Available online at www.sciencedirect.com
ScienceDirect
Biomaterials 28 (2007) 3198–3207
Biomaterials
www.elsevier.com/locate/biomaterials

The effect of three-dimensional demineralized bone matrix on *in vitro* cumulus-free oocyte maturation

Suofeng Ma[a], Hang Lin[a,b], Yiliang Miao[b,c], Xinyong Liu[d], Bin Wang[a,b], Jianwu Dai[a,*]

[a]*Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, No. 3 Nanyitiao, Zhongguancun, Beijing 100080, China*
[b]*Graduate School, Chinese Academy of Sciences, Beijing, China*
[c]*State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China*
[d]*Medical Center for Reproduction, The Central Hospital of Tai'an, Tai'an City, Shandong Province, China*

Received 2 December 2006; accepted 8 March 2007
Available online 19 March 2007

Abstract

The physiological role of cumulus cell surrounding oocytes is particularly important for normal cytoplasmic maturation of oocytes. Collagen-based demineralized bone matrix (DBM) is a valuable biomaterial for the three-dimensional (3-D) cell culture. The present study was designed to determine whether *in vitro* maturation (IVM) of cumulus-free oocytes in mice could be improved by using the 3-D DBM co-culture system. The results indicated that the denuded oocytes cultured in 3-D DBM co-culture system with cumulus cells showed close similarity of cortical granules (CGs) distribution pattern, had more normal maturation-promoting factor (MPF) level and zona pellucida (ZP) hardening level to the *in vivo* matured oocytes, and the best preimplantation development after being activated by *in vitro* fertilization (IVF) or parthenogenetic activation. Thus, 3-D DBM collagen scaffold could serve as a tool for fundamental *in vitro* studies of cells or tissues under the environment that closely assembles the *in vivo* conditions.

Keywords: Co-culture; Demineralized bone matrix; *In vitro* maturation; Oocyte

1. Introduction

In vitro maturation (IVM) techniques have been used to investigate cytochemical and cytogenetic events occurring in maturing oocytes of mammals. Studies have demonstrated that the developmental capacity of *in vivo* matured oocytes is superior to that of the *in vitro* matured ones [1–3]. It is known that oocytes need to undergo cytoplasmic maturation as well as nuclear maturation to become able to support successful fertilization and embryo development [1,4]. Oocytes acquire cytoplasmic maturity after a long series of preparatory processes involving transcription and subsequent translation of transcripts during the meiotic prophase *in vivo* [5]. *In vitro*, however, a premature meiotic resumption without adequate cytoplasmic maturation is induced by transfer of oocytes from follicles into an unsuitable culture system. It is well established that culture conditions, including the medium, supplementations, and the *in vitro* physical environment (such as the oxygen tension, presence of cumulus cells, and the extracellular matrix (ECM) surrounding cumulus cells) all influence oocyte maturation and subsequent embryonic development [6–8].

Oocytes that mature *in vivo* are enclosed with cumulus cells. The physiological role of cumulus cells surrounding oocytes is particularly important for normal cytoplasmic maturation of oocytes because they play essential nurturing roles in the regulation of oocyte metabolism, such as in the use of energy sources [9] and the metabolism of maturation-promoting factor (MPF) [10]. The role of the surrounding cumulus cells in maturation, ovulation, and fertilization of oocytes has been extensively studied [11–14]. However, in some experiments involving micromanipulation of immature oocytes (e.g., germinal vesicle exchange and spermatocyte injection), removal of the surrounding cumulus cells is unavoidable, possibly resulting in compromised ooplasmic maturation and subsequent embryo

*Corresponding author. Tel./fax: +86 010 82614426.
E-mail address: jwdai@genetics.ac.cn (J. Dai).

0142-9612/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2007.03.007

development [15]. The majority of human IVM studies have utilized immature oocytes denuded of surrounding cumulus cells that were obtained after ovarian stimulation with gonadotrophins [2,4]. Denuded oocytes (DOs) exhibit accelerated meiotic resumption *in vitro*, a deficiency in the ability of the cytoplasm to maintain M-phase characteristics while meiosis is progressing, which results in a propensity to spontaneously activate after M-phase arrest, and a lack of coordination between nuclear and cytoplasmic maturation [16]. What contributes to these deficiencies remains to be determined, but the interdependence of oocytes and cumulus cells for their normal development and function is critical [12]. Co-cultures of oocytes and cumulus cells have been employed *in vitro* to restore the support from the surrounding cumulus cells to the oocyte and to probe interactions between the two cell compartments. In those experiments, oocytes were cultured either over monolayers of cumulus or granulosa cells, or along with pieces of mural granulosa cells or follicular shells in several species, including the mouse [17], cow [18], pig [19], and human [8]. However, all those experiments were performed in the two-dimensional (2-D) culture environment, which might be deficient for cumulus cells for performing their physiological roles.

