Zygote **9** (August), pp 193–199. © 2001 Cambridge University Press Printed in the United Kingdom

Cytogenetic analysis of caprine 2- to 4-cell embryos produced *in vitro*

Patricia Villamediana[1], *Francesca Vidal*[2] *and Maria-Teresa Paramio*[1]
Universitat Autònoma de Barcelona, Barcelona, Spain

Date submitted: 11.10.00. Date accepted: 11.01.01

Summary

Prepubertal goat *in vitro* matured/*in vitro* fertilised oocytes produce only a small percentage of blastocysts. The present study examines the incidence of chromosomal anomalies in 2- to 4-cell embryos *in vitro* produced (IVP) from prepubertal oocytes fertilised with the semen of two males. Cumulus–oocyte complexes were obtained by slicing ovaries from slaughtered prepubertal goats. Oocytes were matured in TCM199 supplemented with 20% heat inactivated Donor Bovine Serum (DBS), 10 μg/ml FSH + 10 μg/ml LH + 1 μg/ml 17β-oestradiol for 27 h at 38.5 °C in 5% CO_2 in air. IVM oocytes were inseminated with the sperm from two males prepared using the swim-up and heparin-capacitation procedures. At 24 h post-insemination (hpi) the oocytes were transferred to 100 μl drops of SOF medium for a further 24 h. At 17 hpi a sample of oocytes was stained with lacmoid to evaluate the nuclear stage after fertilisation. The cleavage rate was determined at 24, 36 and 48 hpi and chromosome slides were prepared according to the gradual-fixation technique and stained with Leishman. A total of 1070 2- to 4-cell embryos from prepubertal goat oocytes were studied, but it was only possible to analyse 241 cytogenetically. Of these, 40% exhibited a normal diploid chromosome complement, 59% were haploid and 1% were triploid. There were significant differences between the two males in sperm oocyte penetration and oocyte cleavage but no differences were found in chromosomal anomalies. In conclusion, the low number of embryos karyotyped and the high number of haploid embryos found in this study suggested a high incidence of abnormal fertilised embryos and deficient cytoplasmic maturation of the oocyte which inhibits sperm head decondensation.

Keywords: Cytogenetic, Goat, IVF, Prepubertal

Introduction

The *in vitro* fertilisation of prepubertal goat oocytes shows a high loss rate of embryos during the 2-cell to blastocyst stage (Izquierdo *et al*., 1999). This failure in embryo development could be due to unsuitable culture conditions during *in vitro* maturation, fertilisation and development (IVM, IVF and IVD) or to chromosomal anomalies developing during these processes. According to King (1990) chromosomal abnormalities constitute a major cause of embryonic loss in mammals. The incidence of chromosomal abnormalities in the embryos of domestic animals produced *in vivo* has been estimated at 10.4% in cattle, 6.6% in sheep and 5% in pigs. The most common anomalies were mixploidy (52%) and aneuploidy (21%). This author reported that in embryos about a quarter of the chromosome abnormalities can be attributed to errors in meiosis and the remaining three-quarters occur around the time of fertilisation and early embryonic development.

In sheep, Murray *et al.* (1985, 1986) reported 11% of chromosomally abnormal 2-day embryos in both superovulated and hormonally untreated females. In pigs, Underhill *et al.* (1991), analysing 4-day embryos obtained from hormonally treated prepubertal gilts, found 9–25% abnormal metaphases.

All correspondence to: Maria-Teresa Paramio, Departament de Ciencia Animal i des Aliments, Facultat de Veterinaria, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. Tel: +34 935811456. Fax: +34 935812006. e-mail: teresa.paramio@uab.es
[1]Departament de Ciencia Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
[2]Departament de Biologia Cellular i Fisiologia, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

https://doi.org/10.1017/S0967199401001204 Published online by Cambridge University Press

There are numerous cytogenetic studies on human embryos produced *in vitro* (Wimmers *et al.*, 1988; Papadolpoulos *et al.*, 1989; Pellestor *et al.*, 1994*a*, *b*), but in domestic animals we have found studies on only cattle and pig. In cattle, Iwasaki *et al.* (1989) reported 13.7% of embryos with chromosomal anomalies, which were mainly triploidies. Lechniak (1996) found 23% chromosomal anomalies and Kawarsky *et al.* (1996) 36.3% and 39.3% in 2-day and 5-day embryos, respectively. The most common abnormalities in these studies were aneuploidy and polyploidy. Yadav *et al.* (1993), studying the relation between the timing of oocyte cleavage and the chromosomal anomalies, concluded that late-cleaving embryos (cleavage at 40, 48, and 62 hpi) have more chromosomal anomalies than early-cleaving embryos (24 and 30 hpi).

