Blastocyst morphological characteristics do not correlate with abnormal nuclei in IVP embryos

In experiment 2 the possibility of a correlation between blastocyst morphological characteristics (developmental rate, trophectoderm organization, presence of extruded cells and presence of expansion) and abnormal nuclear morphology was explored (results are shown in Table 2).

		No. of embryos	No. of [illegible]	MN%	NF%	MC%
Developmental Rate (time to blastocyst formation)	6-9 days	15	203.5 ± 21.1[a]	10.9 ± 1.9	4.0 ± 0.5	14.6 ± 2.3
	10-13 days	12	333.0 ± 29.9[b]	8.1 ± 1.3	2.7 ± 0.3	17.6 ± 3.9
Trophectoderm layer	Clearly visible	15	276.6 ± 27.9	10.1 ± 1.2	3.1 ± 0.2	12.5 ± 2.0
	Non visible	12	241.6 ± 33.9	9.0 ± 2.3	3.7 ± 0.7	20.2 ± 3.8
Extruded cells	No	17	279.9 ±26.0	10.4 ± 1.7	3.3 ± 0.5	14.4 ± 1.8
	Yes	10	228.9 ± 36.9	8.4 ± 1.5	3.5 ± 0.4	18.5 ± 4.8
Expansion	No	9	164.2 ± 24.9[a]	10.9 ± 3.2	4.5 ± 0.9[a]	24.5 ± 4.6[a]
	Yes	18	309.4 ± 22.3[b]	8.9 ± 0.9	2.9 ± 0.2 [b]	11.6 ± 1.4[b]

Table 2. Total number of nuclei and relative percentage of micronuclei (MN%), cells showing nuclear fragmentation (NF%) and mitotic cells (MC%) in equine *in vitro*-produced blastocysts subdivided by developmental rate, presence of a visible trophectoderm (TE) layer, extruded cells and expansion. Unless indicated otherwise, data are given as a mean ± s.e.m. Within columns, different superscript letters after values for developmental rate (i.e. time to blastocyst formation), TE layer, extruded cells and expansion separately indicate significant differences ($P<0.05$).

Embryos that developed into a blastocyst later than 9 days post ICSI (n = 15) had more nuclei when they eventually reached the blastocyst stage than embryos that developed into a blastocyst within 9 Days (n = 12) (333.0 ± 29.9 vs. 203.5 ± 21.1; P = 0.002). However, there were no significant differences in MN%, MC% and NF% between these two groups. Moreover, there were no significant differences in number of nuclei, MN%, NF% or MC% between IVP embryos showing a readily discernible trophectoderm layer (n = 15) and embryos in which this feature was absent (n = 12). Similarly, there were no significant differences for any of the parameters investigated between embryos with (n = 10) and without extruded cells (n = 17). However, when nuclear morphology data were analysed in relation to the presence or absence of expansion, IVP embryos classified as showing expansion (n= 18) had more nuclei (309.4 ± 22.3 vs. 164.2 ± 24.9; P < 0.001), a lower MC% (2.9 % ± 0.2 vs. 4.5 % ± 0.9; P = 0.02) and a

lower NF% (11.6 % ± 1.4 vs. 24.5 % ± 4.6; P = 0.002) than embryos that did not started to expand (n = 9).

IVP embryos that develop slowly or exhibit expansion prior to transfer are less likely to result in ongoing pregnancy.

IVP-embryos (n = 66) had a lower likelihood of yielding a pregnancy (41/66: 62%) 7 days after transfer than *in vivo* derived embryos (187/211: 89%; P < 0.001). Within the IVP-embryos, embryos that reached the blastocyst stage later than day 9 post ICSI yielded a lower initial likelihood of pregnancy than embryos that reached the blastocyst stage earlier (3/11, 27% vs 38/55, 69%; P = 0.02). None of the other morphological parameters examined were significantly correlated with differences in likelihood of pregnancy. Interestingly, IVP-embryos showed a similar incidence of pregnancy loss between Days 14 and 40 of gestation (7/41; 17%) as *in vivo* derived embryos (21/187, 11%; P = 0.3). Moreover, the speed of *in vitro* development to a blastocyst did not statistically influence the likelihood of pregnancy loss, although the numbers were too small for meaningful analysis since only 3 of the 11 IVP embryos that reached the blastocyst stage later than day 9 resulted in pregnancy at the first examination. Nevertheless, all 3 of these pregnancies were ongoing at Day 40. Of the IVP embryos that reached the blastocyst stage at Days 7-9, blastocysts that had visibly expanded were more likely to succumb to pregnancy loss between Days 14 and 40 (4/9; 44%) than blastocysts that had not visibly started to expand (3/29, 10%; P = 0.04); the other morphological parameters did not influence pregnancy loss between Days 14 and 40.

DISCUSSION

The results of this study indicate that the *in vitro* culture conditions employed negatively affect the developmental competence and, probably, the integrity of chromosome segregation in equine embryos. Indeed, approximately 10 % of the cells in IVP embryos exhibited one or more MN, whereas less than 1 % of the cells in *in vivo* derived embryos showed similar evidence of aberrant chromosome segregation (Table 1). The higher prevalence of MN in IVP compared to *in vivo* derived embryos suggests that the *in vitro* culture conditions employed compromise the ability of some cells to undergo normal chromosome segregation during mitosis. This is in contrast to mouse embryos where similar numbers of MN were reported in *in vivo* derived and *in vitro* produced morulae [17]. However, the fact that the *in vivo* derived embryos in our study are more advanced in their development than the IVP embryos could partly explain the

reduction in the prevalence of nuclear abnormalities. Indeed, in human embryos, the frequency of nuclear abnormalities has been reported to decrease as embryos develop, with 16% of nuclei abnormal in cleavage stage embryos compared to 5 % in blastocyst stage embryos. The highest percentage (65%) of chromosomal abnormalities was seen in arrested human embryos [16].

Micronuclei are a hallmark of mitotic infidelity in cancer cells and are formed when a chromosome 'lags' behind during anaphase, and becomes far enough detached from the rest of the chromosomes to stimulate the formation of a distinct nuclear envelop rather than joining the rest in the nucleus. Although enclosed within a nuclear envelop, the chromosomes in MN may undergo inadequate DNA replication and are more prone to DNA damage during the following S-phase [36]. When the cell next divides, isolation of the chromosome in the MN leads to a copy number asymmetry during the following mitosis, inducing a new mis-segregation event [36]. Moreover, due to the compromised DNA repair mechanisms, the chromosomes in the MN accumulate DNA damage and can undergo chromosome rearrangements, *de novo* mutations or chromothripsis [36, 37]. A recent time-lapse study of mouse embryos revealed that the outcome of MN formation is different for murine morulae than for cancer cells [17]. These authors found that MN in embryos lack kinetochores and are thus unable to correctly segregate during mitosis, resulting in random inheritance of the MN by only one of the daughter cells. The authors suggest that this form of inheritance, together with the possible absence of chromothripsis may be a mechanism to prevent the incorporation of damaged DNA into the genome. In the present study, MN were identified solely on the base of positive DNA staining combined with their size and position relative to the main nucleus, one limitation of this methodology is that the identity of the additional pieces of DNA as micronuclei is not corroborated, and neither does it allow the study of DNA damage occurring as consequence of micronucleus formation. In the future, more robust studies could analyse a larger number of IVP embryos and combine the immunofluorescent staining of DNA, with markers for the nuclear envelope (e.g. Lamin-B) to verify their identification as micronuclei. Additional staining for DNA double strand breaks (e.g. with phosphorylated γ-H2AX or 53BP1) would also help characterize the consequences of micronucleus formation to DNA integrity in horse embryos.

