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Effect of KnockOut serum replacement on germ cell development of immature testis tissue culture

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ABSTRACT

To compare KnockOut serum replacement (KSR) and fetal bovine serum (FBS) for the development of germ cells. Testicular tissues from Sprague–Dawley rats were cultured for 4 weeks in culture media supplemented with FBS or KSR. Tissue area was measured at the beginning and end of the culturing period. Testicular histology, development of the germ cells, and the diameter of seminiferous tubules were analyzed by hematoxylin and eosin staining. After 4 weeks in culture, apoptosis and expression of the stage-specific spermatogenesis marker genes Kit, Sycp3, and Crisp1 were assayed. Tissues cultured in KSR-supplemented media were able to sustain growth and gradually increase seminiferous tubule diameter during the culture period. In addition, spermatogonia, primary spermatocytes, secondary spermatocytes, and round spermatids were observed after 4 weeks in culture, and reverse transcription-PCR confirmed expression of the marker genes. In comparison, tissues cultured in FBS-supplemented media showed dwindling testicular organization, necrotic seminiferous tubules, and expression of Kit, but inconsistent expression of Sycp3 and Crisp1 KnockOut serum replacement outperforms FBS as a growth media supplements for culturing immature spermatogonial tissue culture.

1. Introduction

There is a long history of studies aimed at understanding the process of mammalian spermatogenesis. In 1915, Goldschmidt made the first report from an *in vitro* experimental investigation of spermatogenesis, which initiated study of the functional roles of tissue and cells for promoting and maintaining sperm development. Methods for testicular organ culture were developed in the 1960s and 1970s. However, difficulties inducing complete meiosis were encountered, resulting in differentiation arrest at the pachytene stage [1–3]. These obstacles led researchers to prefer cell culture over organ culture, but the former still proved challenging for promoting the progression of spermatogonial cells to fertile sperm [4,5]. In comparison, methods for culturing immature testicular tissue *in vitro* have been successful and represent an important method to obtain mature sperm [6].

The advantages of *in vitro* tissue culture arise from the ability to maintain the interactions between cells and the cellular microenvironment that are needed for development *in situ*. Inhibin B secreted by Sertoli cells and testosterone secreted by Leydig cells are important hormones that regulate spermatogenesis by conditioning the cellular microenvironment for germ cell development and differentiation [7–10]. However, it is difficult to consistently obtain mature spermatozoa or sperm cells, particularly from immature testicular tissue, which is likely a result of varying *in vitro* tissue culture conditions [6].

In the present study, we set out to determine whether KnockOut serum replacement (KSR; Gibco of Thermo Fisher Scientific Inc., Waltham, MA, USA) growth media

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supplement is more suitable for immature testicular culture compared with fetal bovine serum (FBS) and to establish a more consistent method for culturing immature testicular tissue and spermatogenesis *in vitro*. Moreover, testicular tissue obtained from rats was used, representing a histologic and pathophysiological model that is more comparable to human testicular tissue. Experimental outcomes presented here represent an improved culture method suitable for studies aimed at testing infertility drugs and the optimal timing of clinical treatment.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were obtained from the Experimental Animal Center, Chongqing Medical University, Chongqing, China. The Animal Ethics Committee of the Institute of Zoology and Chongqing Medical University approved all animal experiments.

2.2. Tissue culture

Sterile 1.5% low-melting point agarose gel was made with distilled water, passed through a 0.22-μm filter, and poured into a six-well plate. Once solidified, the gel was cut into 10 × 10 × 5-mm blocks that were soaked overnight in Dulbecco's modified Eagle's medium F12 (12400016; Gibco) containing KSR (10828028; Gibco) or FBS (SV30087.01, Hyclone; GE HealthCare, Little Chalfont, Buckinghamshire, UK). Sprague–Dawley rats (n = 3) aged 7 days old were euthanized, and the tunica albuginea testes were isolated under sterile conditions. The testes were cut into 1-mm pieces and individually placed on five presoaked gel blocks in six-well tissue culture plates. To determine the optimal amount of KSR, concentrations of 5%, 10%, and 15% KSR-supplemented media were added to the well until flush with the agarose gel blocks such that the testicular tissue was in the liquid–vapor phase. Cultures were maintained in a humidified growth chamber at 34 °C with 5% CO_2. Media were replaced every 4 days, and cultures were maintained for 4 weeks, at which time the areas of cultured tissues were measured. Cultures with an equivalent amount of FBS-supplemented media served as the control group. The experiment was repeated three times with five agarose gel blocks containing testicular tissue explants per condition.

