Reproductive Biology 17 (2017) 180–184

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Original article

Live, full-term mouse pups from oocytes grown and matured *in vitro* with serum substitutes

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ARTICLE INFO

Article history:
Received 11 January 2017
Received in revised form 27 April 2017
Accepted 6 May 2017
Available online 13 May 2017

Keywords:
Serum substitute
Oocyte growth
Oocyte maturation
Zona hardening
Full-term development

ABSTRACT

For *in vitro* growth and maturation of mouse oocytes (IVG-IVM), serum is added to media up to and including the stage of oocyte maturation; this subsequently supports oocytes through fertilization and early embryo development. However, problems may occur with sera, such as batch differences and issues of biosafety. The purpose of the present study was to determine the capacity for fertilization and pre- and post-implantation development of oocytes that underwent IVG-IVM with a serum substitute. Oocyte-granulosa cell complexes from preantral follicles were cultured in medium with either fetal bovine serum (FBS), Serum Substitute Supplement™ (SSS), or Knockout™ Serum Replacement (KSR) for 10 days, and were then allowed to mature for 17 h. Subsequently, more than 90% of oocytes underwent germinal vesicle breakdown (GVBD) and more than 70% reached metaphase II, with no significant difference between the groups. A lower fertilization rate, presumably due to zona hardening, was found in the serum substitute groups. Nevertheless, more than 50% of the inseminated oocytes were fertilized and 35%–45% of them underwent first cleavage and developed to the blastocyst stage. Following embryo transfer, one and four live offspring were produced from the SSS and KSR groups, respectively. The present study demonstrated that murine IVG-IVM oocytes cultured in media with a serum substitute, achieved fertilization *in vitro*, pre- and post-implantation development, and the delivery of live pups, although the efficiency of the process is reduced compared to FBS supplementation.

1. Introduction

The production of mouse offspring through *in vitro* fertilization, preimplantation development, and embryo transfer from oocytes grown and matured *in vitro* has become possible [1–3]. These results have been achieved with the addition of fetal bovine serum (FBS), or fetuin derived from FBS, to culture media for *in vitro* growth and maturation of oocytes (IVG-IVM). However, sera derived from animals have greater batch variation than a single purified product such as serum albumin. It is well known that serum quality influences experimental results, gives questionable reproducibility, and introduces the risk of pathogenic contamination [4,5]. Although the replacement of FBS with bovine serum albumin (BSA) can support oocyte growth and maturation, "zona hardening" may take place and this prevents fertilization [6,7].

Today, serum substitutes that contain at least some serum component are used in the *in vitro* culture of various cells [5]. The use of these substitutes in culture provides no or minimal batch variation, and adequate safety. In the IVG-IVM of oocytes, however, the effect of serum substitution has not been examined. Therefore, its effect on development after oocyte maturation is unclear.

The present study set out to clarify two questions. First, whether oocytes that underwent IVG-IVM with a serum substitute showed similar developmental competence as when FBS was used. Second, whether the oocytes acquired the potential for full-term development. To clarify the above questions, two serum substitutes were adopted in the present study. One was Serum Substitute Supplement™ (SSS), which is commonly used in human assisted reproductive technology (ART) [8–11] and the second was Knockout™ Serum Replacement (KSR), which may be used as a serum substitute for the culture of pluripotent stem cells [12–15].

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http://dx.doi.org/10.1016/j.repbio.2017.05.004
1642-431X/

The efficacy of the serum substitutes for IVG-IVM of mouse oocytes was compared with that of FBS. Furthermore, competence of the oocytes for fertilization after maturation, preimplantation, and post-transplantation embryo development were examined.

2. Materials and methods

2.1. Animals

Mice used for the current study were housed and bred under controlled lighting (14 h light:10 h darkness), temperature, and humidity, with food and water available *ad libitum*. Ovaries were collected from 12-day-old female mice (C57BL/6J × CBA/JN; Clea Japan, Inc., Tokyo, Japan; Charles River Laboratories Japan, Inc., Kanagawa, Japan). Eight to 12-week-old male mice (C57BL/6N × C3H/HeN; Clea Japan, Inc.,) were humanely killed for the collection of sperm from the cauda epididymidis for IVF. Eight to 16-week-old CD-1 (ICR) females (Charles River Laboratories Japan, Inc., Kanagawa, Japan) were used as recipients for embryo transfer.

2.2. Isolation of oocyte-granulosa cell complexes (OGCs)

The OGCs were isolated from 12-day-old mouse ovaries using a basic enzymatic digestion method with slight modification [16]. Briefly, the ovaries were digested in Leibovitz's L-15 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 5% FBS (Japan·Bio serum, Hiroshima, Japan), 1–1.3 mg/ml collagenase type I (Worthington Biochemical Corp., Freehold, NJ, USA) and 0.02% DNase I (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min at 37 °C; OGCs were then collected.