Nevertheless, the apparently higher incidence of MN in IVP than *in vivo* derived equine embryos, is an indication that the former suffer from a higher level of chromosomal instability. Since aneuploidy has been proposed to be the major factor responsible for human embryo developmental arrest and pregnancy failure (King 1990; Munne *et al.* 1994; Margalioth *et al.*

2006; Hodes-Wertz *et al.* 2012; Maxwell *et al.* 2016) this may explain the lower likelihood of pregnancy for IVP compared to *in vivo* derived embryos after transfer into recipient mares. In human IVF, aneuploidy appears to be more frequent in slowly developing embryos [38, 39]. However, we did not see a significant difference in the incidence of MN between IVP-blastocysts developing before or after Day 9 post ICSI. This might be because we did not examine the incidence of MN at the same time point in these two groups of embryos. Instead, we waited until the delayed embryos had developed into a blastocyst. During this "time gap" a process of cell selection could have induced apoptosis in some of the aneuploid cells, thus reducing the apparent incidence of MN.

Unexpectedly, IVP-embryos that developed into a blastocyst after Day 9 post ICSI, showed a higher cell number at blastocyst formation and similar MN%, NF% and MC% to embryos developing more rapidly. That these slowly developing embryos showed a reduced likelihood of pregnancy after transfer (27% vs. 69%) could be a consequence of perturbation of the process of cell lineage segregation. In this respect, mouse embryos showing delayed development have been reported to show a reduced percentage of inner cell mass cells [40]. The present study estimated cell proliferation by counting cells in the M phase of mitosis, this method underestimates the real number of mitotic cells since it does not take account of cells in the G1, S and G2 phases. Future studies should include the use of proliferation markers, such as Ki-67, to more specifically evaluate the relationship between rate of cell proliferation and pregnancy survival in equine IVP embryos.

Similarly to Lewis, Hinrichs [4], we found it challenging to correlate bright field microscope morphological characteristics of IVP-blastocysts with the incidence of nuclear abnormalities. An example of obvious discrepancies between microscopic morphology and incidence of nuclear abnormalities can be seen in Figure 3. Overall this indicates that, although morphology can help in the selection of blastocysts for transfer, it cannot be used to reliably assess IVP-embryo nuclear or chromosomal integrity. Interestingly, although the number was low, embryos that had visibly expanded at the time they were first recognised as a blastocyst showed an increased likelihood of pregnancy loss (44% vs. 10%) compared to embryos that had not yet started to expand. This could be because the expanded embryos had progressed further with development, and that later developmental processes such as cell lineage allocation might be more profoundly disturbed by continued *in vitro* culture. Overall, this suggests that the prompt identification of IVP blastocysts before significant expansion is crucial for satisfactory results

after transfer, even though it may be tempting to wait for expansion as an obvious sign of blastocyst formation.

In conclusion, the results of this study indicate that *in vitro* culture leads to increased incidences of nuclear fragmentation and MN formation in equine embryos, where the latter is a proxy for aneuploidy arising during mitotic divisions. These abnormalities are not, however, reliably correlated with bright field microscope morphological characteristics, and morphological assessment of equine IVP-blastocyst quality remains challenging. Furthermore, our results indicate that IVP embryos showing delayed development (i.e. do not reach the blastocyst stage until Day 10 or later) have a significantly lower likelihood of resulting in a pregnancy after transfer, and that it is important to identify and transfer equine blastocysts before expansion has started, to avoid an increased risk of early pregnancy loss in recipient mares.

Clearly, further studies are needed to investigate the mechanisms that predispose to MN formation and the fate of the cells carrying MN during early development of equine IVP embryos. Based on our observations, it is evident that current *in vitro* culture conditions need optimisation to reduce chromosomal instability and improve the developmental competence of resulting embryos.

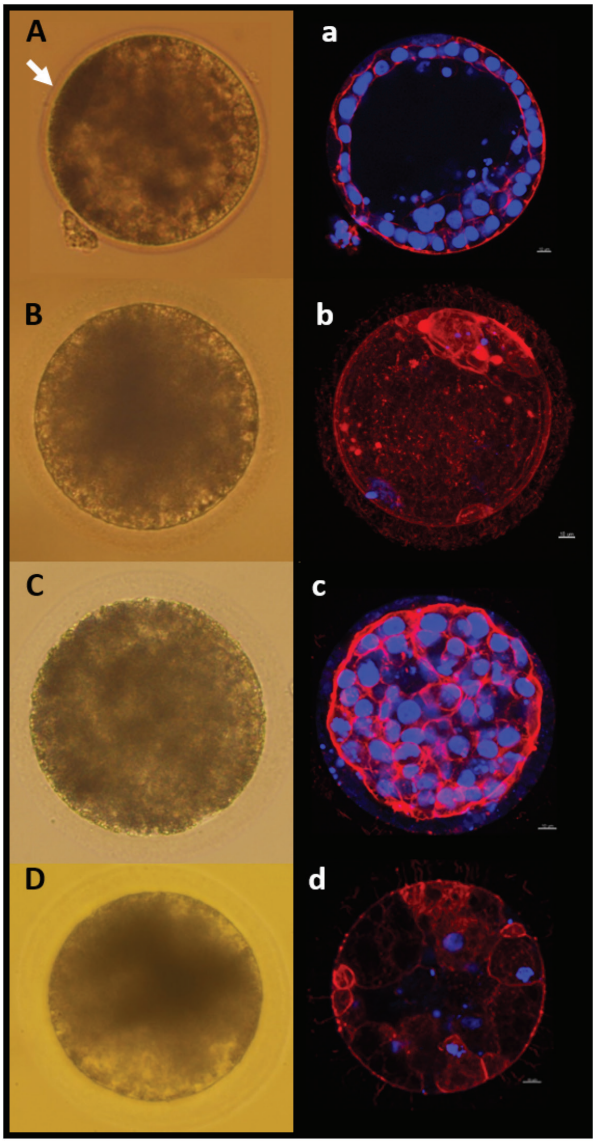

Figure 3. Photomicrographs of *in vitro* produced (IVP) equine blastocysts before fixation under brightfield microscopy (A-D) and after immunofluorescent staining under confocal laser scanning microscopy (a-d; Blue, chromatin; Red, F-actin), illustrating the challenge of scoring IVP equine embryos for quality. A) An embryo scored as exhibiting expansion, a continuous trophectoderm (TE) layer and extruded cells (arrow, presence of a dark area). Confocal microscopy (a) confirmed the brightfield classification. (B) Embryo scored as a blastocyst without expansion, with a continuous and regular TE layer and without extruded cells. Confocal imaging (b) revealed that it was a degenerated embryo, composed only of cells showing nuclear fragmentation. (C) Embryo scored as degenerated, without expansion, with an irregular TE layer and no extruded cells. Confocal microscopy (c) showed that the embryo was an early blastocyst that had just started forming a blastocoele. (D) Embryo classified as degenerated without expansion, an irregular TE layer and no extruded cells. The confocal image (d) demonstrated that it contained only cells showing nuclear fragmentation. Scalebars are set at 10 µm.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest

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Chapter 6

Summarizing Discussion

SUMMARIZING DISCUSSION

The main aim of this thesis was to investigate the effects of advanced maternal age and assisted reproductive techniques on the quality and developmental competence of equine oocytes and embryos. The design of the study arose from previous observations that age-related reduced fertility in mares is an important problem in the equine breeding industry. Reproductive performance in aged mares has improved significantly thanks to better management and the introduction of advanced diagnostic (e.g. ultrasonography, laparoscopy, endocrinological), therapeutic (e.g. various hormonal treatments, uterine cyst ablation, oviductal patency treatment) and assisted reproductive techniques (e.g. artificial insemination, embryo transfer and *in vitro* embryo production [IVEP]). IVEP in particular has undergone gradual but consistent improvement over the past two decades. Despite all these areas of improvement, early pregnancy loss in older mares remains an important issue, causing significant economic losses to the horse breeding industry.

