mM) compared with that in blood stream (3-6 mM). In this study we examined effects of various potassium concentrations in fertilization medium on acrosome reaction, polyspermy, and pronuclear formation. Porcine oocyte-cumulus cell complexes from ovaries were cultured in NCSU23 medium supplemented with 0.6 mM cysteine, 2 μg/mℓ FSH, and 10% porcine follicular fluid for 42 h. After culturing for maturation, oocytes were co-cultured with spermatozoa in Tris-buffered medium containing 0, 3, 6 and 12 mM potassium supplemented with 5 mM caffeine and 0.4% BSA for 6 h. Potassium concentrations were varied by adjusting KCl. Osmolarity and Cl^- concentrations were maintained by adjusting NaCl concentration. Subsequent to IVF oocytes were cultured in NCSU23 supplemented with 0.4% BSA for 6 h. At 12 h after insemination, oocytes were fixed in ethanol and acetic acid (3:1, v/v) for 48 h, stained with 1% aceto-orcein, and examined sperm penetration and male pronuclear formation under phase contrast microscope. In absence of potassium in the fertilization medium, sperm penetration was not observed. Supplement with 3, 6, and 12 mM potassium in the fertilization medium resulted in high incidence of polyspermy. The incidence of polyspermy was significantly ($p<0.05$) higher in the fertilization medium contained 6 or 12 mM K^+ than in that contained 3 mM K^+. The mean number of sperm penetrated in oocytes in medium with 6 and 12 mM potassium was higher ($p<0.05$) than that in medium with 3 mM potassium. There was no difference in proportions (45~57%) of pronuclear formation among different potassium concentrations. Chlorotetracycline (CTC) analysis was used to determine the capacitation and acrosome reaction of spermatozoa incubated for 1.5 and 3 h after preincubation in the various concentrations of potassium. CTC analysis revealed three main fluorescence patterns, uncapacitated F, capacitated B and acrosome reacted AR. For each potassium concentration, there was a significant shift from F to B and B to AR pattern with increasing potassium concentration. These results suggest that extracellular potassium is required for the sperm penetration and addition of extracellular potassium in the fertilization medium affects sperm capacitation and acrosome reaction which result in the high incidence of polyspermy.

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Sexing of Bovine Preimplantation Embryos derived from IVF using Fluorescence *in situ* hybridization(FISH)

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Ⅰ. Construction of Bovine Y chromosome-specific Probe

Sex identification of preimplantation embryos is important to livestock for the control sex ratios. Several methods have been reported for bovine sex determination. Previous work in this area has utilized the polymerase chain reaction(PCR) to amplify Y-chromosome-specific sequences from a single cell in order to determine embryonic sex.

The purpose of the current studies was to develope an rapid, sensitive method for sex determination which use bovine Y chromosome-specific probe for FISH in order to detect this marker in bovine chromosome, sperm and embryos.

Primers were chosen on the genomic DNA nucleotide sequences as published in GenBank. PCR was carried out on 200ng of genomic DNAs in 50㎕ of reaction volume. The gel region containing bovine DNA fragments ranging from 1.4~1.5kb was excised along with the corresponding region and DNA was collected by electroelution onto dialysis membrane. DNA was electroelution and ethanol precipitation. PCR products were reamplified by seeding 50㎕ PCR reaction containing 1㎛M primer and 0.4㎕ of the initial PCR reaction. Reamplified alleles were purified by agarose gel electrophoresis as described above. Next, reamplified DNA were prepared for ligation in vector pBluescripts(2.7kb) by using a subclone ligation kit(Pharmacia). Following established procedures, ligated vectors were cultured overnight in competent E. coli cells. Clones were screened for correct insert site by PCR, utilizing the designed bovine Y-chromosome probe.

Since the genomic nucleotide sequences of bovine or human genes were published in GenBank, it was simple to produce specific fragment by means of PCR and/ or these as probes for FISH. It is concluded that Bovine Y chromosome-specific probe(1.4kb) can be amplified, isolated, and cloned by means of PCR and used as probes for FISH.

II. FISH to place Y chromosome-specific Probe on Bovine chromosome, Sperm and Embryos

The sucess of fluorescence in situ hybridization(FISH) on bovine chromosome, sperm and embryos relies on partial experimental method. Several procedures have been described. A sensitive sex determination using FISH is proposed here. Its advantages and efficiency are evaluated and discussed.