In pigs, according to Prather & Day (1998) the major anomalies found in IVM-IVF-IVD embryos were polyspermy and failure of the oocyte to form a male pronucleus. Wang *et al.* (1999) stained porcine embryos produced *in vitro* with orcein and showed that a large number of them were fragmented (no nucleus in one or more blastomeres).

Embryo chromosomal abnormalities are associated with different factors such as the mother's age, the induction of oocyte maturation and gamete ageing (Santalo *et al.*, 1992), the *in vitro* conditions during fertilisation (Iwasaki *et al.*, 1989; Santalo *et al.*, 1992), the oocyte maturation stage (Plachot & Crozet, 1992) and embryo freezing (Glenister *et al.*, 1987; Van der Elst *et al.*, 1993).

The male has a significant effect on the IVF and *in vitro* embryo production. Spermatozoa from different males showed differences in oocyte penetration rates in rabbit (Brackett & Oliphant, 1975), cattle (Brackett *et al.*, 1982; Ohgoda *et al.*, 1988; Marquant-Le Guienne & Humblot, 1998) and sheep (Fukui *et al.*, 1988).

In our laboratory, testing different IVM-IVF-IVD media with prepubertal oocytes, blastocyst production was never higher than 12% (Izquierdo *et al.*, 1999) – a percentage which is lower than that found by other authors using adult goat oocytes: Crozet *et al.* (1995), 26%; Pawshe *et al.* (1996), 40%; Kestintepe *et al.* (1996, 1998), 39% and 36%, respectively.

The aim of this study was to carry out a cytogenetic investigation of the IVM-IVF-IVD of 2- and 4-cell embryos produced from prepubertal goat oocytes and analyse the chromosomal abnormalities according to the male used, number of embryo cells (2, 3 and 4 cells) and embryo cleavage time (24, 36 and 48 hpi).

Materials and methods

In vitro oocyte maturation

Oocyte recovery and IVM were carried out according to the method used by Mogas *et al.* (1997) in our laboratory. Ovaries were recovered at a local abattoir from prepubertal goats (approximately 2 months old) shortly after slaughter and transported to the laboratory at 35–37 °C in phosphate-buffered saline (PBS: P-4417, Sigma, St Louis, MO) supplemented with 50 mg/l gentamycin. On reaching the laboratory the ovaries were washed four times in PBS plus gentamycin (50 mg/l). The medium used for oocyte collection was TCM199/HEPES (M-2520, Sigma) containing 2.2 mg/ml $NaHCO_3$ and 50 μg/ml gentamycin. Cumulus-oocyte complexes (COCs) were recovered by cutting the surface of the ovaries with a razor blade. Only oocytes possessing evenly granulated cytoplasm with one or more layers of compact cumulus cells were used for maturation. COCs were washed twice in TCM199/HEPES (M-7528, Sigma) supplemented with 0.146 g/l L-glutamine (G-5763, Sigma), 2.75 g/l sodium pyruvate (P-4562, Sigma), 50 μg/ml gentamycin and 20% of heat inactivated Donor Bovine Serum (DBS: CanSera, Rexdale, Ontario, Canada) and once in the maturation medium. The oocyte maturation medium was the washing medium supplemented with 10 μg/ml FSH (Ovagen, Immuno Chemicals Products, Auckland, New Zealand), 10 μg/ml LH (supplied by J.F. Beckers, IRSIA Research Unit, University of Liège, Belgium), 1 μg/ml 17β-oestradiol (E-2257, Sigma) and 20% DBS. The COCs were cultured in groups of 20 in 100 μl culture drops for 27 h at 38.5 °C in 5% CO_2 in air with maximum humidity.