A decline in reproductive success with maternal age is also a well-recognized problem in women. Indeed, the average age at first childbirth in western countries has increased as a result of emancipation and the desire to delay motherhood until career goals have been achieved [1, 2]. In order to overcome this problem, an increasing number of couples resort to assisted reproductive techniques, and in particular *in vitro* embryo production (via conventional *in vitro* fertilization [IVF] or intracytoplasmic sperm injection [ICSI]), in order to achieve parenthood. However, age-related embryonic aneuploidy of meiotic origin is still a major cause of developmental arrest and failure of human IVP embryos to implant.

Given various biological and physiological similarities between women and mares with respect to reproductive processes (e.g. mono-ovulatory; long follicular phase; age-related changes in cycle length, hormone concentrations and fertility; long delay to recruitment of some follicles resulting in extended period of meiotic arrest; reproductive senescence), we speculated that aneuploidy may be a major contributor to the increased incidence of early pregnancy loss in aged mares and after transfer of IVP embryos.

In Chapter 2 we investigated the effect of advanced mare age on meiotic spindle morphology and chromosome alignment. We found that *in vitro* matured MII oocytes from old mares show a higher incidence of chromosome misalignment than oocytes from young mares, with the chromosomes showing greater dispersion from the spindle equator within a significantly thicker metaphase plate. Moreover, although gross spindle morphology did not differ between the two

age groups, MII spindle length was influenced by both maternal age and chromosome misalignment, with advanced mare age associated with a reduced pole-to-pole length in spindles with normally aligned chromosomes, and oocytes with misaligned chromosomes displaying significantly increased pole-to-pole distances. Spindle length is also reported to be affected by chromosome misalignment and maternal age in women [3], and recent reports suggest that regardless of whether it is increased or decreased, spindle length abnormality is associated with a reduced likelihood of normal fertilization, blastocyst formation, and clinical pregnancy [4]. Spindle length is itself determined by ATP-dependent motor proteins and kinases involved in the assembly of microtubular structure and in aligning the chromosomes [5]; the various contributory processes require considerable energy, which is provided by mitochondria. Advanced mare age is associated with mitochondrial damage and a decrease in the mtDNA copy number in *in vitro* matured oocytes [6, 7] and it is, therefore, possible that *in vitro* matured oocytes from old mares may not be able to satisfy the energy requirements for proper spindle assembly and chromosome alignment. However, differences in mitochondrial number between oocytes from young and old mares have only been demonstrated after and not prior to *in vitro* maturation, whereas maternal age-related chromosome misalignment has also been shown in *in vivo* matured oocytes [8]. Since defects in chromosome alignment could also result from loss of centromeric cohesion [9, 10] or SAC dysfunction [11, 12], we focused our attention on the study of these two phenomena (Chapters 3 and 4). Although both *in vivo* and *in vitro* matured MII oocytes from old mares show chromosome misalignment, and this is probably one of the causes of meiosis-related aneuploidy in aged mares, the incidence and origin of aneuploidy in horse oocytes had not previously been reported. We therefore first evaluated the effect of maternal age on the incidence of aneuploidy and on centromeric cohesion in *in vitro* matured oocytes from young and old mares. We found that aneuploidy is a common feature in MII horse oocytes, with an incidence of 15% and 55% in young and old mares respectively, similar to the proportions previously reported in young and older women [13]. Furthermore, 75% of the aneuploid oocytes from aged mares showed an uneven chromatid number, consistent with premature separation of sister chromatids (PSSC) and subsequent random distribution between the oocyte and the first polar body. This result was also comparable to what has been described in older women, where 80% of the aneuploidies detected in the oocytes of aged women are the result of PSSC [14, 15]. It therefore appears that the mare is an interesting, and more natural, animal model than the mouse to study the effect of maternal aging on aneuploidy of meiotic origin.

During the first meiotic division, the two sister chromatids must remain attached and migrate together until anaphase of the second meiotic division is ready to begin, thus the presence of functional cohesion complexes at their centromeres during the completion of the first meiotic division is paramount. Cohesion loss can be evaluated by measuring the interkinetochore distance (iKD: see Chapter 3). When the iKD is increased there is a higher risk that the two sister kinetochores will not act as a single unit during the first meiotic division, and instead bind to microtubules from opposite poles of the spindle (merotelic attachment), this in turn can predispose to premature separation and lead to either PSSC or Reverse Segregation [16, 17]. In chapter three of this thesis, we showed that advanced mare age is associated with an increased iKD in chromosome bivalents in horse oocytes, of a similar magnitude to the increased iKD described in oocytes from older women [18, 19]. Considering that cohesins in mammalian oocytes are synthesized and established around the time of birth and are not subject to further turnover [18-21], one would expect that oocytes from aged mares, that have been in a state of meiotic quiescence for a long period, could suffer from reduced abundance or function of these proteins which could explain the weakened inter-chromatid cohesion. Although we did not measure protein expression, and the gene expression for some cohesins (e.g. REC8 or STAG3) did not vary between mares of different ages, we did find that shugoshin 1 (SGO1) expression was lower in oocytes from aged compared to younger mares. The shugoshin proteins are known to be responsible for protecting chromosomal cohesion. The ring-like cohesin structure is wrapped around both the centromeres and chromosome arms during pro-metaphase and metaphase I; however, by the time that anaphase I is ready to begin, cohesion between the chromosome arms must be released to allow segregation of the homologous chromosomes (Figure 1) [22, 23].

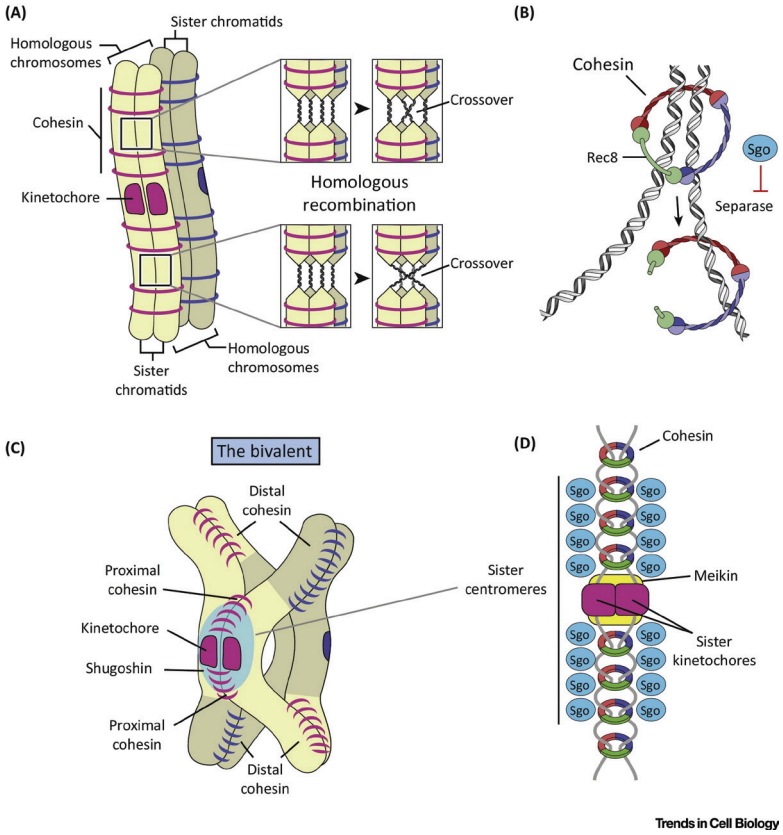

Figure 1. Cohesion of the chromosome bivalent after homologous recombination during meiosis I. (A) Bivalents are held together by having undergone recombination events (crossover) during the replication phase, which gives rise to DNA-links at the crossover sites, and by having the sister chromatids locked together by cohesins. (B) The cohesion complex is composed of three-subunit proteins and acts as a molecular "glue", forming as a ring-like structure that surrounds and holds together the sister chromatids. The shugoshin proteins (Sgo) protect centromeric cohesion by preventing the phosphorylation of Rec8 by Separase. (C) The bivalent structure is guaranteed by the crossover sites and by the presence of cohesin along the chromosome arms (distal cohesin) and at the centromeres (proximal cohesin). (D) Pericentromeric localization of the Sgo proteins is essential for the protection of proximal cohesion from Separase during the meiosis I to II transition. (Modified from Webster A and Schuh M. Mechanisms of Aneuploidy in Human Eggs. Trends in Cell Biology. 2017; 27: 55-68).

