

Chromosome analysis of horse oocytes cultured *in vitro**

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Summary – Oocytes collected from slaughtered mares of unknown reproductive history were cultured in modified Krebs Ringers bicarbonate supplemented with fetal calf serum (20%) and fixed for chromosome analysis. To determine the time required for nuclear maturation, oocytes were fixed either after 12 h ($n = 21$), 24 h ($n = 21$), 48 h ($n = 20$) and 60-96 h ($n = 12$) or without culture ($n = 30$). In all 89% of those suitable for analysis, meiosis was resumed with 59% reaching second metaphase (MII) stage. In the majority of oocytes germinal vesicle breakdown occurred by the end of the 1st 12 h of culture and MII was reached by 24 h. To examine the chromosome features, an additional 113 oocyte-cumulus-complexes were cultured for 24 h before fixation. In all, 7 diakinesis/metaphase I (MI) and 36 MII spreads could be analyzed. Of the MII spreads, 5 (13.8%) were found to be lacking chromosomes, 1 (2.7%) had an excess of chromosomes and 1 (2.7%) was diploid. Compensating for possible artifactual loss of chromosomes, the rate of non-disjunction or anaphase lagging was calculated to be 5.5%. It was concluded that with respect to timing and chromosomal features, nuclear maturation of *in vitro* cultured oocytes in horses resembles that of other domestic animals.

meiosis / oocyte / nuclear-maturation / non-disjunction / horse

Résumé – Analyse des chromosomes d'ovocytes équins cultivés *in vitro*. Les ovocytes équins utilisés lors de cette étude furent prélevés chez des ovaires de juments ayant un statut reproductif inconnu. Ces ovocytes furent cultivés dans une solution de bicarbonate de Ringers modifiée, enrichie de 20% en sérum de veau fœtal (SVF). La culture des ovocytes était achevée par la fixation de ceux-ci en vue d'une analyse chromosomique. Afin de déterminer le temps requis pour la maturation nucléaire, les ovocytes furent fixés après les périodes de culture suivantes: 0 h ($n = 30$), 12 h ($n = 21$), 24 h ($n = 21$), 48 h ($n = 20$) et 60 à 90 h ($n = 12$). Les ovocytes fixés au temps 0 h servirent de groupe contrôle. Une reprise méiotique fut observée chez 89% des ovocytes étudiés et 59% atteignirent le stade de deuxième métaphase (MII). Dans la plupart des cas, la rupture de la vésicule germinale fut observée après 12 h de culture et la MII était atteinte avant 24 h de culture. Afin d'examiner l'aspect morphologique des chromosomes, 113 ovocytes entourés de leurs

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cellules du cumulus furent cultivés pour 24 h avant d'être fixés. On a pu ainsi identifier 7 diakinèse/métaphase I (MI) et 36 MII. Parmi les ovocytes qui atteignirent le stade MII, 5 (13,8%) n'avaient pas de chromosomes présents, 1 (2,7%) avait des chromosomes en surplus et 1 était diploïde. Après avoir compensé les pertes de chromosomes dues aux artéfacts, le taux de non-disjonction chromosomale ou d'anaphase tardive était de 5,5%. Ainsi après culture des ovocytes équiens *in vitro*, on peut conclure que la maturation nucléaire de ceux-ci est semblable à celle des autres espèces domestiques en ce qui concerne la synchronisation et l'apparence des chromosomes.

méiose / ovocyte / maturation nucléaire / non-disjonction / cheval

INTRODUCTION

It has been suggested that chromosome abnormalities are a significant factor accounting for infertility in the mare (Chandley *et al*, 1975). To-date, all reported cases of chromosome abnormalities have been associated with fertility disturbances or congenital defects (Long, 1988). Most cases of aneuploidy, sex-chromosome (Trommerhausen Bowling *et al*, 1987; Long, 1988) and autosome (Power, 1987; Klunder *et al*, 1989) can be attributed to non-disjunction during female or male meiosis. The frequency of non-disjunction and irregular segregation during meiosis can be estimated by examining meiotic chromosomes at the second meiotic metaphase (MII). Several studies of male meiosis have been reported in the domestic species, including 1 in the domestic horse (Scott and Long, 1980). While involving only 8 stallions, the incidence of non-disjunction (3.4%) was similar to that reported for other domestic males (Scott and Long, 1980). Meiosis in the female of the domestic species has also received considerable attention since it was first observed that meiosis resumes when oocytes are removed from immature follicles (Pincus and Enzmann, 1935) and that metaphase I and II preparations can be readily obtained by *in vitro* culture of oocytes collected from slaughtered females.