Probe

The bovine Y-specific probe, 1.4kb pairs in pBluescripts, was made by our laboratory. DNA probe was labeled by nick-translation with biotin-dUTP(BM) according to the instructions of the supplier. Labelled probe was separated from the selection by using spin columns filled with Sephadex G-50. PCR products or plasmid DNA was used for the hybridizations.

Sample preparation

1. Chromosome: bovine metaphase or interphase chromosomes were prepared by standard procedures

2. Sperm decondensation: the bovine spermatozoa were resuspended in a small volume(50-200㎕) of freshly diluted 10mM dithiothritol in 0.05M Tris and incubated at room temperature for 10-50min. The sperm suspension was immediately dropped on lean slides, spread on the surface of the slides with the edge of another slide and air-dried. The preparation were then fixed with ethanol: acetate(3:1) for 20min twice at room temperature, and overnight at -20℃.

3. Embryo spreading: bovine embryos derived from IVM/IVF were washed in PBS for 2min and transferred to a small drop of spreading solution(0.01N HCl 0.1% Tween 20) on a poly-L-lysine-coated glass slide. The slides were left to dry onto the slide. After spreading and dehydration, slides were treated with pepsin(sigma, 100㎕/㎎ in 0.01N HCl) for 20min, 37℃ to remove any remnants of cytoplasm. Slides were rinsed in PBS and fixed for 10min with 1% paraformaldehyde at 4℃. The slide were again rinsed in PBS and dehydrated through an ascending ethanol series.

FISH protocol

1. The slides of chromosome, sperm and embryos were again rinsed in PBS and dehydrated through an ethanol series. An

aliquots of 10㎕ probe solution was added to the slide under a coverslip, and nuclear and probe DNA were denatured simultaneously for 3min, at 75℃. Slides were incubated ar 37℃ for overnight hybridization.

2. The biotin-labelled probes were detected by incubation with avidin-FITC and the signal was amplified by subsequent incubation with biotinylated goat-anti-avidin and a second layer of avidin-FITC. All incubations for the detection of the biotin-labelled probes were followed by washes with 4×SSC/0.05% Tween 20 at room temperature.

3. Finally, slides were mounted in antifade solution containing 0.5㎍ DAPI or 1㎍/㎖ propidium iodide. Nuclei were examined with a Olympus microscope equipped for fluorescence with UV.

These results demonstrate that our bovine Y specific probe comprise a family clustered exclusively on the Y to metaphase, interphase nuclei, sperm and blastomere. Therefore, our 1.4 kilobase DNA probe specific for the Y chromosome can be used as a sensitive sexing method for bovine preimplantation embryos derived from IVF using FISH.

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Cryopreservation of Mouse IVF/IVC Blastocysts by Vitrification

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Vitrification, involves the addition of high concentrations of cryoprotectant and a very rapid freezing rate, circumvents intracellular ice formation, which is a major cause of cell death (Fahy et al., 1984). The first successful vitrification of mannalian embryos was achieved by Rall and Fahy (1985) with eight-cell mouse embryos.

This experimant was designed to fine optimal conditions for obtaining high survival of mouse IVF/IVC blastocysts after vitrification. To produce blastocysts, B6CBA F1(C57BL/6, ♀ × CBA/N, ♂) mouse eggs were inseminated with 1 × 10^6 spermatozoa/ml and cultured at 37℃ in 5% CO_2 in air for 96 h. The rates of fertilization and development to blastocyst stage at day 4 of the eggs were 89.5% and 86.1%, respectively. The IVF/IVC blastocysts were divided into three stages of early, expanded and hatching at day, 4, individually. The vitrification solution used was EFS 40, contained 40% ethylene glycol diluted in DPBS medium containing 30% Ficoll plus 0.5 mol sucrose. The embryos were exposed to 20% ethylene glycol and EFS 40 in two steps at 25℃, vitrified in liquid nitrogen and warmed rapidly. To investigate the toxicity of vitrification solution, when embryos were exposed to the solution (EFS 40) at 25℃, 82.9~88.4~ of blastocysts were re-expanded after 24 h of culture. And there were no significant differences between all three stages group and control group. When the expanded and hatching blastocysts were vitrified in EFS 40 and assessed by the re-expansion of the blastocoel after 24 h of culture, the postwarming survival rates of them (71.9% and 89.5%) were significantly higher than that of the early blastocysts (54.2 %). The resent result shows that vitrification can be routinely used to cryopreserve mouse embryos without loss of viability and it is expected that this simple and efficient