SGO1 is localized at both the centromeres and along the chromosome arms during metaphase of the first meiotic division, and it is thought to protect against Separase-dependent REC8 degradation. SGO1 expression along the chromosome arms is reduced prior to the onset of

anaphase, thereby allowing REC8 removal and, consequently, the release of cohesion between the bivalents. On the other hand, SGO1 persists at the centromeric region preserving centromeric cohesion between the sister chromatids, which makes them behave as single unit and remain together until Meiosis II [24]. Reduced SGO1 expression may lead to reduced SGO1 function and failure to avoid centromeric cohesin removal, leading to premature loss of cohesion between the sister chromatids [25-28]. Precocious sister chromatid segregation could, in turn, result in an unbalanced first meiotic division and the formation of MII oocytes with an uneven number of chromatids, just as we found in oocytes from aged mares. Shugoshin recruitment and localization is controlled by BUB1 [29], a centromere-associated protein that is also considered to be an important SAC component. It has previously been shown that BUB1 gene expression is reduced in oocytes from women of advanced age; moreover, impaired BUB1 function is associated with precocious sister chromatid separation and chromosome mis-segregation both in meiosis and mitosis [30-33], most likely mediated by failure of SGO1 recruitment and SAC activation [34-36]. The reduced SGO1 expression in oocytes from aged mares may lead to PSSC via similar mechanisms to those described for impaired BUB1 function.

The main task of the SAC is to delay the onset of anaphase by inhibiting the activator (CDC20) of its main promotor, Anaphase-Promoting Complex or Cyclosome (APC/C). The APC/C is a ubiquitin protein ligase that, in association with CDC20 (APC/C^{Cdc20}), triggers the degradation of Securin and cyclin B1, both of which are essential for the inhibition of Separase, the enzyme responsible for cleavage of the cohesin REC8. In addition, the SAC-dependent inhibition of the onset of anaphase I allows time for the control machinery to ensure that all bivalents are correctly aligned and bi-oriented, with the kinetochores attached to the MTs linking them to opposite spindle poles. Reduced (more permissive) SAC activity may lead to failure to inhibit the APC/C, and could lead to early activation of Separase and premature progression to anaphase when chromosome alignment on the metaphase plate and establishment of the K-MT attachments are not yet complete; this would lead to a high risk of mis-segregation [37]. In short, SAC dysfunction can also contribute to chromosome mis-segregation during meiosis. In chapter 4, we analysed the effect of maternal age on gene expression for selected SAC components in horse oocytes. Conversely to what has been described in oocytes from aged mice and women, *BUB1* gene expression was similar in oocytes from young and old mares. On the other hand, advanced mare age was associated with a reduction in the expression of other SAC components, namely *MPS1*, *SPC25* and *AURKC*. We next investigated the role of MPS1 in

spindle assembly and chromosome alignment by using a specific inhibitor, CPD5. Oocytes from aged mares showed an increased sensitivity to MPS1 inhibition. More specifically, reduced MPS1 function compromised the ability of *in vitro* matured MII oocytes from aged mares to successfully re-form a bipolar spindle following nocodazole washout, and promoted chromosome misalignment. MPS1 activity is required for the localization of the meiosis specific Aurora kinase, AURKC, which is a component of the chromosomal passenger complex (CPC) and essential for K-MT error correction and spindle microtubule stabilization. Spindle instability results in a higher frequency of chromosome misalignment and mis-segregation and correct K-MT attachment plays a crucial role in correct segregation. K-MT interaction is a highly dynamic and unstable process involving multiple rounds of assembly and disassembly regulated by the Aurora kinases, during both mitosis and meiosis. Aurora-kinase dependent regulation is, in fact, essential for promoting error correction and proper attachments that, in turn, ensure the formation of a metaphase plate with correct alignment of the chromosomes so as to guarantee their separation towards the appropriate spindle poles. Alongside reduced *AURKC* expression, the observed reduction in *MPS1* expression in aged mares may further impair AURKC function, thereby predisposing to spindle instability, faulty K-MT attachment and, consequently, mis-segregation. Furthermore, MPS1 plays a crucial role in the SAC dependent inhibition of the APC/C. By phosphorylating the outer-kinetochore protein KLN1, MPS1 induces the recruitment of BUB1 and MAD1, and the sequential activation of the other SAC components to the unattached kinetochores, leading to the formation of the MCC complex responsible for the SAC-dependent block of the APC/C activator CDC20. Reduced MPS1 activity could also impair the recruitment of BUB1, and potentially lead to a SGO1-dependent lack of cohesion protection, which could result in premature activation of Separase-dependent REC8 degradation and PSSC. Although in the experiments described in Chapters 2 and 4, we observed frequent chromosome misalignment in MII oocytes from aged mares, the lack of a specific kinetochore stain did not allow us to establish whether the mis-aligned chromosomes were bivalent, univalent or single chromatids, we could therefore neither confirm nor exclude a direct effect of MPS1 on cohesion loss. Recent findings in oocytes from aged mice suggest that an aging-related decrease in SAC function is the cause of increased APC/C-mediated Securin destruction in the MI-to-MII transition, resulting in increases in the iKD and PSSC [37]. In particular, MII oocytes from aged mice display low levels of Securin compared to oocytes from young animals, whereas no age-related differences were seen in GV or MI oocytes. To test the role of the SAC in increased APC/C-mediated Securin destruction, Nabti et *al.* (2017) inhibited the SAC using an MPS1 inhibitor (AZ3146) in young oocytes undergoing

the MI-to-MII transition; this resulted in reduced levels of Securin in the MII oocytes and a phenotype similar to that observed in old oocytes (increased iKD and PSSC). Interestingly, overexpression of MPS1 partially reversed the increase in iKD and reduced PSSC in old mice. These findings agree with our hypothesis regarding the possible involvement of MPS1 in the mechanisms underlying age-related loss of chromosome cohesion and aneuploidy in horse oocytes; however, further investigations are needed to prove their interdependence. A possible approach would be to measure the iKD and chromatid counts in equine oocytes matured in the presence of an MPS1 inhibitor. Or, more interestingly, a live imaging study of oocytes expressing fluorescent proteins that label the DNA, kinetochores and SGO1. Time lapse imaging of the meiotic divisions in oocytes in the presence or absence of an MPS1inhibitor, would allow us to evaluate the role of MPS1 in the recruitment and function of the shugoshin proteins. Moreover, if this was combined with producing a chromosome spread after maturation, we could measure the iKD and the incidence of aneuploidy in the same oocytes. That MPS1 has been proven to be required for the recruitment of BUB1/SGO1 and SGO2 in mitosis and meiosis [38, 39], further encourages this approach.

At this point, starting from our initial hypothesis that aging-associated aneuploidy may explain reduced fertility in older mares, we can at least conclude that maternal aging clearly affects the incidence of segregation errors and aneuploidy in *in vitro* matured horse oocytes, and may explain the higher incidence of early pregnancy loss.