In the mare, very few descriptions of nuclear maturation (meiosis) of oocytes matured *in vitro* or *in vivo* have been reported (Webel *et al*, 1977; Fulka and Okolski, 1981; King *et al*, 1987). In fact, very little is known of the events leading to fertilization in this species. Here we report on the timing of nuclear maturation and the meiotic chromosomes of horse oocytes collected at slaughter and cultured *in vitro*. Preliminary observations from this study have been previously reported in abstract form (Desjardins *et al*, 1985).

MATERIALS AND METHODS

Ovaries were recovered from mares within 20 min of slaughter and were kept warm (25°C-35°C) throughout the manipulation. The content of antral follicles (> 5 mm) was aspirated into 20 cc syringes through 18 gauge needles. The follicular fluid was transferred into heparinized petri-plates and oocyte-cumulus-complexes located under a dissection microscope, transferred to sterile disposable 5 cc plastic tubes containing modified Krebs Ringers bicarbonate (KRb) solution (Fukui *et al*, 1982) and maintained at 30°C-37°C while being transported to the laboratory. Representative control oocytes were selected at the slaughter house and transferred into cold PBS (4°C). At the laboratory, the oocyte-cumulus complexes were transferred to fresh medium. Only oocytes with at least 1 layer of cumulus

cells were used. Oocyte-cumulus complexes were then cultured in KRb enriched with 20% fetal calf serum. At the end of culture the cumulus cells were removed by treatment with a mixture of trypsin ($1\mu\text{g/ml}$) and pronase ($1\mu\text{g/ml}$) and hyaluronidase ($1\mu\text{g/ml}$) and the oocytes were fixed individually on slides (King *et al*, 1979). Control oocyte-cumulus complexes were examined, their cumulus cells dispersed and they were fixed without culture. The slides were stained with aceto-orcine and examined. The bivalents (MI) and univalents (MII) were counted and when possible, karyotypes were made.

The study was performed in 2 parts. In the first, oocytes were fixed after 12, 24, 48 and 60 to 96 h to determine the timing of nuclear maturation. In the second, oocytes were cultured for 24 h to obtain additional MII spreads for chromosome analysis.

RESULTS

Part I

In total 104 oocytes were collected, of which 74 were cultured before fixation and 30 were fixed without culture. Of those cultured, 62% (46/74) could be analyzed. Of these, 89% (41/46) had resumed meiosis and 59% (27/46) reached second metaphase. The meiotic stage after fixation in relation to the culture period is summarized in table I. Germinal vesicle breakdown (GVB) and the reappearance of the chromosomes (figs 1 and 2) was completed in 75% (9/12) of the analyzable oocytes after 12 h in culture and in all but 1 (13/14) after 24 h culture. The resumption of meiosis was characterized by the reappearance of diffuse chromatin filaments with remnants of the nuclear membranes (fig 1) and nucleoli (fig 2). At MI (fig 3) and MII (fig 4) a modal value of 32 bivalents and univalents, respectively, was observed while the chromosomes generally resembled those of most mammals during meiosis. Occasionally, 2 metaphase spreads, the secondary oocyte metaphase and presumably the polar body metaphase, were observed in the same oocyte (fig 4).

Table I. Summary of the stage of nuclear maturation in oocytes fixed after various culture periods. *degenerated, no chromatin observed or chromatin present but stage of meiosis not recognizable.

Culture time (h)	No oocytes	GV	diplotene/ diakinesis	MI	MII	Not* analyzable
0	30	1	7	3	4	15
12	21	3	7	1	1	9
24	21	1	0	3	10	7
48	20	0	2	0	9	9
60-96	12	1	1	0	7	3
Total	104	6	17	7	31	43

