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Application of a microfluidic sperm sorter to *in vitro* production of dairy cattle sex-sorted embryos

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ABSTRACT

Viable sperm from sex-sorted semen without centrifugal treatment was separated by a microfluidic sperm sorter (MFSS) for IVF to improve *in vitro* embryo production of dairy cattle. The MFSS was originally developed to isolate motile human sperm by two laminar flows in the micro-channel (there are four chambers in an MFSS. Chamber A is the inlet for semen, chamber B is the inlet for the medium, chamber C is the exit chamber for motile sperm, and chamber D is the outlet for nonmotile sperm). Sex-sorted sperm were adjusted to 1×10^7 spermatozoa/mL (2 million cells/dose, sperm motility was 30% above after thawing). In a first experiment, diluted sex-sorted semen was mixed with modified Medium199(mM199) containing 5-mM caffeine for 5 minutes, resulting in variations in sperm concentration and quality parameters at chambers A, C, and D. In a second experiment, medium containing sperm from three MFSS chambers was collected and mitochondrial activity of the sperm was determined by flow cytometry, the relative activity of sperm mitochondria in chamber C (1.56 ± 0.03) was the highest in three observation areas ($P < 0.05$). Thus, sperm motility and mitochondrial activity of sperm was high in chamber C. In a third experiment, different concentrations of sperm were added to chamber A and dairy cattle IVM oocytes were placed in chamber C, where motile spermatozoa will accumulate, with mM199 containing 5-mM caffeine for 5 minutes, and then cultured in caffeine-free mM199 for 8 hours. The results showed that sperm penetration rate, the monospermic penetration rate, and blastocyst rate of the 10×10^6 group (10×10^6 sperm/mL) were higher than in the 1×10^6 and 5×10^6 groups ($P < 0.05$). In the last experiment, we compared sperm penetration in the MFSS-IVF system with a modified standard IVF method (cocultured in droplets for 8 hours). The normal fertilization index (the ratio of monospermic oocytes to the number of oocytes examined) 8 hours after insemination was higher in the MFSS-IVF system than the modified standard IVF system ($P < 0.05$). Developmental competence of fertilized oocytes to the blastocyst stage was also higher in the MFSS-IVF system (40.12% ± 2.61%) than the modified standard IVF technique (24.55% ± 4.54%). These results demonstrate that a short coculture of dairy cattle oocytes with isolated motile sex-sorted spermatozoa gradually accumulated in the MFSS device improves the efficiencies of normally produced fertilized embryos and blastocyst formation.

1. Introduction

Sperm sex-sorting is an effective way to control the sex of offspring in dairy cattle by *in vivo* fertilization or IVF [1,2].

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An important reason for the use of sexed semen technology in *in vitro* embryo production in cattle is the need for using a small number of sperm during IVF. However, the efficiency of *in vitro* production of sex-sorted bovine embryos using sex-sorted semen is affected by many factors, resulting in lower fertilization, cleavage, and blastocyst rates (20%–40%) [3–7].

Sperm motility is affected by bisbenzimide staining, the separation process, and long residence time *in vitro*, when semen is separated by flow cytometry [3,8,9]. Furthermore, centrifugation appears to diminish the quality of spermatozoa before IVF [10,11]. This leads to lower fertilization, cleavage, and blastocyst rates [9,12]. In addition, a high incidence of polyspermic penetration is an obstacle to obtain normal zygotes after IVF of mammalian oocytes after IVM [13–15]. Simultaneous sperm penetration and delay of a zona reaction at first sperm penetration may be an important reason for polyspermic penetration *in vitro* [13,16]. In standard IVF of cattle, 30 to 50 denuded or cumulus-enclosed oocytes are cocultured with 1 to 10×10^4 spermatozoa (final concentration of sperm is 2.5–5.0×10^5 cells/mL) in a droplet of IVF medium containing caffeine for several hours [17]. As caffeine, a cyclic-nucleotide phosphodiesterase inhibitor, increases intracellular cyclic adenosine monophosphate concentration [18] and induces sperm capacitation and a spontaneous acrosome reaction [19], a number of spermatozoa with induced capacitation simultaneously try to penetrate the oocytes. Furthermore, heparin, a glycosaminoglycan commonly used as a blood anticoagulant, stimulates sperm capacitation in bulls. Consequently, the incidence of polyspermic penetration increases significantly if caffeine is added to IVF media [20,21]. However, in cow oviducts because spermatozoa at the utero-tubal junction undergo induced capacitation and start to enter the site of fertilization, oocytes should gradually meet capacitated spermatozoa.