In Chapter 5, we directed our attention to investigating post-zygotic aneuploidy in *in vivo* and *in vitro* produced embryos, and the association with the likelihood of pregnancy after transfer. We indirectly quantified aneuploidy in IVP and *in vivo* derived embryos by evaluating the incidence of micronucleus (MN) formation, i.e. small nucleus-like structures that can originate from chromosomes that lag behind during anaphase and subsequently separate from the main group of chromosomes at the time of nuclear envelope reformation. Micronuclei are considered to be reliable indicators of chromosomal aneuploidy, and an increase in their incidence is associated with embryo developmental arrest and miscarriage. In our studies, IVP horse embryos displayed a higher incidence of MN than *in vivo* derived early and expanded blastocysts. This may explain the impaired developmental competence of IVP embryos and their lower likelihood of yielding a viable pregnancy. In an attempt to establish selection criteria that could be used to predict pregnancy establishment and survival after ET of IVP embryos, we investigated whether there were any correlations between brightfield morphological characteristics of the embryos, their speed of development and the incidence of MN. None of

the analysed morphological phenotypes (development of a visible trophectoderm, presence of extruded cells, blastocoel expansion and speed of development) were correlated with nuclear status or the incidence of MN. In fact, while morphological assessment is considered a reliable indicator of the quality of IVP embryos in other species, the scoring system currently used for equine IVP embryos was unable to predict either the incidence of MN or apoptotic nuclei in the embryos or the likelihood of pregnancy after transfer to a recipient mare. However, we did identify specific embryonic features that correlated with pregnancy establishment and survival. In this respect, IVP embryos showing delayed development (i.e. did not reach the blastocyst stage until Day 10 or later) had a significantly lower likelihood of resulting in a pregnancy after transfer than embryos that developed into blastocysts before Day 9, even though the former showed a higher cell number at the time of blastocyst formation and a similar incidence of MN. Since blastocysts that showed delayed development have been under culture conditions for longer time, it is possible that they may have been exposed to stressors that could cause perturbation of their cell lineage segregation processes, impairing embryonic developmental competence and the ability to establish a pregnancy. In addition, embryos that had visibly expanded when first identified as a blastocyst showed an increased likelihood of embryonic death after establishing pregnancy (44% vs 10%) compared to embryos that had not yet started to expand. This could similarly be explained by the fact that expanded embryos had progressed further with their development, and that later developmental processes such as cell lineage allocation may be more profoundly disturbed by continued *in vitro* culture. Thus, although they resulted in pregnancy at the first examination, they were not able to develop further. Overall, the speed of development of IVP embryos significantly influenced the likelihood of pregnancy after transfer; therefore, prompt blastocyst identification before expansion may be crucial to avoid an increased risk of early pregnancy loss in recipient mares. Unfortunately, morphological assessment of equine IVP blastocyst quality remains challenging and, based on our observations, it is evident that current *in vitro* culture conditions need optimisation to reduce the incidence of aneuploidy and improve the developmental competence of resulting embryos. The mechanisms that predispose to MN formation, and the fate of the cells carrying MN, during early development of equine IVP embryos is still unknown. However, while the presence of the MN is an indicator of mitotic-derived chromosomal instability, their absence is no guarantee of euploid embryos. More advanced analytical techniques, such as single cell DNA sequencing, should be used for a more specific study on the incidence of aneuploidy in IVP embryos. Of course, embryonic aneuploidy may also be of meiotic origin and, as yet, we don't know whether the aneuploidy observed in MII oocytes from aged mares may or may not have an impact on

the incidence of aneuploidy in IVP embryos. However, a recent retrospective study reported no influence of mare age on the blastocyst rate, pregnancy or early embryonic loss rates after embryo transfer in a horse OPU-ICSI program [40]. We speculate that aneuploid oocytes may be unable to complete fertilization, or that they may be more prone to early mitotic division errors and unable to proceed further than the early cleavage stages. Due to limited material and the non-homogeneous distribution, donor age was not considered during the analysis of the results of Chapter 5. A further step would be to look at the differences and incidence of aneuploidy in IVP embryos from young and old mares. However, suboptimal *in vitro* culture conditions may influence blastocyst development and pregnancy rates with a similar impact regardless of the age of the donor mare, possibly explaining why advanced maternal age is associated with reduced fertility in all breeding techniques except OPU-ICSI [40]. Clearly, the pursuit of further improvements in *in vitro* culture techniques must go on, while future investigations are needed to better clarify the fate of age-related meiotic aneuploidy in horse oocytes. Time-lapse imaging of live sperm-injected oocytes from young and old mares, labelled for DNA and kinetochores, could be an interesting approach to better study the origin of post zygotic aneuploidy. Moreover, the combination of this technique with single cell DNA sequencing of the resulting blastomeres would not only allow us to investigate the origin of aneuploidy in *in vitro* produced embryos, but also its consequences for the viability of the daughter blastomeres.

In conclusion, the studies presented in this thesis show that advanced mare age is associated with an increased incidence of chromosome misalignment and aneuploidy in *in vitro* matured horse oocytes, which may arise from loss of chromosomal centromeric cohesion and defective MPS1 function. In addition, *in vitro* culture of horse embryos leads to an increased incidence of nuclear fragmentation and MN formation, where the latter is a proxy for mitotic-derived aneuploidy. All of these effects could contribute to the higher incidence of EPL seen both in aged mares and after transfer of IVP embryos; current *in vitro* embryo culture conditions clearly impact embryo quality regardless of donor mare age, and need to be optimized to reduce chromosomal instability and enhance embryo developmental competence.

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Summary

SUMMARY

During the last 20 years, the potential advantages of modern assisted reproductive techniques (ARTs), in particular, *in vitro* embryo production, have attracted increasing interest from the horse breeding industry. This has in large part been stimulated by appreciable improvements in both the efficiency and success of *in vitro* production of horse embryos. However, despite the improvements, the likelihood of producing a live foal varies markedly between centers. In particular, the likelihood of pregnancy after transfer of an IVP embryo ranges between 50 % and 80 %. In addition, 16 to 25 % of these pregnancies succumb to early pregnancy loss (EPL) before day 60 of gestation. In fact, EPL is one of the major sources of inefficiency and economic loss to the horse breeding industry in all its formats. Among the possible predispositions to EPL, a maternal age-related decline in oocyte and embryo quality is considered a significant contributor.

Advanced maternal age is known to be a cause of reduced fertility in both mares and women. Indeed, pregnancy loss in women above approximately 35 years of age is associated with an increased incidence of embryonic aneuploidy (i.e., an embryo showing an abnormal number of chromosomes), which represents the primary cause of developmental arrest, implantation failure and miscarriage. Since it has been reported that *in vivo* matured equine oocytes show defects of chromosome alignment within the meiotic spindle, which could potentially lead to segregation errors, and equine embryos are also known to suffer from aneuploidy, we hypothesized that embryonic aneuploidy may be an important cause of the increased incidence of EPL in aged mares. The purpose of the studies described in this thesis was therefore to investigate the effect of advanced maternal age on the quality and developmental competence of equine oocytes and embryos.

In **Chapter 1** we reviewed the mechanisms by which aneuploidy can arise in oocytes and embryos, highlighting the potential impact of both maternal age and *in vitro* embryo production. The molecular mechanisms implicated in the mis-regulation of chromosome alignment and segregation include 1) loss of cohesion between sister chromatids; 2) abnormalities in chromosome-spindle attachment and 3) reduced stringency of the Spindle Assembly Checkpoint (SAC) mechanism.

In the following chapters, we evaluated how advancing maternal age might contribute to an increase incidence of chromosome segregation abnormalities by investigating the inter-chromosomal cohesion and inhibition of SAC proteins in the dynamics of spindle formation

and chromosome alignment. In addition, we indirectly estimated the incidence of chromosome instability in *in vitro* produced equine embryos by evaluating the frequency of micronucleus formation as a proxy for aneuploidy. Finally, we searched for correlations between the morphological characteristics of IVP embryos, the incidence of micronuclei and other abnormalities of nuclear form, and success of pregnancy after embryo transfer.