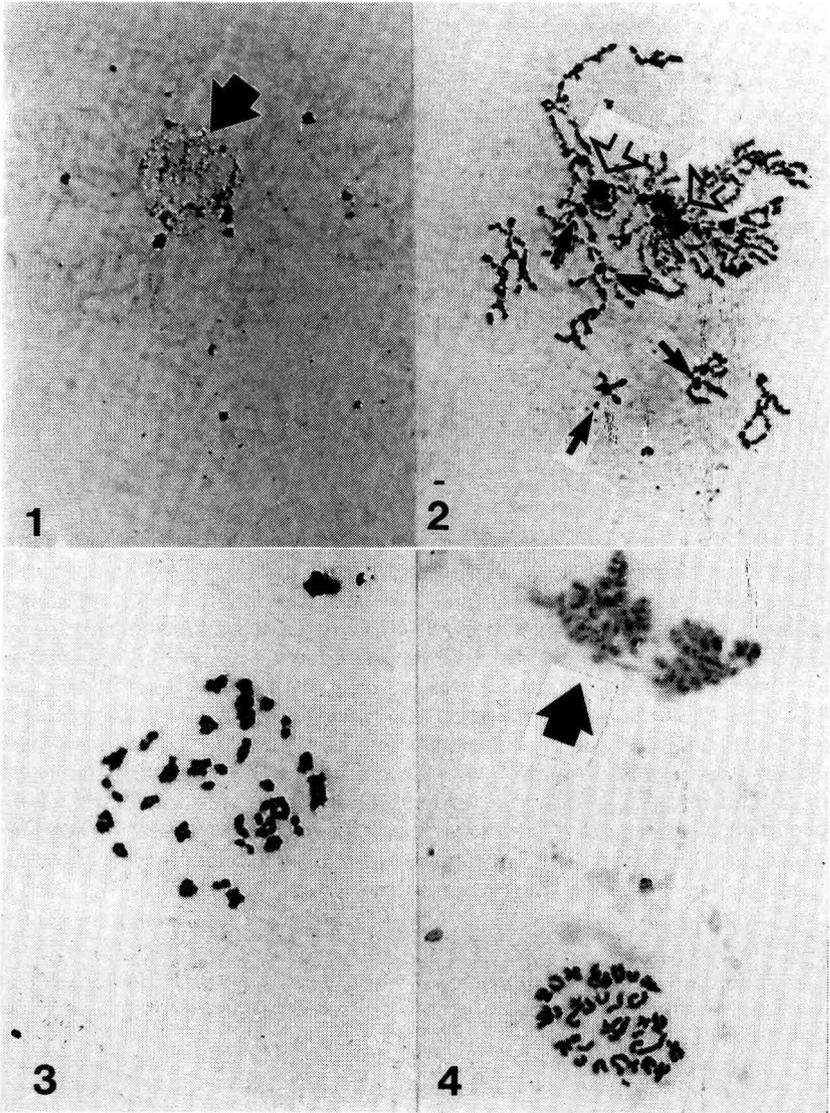


Fig 1-4. Photographs of chromosome preparation from horse oocytes cultured *in vitro* showing different stages of meiosis.

Fig 1. Germinal vesicle breakdown (GVB), note the remnants of nuclear envelope (arrows).

Fig 2. Early diakinesis, note the condensed chromatin in the centromeric regions of some chromosomes (arrows) and remnants of the nucleolus (open arrow).

Fig 3. First meiotic metaphase (MI).

Fig 4. Second meiotic metaphase (MII) and condensed chromatin of polar body (arrow).

Part II

A total of 113 oocyte-cumulus-complexes were cultured for 24 h. After fixation, 39% (44/113) could be analyzed. Of these, 93% (41/44) had resumed meiosis, 11% (5/44) were diplotene/early diakinesis stage, 18% (8/44) were diakinesis/MI stage and 68% (30/44) were MII stage. Two of the MII stage oocytes also presented presumptive polar body metaphase spreads.

By combining part I and II, a total of 14 diakinesis/MI were examined of which 5 had 32 bivalents, 2 had less than 32 and 7 were not of sufficient quality to be accurately counted. Sixty-one MII stage oocytes were examined, of these 29 had 32 univalents (haploid), 5 had less than 32 (hypohaploid), 1 had 33 (hyperhaploid), 1 had 64 (diploid) and 25 were not of sufficient quality to be counted. In addition to these, 5 metaphase spreads of presumptive polar bodies were examined, (1 with 32 univalents and 1 less than 32 univalents; 3 were uncountable).

The hyperhaploid metaphase contained 33 chromosomes which included an extra acrocentric chromosome. The spreads with 31 chromosomes were both missing a bi-armed chromosome while those with 30 or less chromosomes were missing both acrocentric and bi-armed chromosomes. The 5 hypohaploid spreads contained 31, 31, 30, 29, and 26 chromosomes respectively. The hypohaploid presumptive polarbody (25 chromosomes) was in the same oocyte as an MII spread with 31 chromosomes. The karyotype of a haploid, hypohaploid and hyperhaploid oocytes is shown in figures 5, 6 and 7.

