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Effect of extracellular matrix on bovine spermatogonial stem cells and gene expression of niche factors regulating their development in vitro

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ABSTRACT

Extracellular matrix (ECM) could influence cells function through providing structural and functional networks facilitating the cellular interactions. The present study was conducted to evaluate the effect of culture on ECM versus plastic on bovine spermatogonial stem cells (SSCs) and growth factors regulating their development. Following isolation, bovine testicular cells were cultured on ECM-coated or uncoated (control) plates for 12 days. The colonization of SSCs was assessed by inverted microscope and the gene expression was evaluated using quantitative real-time PCR. The colonization rate was greater in ECM than the control group ($P < 0.05$). The expression of markers of undifferentiated spermatogonia increased in response to conventional culture ($P < 0.05$). Conversely, the expression of *ckit* as a marker for differentiated spermatogonia was reduced following culture in the control and ECM groups ($P < 0.05$), but this decrease was less in ECM group ($P < 0.05$). Accordingly, while cells cultured on uncoated plates had greater expression of markers of undifferentiated spermatogonia ($P < 0.05$), cells cultured on ECM-coated plates showed higher expression of *ckit* ($P < 0.05$). Moreover, culture on ECM resulted in higher expression of kit ligand (*Kitlg*; $P < 0.05$), whereas culture on plastic led to greater expression of glial cell line-derived neurotrophic factor (*Gdnf*; $P < 0.05$). In conclusion, the present study revealed that the permissive effect of ECM on bovine SSCs differentiation in vitro, which was probably mediated through upregulation of KITLG expression. Moreover, the results imply that GDNF might contribute to germ cells self-renewal during conventional culture.

1. Introduction

Spermatogenesis is an intricate and tightly-regulated process of cell proliferation and differentiation leading to production of mature spermatozoa from spermatogonial stem cells (SSCs) (Oatley and Brinster, 2008; Russell et al., 1990). Maintenance of spermatogenesis depends on capability of SSCs to both self-renew and differentiate (Oatley and Brinster, 2008). Self-renewal of SSCs helps the male maintain the reservoir of germ cells providing the foundation for continual production of spermatozoa (Oatley and Brinster, 2008). On the other hand, over the course of differentiation, SSCs, which are referred to A_{single} (A_s)

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spermatogonia, develop into other generations of spermatogonia including A_{paired} (A_{pr}), $A_{aligned}$ (A_{al}), A_1, A_2, A_3, A_4, intermediate and type B spermatogonia, which would further undergo meiosis to differentiate into primary and secondary spermatocytes, leading to production of spermatids and subsequently spermatozoa (Oatley and Brinster, 2008; Russell et al., 1990). In this context, THY1 (Kubota et al., 2003; Oatley and Brinster, 2008; Reding et al., 2010), BCL6B (Oatley et al., 2006, 2007; Reding et al., 2010) and GFRA1 (He et al., 2007) are known as markers of undifferentiated spermatogonia, whereas cKIT is considered as a marker for differentiated spermatogonia (Izadyar et al., 2003; Schrans-Stassen et al., 1999).

Regulation of spermatogenesis, and the balance between SSCs self-renewal and differentiation are under the influence of a specialized microenvironment, called niche, encompassing the SSCs and other generations of cells involved in the process of spermatogenesis (Oatley and Brinster, 2008). The niche consists of different somatic cells including Sertoli cells, peritubular myoid cells and Leydig cells, governing the spermatogenesis by producing various growth factors (Oatley and Brinster, 2008). Additionally, it is comprised of extracellular matrix, which is made up of a complex network of macromolecules with different structural and functional properties modulating the function and development of cells (Hynes, 2009; Watt and Huck, 2013).

The culture of cells on ECM provides an environment partly resembling the normal tissue (Abbott, 2003; Rosso et al., 2004; Zhang, 2004), which allows the initiation of SSCs differentiation in vitro (Lee et al., 2006, 2007). This phenomenon has been attributed to the impact of ECM on the interaction between somatic cells, particularly Sertoli cells, and germ cells as well as to the effect of ECM on the function of Sertoli cells (Lee et al., 2006). In this context, Hadley et al. (1985) reported higher secretion of total protein, androgen binding protein, transferrin, and type I collagen by Sertoli cells cultured on ECM as compared with those cultured on plastic.

Glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2) and kit ligand (KITLG) are of the most important growth factors which have been indicated to contribute to regulation of SSCs development (Ishii et al., 2012; Meng et al., 2000; Sato et al., 2012). GDNF, a member of transforming growth factor β family, has been shown to play a pivotal role in spermatogenesis and to be responsible for SSC self-renewal in various mammalian species (Hofmann et al., 2005; Johnston et al., 2011; Kubota et al., 2004; Meng et al., 2000) including bovine (Aponte et al., 2006; Oatley et al., 2004). FGF2 has also been indicated to stimulate the proliferation and self-renewal of both somatic and germ cells (Ishii et al., 2012; Kubota et al., 2004). By contrast, KITLG has been found to be essential for SSCs differentiation (Sato et al., 2012; Zhang et al., 2011).

The present study was conducted to evaluate the effect of culture on ECM on SSCs and the gene expression of growth factors involved in their development during in vitro culture.

2. Materials and methods

2.1. Animals and testicular biopsy

Animal Ethics Committee at University of Tehran approved the present study in terms of animal welfare and ethics. To obtain testicular tissue, Holstein calves ($n = 8$; 5 calves were used for evaluation of colonization and 3 calves were used for gene expression analysis), aged 3–5 months, were subjected to testicular biopsy as previously described (Shafiei et al., 2013). In brief, testicular biopsy was performed under sedation with xylazine (0.2 mg/kg; Alfasan, Holland) and local anesthesia with lidocaine (Aburaihan Pharma Co., Iran). Following incision, the testicular tissue was obtained and placed into a 15 ml tube containing Dulbecco' Minimal Essential Medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Sigma–Aldrich) and antibiotics (100 IU/ml Penicillin and 100 μg/ml streptomycin; Gibco). The specimen was subsequently transferred on ice to the laboratory within 2 h.

2.2. Cell isolation

Cell isolation was implemented using a two-step enzymatic isolation procedure, as previously described by Izadyar et al. (2002) with minor modification. In brief, the testicular tissue was washed three times in DMEM containing antibiotics and was minced into small pieces by a sterile scissor. The minced testicular tissue was incubated in DMEM containing 1 mg/ml collagenase (Sigma–Aldrich), 1 mg/ml hyaluronidase (Sigma–Aldrich), 1 mg/ml trypsin (Sigma–Aldrich) and 5 μg/ml DNase (Fermentas, Germany) at 37 °C in a shaker incubator with 80 cycles per minute for approximately 60 min. The digested testicular tissue was washed three times with DMEM and the supernatant was disposed after each washing, leading to isolation of seminiferous tubules. During the second step of enzymatic digestion, the seminiferous tubules were incubated at 37 °C in DMEM containing 1 mg/ml collagenase, 1 mg/ml hyaluronidase and 5 μg/ml DNase until disintegration of the seminiferous tubules and separation of the constituent cells. Individual cells were isolated from the remaining tubule fragments by centrifugation at $30 \times g$ for 2 min. Following filtration through 77 and 55 mm nylon filters, the cells were pelleted. The pellet was re-suspended in the DMEM containing antibiotics and 10% knock-out serum replacement (KSR, Gibco).

2.3. Cell culture

Wells used for the control group were uncoated. Wells used for the extracellular matrix (ECM) group were coated with ECM gel (Sigma–Aldrich) as the manufacturer indicated. The ECM gel was prepared from Engelbreth–Holm–Swarm (EHS) mouse sarcoma and was composed of laminin as the major component, collagen type IV, heparan sulfate proteoglycan, entactin and other minor components. In the experiments related to assessment of gene expression and colonization, 6 and 24-well plates were used, respectively. In the experiments associated with evaluation of gene expression and colonization,