2.3. Tissue area measurement

Cultured tissues were observed under an inverted microscope (CKX41; Olympus, Shinjuku, Japan), and areas were measured from captured images (NIS-Elements DR software; Nikon Corp., Tokyo, Japan). The area was calculated as the average of the five specimens from three rats at each time point.

2.4. Histology and apoptosis

Three testicular tissue cultures were collected weekly from each treatment and fixed with modified Davidson's fluid for 24 hours, followed by conventional dehydration, paraffin embedding, and sectioning to 4-μm thickness. Sections were stained with hematoxylin and eosin, and the germ cell development and seminiferous tubule diameter were evaluated. The cross-sectional diameter of 50 randomly selected seminiferous tubules was measured at 400 × magnification. The data are presented as an average of three samples (one from each rat).

Samples taken at 4 weeks were probed for apoptosis using the *In situ* Cell Death Detection Kit, POD (11684817910; Hoffmann–La Roche, Basel, Switzerland). Sections were examined under an inverted microscope (CKX41; Olympus), and five images of each sample (at 400 × magnification) were captured and analyzed using NIS-Elements DR software (Nikon).

Table 1
PCR primer sequences.

Gene	Primer sequence, 5′→3′	Melting temp. (°C)	Length (bp)
β-actin	Forward: GAGATTACTGCCCTGGCTCCTA Reverse: GACTCATCGTACTCCTGCTTGCTTG	56	150
Kit	Forward: ACAGGACGCCTACTAACAGA Reverse: ATCAAATGTCACGGAAGCAC	57	164
Sycp3	Forward: TTTCAAAGCCAGTAACCA Reverse: CTTTCATTCTCTGGCTCT	52	224
Crisp1	Forward: AGGTGCATTTACAATCACA Reverse: GATTACAGAAGACCACGA	54	105

2.5. RT-PCR and quantitative (q)RT-PCR

Total RNA from testicular tissue was isolated from five explants from three rats at each time point using an RNA Extraction Kit (RP-1202; Beijing BioTeke Corporation, Beijing, China) at the time of collection and weekly thereafter for four consecutive weeks. RNA was reverse

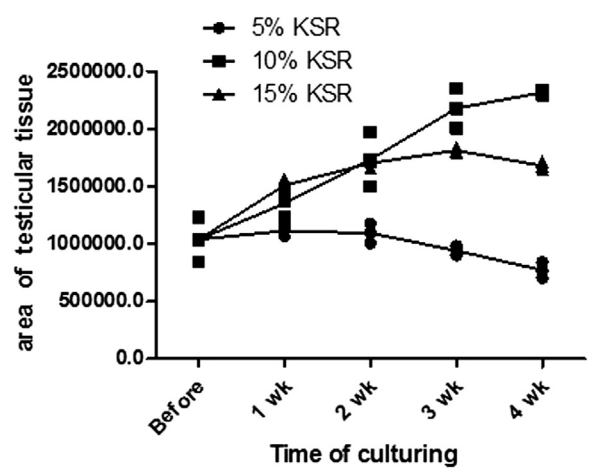

Fig. 1. Area of testicular tissue in different concentrations of fetal bovine serum. In 5% KnockOut serum replacement (KSR) group, the gross testicular area did not significantly increase in the first 2 weeks and appeared atrophy from the beginning of the second week. In 15% KSR group, the growth is rapid in the first week, but the growth rate became slow from second week, and testicular tissue showed gradual shrinking of the gross testicular area in the fourth week. In 10% KSR group, a sustained growth state was seen.