DISCUSSION

While female meiosis in the domestic horse has only been previously described using whole mount techniques, our observations on air-dried horse oocytes show that the stages of germinal vesicle breakdown, resumption and completion of meiosis resemble those of most domestic mammalian species (McGaughey and Chang, 1968; Jagiello *et al*, 1974; King *et al*, 1986; Madison, 1988). GVB in the majority of oocytes surrounded by at least 1 layer of cumulus cells was completed within 12 h of initiation of culture which is similar to the time required for GVB in cattle (Motlik *et al*, 1978) and sheep (Moor and Crosby, 1985; Madison, 1988). However, Fulka and Okolski (1981) reported that the time required for completion of *in vitro* oocyte nuclear maturation is longer in horses than in cattle or sheep more closely resembling that of pig (McGaughey and Polge, 1971). Our observations suggest, that under the conditions described here, the majority of oocytes which resume meiosis do reach MII within 24 h of initiation of culture, and in this respect nuclear maturation in the horse resembles that of cattle (King *et al*, 1986) and sheep (Madison, 1988). Some controversy concerning the stage of nuclear maturation of oocytes at ovulation exists in the literature; Hamilton and Day (1945) reported that oocytes are ovulated before MII while Van Niekerk and Gerneke (1966) suggested that MII occurs before ovulation. Working with air-dried oocytes King *et al* (1987) observed only MII stages in preovulatory oocytes while Webel *et al* (1977) observed both MI and MII stages in whole mount preparations of ovulated oocytes. The observation of 4 oocytes at MII stage in the uncultured groups in the present study are in agreement with the contention that oocytes can reach MII stage in the follicle.

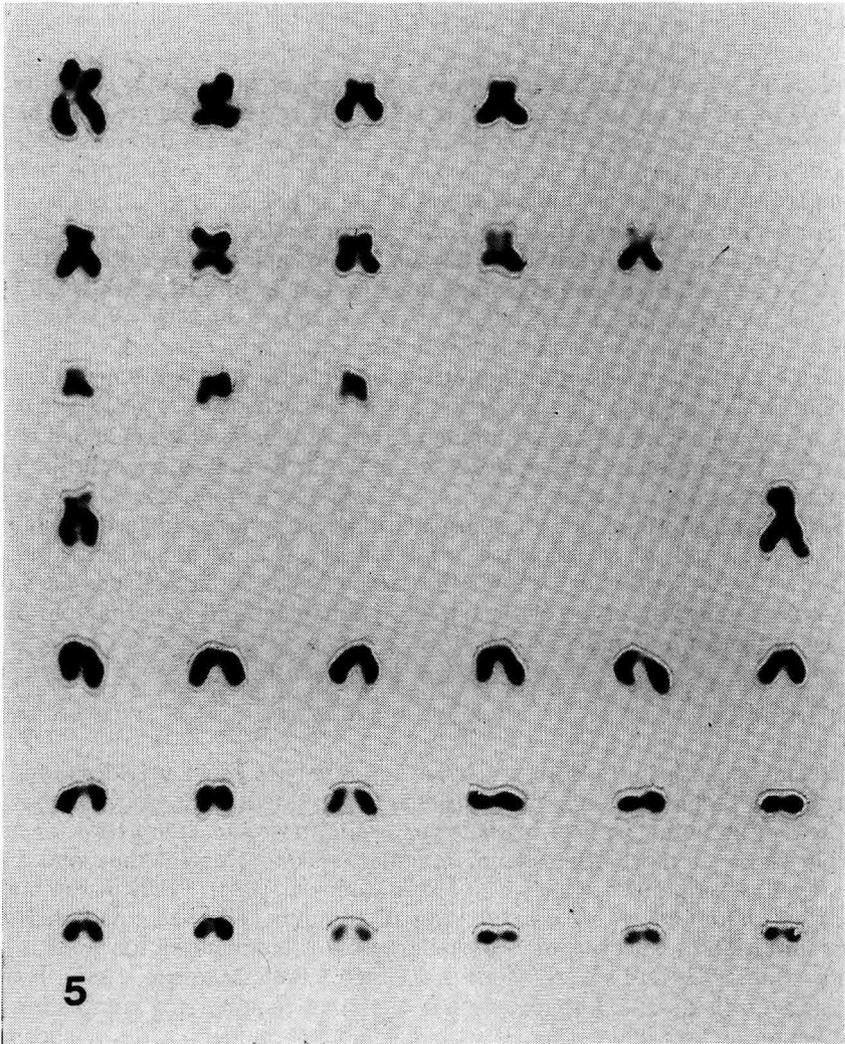


Fig 5-7. Karyotype from MII spreads. The chromosomes are arranged according to size and morphological groups although identification of individual chromosomes is not possible.