In **Chapter 2,** we investigated the influence of maternal age on spindle morphology, morphometry and chromosome alignment in *in vitro* matured oocytes collected from young (≤ 14 y.o.) and old (≥ 16 y.o.) mares. Oocytes from old mares showed a higher incidence of chromosome misalignment (47 % vs 5 % of oocytes had at least one misaligned chromosome; $P < 0.001$) and a thicker metaphase plate (mean ± s.d.: 5.8 ± 1.0 µm vs 4.9 ± 0.9 µm; $P = 0.004$) than those from young mares. Furthermore, although neither spindle morphology not morphometry were influenced by mare age, spindle length was increased in oocytes showing chromosome misalignment (mean ± s.d.: 25.3 ± 6.1 µm vs 20.8 ± 3.3 µm; $P = 0.05$), irrespective of mare age. We concluded that advanced mare age is associated with a compromised ability to correctly align the chromosomes on the metaphase plate, which may predispose to aneuploidy. Indeed, if the chromosomes are not properly aligned at the spindle equator before the onset of anaphase, they may segregate and distribute incorrectly between the oocyte and the polar body, resulting in hypo- or hyperploidy.

In **Chapter 3**, we investigated whether aging in mares predisposes to loss of chromosomal cohesion in their oocytes and thereby to aneuploidy. To this end, we evaluated the effect of advanced mare age on gene expression for selected cohesin components (REC8, STAG3, SGO1, SGO2, WAPL), and on the incidence of aneuploidy and the strength of chromosome centromere cohesion by, respectively, counting the number of chromatid-kinetochore units and measuring the inter-kinetochore distance (iKD) in *in vitro* matured oocytes from young (≤ 14 y.o.) and old (≥ 16 y.o.) mares. Gene expression for the centromeric cohesion protector, SGO1, was reduced in oocytes from aged mares. In addition, *in vitro* matured oocytes from aged mares showed a higher incidence of aneuploidy than oocytes from young mares (54.6 % versus 15.2%; $P < 0.05$) and an increased iKD (mean ± s.d.; 1.96 ± 0.74 versus 1.33 ± 0.40 µm; $P < 0.0001$). Furthermore, 70% of aneuploid oocytes from old mares had an uneven number of chromatids, consistent with unbalanced premature separation of sister chromatids (PSSC) during meiosis I. We concluded that advanced mare age predisposes to weakening of centromeric cohesion, predisposing to premature separation and random segregation of the sister chromatids during

the first meiotic division. The age-related increase in aneuploidy observed in horse oocytes may explain the reduced fertility and increased incidence of EPL observed in old mares.

In **Chapter 4**, we investigated whether the age-related increase in chromosome mis-segregation in horse oocytes may be attributable to a defective Spindle Assembly Checkpoint (SAC) control machinery. We first evaluated gene expression for the main SAC components (MAD1, MAD2, BUB1, BUB3, BUB1R, MPS1, NDC80, SPC25, AURKB, AURKC) in *in vitro* matured oocytes from young (≤ 14 y.o.) and old (≥ 16 y.o.) mares and found gene expression for MPS1, SPC25 and AURKC to be reduced in oocytes from old compared to young mares. On the basis of these results, we investigated the importance of MPS1 and AURKB/C to formation of a normal meiotic spindle by analysing spindle morphology and chromosome alignment of *in vitro* matured MII oocytes from old and young mares, after nocodazole-induced microtubule depolymerization and spindle reassembly in the presence of specific MPS1 and AURKB/C inhibitors (respectively, CPD5 and ZM447439). MII oocytes from old mares showed a higher incidence of spindle abnormalities after exposure to the MPS1 inhibitor CPD5, while AURKB/C inhibition resulted in severe impairment of spindle reformation, independent of mare age. Moreover, although oocytes from old mares tended to show a higher incidence of chromosome misalignment when compared to those from young mares, after the inhibitor treatments there was no significant increase in the incidence of misalignment within an age group. We concluded that reduced expression of the key SAC component MPS1 in oocytes from aged mares, may contribute to spindle instability which, in turn, could result in mis-segregation leading to aneuploidy.

In **Chapter 5**, we indirectly quantified and compared post-zygotic aneuploidy in *in vitro* and *in vivo* produced embryos by evaluating nuclear morphology and, in particular, the frequency of micronucleus formation (MN). We also investigated whether associations exist between bright field morphological characteristics of IVP embryos, the incidence of nuclear abnormalities and the likelihood of pregnancy. IVP embryos showed a higher incidence of MN than *in vivo* derived embryos (10 % vs 1 %; P = 0.05). Micronuclei are considered an indicator of chromosomal instability and this evidence of increased instability may, in part, explain the lower rates of pregnancy survival after transfer of IVP compared to *in vivo* embryos. Moreover, the increased incidence of MN formation in IVP embryos suggests that current *in vitro* culture conditions are sub-optimal and need further improvement. However, nuclear morphology and frequency of MN in IVP embryos were not correlated with any of the bright field morphological characteristics. On the other hand, IVP embryos that showed delayed development (i.e., they

reached the blastocyst stage after Day 9 of *in vitro* culture) were less likely to yield a pregnancy than embryos reaching the blastocyst stage before Day 9 (27 % vs 69 %), while embryos that showed signs of blastocoele expansion at the time of transfer were more likely to undergo subsequent embryonic death (44 % vs 10 %). In short, the speed of embryo development was the only parameter that correlated with pregnancy survival; moreover, morphological classification of the quality of equine IVP embryos remains a challenging process.

The major conclusion of this thesis is that advanced mare age is associated with an increased incidence of chromosome misalignment and aneuploidy in *in vitro* matured oocytes, most likely originating from a combination of weakened chromosomal centromeric cohesion and defective SAC function. In addition, *in vitro* culture of equine embryos leads to an increased incidence of MN formation, which is a proxy for mitotic-derived aneuploidy. These deficits almost certainly contribute to the reduced fertility and higher incidence of EPL observed in aged mares and after transfer of IVP embryos.

Samenvatting

SAMENVATTING

Gedurende de afgelopen 20 jaar, hebben de potentiele voordelen van mordene geassisteerde reproductietechnieken (ART), in het bijzonder *in vitro* embryo productie, de aandacht getrokken van de paarden fokkerij. Dit werd voornamelijk gestimuleerd door merkbare verbeteringen in zowel de efficientie als het succes van *in vitro* productie van paardenembryo's. Echter, ondanks verbeteringen, verschilt de aannemelijkheid op het verkrijgen van een levend veulen duidelijk tussen centra. In het bijzonder, de kans op dracht na het overplaatsen van een IVP embryo varieert van 50 % tot 80 %. Daarnaast resulteert 16 % tot 25 % van deze drachten in vroeg embryonale sterfte (VES) voor dag 60 van de dracht. In feite, VES is een van de voornaamste oorzaken van inefficientie en economisch verlies voor de paardenfokkerij in alle opzichten. Onder de mogelijk predisponerende factoren voor VES, wordt maternale leeftijdsgerelateerde afname in eicel en embryo kwaliteit beschouwd als een significante bijdrager.

Gevorderde maternale leeftijd is een bekende oorzaak van verlaagde fertiliteit bij zowel merries als vrouwen. Zo worden miskramen bij vrouwen boven de 35 jaar geassocieerd met een verhoogd voorkomen van embryonale aneuploïdie (bijv., een embryo met een afwijkend aantal chromosomen), wat de voornaamste oorzaak is voor stagnatie in ontwikkeling, innestelingsproblemen en miskramen. Omdat het bekend is dat in vivo gematureerde paardeneicellen afwijkingen in de positie van chromosomen in de meiotische spoelfiguur vertonen, wat potentieel kan leiden tot segregatie fouten, en dat paardenembryo's ook aneuploïdie vertonen, hebben wij de hypothese gesteld dat embryonale aneuploïdie een belangrijke oorzaak is van de verhoogde VES in oudere merries. Het doel van de studies die in deze thesis beschreven zijn, was daarom het onderzoeken van het effect van gevorderde maternale leeftijd op de kwaliteit en ontwikkelingscompetentie van paarden eicellen en embryo's.