Sperm capacitation and *in vitro* fertilisation

Fresh semen was collected by artificial vagina from two males. Male 1 was a 2-year-old Malagueño breed and male 2 was a 5-year-old Murciano-Granadino breed. Sperm motility was evaluated by light microscopy and ejaculates with more than 70% motile sperm were used. From each male, an aliquot of sperm suspension (70 μl) was layered under 2 ml of capacitation medium in two tubes for a swim-up procedure according to the method of Parrish *et al.* (1986). The capacitation medium used was Defined Medium modified by Younis *et al.* (1991), referred to as mDM here. The top layer (0.6 ml) of medium was collected from each tube after 1 h of incubation at 38.5 °C. The pooled medium containing highly motile spermatozoa was centrifuged (200 *g*) for 10 min. Simultaneously, sperm concentration was measured with a Thomas Camara cell. The pellet was resuspended in sufficient mDM medium to yield a concentration of 160×10^6 total

https://doi.org/10.1017/S0967199401001204 Published online by Cambridge University Press

sperm/ml. An equal volume of a 100 µg/ml heparin (Sigma, H-3393) solution made with mDM was added to the sperm suspension to yield final sperm and heparin concentrations of 80×10^6 cells/ml and 50 µg/ml, respectively. The heparin-treated sperm were incubated for 45 min at 38.5 °C.

In vitro fertilisation and culture

Drops of 100 µl of fertilisation medium under oil were prepared with TALP-fert medium modified by Parrish *et al.* (1986) supplemented with hypotaurine (1 µg/ml; Sigma, H-1384) in advance of oocyte–spermatozoa culture, placed in the incubator and allowed to equilibrate for approximately 1–2 h. Oocytes cultured for 27 h were washed once with TALP-fert medium and groups of 20 COCs were put into IVF drops. A 5 µl aliquot of the heparin-treated sperm was then added to the oocytes in each drop to give a final sperm concentration of 3.5 to 4×10^6 cells/ml (Palomo *et al.*, 1995). After insemination, oocytes and sperm were incubated at 38.5 °C under 5% CO_2 in air.

At 24 hpi, spermatozoa and cumulus cells were removed from oocytes using a pipette. All presumptive zygotes and early 2-cell embryos were washed several times in culture medium and placed in microdrops (100 µl) of Synthetic Oviductal Fluid medium (mSOF; Takahashi & First, 1992) under oil at 38.5 °C in 5% CO_2 and humidified air for 24 h.

Evaluation of the IVF and cleavage

To evaluated the nuclear stage after fertilisation, at 17 hpi a sample of approximately 20 oocytes per replicate was separated from the sperm by agitation and fixed in acetic acid:ethanol (1:3, v/v) at 4 °C for 24 h. Samples were stained with 1% lacmoid in 45% acetic acid solution and examined under a phase-contrast microscope.

Oocytes containing a sperm tail in the cytoplasm were considered to be fertilised and were classified as: normally fertilised or 2PN+T (female pronucleus, male pronucleus and sperm tail), polyspermic (PS; oocytes with two or more sperm tails with decondensed or non-decondensed heads or with more than two pronuclei) and asynchronous (AS; asynchronous development of male and female pronuclei, in which generally the head of the spermatozoon is non-decondensed and the female pronucleus is well formed).

The cleavage rate (number of cleaved oocytes or >2-cell embryos/oocytes inseminated) was evaluated at 24, 36 and 48 hpi and embryos were classified as 2/3 cells or 4 cells.

Evaluation of parthenogenetic activation

To investigate the incidence of spontaneous parthenogenetic activation in the oocytes of prepubertal goats under our system of IVM and IVF the following experiment was carried out. Two hundred and ninety-nine IVM oocytes were divided into three groups: (a) the oocytes were incubated in 100 µl drops of IVF medium supplemented with hypotaurine (1 µg/ml; H-1384, Sigma); (b) the oocytes were incubated in 100 µl drops of IVF medium supplemented with hypotaurine (1 µg/ml; H-1384, Sigma) plus 5 µl of sperm capacitation medium with heparin (50 µg/ml; H-3393, Sigma) without sperm; (c) the same fertilisation protocol was used as described for (b) but with dead spermatozoa. A sample of approximately 15 oocytes per experiment was taken, in the same way as for the IVF protocol, after 17 h of culture, and processed and stained in lacmoid. At 24 h of culture in the IVF medium, the oocytes were transferred to 100 µl drops of mSOF and incubated for a further 24 h.