Because of the above idea, unique IVF systems, such as the climbing-over-the wall method [22] and straw-IVF method [23], have been developed. Owing to limited improvements in these methods, however, development of a new simple IVF method is required for a regular supply of normal embryos *in vitro*. Recently, a micro-channel has been used as a new tool for biological research and applied to artificial reproductive technologies during IVM of pig oocytes [24], human IVF [25], and early development of mammalian embryos [26]. In pigs, the normal fertilization index and developmental competence of fertilized oocytes to the blastocyst stage was higher in the MFSS (microfluidic sperm sorter)-IVF system than the standard IVF system [27]. In the MFSS, two parallel laminar flows stream in a microchannel and then separate in different directions. Only motile spermatozoa swim across the contacting surface area of the laminar flows, deviate from the initial stream line into the media stream for collection, and gradually accumulate in the collection chamber. If dairy cow oocytes can be cocultured with motile spermatozoa in the MFSS chamber, the gradual accumulation of motile and uninjured spermatozoa could be induced at the meeting place with oocytes without centrifugation. Consequently, this may improve the efficiency of obtaining monospermic embryos.

The aim of this study was to systematically improve the efficiency of bovine IVF production using sex-sorted sperm by an MFSS. We examined if the application of the MFSS to coculture dairy cattle oocytes with isolated highly motile spermatozoa improved the efficiency of production of normal fertilized sex-sorted embryos.

2. Materials and methods

2.1. Chemicals and culture media

All chemicals were purchased from Sigma–Aldrich (China [Mainland]) unless otherwise noted.

The medium used for collecting and washing cumulus-oocyte complexes (COCs) was modified M199 medium, which contained M199 (Earl's salts; Gibco, Grand Island, USA, lot No. 11146397) supplemented with 2-mM $NaHCO_3$ (Sigma, USA, lot No. 011M01472V), 10-mM HEPES (Sigma, USA, lot No. 81M5435V), 0.1% (wt:vol) polyvinyl alcohol (Sigma, USA, lot No. BCBG1529 V), 25-μg/mL gentamicin, and 65-μg/mL potassium penicillin G.

The IVM medium contained M199 supplemented with 2-mM $NaHCO_3$, 0.01-IU/mL FSH (Ningbo Sansheng Pharmaceutical Co., Ltd, lot No. 120216), 0.01-IU/mL LH (Ningbo Sansheng Pharmaceutical Co., Ltd, lot No. 120510), 1-μg/mL 17β-Estradiol (Sigma, Lot No. 021M8707V), 10% FBS (Zhejiang Tianhang Biological Technology Co., Ltd. lot No. 120104), 25-μg/mL gentamicin, and 65-μg/mL potassium penicillin G. Embryo culture medium contained M199 supplemented with 2-mM $NaHCO_3$, 5-mg/mL BSA, 25-μg/mL gentamicin, and 65 μg/mL potassium penicillin G.

The medium for IVF was essentially the same as that used by Brackett and Oliphant (1982) for the fertilization of cow oocytes *in vitro* [28], i.e., BO medium.

All media were equilibrated at 39 °C in an atmosphere of 5% CO_2 in air overnight before use.

2.2. Preparation of sex-sorted sperm and sperm quality analysis

Frozen-thawed sex-sorted sperm (X-chromosome bearing) was used from Holstein bulls (Shanghai Dairy Cattle Breeding Center, LTD. China.), frozen at 2×10^6 sperm per 0.25-mL straw. The semen contained at least 30% progressively motile sperm after thawing. Straws of semen were thawed in water at 38 °C to 39 °C for 30 seconds. Frozen-thawed sex-sorted sperm was used in each experiment. The concentration of the sperm was adjusted to 10×10^6 cells/mL with Bracket and Oliphant (BO) medium and used for experiments.

Five minutes after the start of flow, 5 μL of media from chambers A, C, and D in the MFSS were collected, and then, quality of the parameters (motility; straight line velocity, VSL; curvilinear velocity, VCL; average path velocity, VAP; amplitude of lateral head displacement, ALH; beat/cross frequency, BCF; linearity, LIN; mobile of average degree, MAD; and straightness, STR) of the sperm were determined using a computer assisted sperm analysis system (CASA, Nanning Song Jing Tianlun Bio-technology Co., Ltd.)