Fig 5. Karyotype of a haploid metaphase with 32 (18 acrocentrics and 14 biarmed) chromosomes.

Non-disjunction at the first meiotic division leads to hypohaploid or hyperhaploid MII spreads, theoretically in equal proportions, and fertilization of such oocytes would lead to aneuploid zygotes. Indeed, several cases of sex chromosome aneuploidy 63XO, 65XXX, 65XXY (Long, 1988) and 1 case of trisomy of autosome 23 (Klunder *et al*, 1989) have been reported in adult horses. Non-disjunction

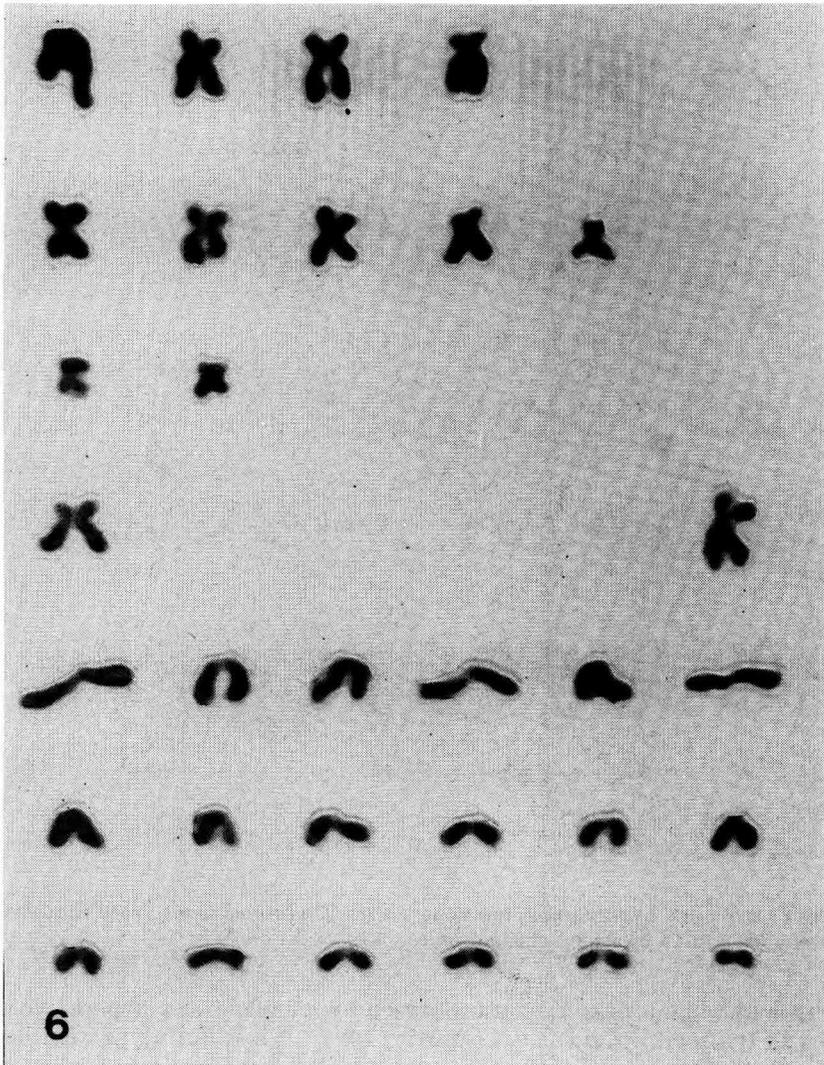


Fig 6. Karyotype of a hypohaploid metaphase with 31 chromosomes. Note that there are 18 acrocentrics and only 13 bi-armed chromosomes.

in the oocytes examined here was evidenced by the presence of hypohaploid (13.9%) and hyperhaploid (2.7%) MII spreads. The unequal ratio of hypohaploid to hyperhaploid (5:1) can be interpreted in at least 2 ways. Firstly, non-disjunction may have been accompanied by anaphase lagging so that there was a loss of chromosomes during meiosis rather than unequal distribution between oocyte and polar body, and secondly, during fixation chromosomes were artifactually lost. Support for the first interpretation comes from zona-free hamster oocytes penetrated by human sperm where an excess of hypohaploid oocyte complements