Cytogenetic analysis

The embryos were analysed according to the methodology of Iwasaki *et al.* (1992). Briefly, embryos were cultured for a further 12 h in the same culture medium (mSOF) supplemented with 0.1 µg/ml of colchicin (C-3915, Sigma). Then, the embryos were treated with pronase at 0.5% (P-8811, Sigma) to digest the zonae pellucidae and placed in hypotonic medium at room temperature (20 min in sodium citrate at 0.9%). Chromosome fixing was carried out in two phases. The first fixing was in a solution of acetic acid, methanol and hypotonic solution (5:15:8) for 3 min. The second fixing was in a mixture of acetic acid and methanol (1:3) for 30 min. After fixing, the embryos were transferred to previously degreased slides (ether:ethanol, 7:3 v/v). The chromosomal preparations were stained with Leishman (Panreac, Cod 251378, Montplet & Esteban, Barcelona, Spain) at 20% Leishman buffer (BDH Laboratory Supplies, Poole, UK) pH 6.8, for 8 min. The slides were analysed using an Olympus BX-60 microscope to determine the ploidy of the embryos (2n=60 chromosomes) by counting the number of chromosomes.

Statistical analysis

Comparisons of the proportions of fertilised oocytes and developed embryos were made using chi-square analysis. Differences were considered significant at values of $p<0.05$. The CATMOD (Categorical Modeling) procedure of SAS was used, eliminating the effects and interactions that were not significant.

https://doi.org/10.1017/S0967199401001204 Published online by Cambridge University Press

Results

Results of fertilisation and cleavage rate of oocytes from prepubertal goats inseminated with two different males are summarised in Table 1. The oocytes inseminated from male 1 showed significantly higher (p<0.05) results for total sperm penetration, polyspermy, asynchrony and cleavage rate than oocytes inseminated from male 2.

The cytogenetic characteristics of 2/3-cell embryos and 4-cell embryos from prepubertal goat oocytes are summarised in Table 2. Of the total of 1070 fixed embryos only 22.5% could be cytogenetically analysed. Of these 241 analysed embryos, 39.8% showed a normal number of chromosomes (2n=60), 58.9% were haploids and only 3 embryos were triploids. The effect of the males was not statistically significant.

Regardless of the male used, the percentage of haploid embryos was higher (p<0.05) in 4-cell embryos than 2/3-cell embryos. Of all embryos only 18 were 3-cell embryos and of these 77.7% (14/18) were haploids.

We did not find differences between diploid embryos obtained at 24, 36 and 48 hpi.

The Mitotic Index (MI=number of metaphases/number of blastomeres analysed × 100) was 72.22 ± 25.56 for the 2-cell embryos, which is 1.50 ± 0.50 metaphases per embryo. In 3-cell embryos the MI was 48.15 ± 17.04, which is 1.44 ± 0.51 metaphases per

Table 1 Results of *in vitro* fertilisation and cleavage rate of prepubertal goat oocytes inseminated with two males

	17 h post-insemination					48 h post-insemination	
Male	No. of oocytes[a]	%Pen[b] (n)	%2PN+T[c] (n)	%PS[d] (n)	%Asyn[e] (n)	No. of oocytes[f]	% Cleavage[g] (n)
1	156	91.0[h] (142)	26.9 (42)	26.3[h] (41)	37.8[h] (59)	1150	54.2[h] (623)
2	203	41.9[i] (85)	21.7 (44)	5.9[i] (12)	14.3[i] (29)	1847	40.9[i] (755)

[a]A sample of fixed and tested oocytes at 17 h post-insemination.
[b]Total oocytes penetrated by one or more sperm.
[c]Two pronuclei + tail spermatozoa.
[d]Polyspermic.
[e]Asynchronous.
[f]Number of oocytes inseminated allocated in culture.
[g]Embryos of 2 to 4 cells.
[h,j]Values in a column with different superscripts are significantly different (χ^2, p<0.05).

Table 2 Cytogenetic characteristics of 2/3- and 4-cell embryos obtained at 24, 36 and 48 h post-insemination for males 1 and 2

	24 h post-insemination				36–48 h post-insemination				Total			
No. of embryo cells	2n	1n	3n	Total embryo	2n	1n	3n	Total embryo	2n	1n	3n	Total embryo
Male 1												
2/3 cell	38	45	1	84	7	10	–	17	45	55[b]	1	101
>4 cell	4	5	–	9	9	31	–	40	13	36[a]	–	49
Total	42	50	1	93	16	41	–	57	58	91	1	150
Male 2												
2/3 cell	16	16	2	34	10	11	–	21	26	27	2	55
>4 cell	1	10	–	11	11	14	–	25	12	24	–	36
Total	17	26	2	45	21	25	–	46	38	51	2	91
Total	59	76	3	138	37	66	–	103	96	142	3	241

2n = diploid, 1n = haploid, 3n = triploid.
[a,b]Values in a column with different superscripts are significantly different (χ^2, p<0.05).

https://doi.org/10.1017/S0967199401001204 Published online by Cambridge University Press

embryo, and for the 4-cell embryos the MI was 31.73 ± 11.56, which is 1.70 ± 0.59 metaphases per embryo.

