Optimal Milieu for Culturing Porcine Sertoli Cell

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ABSTRACT

The purpose of the present study was to establish culture conditions for the *in vitro* study of the neonatal piglet Sertoli cell. Isolation for the culture of Sertoli cell was established using collagenase and pancreatin digestion of testicular tissues. The effects of various culture media, fetal bovine serum (FBS), follicular stimulating hormone (FSH), epidermal growth factor (EGF) and insulin-transferrin-sodium selenite (ITS) on growth of neonatal piglet Sertoli cells were investigated. The mitogenic effects of Dulbecco's modified Eagle's medium + Ham's F-12 medium was higher than other media used in this experiment. The addition of 1% FBS in cultures was necessary for attachment of Sertoli cell clusters. However, except FBS and EGF, FSH and ITS did not stimulate Sertoli cell proliferation. When Sertoli cells isolated from neonatal piglets were cultured in Dulbecco's modified Eagle's medium + Ham's F-12 medium supplemented with 1% FBS, FSH, EGF and ITS, the yield and plating efficiency of Sertoli cells were largely increased. Confluency of Sertoli cells was reached as early as 4 days of culture. The method described here reduces or eliminates many of the drawbacks of the conventional procedures used to isolate and culture of Sertoli cells, thus providing a useful tool in studies of growth kinetics and regulation of cell proliferation *in vitro*.

(Key words : Sertoli cell, Fetal bovine serum, Follicular stimulating hormone, Insulin-transferrin-sodium selenite, Epidermal growth factor (EGF), Mitogenic effects)

INTRODUCTION

Sertoli and peritubular cells contribute to the formation of a selective permeability barrier responsible for the maintenance of a unique environment within the seminiferous tubule. The present knowledge regarding Sertoli cell function and its hormonal regulation is derived primarily from *in vitro* studies of cultured Sertoli cell isolated from testes of immature animals. Prepubertal testes contain fewer germ cells and less-differentiated Sertoli cells. This reduces the difficulties in isolation because of the dilution of Sertoli cell by germ cells and favors the attachment of Sertoli cell to a substratum and their adaptation to *in vitro* conditions. Sertoli cells undergo structural and functional maturation during pubertal development (Heckert and Griswold, 1992; Gondos and Berndtson, 1993). During establishment of complete spermatogenesis, functional interaction develops between germ cells and Sertoli cell that is essential for normal spermatogenesis in adult animals (Sharpe, 1993). In the last few years, pig as well as rat and hamster Sertoli cells have been extensively used to investigate their secretory products and their role in the regulation of the function of other testicular cells (Parvinen, 1982; Saez *et al.*, 1985). Secretory products of Sertoli cell serve as a nutritional source for the growth and development of germ cells (Mita and Hall, 1982; Jutte *et al.*, 1983). Protein secretion by the Sertoli cell has been shown to vary with testicular maturation and the presence of germ cells in the vicinity of Sertoli cell (Jegou *et al.*, 1988).

A number of methods for isolation of Sertoli cell have been devised and several studies have shown that under these experimental conditions Sertoli cell maintain *in vitro* many structural and biochemical characteristics, as well as functional activities observed *in vivo* (Steinberger and Jakubowiak, 1993). The most commonly used procedures for the isolation of Sertoli cell include a sequential enzymatic digestion of the testicular tissue, ending up with tissue fragments (Dorrington *et al.*, 1975; Steinberger *et al.*, 1975; Welsh and Wiebe, 1975; Rich *et al.*, 1983). There are some reports in the literature of the successful isolation of relatively pure Sertoli cell from mature rat testes. A potential problem with culturing the cells is that isolated cells may lose differentiated function during the culture period, thus making it difficult to interpret results. In the case of Sertoli cell, differentiated function is not easy to define, and thus loss of differentiated function is not easy to assess. Immature testes have been used as the source of Sertoli cell primarily because of the high purity that can be obtained. Concerning Sertoli cell primary cultures, most studies have been oriented to investigate

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the effects of hormone-supplemented serum-free media on Sertoli cell functions (Saez and Jaillard, 1986), but the mitogenic effect of such media has been investigated in only a few cases (Rich *et al.*, 1983; Borland *et al.*, 1984). Moreover, all of the above studies were limited to Sertoli cells from rats, hamsters and monkeys. So, it therefore seemed of interest to determine the optimal culture conditions for porcine Sertoli cell. In our present work, we have investigated the effect of several factors on pig Sertoli cell growth and function and showed that FBS and EGF are the most active factors studied.