2.3. Culture of OGCs

OGC culture was performed using methods described previously [16]. Briefly, the OGCs were cultured on collagen-coated membrane inserts (Corning Inc., Corning, NY, USA) in 4 ml of MEM alpha GlutaMAX (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 5% FBS, 100 mIU/ml recombinant human FSH (Merck & Co. Inc., Kenilworth, NJ, USA), 10 mIU/ml human LH (AbD Serotec, MorphoSys UK Ltd., Oxford, UK), 50 ng/ml recombinant human insulin-like growth factor I (Thermo Fisher Scientific), and 50 ng/ml recombinant murine stem cell factor (PeproTech Inc., Rocky Hill, NJ, USA) in 6-well dishes under 5% CO_2, in air, at 37 °C for 10 days. For trial cultures, SSS (Irvine Scientific, Santa Ana, CA) or KSR (Thermo Fisher Scientific) were used in place of FBS. Half of the medium was changed every other day until day 8 and daily thereafter.

2.4. Hormone measurements

Used culture media were collected at the time of medium exchange and stored at −30 °C until needed for estradiol-17β and progesterone assays. The samples were measured by liquid chromatography coupled with tandem mass spectrometry (LC−MS/MS) using outsourcing services of ASKA Pharmaceutical Medical Co., Ltd.

2.5. Electron microscopy

Three samples per experimental specimen were fixed with 2% paraformaldehyde or 2% glutaraldehyde in 0.1 M phosphate buffer (PB) at 4 °C overnight, and then postfixed with 2% osmium tetroxide in 0.1 M PB at 4 °C for 2 h. The samples were dehydrated and embedded in epoxy resin. Sliver sections (70 nm) were stained with 2% uranyl acetate at room temperature for 15 min followed by lead citrate for 3 min. The grids were observed under a transmission electron microscope (JEM-1400Plus; JEOL Ltd.) at an acceleration voltage of 80 kV. Digital images (2048 × 2048 pixels) were taken with a CCD camera (Olympus Soft Imaging Solutions GmbH).

2.6. IVM of oocytes

IVM of oocytes was performed on day 10 of OGC culture. The OGCs were stimulated to mature for 17 h by the addition of 1.5 IU/ml hCG (ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) and 5 ng/ml recombinant human epidermal growth factor (Thermo Fisher Scientific). The nuclear maturity of oocytes was evaluated as GV when the germinal vesicle was recognizable, as GVBD (germinal vesicle breakdown) when the GV was absent, or as metaphase II (MII) when the first polar body was extruded.

2.7. IVF of oocytes

For capacitation, spermatozoa collected from the cauda epididymides of male mice were incubated for 1.5 h in 200 μl droplets of TYH medium under paraffin oil. Insemination was conducted in 100 μl droplets of TYH medium for 4 h. Sperm concentration was adjusted to 2–3 × 10^7/ml.

2.8. Culture of embryos

After IVF, zygotes were cultured in 10 μl droplets (5 embryos/droplet) of KSOM/AA medium [17] for further embryo

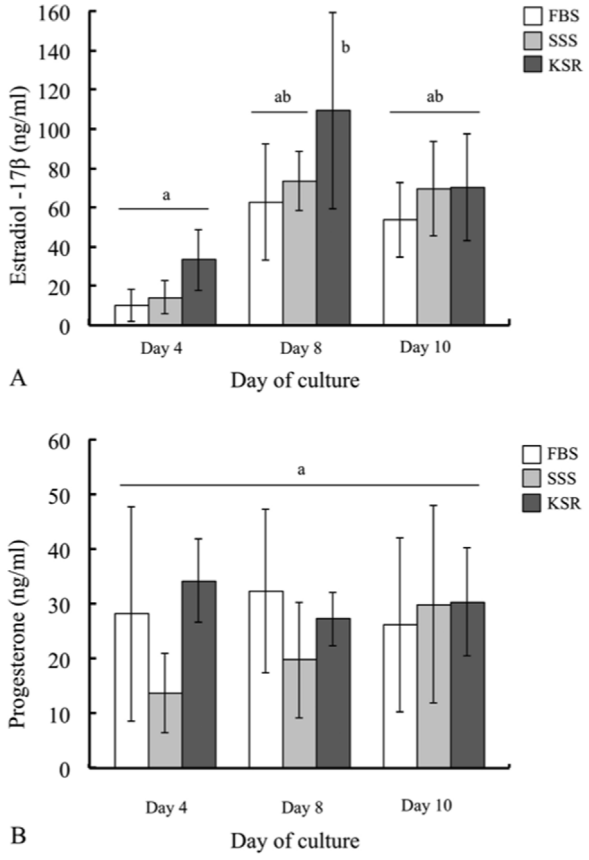

Fig. 1. Concentration of steroid hormones in used culture media during culture. Half of the fresh medium was changed every other day until day 8, and daily thereafter. (A) Estradiol-17β concentrations. (B) Progesterone concentrations. Values are mean ± S.D. (n = 5).