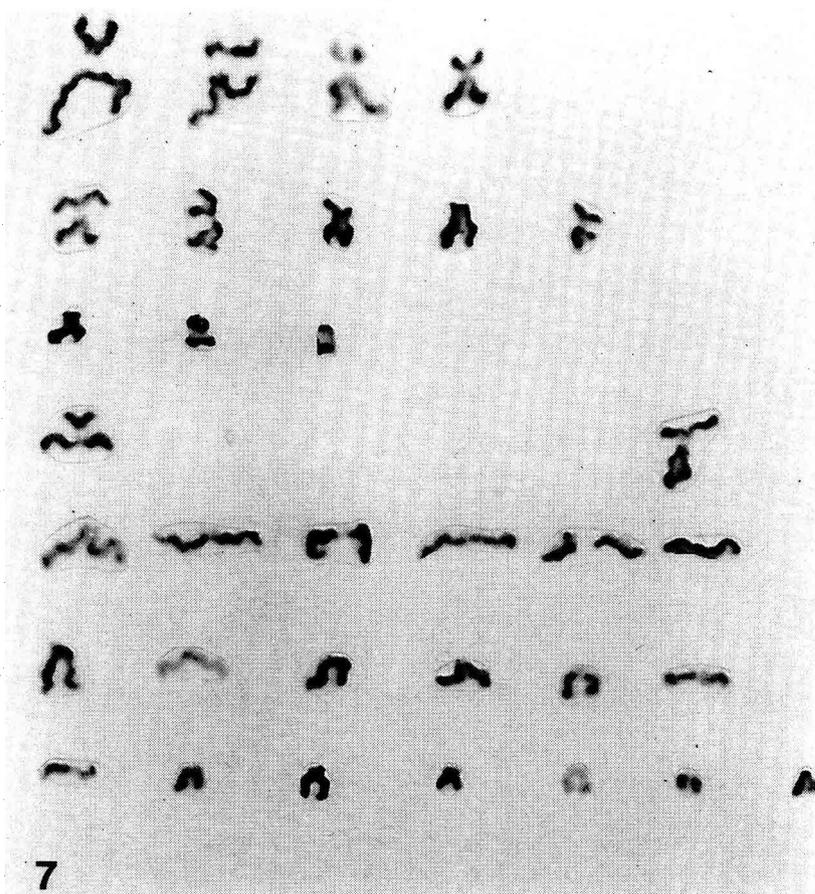


Fig 7. Karyotype of a hyperhaploid metaphase with 33 chromosomes. Note that there are 19 acrocentrics and 14 bi-armed chromosomes.

were observed while a near 1:1 ratio between hypohaploid and hyperhaploid was observed in the sperm complement (Martin, 1984). If loss during fixation had occurred it should have equally affected both complements. It might also be argued that since reported cases of X-chromosome monosomy outnumber X-chromosome trisomy in horses (Trommerhausen Bowling, 1987) there is a prevalence of chromosome loss during meiosis. However, the frequency of reports of 63XO mares may be related to the distinctive phenotypic features of these animals which motivates referral for karyotype analyses (Long, 1988). Observations on unselected populations of mares (Walker and Bruere, 1979; Long, 1988) and embryos (Romagnano *et al*, 1987), while limited, do not indicate high rates of non-disjunction or anaphase lagging during meiosis *in vivo*. Support for the second interpretation comes from the observation of an oocyte with MII and polar body spreads which were both hypohaploid. Non-disjunction would be expected to lead to a hyperhaploid and a hypohaploid metaphase while anaphase lagging would lead to a hypohaploid and

a haploid metaphase. Also, oocytes with MI spreads with less than 32 bivalents indicate technical loss since it has been recognised that pre-meiotic non-disjunction or anaphase lagging of pairs of chromosomes is very rare (Hulten *et al*, 1985). If it is assumed that the majority of hypohaploids occur due to loss during fixation but that there is a 1:1 ratio between hypo- and hyperhaploid MII, a conservative estimation of non-disjunction would be 5.5% or twice the frequency of hyperhaploids. This rate is similar to the reported 3.4% non-disjunction in the stallion (Scott and Long, 1980), 5.2% in men (Martin, 1984), 4% in human oocytes (Martin *et al*, 1986) and 3.3% in hamster oocytes (Martin, 1984).

From this study, it was concluded that oocyte-cumulus complexes collected from slaughtered mares are capable of nuclear maturation *in vitro* and provide suitable material for chromosomal analysis. The chromosomal features and the rate of non-disjunction and/or anaphase lagging during female meiosis are similar to those of other mammalian species.

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