Centrosome changes during meiosis in horse oocytes and first embryonic cell cycle organization following parthenogenesis, fertilization and nuclear transfer

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Abstract

Various types of cell cycle organization occur in mammals. In this study, centrosome changes during meiosis in horse oocytes, and first cell cycle organization following fertilization, parthenogenesis and nuclear transfer, were monitored. Cumulus oocyte complexes harvested from horse ovaries obtained from slaughtered mares were cultured *in vitro*. Meiotic oocytes of germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I and II (MI and MII) stages were selected at various set times during *in vitro* maturation. Embryos at the first cell cycle stage were generated by subjecting MII stage oocytes to fertilization by intracytoplasmic sperm injection (ICSI), parthenogenetic treatment or nuclear transfer. Centrosome changes during meiosis and the first cell cycle organization were detected by indirect immunofluorescent staining, using a mouse anti- α -tubulin antibody for microtubules and a rabbit anti- γ -tubulin antibody for centrosomes. These examinations showed that the centrosomes of the horse oocyte reorganize themselves from the beginning of GV stage to leave only PCM of γ -tubulin surrounding both poles of the MI and MII stage spindles. These MII oocytes can organize the separation of metaphase chromosomes during the first embryonic cell cycle by parthenogenetic treatment. When the MII oocytes were subjected to ICSI or nuclear transfer, one or two red-stained centrosomes of γ -tubulin were introduced by the fertilising spermatozoon or the donor cell which associated with the sperm chromatin in the fertilized embryos and with the donor cell chromatin and microtubules in the cloned embryos. This finding suggests that centrosomes are not an essential component in the formation of the metaphase spindle during meiotic maturation of horse oocytes, but they can be introduced from the spermatozoon or donor cell and are necessary for the organization of normal embryonic development.

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Introduction

The centrosomes are found at the poles of mitotic spindles, which are microtubule-organizing centres (MTOC) of mammalian cells during the cycle of somatic division (Kirschner & Mitchison 1986, Mazia 1987, Nurse 1990, Karp 1999). Centrosomes are typically composed of two elements, centrioles and pericentriolar material (PCM). Although centrioles are part of centrosomes, they may not be essential for the meiotic spindles when oocytes are generated from germ line cells and their microtubules are still organized by PCM, which occurs during mammalian oogenesis (Laemmli 1972, Szollosi *et al.* 1972, Maro *et al.* 1985, Taieb *et al.* 1997, Karp 1999).

The patterns of centrosome inheritance during gametogenesis and fertilization differ among the different

mammalian species and very little information relevant to this subject is available for the horse. Mouse oocytes are strongly supported by the presence of a population of non-spindle associated microtubule organizing centres which are not present in other mammalian species of pig, bovine and horse, in which the reconstitution of the centrosome inheritance may therefore differ (Schatten 1994, Simerly *et al.* 1999, Dai *et al.* 2000, Tremoleda *et al.* 2001, Shin *et al.* 2002). Furthermore, attempts to produce horse embryos by *in vitro* techniques have produced much lower success rates than in other domestic animal species and we still do not understand the reason why, especially in relation to first cell cycle organization following fertilization by ICSI or reconstruction by nuclear transfer (Hinrichs *et al.* 1993, Navara *et al.* 1996, Li *et al.*

2001, 2002, 2003, Choi 2002, 2003, Woods *et al.* 2003, Galli *et al.* 2003). Therefore, in the present experiment, we studied the behaviour of centrosomes during the meiotic cell cycle, and their function at the first embryonic cell cycle, in embryos produced *in vitro* by parthenogenesis, ICSI-fertilization and nuclear transfer.