MATERIALS AND METHODS

Reagents

Unless otherwise specified, chemicals used for isolation and culture of Sertoli cell were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of Sertoli Cell

Mechanical Dispersion

Testes castrated from 3 days old regardless strain of piglet were immediately washed and placed in cold (4℃) calcium and magnesium-free PBS (containing 100 U/ml of penicillin-G and 100 μg/ml of streptomycin sulphate, pH 7.4). Within 2 hr of castration, testes were decapsulated in a sterile dish (60×15 mm). Decapsulated tissue was washed twice and cut into pieces (3~5 mm). Minced tissue was suspended in PBS (2 g tissue/50 ml) in a glass-stoppered 100 ml bottle and shaken vigorously for 1 min to disperse the tissue. The tissue was settled on ice for 5 min, and the supernatant was removed. This procedure was repeated four times to mechanically remove red blood cells and free Leydig cells.

Enzymatic Digestion

Sertoli cells were isolated by enzymatic digestion of the seminiferous tubules using modifications of the procedure described by Welsh and Wiebe (1975) and Majumdar *et al.*, (1995). Enzymatic digestion for each developmental stage was customized in order to obtain optimum yields of Sertoli cell. The pellet obtained from the final wash was resuspended in 50 ml of PBS containing 5 mg/ml of collagenase (type I, C-0130, Sigma). Digestion was then carried out at 32℃ in a shaking water bath (160 oscillations/min) for 30 min until an aggregate of tissue appeared. After the cluster of tissue resulting from collagenase digestion was discarded, the remaining suspension containing free tubules, Sertoli cell, germ cells, and dispersed Leydig cells was washed twice at unit gravity. Gentle agitation between washes was provided with use of a wide mouth Pasteur pipette. The supernatant was discarded after each wash. After the third wash, the pellet was suspended in 50 ml of PBS for the second enzymatic digestion, which was performed with pancreatin (5 mg/ml; P-3292, Sigma) and digested for 15~20 min in a shaking (160 oscillations/min) water bath at 32℃ until another single aggregate of tissue was formed. This aggregate composed of peritubular cells was discarded immediately after completion of the enzymatic digestion. The suspension of Sertoli cell-germ cell clusters left in the bottle was transferred to a 15-ml sterile centrifuge tube and pelleted by centrifugation at 150~200 ×g for 3~4 min (depending on density of suspension). This procedure was repeated at least eight times to remove the majority of the germ cells.

The final pellet was resuspended in 20 ml PBS, gently layered on 250 ml of cold PBS in a 500-ml sterile glass beaker, and allowed to stand for 25~30 min on a motionless surface. This procedure produced sedimentation of the major portion of Sertoli cell clusters at the bottom of the beaker. Subsequently, 100 ml of supernatant was gently aspirated from the beaker and checked under a microscope for the presence of small clusters of Sertoli cell or of Sertoli cell plus germ cells. Another 100 ml of PBS was screened identically. Any fraction of the suspension occupying more than 10% of the microscopic field (at 100× magnification) with clusters was distributed in aliquots of 30~35 ml in centrifuge tubes (50-ml capacity) and centrifuged at 150 ×g for 2 min. If a pellet was formed, it was added to the Sertoli cell fraction remaining at the bottom of the beaker. At this point, the suspension in the beaker was shaken gently and distributed equally in two 50-ml centrifuge tubes and centrifuged at 200 ×g for 4 min.

Plating

For culturing of Sertoli cells, the final pellet obtained from the procedure described above was re-suspended in 5 ml of culture medium, and the concentration of clusters was adjusted to 2000 clusters/2 ml by means of a hematocytometer. Cell suspension was distributed 1 ml/test tube and centrifuged for 5 min. the final pellet obtained was diluted with five different culture media. The day of plating was considered Day 0 of culture, and the entire culture was conducted at 37℃ in a CO_2 incubator (5% CO_2, 95% air).

Removal of Contaminated Cells

To increase the purity of the Sertoli cell, cultured cells were subjected to hypotonic shock in each trail at 37℃ with 20 mM Tris-HCl (pH 7.2) for 5 min on day 3 of culture to remove germ cells (Galdieri *et al.*, 1981). Germ cells damaged by this procedure were removed by washing three times with 1 ml culture medium per

well.