Parthenogenesis

After 48 h in culture none of the 299 IVM oocytes was classified as parthenogenetic according to their morphology (no blastomeres). The sample of oocytes analysed after 17 h in fertilisation medium did not show parthenogenetic activation either (no signs of pronuclei or the second polar body).

Discussion

The results of this study show that a larger percentage of 2- and 4-cell embryos from prepubertal goats obtained under our protocol of IVM/IVF/IVD are haploids. The male, the number of embryo cells and the time of oocyte cleavage do not modify this percentage.

At least one metaphase plate was analysed in 22.5% (241/1070) of the *in vitro* embryos used in this study. In the literature the mean of the cytogenetically analysable embryos, produced *in vivo*, varies from 38% to 80% in pigs (King, 1990; Underhill *et al.*, 1991); from 43.2% to 51% in cattle; and is 34% in sheep (King, 1990). In bovine embryos produced *in vitro* the percentage of karyotyped embryos is 15% in 2- to 8-cell embryos and 82% of >32-cell embryos (Kawarsky *et al.*, 1996). In human *in vitro* produced embryos, Papadopoulos *et al.* (1989) were able to karyotoype 47% of embryos. However, with embryos of poor morphology (grade IV) only 26% were karyotyped (Pellestor *et al.*, 1994*b*), and of embryos not destined for replacement in the uterus 23% were successfully karyotyped (Wimmers *et al.*, 1988). The low percentage of cytogenetically analysable embryos could be a sign of poor-quality embryos. Wang *et al.* (1999), using prepubertal porcine embryos produced *in vitro*, found that 24% of 2-cell, 63% of 3-cell and 37% of 4-cell embryos were fragmented. These authors showed that fragmentation is the main embryo anomaly. In conclusion, the low percentage of embryos karyotyped in our study could be due to: (a) blocking and degeneration of the embryos before treatment with colchicin, as has also been observed by Pellestor *et al.* (1994*b*) and Lechniak *et al.* (1998), (b) asynchronism of the cellular divisions of these embryos, which gives a large number of interphasic nuclei; and (c) fragmented embryos produced under our *in vitro* conditions.

In our study, the major chromosomal anomaly was haploidy. Haploidy has been observed in the embryos of domestic animals and humans with frequencies of 1% to 22%. In cattle, the rate of haploid embryos ranges from 1.1% to 8.3% (King & Picard, 1985; King, 1991). In pigs, haploid embryos accounted for 12.2% of embryos (Underhill *et al.*, 1991), in sheep, 2.7% (Murray *et al.*, 1985) and in human embryos, 2.6% (Jamiesson *et al.*, 1994) and 11.8% (Pellestor *et al.*, 1994*b*). The highest percentage of haploidy reported until now – 22% – was observed by Macas *et al.* (1993) when analysing mouse blastocysts from oocytes inseminated by multiple injection in the perivitelline space. The principal chromosomal anomaly found in bovine embryos produced *in vitro* is mixploidy. The reason could be that, according to Kawarsarki *et al.* (1996), mixploidy was seen only in embryos that had developed beyond the 8-cell stage and ours were 2- to 4-cell embryos. One of the causes of polyploidy in embryos is polyspermic penetration. In prepubertal goat oocytes, evaluated at 17 hpi, 23% (53/227) of all oocytes penetrated by sperm were polyspermic but only 3 embryos of 241 showed polyploidy. The high incidence of haploidy and the low incidence of polyploidy could be caused by abnormal or incomplete cytoplasmic maturation, which would inhibit sperm head decondensation and male pronuclei formation. We demonstrated experimentally that these haploid embryos do not have a parthenogenetic origin caused by the culture medium, which indicates that the oocyte must be activated by spermatozoal penetration in order to complete the second meiotic division and continue development, although the spermatozoon does not contribute, through its genetic material, to the development of the embryo.