Materials and Methods

Preparation of oocytes

Horse ovaries were obtained from a commercial abattoir and cumulus oocyte complexes (COCs) were recovered by scraping the walls of follicles 0.5–3.0 cm in diameter. Meiotic oocytes of germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I and II (MI and MII) stages were prepared from groups of 20–30 COCs cultured *in vitro* for 4, 12, 20 and 28–30 h, respectively . Culture conditions were at 38°C in 5% CO₂-in-air in TCM199 (Sigma Chemicals, St Louis, USA) supplemented with 10% v/v heat inactivated fetal bovine serum, 10 µg/ml FSH, 5 µg/ml LH, 1 µg/ml oestradiol and 200 ng/ml IGF-I (all from Sigma). The same medium was used to culture the oocytes after parthenogenetic treatment, fertilization by ICSI and reconstruction by nuclear transfer.

Parthenogenesis, ICSI-fertilization and nuclear transfer treatments

MI oocytes were subjected to parthenogenetic stimulation, ICSI-fertilization or nuclear transfer using somatic cells, as described previously by Li *et al.* (2002, 2003 2004). For ICSI-fertilization, MII oocytes that had been matured for 28–30 h *in vitro* had their cumulus cells removed before a whole sperm was injected into their cytoplasm. MII stage oocytes were also used for nuclear transfer and their nuclei were removed before a somatic cell was injected into the cytoplasm. After both sperm injection and nuclear transfer, the oocytes were activated chemically by immersing them in PBS containing 5 µM ionomycin for 5 min, followed by culture for 4 h in TCM199 medium containing 5 µg /ml cytochalasin B (Sigma) and 10 µg/ml cycloheximide (Sigma). To induce parthenogenesis, MII stage oocytes were subjected to the same culture conditions applied to activate the reconstructed oocytes. Groups of 5–10 treated nuclear transfer oocytes in the 3 experiments were then cultured in 500 µl drops of development medium at 38°C in an atmosphere of 5% CO₂-in-air. The treated oocytes in each group were fixed 2–4, 12–15 and 20–24 h, respectively, after activation to observe their first mitotic cell cycle.

Centrosome, microtubule and chromatin analyses

Oocytes were selected at various stage of meiosis, or after parthenogenetic stimulation, ICSI-fertilization or nuclear transfer. They were fixed initially by immersion for 1 h at

38°C in M medium (Simerly & Schatten 1993) followed by 2.5% paraformaldehyde for 30 min. Their microtubules and centrosomes were labeled by incubating the fixed oocytes for 90 min at 38°C in a mixture of a mouse anti-α-tubulin antibody (Sigma) and a rabbit anti-γ-tubulin antibody (Sigma), all diluted 1:250 in PBS containing 3 mg ml⁻¹ BSA. They were then incubated for a further 1 h in a blocking solution of 10% v/v goat serum in PBS after which they were exposed for 1 h at 38°C to the respective second antibody, each diluted 1:250 in PBS containing 0.5% Triton X-100 and 0.5% BSA. FITC-conjugated goat anti-mouse globulin (Sigma) stained the microtubules green and Alexa Flour 568-conjugated goat anti-rabbit globulin (Sigma) stained the centrosomes red. The stained oocytes were then mounted under a coverslip in an anti-fade mounting medium containing TOTO3 (Sigma; stains chromatin blue) and examined by confocal microscopy.

All the animals used in these experiments were licensed (Project no. PPL 80/1442) and maintained (Certificate of Designation no. PCD 80/9044) under the provision of the Animals (Scientific Procedures) Act 1986, and under the supervision of the Home Office Experimental Animal Inspectorate.

