AN IMPROVED METHOD FOR CHROMOSOME PREPARATIONS FROM BOVINE OOCYTES AND ZYGOTES OBTAINED BY *IN VITRO* MATURATION AND FERTILIZATION TECHNIQUES

UN MÉTODO OPTIMIZADO PARA EL ANÁLISIS CROMOSÓMICO DE OVOCITOS Y CIGOTOS OBTENIDOS MEDIANTE TÉCNICAS DE MADURACIÓN Y FECUNDACIÓN *IN VITRO*

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PALABRAS CLAVE ADICIONALES

Citogenética. Vacuno. Ovocito. Cigoto. Fecundación *in vitro.*

SUMMARY

Matured bovine oocytes and zygotes obtained by *in vitro* maturation and fertilization techniques (IVM and IVF) were cytogenetically prepared by using an improved method for chromosome preparations.

The method, which involves use of trypsinized-hypotonic solution plus a vortex-agitated system and very cold two-step fixation process, contributes to weaken the zona pellucida and allows the swelling of oocytes and zygotes which helps the subsequent spreading of the chromosomes. This method permits to obtain preparations of good quality for examining the number and morphology of the chromosomes of oocytes and zygotes for any meiotic and mitotic stage.

RESUMEN

Se logró optimizar un método rápido para analizar citogenéticamente ovocitos y cigotos de bovino obtenidos mediante técnicas de maduración y fecundación *in vitro* (MIV y FIV).

El método, el cual utiliza una solución hipotónica con tripsina, un sistema de agitación mediante vortex y un sistema doble de fijación en frío, contribuye a eliminar fácilmente la zona pelúcida y a hinchar a los ovocitos y cigotos favoreciendo posteriormente la extensión de los

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cromosomas. Este método permite obtener preparaciones de muy buena calidad para el análisis tanto del número como de la morfología de los cromosomas de los ovocitos y cigotos en cualquier estadío meiótico y mitótico.

INTRODUCTION

The increasing interest in reproductive cytogenetics that used various embryo manipulation procedures calls for a reliable, simple and constant method for chromosomal analysis of early embryos of domestic mammals.

The first effective air drying method for making chromosome preparations from pre-implantation mouse embryos was devised by Tarkowski (1966). Although widely used, not only with mouse but also with other mammalian species, it requires considerable skill to obtain high quality preparations and is particularly difficult to use on zona-free embryos or isolated blastomeres, which are difficult to handle by this method. Several investigators have modified the method of Tarkowski for chromosome preparations of mouse embryos (Garside and Hillman, 1985) and other mammalian species such as pig (McFeely, 1966), and bovine (King *et al.*, 1979). Earlier modifications, which made possible a higher rate of analyzable metaphases, concerned primary the improvement of the fixation process (Kamiguchi *et al.*, 1976). However, some considerable technical difficulties, especially in cases of pre-implantation embryos, have been encountered with all these methods.

In this study, we would like to provide a simplified, reliable method for the demonstration of chromosomes of bovine oocytes and zygotes obtained by *in vitro* maturation and fertilization techniques, which may be called a trypsinized-hypotonic and double fixation method, and is believed to have advantages over the previous one in that it constantly prepares clear and well-spread metaphase plates.

MATERIALS AND METHODS

PRODUCTION OF *IN VITRO*-MATURED BOVINE OOCYTES AND ZYGOTES

Bovine matured oocytes and zygotes were produced by following an IVM-IVF technique. Briefly, cumulus-oocyte complexes (COC) were obtained by aspirating immature follicles (<7 mm) from ovaries collected at slaughter. Selected COC were cultured for maturation in 1 ml droplets of TCM-199 medium supplemented with 1 IU/ml PMSG and 0.5 IU/ml HCG according to the procedure described by Ocaña *et al.* (1994). After 24 h of culture, the expanded COC were inseminated with spermatozoa selected by a modified swim-up through Percoll gradient system (Utsumi *et al.*, 1991). Briefly, 2 ml of 45 p.100 Percoll solution was placed on 2 ml of 90 p.100 Percoll in a 10-ml test tube. Four 0.5 ml-straw of frozen semen was thawed in 37ºC water, and 2 ml of semen were deposited on the upper layer of the percoll gradient solution. The semen was centrifuged for 15 min at 700 g and the sedimented spermatozoa displaying good motility in the bottom of tube were resuspended in 1 ml of H-TALP medium containing 0.6 p.100 BSA (Sigma), and 100 µg/

ml heparin (Sigma) and were incubated for 15 min in a CO_2 incubator for capacitation. 300 µl of the capacitated sperm suspension were introduced into 1 ml of freshly prepared fertilization medium (TCM-199 supplemented with 10 p.100 FCS), containing 20-40 matured oocytes at a concentration of 1-2x106 total spermatozoa/ml and cultured for 24 h at 39ºC under 5 p.100 CO_2 in air.