RESULTS

Experiment 1. Effect of Various Basic Media on Sertoli Cell Culture

Experiments were designed to develop suitable culture condition for Sertoli cell cultures from neonatal piglet testes. Firstly, isolated Sertoli cells were cultured in fresh culture medium (TCM199, DMEM, EMEM, Ham's F-12 and DMEM-Ham's F-12) without any supplementation. Even though growth of Sertoli cell was started very slowly from day 1 in TCM199, DMEM, and EMEM, but they remained constant in number and morphology till day 7 as shown in Fig. 1. However, in Ham's F-12 medium alone and DMEM-Ham's F-12 medium (1:1), the cellular growth was faster than other media and it is also to be noted that Sertoli cell cultures showed significant growth and well-defined morphological structure compared to other culture media.

Experiment 2. Effect of FBS on Sertoli Cell Culture

In order to find out the effect of FBS on Sertoli cell growth, 1 % FBS was added in the fresh culture medium, which remarkably enhanced the attachment and growth of Sertoli cell (2-3-fold). The mitogenic activity of 1% FBS was not potent for Sertoli cell growth in TCM199, DMEM and EMEM, whereas, it was more potent in Ham's F-12 medium and DMEM-Ham's F-12 medium (1:1) and supported to reach semi-confluent monolayer on day 7(Fig. 2).

Experiment 3. Effect of FSH on Sertoli Cell Culture

Evaluation of cultures containing fresh medium supplemented with FSH indicated no remarkable role for growth of Sertoli cell than fresh medium containing FBS only (Fig. 3).

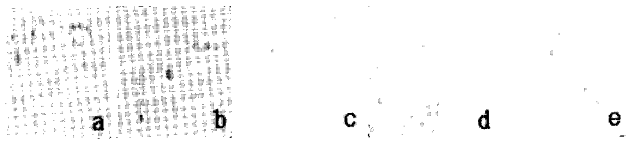

Fig. 1. Effect of various culture media on Sertoli cell culture from piglet testes. Sertoli cells were cultured in a. TCM199, b. DMEM, c. EMEM d. Ham's F-12 medium and e. DMEM-Ham's F-12 medium (1:1) for 7 days (a, ×600; b, c, d and e, ×375).

Fig. 2. Effect of FBS on Sertoli cell culture from piglet testes. Sertoli cells were cultured in a. TCM199, b. DMEM, c. EMEM d. Ham's F-12 medium and e. DMEM-Ham's F-12 medium (1:1) containing 1% FBS for 7 days lacking monolayer. (a, c, d and e, × 375; b, × 600).

Fig. 3. Effect of FBS and FSH on Sertoli cell culture from piglet testes. Sertoli cells were cultured in a. TCM199, b. DMEM, c. EMEM, d. Ham's F-12 medium and e. DMEM -Ham's F-12 medium (1:1) containing 1% FBS and FSH for 7 days. (a, b and c, × 400; d and e, × 375).

Any medium and supplementation of FBS and FSH could not support to form Sertoli cell monolayer. Because the number of cell started growth were too low, it was impossible to represent their morphology under low magnification. So, high magnification had chosen to display the morphological structure of Sertoli cell.

Experiment 4. Effect of Growth Factor on Sertoli Cell Culture

Finally, isolated Sertoli cells were cultured in DMEM -Ham's F-12 (1:1) supplemented with EGF and ITS. Culture characteristics of isolated Sertoli cell were determined on Day 7. Before hypotonic shock Sertoli cell cultures were contaminated with Leydig cells, germ cells and peritubular cells (Fig. 4a). Most of the conta-

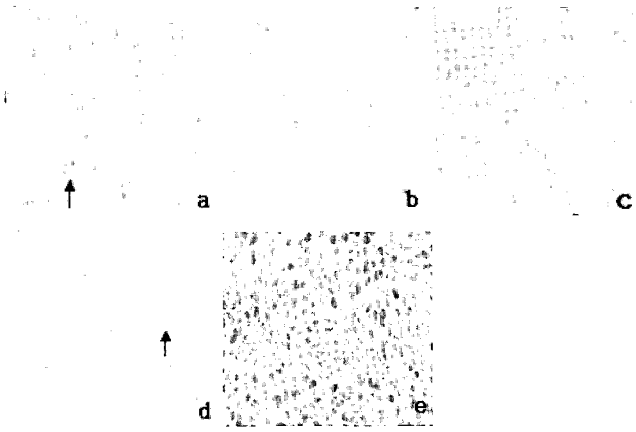

Fig 4. Morphological characteristics of enzymatically isolated neonatal piglet Sertoli cell confluent monolayer cultured in DMEM-Ham's F-12 medium (1:1) containing FBS, EGF, ITS, FSH, Penicillin-G and Streptomycin Sulfate on different culture days. a) Sertoli cells displayed wide clear spread-out cytoplasm (arrow head) along with other contaminant cells in the culture before hypotonic shock on day 3, b) Sertoli cells only culture was obtained after hypotonic treatment on day 4, c) Confluent monolayer of Sertoli cells from neonatal piglets on day 7, d) Methylene blue staining of a monolayer of Sertoli cells after day 7. Arrow showing the dead cell stained with trypan blue. e) Confluent monolayer of Sertoli cells stained with aceto-orcien stain on 8 days. (a, b and c, × 375; d, × 600; e, × 150).