The ECM provides a three-dimensional (3-D) support to organize cellular architecture and to regulate tissue development. The importance of the ECM and a 3-D environment when studying cell behavior and function *in vitro* was well-established [20]. The ECM influences a multitude of cell functions, including morphogenesis, survival, migration, proliferation, communication, metabolism, and response to external stimuli [21,22]. Therefore, it is equally important to culture cells in an *in vitro* environment that assembles the 3-D environment *in vivo* [8]. In fact, in comparison with the 2-D environment, the 3-D environment results show more similar cell behavior, signaling, and gene expression profiles than those observed in living tissues [23,24]. ECM composition is known to affect granulosa cells with behavior and function *in vitro* [25–27]. In addition, follicles and intact cumulus–oocyte complexes (COCs) from the mouse [28], pig [29], cow [30], and human [31] were embedded in a 3-D collagen gel. Another biomaterial, alginate-ECM gel, was also used to culture mouse follicles [32]. Tissue engineering matrices may be useful for the development of systems for the IVM of oocytes, which are needed to preserve reproductive potential for women facing infertility resulting from chemotherapy or other ovarian disorders (e.g., premature ovarian failure) [32].

However, a 3-D system making use of an ECM has not been applied to the co-culture of denuded mouse oocytes and cumulus cells. The present study was designed to determine whether IVM of cumulus-free oocytes in mice could be improved by using the 3-D demineralized bone matrix (DBM) co-culture system. Using different IVM systems, we assessed the quality of oocytes by several cytochemical parameters and the subsequent development of embryos constructed by *in vitro* fertilization (IVF) or parthenogenetic activation.

2. Materials and methods

2.1. Processing of DBM and cell compatibility test

DBM was provided by Zhenghai Biotechnology Inc (Shandong, China). DBM collagen scaffold was made from the spongy bone of bovine. Briefly, the spongy bones were separated and cut into 1 cm × 0.5 cm × 0.5 cm sizes. Then the samples were soaked in acetone for 48 h to remove the fatty composition. Subsequently, the 0.6 M HCl was utilized to demineralize the spongy bones followed by ddH_2O being washed completely, and then being freeze-dried [33]. Prior to use, all the samples were cut into suitable sizes and sterilized by Co60 radiation.

In vitro cell compatibility was tested by direct co-incubation of DBM and fibroblasts. Cells were seeded at a concentration of 1×10^6 cells/ml onto a 24-well culture plate (Costar No. 3524, Corning-Costar Corporation, Cambridge, MA, USA) in which DBM had been placed. After 24 h of incubation at 37 °C in 5% CO_2, the cells were visualized by FDA dyeing through a Zeiss Axiovert 200 inverted microscope (equipped with optical and fluorescence detection light).

2.2. Collection of oocytes and cumulus cells, IVM of immature oocytes

Immature GV oocytes were collected from the ovaries of B6D2F1 female mice (6–8 weeks after birth) injected with 7.5 IU pregnant mare's serum gonadotrophin (PMSG) 46–48 h previously. The large Antral follicles on the ovary were ruptured in M2 medium for COCs. Only oocytes >70 μm in diameter with a homogenous cytoplasm and >3 layers of cumulus cells were selected for experiments. After being freed from cumulus cells by repeated pipetting in M2 containing 0.1% hyaluronidase (Sigma), the DOs were subjected to IVM. All cumulus cells were collected for use in co-cultures after their removal from COCs in preparation for DOs. The medium containing the removed cumulus cells was collected into a conical tube with an equal volume of warm (37 °C) M2. Following centrifugation at 150*g* for 5 min, the cell pellet was resuspended in 1.5 ml of warm maturation media described below. Cells were counted using a hemacytometer, and only cell preparations with a viability ⩾90% as assessed by Trypan Blue exclusion were used.

In vivo matured MII oocytes were retrieved from the oviducts of superovulated females following injection with 7.5 IU PMSG and 7.5 IU human chorionic gonadotrophin (HCG; Tianjin Laboratory Animal Centre, Tianjin, China), 48 h apart. Approximately 14 h after the HCG injection, cumulus-enclosed oocytes were collected from the oviductal ampullae and released from the cumulus cells by treatment with 0.1% hyaluronidase in M2 medium.

Immature oocytes were matured *in vitro* in a microdrop culture, 2-D co-culture, or 3-D co-culture system. The maturation media were TCM-199 (Gibco, Grand Island, NY, USA) containing 10 IU/ml PMSG (Tianjin Laboratory Animal Centre, Tianjin, China) supplemented with 10% fetal calf serum (FCS, Gibco), 100 IU/ml penicillin, and 0.05 mg/ml streptomycin. All cultures were processed at 37 °C in a humidified atmosphere of 5% CO_2 in air.

For the microdrop group, DOs were cultured for 14 h in groups of 20–30 in 100 μl microdrops of maturation media, covered with mineral oil. For the cumulus cells 2-D co-culture group, the final cumulus cells suspension (2×10^6 cells/ml) was added to wells of 96-well culture plates (100 μl suspension per well) about 24 h after seeding; the unattached and dead cells were removed by gently rinsing the wells with fresh culture medium; then 20–30 DOs were added after the medium was renewed and cultured for a further 14 h. For the cumulus cells 3-D co-culture group, one piece sterilized DBM (0.4 cm × 0.4 cm × 0.25 cm sizes) was immersed into a well of the 96-well culture plates, and the cumulus cells suspension (1×10^6 cells/ml) was slowly dropped to DBM (200 μl suspension per well)