Results

Meiotic organization

A total of 286 COCs were used in the study and 63, 56, 67 and 100 oocytes respectively were analysed for GV, GVBD, MI and MII stages, which included the treatments for parthenogenesis, ICSI and nuclear transfer (Table 1). Staining with TOTO3 for DNA, and both positive and negative controls when staining with the mouse anti-α-tubulin antibody (Sigma) for microtubules, and a rabbit anti-γ-tubulin antibody (Sigma) for centrosomes, are shown in Fig. 1. The meiotic organisation of the horse oocyte from GV to the MII stage is shown in Fig. 2. Microtubule organisation is not seen during the condensation of chromatin DNA that occurs at the beginning of meiosis at the GV stage (Figs 2A and B). It appeared, and it forms the spindle net, at the MI and the MII stages (Figs 2E–G). The centrioles are not seen during the whole meiotic process when there are only some PCM structures of γ-tubulin surrounding both poles of the MI and MII stage spindles. This is associated with chromosome separation to release the

Table 1 Horse oocyte maturation rates at different times of *in vitro* culture.

Time in culture (h)	No. COCs used	No. oocytes (%)			
		GV	GVBD	MI	MI
4	55	37 (67)	16 (29)	2 (4)	0
12	46	8 (18)	17 (37)	21 (45)	0
20	47	4 (9)	12 (26)	26 (56)	5 (11)
28–30	138	14 (10)	11 (8)	18 (13)	95 (69)

Figure 1 Staining test for DNA, microtubules and centrosomes. (A) DNA was stained by TOTO3; (B) microtubules and centrosomes were stained with mouse anti- α -tubulin antibody; (C & D) microtubules and centrosomes were stained with a rabbit anti- γ -tubulin antibody. C, positive; D, negative control, respectively.

Figure 2 Meiotic cell cycle organisation in horse oocytes from G1 to MII stages. The inset shows γ -tubulin staining on each photograph. The oocytes were stained for microtubules; α -tubulin, FITC-green; γ -tubulin, Texas-red and chromatin, TOTO3-blue. The G1 stage oocyte shows condensed chromatin (A, GV I) and it has begun to form chromosomes (B, GV II). Microtubules are seen in pro-metaphase (C) which circle the chromatin before passing into the first metaphase stage (D), without any activity in the centrosome throughout this period (A–D). The red γ -tubulin material occurs at the spindle poles in the first metaphase stage (E), and it persists until the pro- (F) and second metaphase (G) stages.

first polar body and, subsequently, the meiotic cycle stops at the MII stage (Fig. 2G).

First cell cycle organisation in parthenogenetic and fertilized oocytes

The chromosomes of MII oocytes separated following parthenogenetic treatment and the first cell cycle of embryonic development were organised under the actions of PCM (Figs 3A1–A3), but not the centrioles. In the case of fertilization by ICSI, the centrosomes of the spermatozoon (Fig. 3B1) reorganised soon after the latter was injected into the MII oocyte (Fig. 3B2) and the first cell cycle of embryonic development was organised following formation of the male and female pronuclei (Figs 3B3 and

B4). In this case, the zygote or early embryo was constructed by the spermatozoon and oocyte each contributing half the genomic DNA while the centrosome came from the oocyte in the form of microtubules (Figs 3B3 and B4).

First cell cycle organisation in cloned embryos

The cell cycle organisation of cloned embryos is shown in Fig. 3. The centrosomes of the somatic cell (Fig. 3C1) injected into the cytoplasm of the enucleated MII oocyte were not observed following nuclear reprogramming (Fig. 3C2). However, more time was needed to generate microtubules than was required after fertilization, which was organised by one or two red-stained centrosome structures of γ -tubulin (Fig. 3C3). The generation of cen-