In **Hoofdstuk 1** hebben we gekeken naar de mechanismen waardoor aneuploïdie in eicellen en embryo's kan ontstaan, waarbij de potentiele impact van zowel maternale leeftijd als in vitro embryo productie zijn uitgelicht. De moleculaire mechanismen betrokken bij de mis-regulatie van chromosoom positionering en segregatie bevatten 1) verlies van cohesie tussen zuster chromatiden; 2) afwijkingen in chromosoom – spoelfiguur binding en 3) minder sterke regulatie van het Spindle Assembly Checkpoint (SAC) mechanisme.

In de volgende hoofdstukken hebben we geevalueerd hoe maternale leeftijd kan bijdragen aan een toename van chromosoom segregatie afwijkingen. Dit hebben we gedaan door de inter-chromosomale cohesie te onderzoeken en daarnaast door het bestuderen van de vorming van de spoelfiguur en positionering van de chromosomen met behulp van remming van SAC eiwitten. Bovendien hebben wij indirect de incidentie van chomosoom instabiliteit in *in vitro* geproduceerde paardenembryo's geschat, door het evalueren van de frequentie van micronucleus vorming als een een indicator voor aneuploïdie. Tot slot, hebben we gezocht naar correlaties tussen morfologische kermerken van IVP embryo's, het voorkomen van micronuclei en andere nucleaire abnormaliteiten, en de kans op succesvolle dracht na embryo overplaatsing.

In **Hoofdstuk 2** hebben we de invloed van maternale leeftijd op de morfologie en de morfometrie van de spoelfiguur en positionering van de chromosomen onderzocht in *in vitro* gematureerde eicellen die zijn verkregen uit jonge (≤ 14 j.o.) en oude (≥ 16 j.o.) merries. Eicellen van oude merries tonen een hogere incidentie van verkeerde positie van de chromosomen op de spoelfiguur (47 % vs. 5 % van de eicellen had tenminste 1 chromosoom verkeerd gepositioneerd; $P < 0.001$) en een dikkere metafase plaat (gemiddeld ± s.d.: 5.8 ± 1.0 µm vs. 4.9 ± 0.9 µm; $P = 0.004$) dan die van jongere merries. Ondanks dat zowel de morfologie als de morfometrie van de spoelfiguur niet werd beinvloed door de leeftijd van de merrie, werd wel een toename van de lengte van de spoelfiguur waargenomen bij eicellen die een verkeerde positie van de chromosomen vertoonden (gemiddeld ± s.d.: 25.3 ± 6.1 µm vs. 20.8 ± 3.3 µm; $P = 0.05$) onafhankelijk van de leeftijd van de merrie. We concludeerden dat gevorderde leeftijd van de merrie wordt geassocieerd met een aangetast vermogen om de chromosomen correct op de metafase plaat te positioneren, wat kan predisponeren tot aneuploïdie. In feite, als de chromosomen niet correct zijn gepositioneerd op de spoelfiguur voor de start van de anafase, kunnen ze zich incorrect scheiden en verdelen tussen de eicel en het poollichaam, wat kan resulteren in hypo- of hyperploïdie.

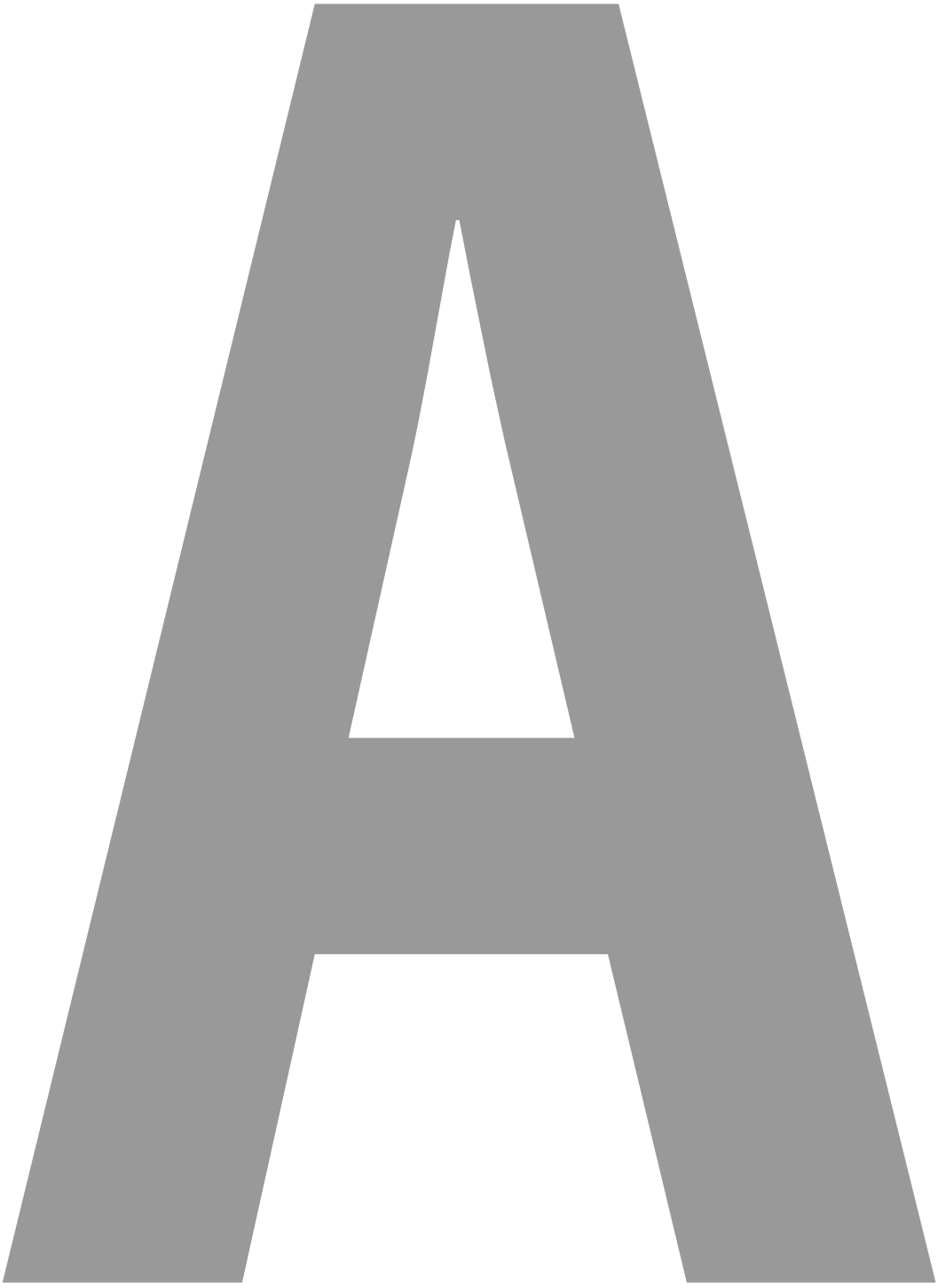
In **Hoofdstuk 3**, hebben we onderzocht of veroudering in merries predisponeert tot verzwakte chromosomale cohesie in eicellen en daarmee tot aneuploïdie. Hiervoor hebben we geevalueerd wat het effect is van gevorderde leeftijd van de merrie op de genexpressie van geselecteerde cohesie componenten (REC8, STAG3, SGO1, SGO2, WAPL), op het voorkomen van aneuploïdie en op de kracht van chromosoom-centromeer cohesie door, respectievelijk, het tellen van de chromatide-kinetochoor eenheden en het meten van de inter-kinetochoor afstand (iKD) in *in vitro* gematureerde eicellen van jonge (≤ 14 j.o.) en oude (≥ 16 j.o.) merries. Gen expressie van de centromere cohesie beschermer, SGO1, was verlaagd in eicellen van oudere