Technique for chromosomes preparation of oocytes and zygotes

Trypsin and hypotonic treatment. At the end of the culture period for maturation and/or fertilization, matured oocytes or zygotes (a total of 480 oocytes and 210 embryos were processed using the control method, while 500 oocytes and 200 embryos were processed using the two-step method) were transferred to 3-ml conical tubes and vortex-agitated for 2-5 min in trisodium citrate (0.88 p.100) and trypsin (0.02 p.100) hypotonic solution which was previously prepared and stored at 39ºC until required for hypotonic treatment of the oocytes/zygotes. After a slight agitation to remove the *cumulus oophorus* cells and weak the zona pellucida, denuded oocytes and zygotes were placed into culture plate containing 2 ml of the same hypotonic solution without trypsin for 30 min at 39ºC.

Fixation. The oocytes were placed into culture plate containing 1 ml of a very cold-initial fixing solution of 1:1 methanol:acetic acid for 5 min. This fixing solution was prepared on the day of use and maintained in the freezing compartment of a refrigerator at -20ºC until required for fixing of oocytes/zygotes. Then, oocytes and zygotes were fixed in a second fixing solution of 3:1 methanol:acetic acid for 24 h. The second fixation time can be variable between 2 to 24 h without affecting to fixation of the cells.

Spreading. Finally, the oocytes were mounted on slides, stained with 5 p.100 Giemsa onto Soremsen buffer solution (Ph=6,8) and examined with the light microscope at 1500 x magnification for evaluation of meiotic stage.

Criteria for maturation and fertilization

Oocytes were morphologically evaluated for stage of maturation after culture. The meiotic progress of oocytes was classified as follows: (1) Germinal vesicle stage: an intact nuclear membrane with the chromatin meiotically inactive in which the chromatin is lightly condense; (2) Metaphase I: the nuclear membrane broken and a chromatin pattern characteristic of an oocyte resuming meiosis; (3) Metaphase II: a polar body present within the perivitelline space with maternal chromatin complement identified in the oocyte and (4) Degeneration: oocyte showing obvious degenerative changes such as vacuolated or fragmented cytoplasm or scattered chromatin complement. For fertilization stage, oocytes were classified as follows: (1) oocytes without both male and female pronuclei were judged as unfertilized; (2) oocytes with both male and female pronuclei and with residual sperm-tail were defined as normally fertilized; (3) oocytes with more than two

Table I. *Percentages of oocytes and embryos karyotyped using different methods.* (Porcentajes de ovocitos y embriones cariotipados usando diferentes métodos).

Method	No of trials	No of processed cells		No of karyotyped cells (p.100)	
		Oocytes	Embryos	Oocytes	Embryos
Control	5	480	210	300 (62.5)[a]	125 (59.5)[a]
Two-step	5	500	200	434 (86.8)[b]	156 (78)[b]

[a,b]Values with different superscripts within each column are statistically different ($p< 0.05$). Chi-square test.

pronuclei and decondensed sperm heads were considered to be polyspermic; (4) oocytes with a cytoplasm vacuolated or fragmented were considered to be degenerated; (5) fertilized oocytes with a diploid chromosomes group were considered to be zygotes.

RESULTS AND DISCUSSION

The percentajes of oocytes and zygotes karyoryped (86.8 and 78 p.100 respectively, **table I**) using the two-step method were significantly higher ($p<0.05$) than those observed in the

Table II. *Method for karyotyping bovine oocytes and zygotes developing from* in vitro *maturation and fertilization techniques.*(Método para cariotipar ovocitos y cigotos de bovino obtenidos mediante técnicas de maduración y fecundación *in vitro*).