In our study significant differences were observed in the results for total embryo penetration and cleavage rate obtained with ejaculates from different males. Iwasaki *et al.* (1989) found differences between the two males used, in both the penetration rate and the cytogenetic analysis. When evaluating bovine embryos produced *in vitro* using three bulls of different breeds, Kawarsky *et al.* (1996) did not find differences in the penetration rate, cleavage rate or embryo development rate beyond the 8-cell stage, although differences were observed by cytogenetic analysis (aneuploidy and total anomalies). In our study the superiority of male 1 as regards total penetration rate is due to the higher proportion of polyspermic and asynchronous oocytes. Crozet (1993) confirms that there are great differences in IVF results obtained from ejaculates of different males, although adjusting the IVF conditions for each male might reduce this difference. Our results seem to indicate that although male 1 was superior in terms of total penetration and oocyte cleavage, these results were not reflected in the cytogenetic characteristics of the embryos.

In this study we observed (data not presented) that 2-cell embryos cleaving at 24 hpi progressed to the next cellular stage in a higher proportion than those cleaving at 36–48 hpi. Shoukir *et al.* (1997) using IVF human embryos and Yadav *et al.* (1993) in bovine IVF embryos

https://doi.org/10.1017/S0967199401001204 Published online by Cambridge University Press

found that embryos cleaving earlier produced a higher gestation rate and fewer chromosomal anomalies, respectively, than embryos cleaving later (42–62 hpi).

We conclude, from our experiments on the control of parthenogenesis, that our culture system did not activate the oocytes. We therefore reject the possibility that the high incidence of haploidy found in our study could be the result of parthenogenesis. In our study, the characteristics of the zygotes examined at 17 hpi and the large number of haploid embryos lead us to the conclusion that the origin of the haploidy is deficient cytoplasmic maturation of the oocyte which would inhibit the transformation of the sperm head. This would be more evident in the case of polyspermic oocytes. In spite of the deficient maturation of the oocytes, our IVM protocol is widely used in goats (De Smedt *et al.*, 1992; Pawshe *et al.*, 1996; Keskintepe *et al.*, 1998) and cattle (Revel *et al.*, 1995; Arlotto, 1998).

In conclusion, the low number of embryos available to be karyotyped and the high number of haploid embryos found in this study – comparatively higher than that observed by other authors in embryos produced *in vitro* – seems to show incomplete cytoplasmic maturation of prepubertal goat oocytes, including the failure of the oocyte to form a male pronucleus after fertilisation and its inability to develop to the blastocyst stage.

Acknowledgement

This study was supported by CICYT (project no. AGF96–1091–CO2–01).

References

Almedia, P.A. & Bolton, V.N. (1996). The relationship between chromosomal abnormality in the human preimplantation embryo and development *in vitro*. *Reprod. Fertil. Dev.* **8**, 235–41.

Arlotto, T. (1998). The effect of hormonal supplementation of steer serum during bovine oocyte maturation on subsequent *in vitro* embryo development. *Theriogenology* **49**, 305.

Brackett, B.G. & Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* **12**, 260–74.

Brackett, B.G., Bousquet, D., Boice, M.L., Donawick, W.J., Evans, J.F. & Dressel, M.A. (1982). Normal development following *in vitro* fertilization in the cow. *Biol. Reprod.* **27**, 147–58.

Catalá, V., Estop, A.M., Santaló, J. & Egozcue, J. (1988). Sexual immaturity and maternal age: incidence of aneuploidy and polyploidy in first-cleavage mouse embryos. *Cytogenet. Cell Genet.* **48**, 233–7.

Crozet, N. (1993). Fertilization *in vivo* and *in vitro*. In *Reproduction in Mammals and Man* (ed. C. Thibault, M.C. Levasseur & R.H.F. Hunter), pp. 327–47. Paris: Ellipses.

Crozet, N., Ahmed-Ali, M. & Dubos, M.P. (1995). Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture *in vitro*. *J. Reprod. Fertil.* **103**, 293–8.

De Smedt, V., Crozet, N., Ahmed-Ali, M., Martino, A. Cognie, Y. (1992). *In vitro* maturation and fertilization of goat oocytes. *Theriogenology* **37**, 1049–60.

Fukui, Y., Glew, A.M., Gandolfi, F. & Moor, R.M. (1988). Ram-specific effects on *in vitro* fertilization and cleavage of sheep oocytes matured *in vitro*. *J. Reprod. Fertil.* **82**, 337–40.

Glenister, P.H., Wood, M.J., Kirby, C. & Whittingham, D.G. (1987). Incidence of chromosome anomalies in first-cleavage mouse embryos obtained from frozen-thawed oocytes fertilized *in vitro*. *Gamete Res.* **16**, 205–16.