Figure 3 First cell cycle organisation of horse oocytes following parthenogenetic stimulation, ICSI-fertilization and nuclear transfer treatments. The oocytes were stained for microtubules; α -tubulin, FITC-green; γ -tubulin, Texas-red and chromatin, TOTO3-blue. The inset shows γ -tubulin staining on each photograph. A1–A3, a MII oocyte (A1) that was activated parthenogenetically showing separation of the metaphase chromosomes after movement of the microtubules to the spindle poles (A2 and A3). Red stained γ -tubulin is more concentrated at the later stage of the first cell cycle, but it has not yet formed centrosomes (A3). B1–B4; a centrosome-like structure of red staining γ -tubulin is seen in the neck of the horse spermatozoon (B1). It reorganises soon after being injected into the cytoplasm (B2), followed by decondensation of the sperm chromatin DNA. A red stained deposit of γ -tubulin is seen again with the formation of the male pronucleus (B3). The centrosome reforms with a regular outline following male and female pronuclear fusion and this subsequently develops into the pre-metaphase stage at the first mitotic cell cycle of the fertilized oocyte (B4). C1–C4; a centrosome-like structure of red staining γ -tubulin is seen in the somatic cell nucleus (C1). When the cell fuses with the enucleated oocyte, the centrosome reorganises (C2) and this situation persists through to the formation of the pseudo-pronuclei (C3). Two red spots, indicating reforming centrosomes, are seen during the pre-metaphase stage at the first mitotic cell cycle (C4).

trosomes provided by the somatic cell was associated with condensation of the chromatin DNA and metaphase chromosome formation at the first cell cycle of embryonic development (Fig. 3 C4).

A total of 30–50 oocytes were evaluated in each of the three treatments, parthenogenesis, ICSI and nuclear transfer. Details of first cell cycle organisation in the horse embryos created by ICSI, or reconstructed by nuclear transfer, are summarised in Figs 4A and B.

Discussion

Meiosis in the germ line halves the ploidy of the gametes, ensuring that the ploidy of the organism is maintained in the next generation. Similarly, the number of centrosomes must also be reduced during gametogenesis or fertilization to maintain the correct number of centrosomes in the zygote (LeGuen & Crozet 1989, Taieb *et al.* 1997).

Centrioles are cylinders of microtubule triplets required for the generation of ciliary flagellar axonemes, and the PCM is an amorphous cloud that surrounds the centrioles and organizes the microtubules (Karp 1999). γ -tubulin, a new member of the tubulin superfamily that functions in microtubule nucleation, has been localized at the spindle poles and cytoplasmic MTOCs in mouse metaphase II-arrested oocytes (Oakley & Oakley 1989, Joshi *et al.* 1992, Palacios *et al.* 1993). Therefore, despite the absence of a definitive centrosome, mouse meiotic spindles contain foci of PCM, including γ -tubulin, which probably nucleates meiotic spindle microtubules at the poles. On the other hand, cytoplasmic MTOC are not present in MII oocytes of pigs, sheep and cows, in contrast to mouse MII oocytes in which they have been found at the spindle poles and in the cytoplasm. Furthermore, several PCM foci can be seen at the acentriolar meiotic spindle poles of mouse oocytes, as well as in the cytoplasm (Maro *et al.* 1985, LeGuen & Crozet 1989, Araki *et al.* 1996, Kim *et al.* 1996, Lee *et al.* 2000). Our results showed that, from the GV to the MII stage of meiosis in horse oocytes, there is only a PCM of γ -tubulin surrounding both poles of the MI and MII stage spindles (Figs 2A–G). These findings are similar to oogenesis in women and cows, but differ from that in mice (Simerly *et al.* 1999). In parthenogenetically activated oocytes, centrioles were also absent from both poles of the microtubular spindles following their further development after separation of the chromosomes (Figs 3A1–A3). However, red stained PCM of γ -tubulin did remain associated with the microtubules (Figs 3A1–A3). These findings indicate that centrioles are not an essential component in the formation of the metaphase spindle during meiotic oogenesis in the horse.