Time	Procedure	Purpose
2-5 min	Trypsin (0.02 p.100) plus hypotonic treatment (0.88 p.100 Na-citrate) plus vortex-agitated system	Disggregate zona pellucida swell cells disperse chromosomes
30 min	Hypotonic treatment (0.88 p.100 Na-citrate)	Swell cells and disperse chromosomes
5 min	Add fixative (methanol:acetic acid 1:1) dissolve zona pellucida	Fix and disperse cells;
24 h	Add Carnoys fixative (methanol:acetic acid 3:1)	Fixation of cells and attachment to slide.
	Drop cell suspension onto cold, wet slides	Attach cells to slide
13-30 min	Stain (Giemsa 5 p.100 onto Sorensen buffer solution,Ph=6,8)	
20 min	Microscopic examination (1500 x)	

Total time: 26 h (including an approximation of time required for microscopic examination).

control method (62.5 and 59.5 p.100 respectively).

When the oocytes and zygotes were placed into a trypsinized-hypotonic solution of sodium citrate plus trypsin, the cell body was considerably swollen, to fill up the periviteline space, and the swollen cell body began to escape from the zona pellucida. This escape was achieved through a slit made in the zona pellucida which did not rupture the cytoplasmic membrane, but did usually cause loss of nuclear material. In **table II** is shown the development of the method and the employed total time for displaying chromosomes, of bovine oocytes and zygotes which were obtained by *in vitro* maturation and fertilization techniques.

The development of a trypsinized-hypotonic treatment for chromosome analysis of oocytes and zygotes obtained by *in vitro* maturation and fertilization techniques was the most important outcome of this study. Hsu (1952) and Hsu and Pomerat (1953) reported the importance of the hypotonic treatment as a means of producing a good spreading of chromosomes from vertebrate cells. Holmquist and Motara (1987) reported that in a hypotonic environment, cell actively absorbs water to equilibrate its salt balance. The mechanism by which hypotonic salt solution exerts its influence on the dividing cells to spread their chromosomes has been explained by its effectiveness in desintegrating the mitotic spindles (Hsu and Pomerat, 1953), or loosening the interchromosomal connections (Klasterska and Ramel, 1978). The composition of the hypotonic solution for chromosome preparations of embryos, isolated blastomeres and zygotes differed among authors. Kamiguchi *et al*. (1976) used 30 p.100 calf serum in their procedure, Tarkowski (1966) used 1 p.100 sodium citrate, Popescu *et al*. (1982) and Long (1977) used KCl. However we used a combination of trypsin plus sodium citrate, accompained with vortex-agitated system during short-term treatment. In addition, several authors have employed some enzyme type, such as actinase (Iwasaki *et al*., 1988), trypsin (Hare *et al*., 1976; Yoshizawa *et al*., 1990), pronase (Angell *et al*., 1983) to weaken the zona pellucida of the zygotes and embryos. However, they used the enzyme treatment prior to hypotonic treatment. Our method has the advantage, with regard to other methods, that enzyme and hypotonic treatment are only realized at one-step treatment. The hypotonic solution action is very intensive due to the fact that trypsin quickly weakens the zona pellucida,

Figure 1. *Metaphase plate from an* in vitro *matured oocyte showing a haploid chromosome complement (n=30). The arrow indicates the X chromosome.* (Metafase de un ovocito madurado *in vitro* mostrando una dotación cromosómica haploide. La flecha indica el cromosoma X).

Figure 2. *Metaphase plate from a zygote showing a diploid chromosome complement (2n=60). The arrows indicate the X chromosomes.* (Metafase de un cigoto mostrando una dotación cromosómica diploide. Las flechas indican los cromosomas X).

Figure 3. *Metaphase plate from a zygote showing a hypodiploid chromosome complement (2n=53). The arrows indicate the X chromosomes.* (Metafase de un cigoto mostrando una dotación cromosómica hipodiploide. Las flechas indican los cromosomas X).

thus permiting the penetration of the hypotonic solution into the cell, and the subsequent breakage of the interchromosomal connections and dispers of the chromosomes **(Figures 1, 2, 3)**. All these mechanisms were favoured by the vortex-agitated system which facilitated the trypsin-digestive action. In our technique, the trypsin concentration has to be exactly controlled at 0.02 p.100 and the exposure time at 5 min as well. Trypsin concentrations or exposure time higher than 0.02 p.100 or 5 min respectively produced an intensive digestion of the zona pellucida determining the loss of both cytoplasmic and nuclear material.

The two-step fixing process used in our study is similar to the double fixation method devised by Fujimoto *et al.* (1975) and Yoshizawa *et al.* (1990). Our two-step process has the advantage over the one-step method of fixing slides in that the prefixed oocytes or zygotes were not washed away, and so not lost, during the second fixing.

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