merries. Bovendien vertoonden in vitro gematureerde eicellen van oudere merries een hogere incidentie van aneuploïdie dan eicellen van jonge merries (54.6 % vs. 15.2%; $P < 0.05$) en een hogere iKD (gemiddeld ± s.d.; 1.96 ± 0.74 vs. 1.33 ± 0.40 μm; $P < 0.0001$). Daarnaast had 70 % van de eicellen van oude merries met aneuploïdie een oneven aantal chromatiden, consistent met ongebalanceerde premature scheiding van zuster chromatiden (PSSC) tijdens meiose I. We concludeerden dat gevorderde leeftijd van merries predisponeert tot verzwakte centromere cohesie, wat ze vatbaarder maakt voor premature en willekeurige scheiding van zuster chomatiden tijdens de eerste meiotische deling. De leeftijds gerelateerde toename in aneuploïdie die werd gezien in paarden eicellen zou de afgenomen fertiliteit en verhoogde incidentie van VES in oudere merries kunnen verklaren.

In **Hoofdstuk 4** hebben we onderzocht of de leeftijdsgerelateerde toename in het incorrect scheiden van chromosomen in paardeneicellen kan worden toegeschreven aan een defecte Spindle Assembly Checkpoint (SAC) controlemechanisme. Allereerst hebben we de genexpressie van de voornaamste SAC componenten (MAD1, MAD2, BUB1, BUB3, BUB1R, MPS1, NDC80, SPC25, AURKB, AURKC) in in vitro gematureerde eicellen van jonge (≤ 14 j.o.) and oude (≥ 16 j.o.) merries geevalueerd. We hebben gevonden dat de genen voor MPS1, SPC25 en AURKC verlaagd tot expressie komen in eicellen van oude merries in vergelijking met die van jonge merries. Op basis van deze resultaten hebben we de rol van MPS1 en AURKB/C voor de formatie van een normale meiotische spoelfiguur onderzocht, door het analyseren van de morfologie van de spoelfiguur en de positionering van de chromosomen van *in vitro* gematureerde MII eicellen van oude en jonge merries, na door nocodazol geinduceerde depolymerisatie van microtubuli en opnieuw formeren van de spoelfiguur in de aanwezigheid van specifieke MPS1 en AURKB/C remmers (respectievelijk, CPD5 and ZM447439). MII eicellen van oude merries vertoonden een hogere incidentie van afwijkingen aan de spoelfiguur na blootstelling aan de MPS1 remmer CPD5, terwijl AURKB/C remming resulteerde in een ernstige beperking van de hervorming van de spoelfiguur onafhankelijk van de leeftijd van de merrie. En hoewel de eicellen van oude merries neigen naar een hogere incidentie van incorrecte positie van chromosomen in vergelijking met deze van jonge merries, was er geen significante toename in de incidentie van incorrecte positie binnen een leeftijdsgroep na de inhibitie behandelingen. We concludeerden dat verlaagde expressie van het belangrijkste SAC component MPS1 in eicellen van oude merries kan bijdragen aan instabiliteit van de spoelfiguur wat, op zijn beurt, zou kunnen resulteren in incorrecte scheiding en kan leiden tot aneuploïdie.

In **hoofdstuk 5** hebben we post zygotische aneuploïdie indirect gekwantificeerd en vergeleken door het evalueren van nucleaire morfologie en, in het bijzonder, de frequentie van micronuclus formatie (MN) in *in vitro* en *in vivo* geproduceerde embryos. Daarnaast hebben we ook onderzocht of er overeenkomsten zijn tussen morfologische kenmerken van IVP embryo's onder de microscoop, de incidentie van nucleaire afwijkingen en de succeskans op dracht. IVP embryo's vertoonden een hogere incidentie van MN dan *in vivo* verkregen embryo's (10 % vs. 1 %; P = 0.05). Micronclei worden gezien als een indicator voor chromosomale instabiliteit en dit bewijs voor verhoogde instabiliteit kan, gedeeltelijk, verklaren waarom de succeskans op dracht na overplaatsing van een embryo lager is bij IPV embryo's in vergelijking met in vivo embryo's. Daarnaast suggereert de verhoogde incidentie van MN formatie in IVP embryo's dat de huidige *in vitro* kweekomstandigheden niet optimaal zijn en verdere verbeteringen behoeven. Nucleaire morfologie en frequentie van MN in IVP embryo's waren echter niet gecorreleerd met een van de microscopisch morfologische kenmerken. Aan de andere kant resulteerden IVP embryo's die een vertraagde ontwikkeling vertoonden (d.w.z., zij bereikten het blastocyst-stadium na dag 9 van de *in vitro* kweek) minder snel in een dracht dan embryo's die het blastocyst stadium vóór dag 9 bereikten (27 % vs. 69 %), terwijl embryo's die tekenen van blastocoele expansie vertoonden op het moment van overplaatsing meer kans hadden op latere embryonale sterfte (44 % vs. 10 %). Kortom, de snelheid van embryonale ontwikkeling was de enige parameter die correleerde met de overleving van de dracht; bovendien blijft de morfologische classificatie van de kwaliteit van IVP embryo's van paarden een uitdagend proces.

De belangrijkste conclusie van deze thesis is dat gevorderde leeftijd van de merrie is geassocieerd met een verhoogde incidentie van incorrecte positionering van chomosomen en aneuploïdie in in vitro gematureerde eicellen, waarschijnlijk afkomstig van een combinatie van een verzwakte chomosomale centromeer cohesie en defecte SAC functie. Bovendien, leidt in vitro kweek van paardenembryo's tot een verhoogde incidentie van MN formatie, wat een indicator is voor mitotisch afgeleide aneuploïdie. Deze tekortkomingen dragen vrijwel zeker bij aan de verminderde fertiliteit en hogere incidentie van VES die waargenomen wordt bij oudere merries na overplaatsing van IVP embryo's.



Appendices

CURRICULUM VITAE

Marilena Rizzo was born on the 30th of June 1987 in Caccamo, Italy. After obtaining her scientific diploma in 2006, at Liceo Scientifico Nicolo Palmeri in Termini Imerese, she started her Veterinary Medicine training at the University of Messina (Italy), and graduated in 2013. In the same year she started a PhD in Veterinary Clinical Sciences (also via University of Messina) and began a collaboration with the Faculty of Veterinary Medicine - Section Reproduction (Utrecht University), subsequently evolved into a registration as an external PhD student. In 2015 she started a 4 years Residency program at Utrecht University's Equine Fertility Center for the European College of Animal Reproduction (ECAR) with sub-specialty Biotechnology of Reproduction (equivalent to Clinical Embryologist training). In 2017 she successfully discussed her thesis on "Effect of age and media on the quality and developmental abilities of equine oocytes from slaughterhouse ovaries" and obtained the PhD degree at the University of Messina. To date she also has been working and involved in all the standard operating procedures (SOPs) for equine *in vitro* embryo production and clinical activity at the Department of Clinical Sciences – Section Reproduction (Utrecht University), and completed her second PhD research on chromosomal segregation errors during meiosis in oocytes and early cleavage divisions in embryos, with particular emphasis on the effect of maternal aging. Marilena is awaiting the exam of specialization for the European College of Animal Reproduction – Biotechnology of Reproduction, and she is currently looking for new career opportunities.

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Oral Presentations

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