Gordon, I. (1994). *Laboratory Production of Cattle Embryos.* Wallingford, Oxon, UK: CAB International.

Iwasaki, S., Shioya, Y., Masuda, H., Hanada, A. & Nakahara, T. (1989). Incidence of chromosomal anomalies in early bovine embryos derived from *in vitro* fertilization. *Gamete Res.* **22**, 83–91.

Iwasaki, S., Hamano, M., Kuwayama, M., Yamashita, H., Ushijima, S., Nagaoka, S. & Nakahara, T. (1992). Developmental changes in the incidence of chromosome anomalies of bovine embryos fertilized *in vitro*. *J. Exp. Zool.* **261**, 79–85.

Izquierdo, M.D., Villamediana, P. & Paramio, M.T. (1999). Effect of culture media on embryo development from prepubertal goat IVM-IVF oocytes. *Theriogenology* **52**, 847–61.

Jamiesson, M.E., Coutts, J.R.T. & Connor, J.M. (1994). The chromosome constitution of human preimplantation embryos fertilized *in vitro*. *Hum. Reprod.* **9**, 709–15.

Kawarsky, S.J., Parvathi, K.B., Stubbings, R.B., Hansen, P.J. & King, W.A. (1996). Chromosomal abnormalities in bovine embryos and their influence on development. *Biol. Reprod.* **54**, 53–9.

Keskintepe, L., Luvoni, G.C., Bassiony, M.M. & Brackett, B.G. (1996). Procedural improvements for *in vitro* production of viable uterine stage caprine embryos. *Small Ruminant Res.* **20**, 247–54.

Keskintepe, L., Simplicio, A.A. & Brackett, B.G. (1998). Caprine blastocyst development after *in vitro* fertilization with spermatozoa frozen in different extenders. *Theriogenology* **49**, 1265–74.

King, W.A. (1990). Chromosome abnormalities and pregnancy failure in domestic animals. *Adv. Vet. Sci. Comp. Med.* **34**, 229–50.

King, W.A. (1991). Cytogenetics of bovine embryos produced from oocytes matured and fertilized *in vitro*: an update. In *Seventh North American Colloquium on Domestic Animal Cytogenetics and Mapping*, pp. 15–19.

King, W.A. & Picard, L. (1985). Haploidy in preattachment bovine embryos. *Can. J. Genet. Cytol.* **27**, 189–94.

Lechniak, D. (1996). The incidence of polyploidy and mixploidy in early bovine embryos derived from *in vitro* fertilization. *Genetics Selection Evolution* **28**, 321–8.

Lechniak, D., Cieslak, D. & Sosnowski, J. (1998). Cytogenetic analysis of bovine parthenotes after spontaneous activation *in vitro*. *Theriogenology* **49**, 779–85.

Macas, E., Rosselli, M., Imthurn, B. & Keller, P.J. (1993). Chromosomal constitution of mouse blastocysts derived from oocytes inseminated by multiple sperm insertion into the perivitelline space. *J. Assist. Reprod. Genet.* **10**, 468–75.

https://doi.org/10.1017/S0967199401001204 Published online by Cambridge University Press

Marquant-Le Guienne, B. & Humblot, P. (1998). Practical measures to improve *in vitro* blastocyst production in the bovine. *Theriogenology* **49**, 3–11.

Mogas, T., Palomo, M.J., Izquierdo, M.D. & Paramio, M.T. (1997). Developmental capacity of *in vitro* matured and fertilized oocytes from prepubertal and adult goats. *Theriogenology* **47**, 1189–203.

Munné, S., Alikani, M., Tomkin, G., Grifo, J. & Cohen, J. (1995). Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertil. Steril.* **64**, 382-91.

Murray, J.D., Boland, M.O., Moran, C., Sutton, R., Nancarrow, C.D., Scaramuzzi, R.J. & Hoskinson, R.M. (1985). Occurrence of haploid and haploid/diploid mosaic embryos in untreated and androstenedione-immune Australian Merino sheep. *J. Reprod. Fertil.* **74**, 551–5.

Murray, J.D., Boland, M.O. & Moran, C. (1986). Frequency of chromosomal abnormalities in embryos from superovulated merino ewes. *J. Reprod. Fertil.* **78**, 433–7.