In most species, the spermatozoon contributes the centrioles and the oocyte contributes the PCM to the zygotic centrosomes. However, there are some exceptions to this paternal inheritance of centrioles rule; for example, spermatogenesis in mice and parthenogenetic development in

wasps (Messinger & Albertini 1991, Schatten 1994). It has been generally understood that both duplication of the centrosome and variations in its microtubule-nucleating capacity are driven by cell cycle-dependent changes in the cytoplasmic environment (Nurse 1990, Pereira *et al.* 2000). In the present experiment, the presence of additional centrosomes was confirmed by γ -tubulin staining in both the horse spermatozoon and in somatic cells, concentrated respectively at the neck of the spermatozoon and the cytoplasm surrounding the nucleolus of the somatic cell (Figs 3B1 and C1; Figs 4A and B). When a spermatozoon was injected into the cytoplasm of an MII oocyte, the centrosome could not be detected after 4–6 h but it then reorganized itself again, becoming surrounded by microtubules following DNA decondensation in the sperm head (Figs 3B2 and B3). This result provided evidence that γ -tubulin of the sperm centrosome may induce the programming of the first cell cycle, from the MII stage oocyte into the zygote and subsequently into the embryo, by the organization of microtubules and chromatin (Figs 3B3 and B4; Fig. 4A).

Nuclear transfer is a relatively new reproductive technique in mammals. It has shown that breakdown of the nuclear envelope, premature chromosome condensation and swelling of the nucleus resulting in nuclear reprogramming, are all prerequisites for first cell cycle organization and further development of embryos created by nuclear transfer (Galli *et al.* 2003, Woods *et al.* 2003, Li *et al.* 2004). In nuclear transfer, the general idea is to replace the metaphase chromatin of the MII oocyte by the nucleolus of a somatic cell recovered from the donor animal. Briefly, the microtubules in the MII oocyte have been removed by the process of enucleation so the first cell cycle organization will progress differently compared with that following fertilization or parthenogenesis.

Previous studies have indicated that, after removal of the centrosome, both somatic and embryonic cells can regenerate a MTOC, but they do not regenerate centrioles even though the cytoplasm (in the case of zygotes) contains enough subunits to assemble many complete centrosomes (Bloecher & Tatchell 2000). Our results related to the organization of first cell cycle in the reconstructed oocytes (Figs 3 C1–C4) showed that the centrioles of the somatic cell (Fig. 3C1) degenerated in the cytoplasm of the enucleated oocyte, within a similar period of 4 h to that of DNA decondensation of the sperm head following ICSI-fertilization (Figs 3B2 and C2). However, more time was needed to generate microtubules (12 h), which were organized by 1 or 2 red-stained centrosome structures of γ -tubulin (Fig. 3C3, Fig. 4B). Furthermore, this appeared to be associated with the introduced donor chromatin following nuclear transfer (Figs 3C3 and C4; Fig. 4B). Thus, the suggestion arises that the centrioles which participate in the reorganization of the chromatin during the first cell cycle in cloned horse embryos may be contributed by the somatic donor cell (Fig. 4B). However, our study was not able to disprove the possible participation of the oocyte

A: Fertilization

B: Nuclear transfer

Figure 4 Visualisation of microtubules and centrosome- γ -tubulins during the first cell cycle organisation of the horse oocyte following ICSI-fertilization and reconstruction by nuclear transfer. (A) ICSI-fertilization; the centrosome reorganises as soon as the spermatozoon is injected into the MII oocyte, but then re-appears following organization of the microtubulues into the first cell cycle of mitosis. (B) Nuclear transfer; the centrosome reorganises following fusion of the donor cell with the enucleated cytoplasm, and then re-appears following nuclear reprogramming into the first cell cycle of mitosis.

cytoplasmic tubulin, which can be recruited during reconstitution of the zygotic centrosome, as seen in human and bovine early zygotes (Schatten 1994, Simerly *et al.* 1999). We propose that this block to microtubule reorganization may be an underlying reason for the lower success rates achieved for *in vitro* production of horse embryos, both following fertilization by ICSI and reconstruction by nuclear transfer (Li *et al.* 2003, 2004, Choi *et al.* 2003, Woods *et al.* 2003, Galli *et al.* 2003).

Taken together, the findings indicate that the techniques used presently to produce horse embryos *in vitro* need further investigations at the molecular level, combined with additional studies of significant morphological changes.

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