Ohgoda, O., Niwa, K., Yuhara, M., Takahashi, S. & Kanoya, K. (1988). Variations in penetration rates *in vitro* of bovine follicular oocytes do not reflect conception rates after artificial insemination using frozen semen from different bulls. *Theriogenology* **29**, 1375–81.

Palomo, M.J., Mogas,T., Izquierdo, M.D. & Paramio, M.T. (1995). Effect of heparin and sperm concentration on IVF of prepubertal goat oocytes. *Theriogenology* **43**, 292.

Papadopoulos, G., Templeton, A.A., Fisk, N. & Randall, J. (1989). The frequency of chromosome anomalies in human preimplantation embryos after *in vitro* fertilization. *Hum. Reprod.* **4**, 91-8.

Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Crister, E.S., Eyeston, W.H. & First, N.L. (1986). *In vitro* fertilization with frozen thawed semen. *Theriogenology* **25**, 591–600.

Pawshe, C.H., Palanisamy, A., Taneja, M., Jain, S. & Totey, S.M. (1996). Comparison of various maturation treatments on *in vitro* maturation of goat oocytes and their early embryonic development and cell numbers. *Theriogenology* **46**, 971-82.

Pellestor, F., Girardet, A., Andréo, B., Arnal, F. & Humeau, C. (1994*a*). Relationship between morphology and chromosomal constitution in human preimplantation embryo. *Mol. Reprod. Dev.* **39**, 141–6.

Pellestor, F., Dufour, M.C. & Humeau, C. (1994*b*). Direct assessment of the rate of chromosomal abnormalities in Grade IV human embryos produced by *in vitro* fertilization procedure. *Hum. Reprod.* **9**, 293–302.

Plachot, M. & Crozet, N. (1992). Fertilization abnormality in human *in-vitro* fertilization. *Hum. Reprod.* **7** (Suppl. 1), 89–94.

Prather, R.S. & Day, B.N. (1998). Practical considerations for the *in vitro* production of pig embryos. *Theriogenology* **49**, 23–32.

Revel, F., Mermillod, P., Peynot, N., Renard, J. & Heyman, Y. (1995). Low developmental capacity of *in vitro* matured and fertilized oocytes from calves compared with that of cows. *J. Reprod. Fertil.* **103**, 115–20.

Santaló, J., Badenas, J., Calafell, J.M., Catalá, V., Munné, S., Egozcue, J. & Estop, A.M. (1992). The genetic risk of *in vitro* fertilization techniques: the use of animal model. *J. Assist. Reprod. Genet.* **9**, 462–74.

Shoukir, Y., Campana, A., Farley, T. & Sakkas, D. (1997). Early cleavage of *in-vitro* fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. *Hum. Reprod.* **12**, 1531–6.

Sirard, M.A. & Lambert, R.D. (1985). *In vitro* fertilization of bovine follicular oocytes obtained by laparoscopy. *Biol. Reprod.* **33**, 487–94.

Takahashi, Y. & First, N.L. (1992). *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**, 963–78.

Underhill, K.L., Downey, B.R., McFarlane, C. & King, W.A. (1991). Cytogenetic analysis of day-4 embryos from PMSG/hCG-treated pubertal gilts. *Theriogenology* **35**, 779–84.

Van der Elst, J., Nerinckx, S. & Van Steirteghem, A.C. (1993). Association of ultrarapid freezing of mouse oocytes with increased polyploidy at the pronuclear stage, reduced cell numbers in blastocysts and impaired fetal development. *J. Reprod. Fertil.* **99**, 25–32.

Wang, W.H., Abeydeera, L.R., Prather, R.S. & Day, B.N. (1999). Morphological evaluation and actin filament distribution in porcine embryos produced *in vitro* and *in vivo. Biol. Reprod.* **60**, 1020–8.

Wimmers, M.S. & Van der Merwe, J.V. (1988). Chromosome studies on early human embryos fertilized *in vitro. Hum. Reprod.* **3**, 894–900.

Yadav, B.R., King, W.A. & Betteridge, K.J. (1993). Relationships between the completion of first cleavage and the chromosomal complement, sex, and development rates of bovine embryos generated *in vitro. Mol. Reprod. Dev.* **36**, 434–9.

Younis, A.I., Zuelke, K.A., Harper, K.M., Oliveira, M.A.L. & Brackett, B.G. (1991). *In vitro* fertilization of goat oocytes. *Biol. Reprod.* **44**, 1177–82.

https://doi.org/10.1017/S0967199401001204 Published online by Cambridge University Press