minated cells were removed from Sertoli cell cultures after hypotonic shock (Fig. 4b). Examination of the cultures under a phase-contrast microscope revealed that Sertoli cell from immature piglets formed a confluent monolayer (Fig. 4c) by day 7 of culture. Cells from at least 3 wells per plate were stained with trypan blue to determine viability (Fig. 4d). Cells were stained with aceto-orcien for 1 hr at room temperature and washed with distilled water. In order to enhance the visualization of nuclei and plasma membranes, cells were kept overnight in water at room temperature before microscopic examination (Fig. 4e).

DISCUSSION

Although procedures for culture of Sertoli cell from different animals have been reported earlier, the present study describes in detail for the first time the establishment of a set of customized conditions for the culture of Sertoli cell harvested from neonatal piglets. The present study showed that the most optimal culture medium for growth and formation of monolayer in neonatal piglet Sertoli cell was combination of DMEM and-Ham's F-12 medium. Neonatal piglet Sertoli cell multiplied to form confluent layers by day 4 of culture, suggesting that such cells are highly mitogenic after isolation from their normal tubular environment. Application of DMEM + Ham's F-12 medium for culturing Sertoli cells has already been established in hamsters and monkeys (Majumder *et al.,* 1995, 1998). Sertoli cells from immature hamsters formed a confluent monolayer in DMEM + Ham's F-12 medium by day 7 (Majumder *et al.,* 1995). After 8 days of culture, Sertoli cells from testes of juvenile monkeys formed confluent monolayer, whereas Sertoli cells from the testes of adult monkeys appeared to occupy only 40~60% of the culture surface (Majumder *et al.,* 1998). Here it should be noted that in this species, mitotic activity of Sertoli cell *in vivo* does not stop until the initiation of puberty.

From our previous study it was observed that TCM 199 has stimulatory effect on oviduct epithelial cell proliferation, whereas, DMEM + Ham's F-12 medium has no/a little mitogenic effect (unpublished data). On the other hand, for epididymal epithelial cell culture combination of DMEM and Ham's F-12 medium was more suitable than TCM 199 (unpublished data). From those studies it is assumed that there might be a number of components present in culture media, which are specific for *in vitro* differentiation and growth of cell. Combination of DMEM and Ham's F-12 medium can support the formation of Sertoli cells monolayer because it contains higher concentration of the amino acids, specially, L-cystene, L-isoleucine and L-threonine, glucose, vitamin B_{12} along with 25 mM HEPES effective buffering system than EMEM and TCM 199. It is generally assumed that components of the DMEM and-Ham's F-12 medium improve cell quality by removing toxic compounds, by supplying small molecular weight metabolites.

For growth and differentiation of almost all somatic cell types *in vitro,* essential nutrients such as, growth factors, hormones, and transport proteins are required in small quantities. Those compounds are usually provided by fetal bovine serum (FBS). Supplementation of serum is also important for attachment and spreading of Sertoli cell from testes of immature rodents (Mather and Sato, 1970). In agree with previous study (Mather and Sato, 1970), our data showed that the addition of 1% FBS for the first 24 h of culture remarkably enhanced the attachment of clusters of Sertoli cells, and therefore only cultures derived in this manner were characterized. However, serum does not seem to offer any advantage over serum-free culture medium for Sertoli cell cultures from adult hamsters (Mather and Sato, 1970).

Present study showed that the mitogenic activities of FSH and ITS were absent in Sertoli cell cultures. When EGF was associated with FSH and ITS, Sertoli cell proliferation was significantly higher than observed with FSH alone. FSH enhances the stimulatory effect of insulin and FGF (fibroblast growth factor) on DNA synthesis, apparently blocks the effects of both factors on Sertoli cell proliferation (Jaillard *et al.,* 1987). Previous work has shown that EGF is mitogenic for both mouse (Mather and Sato, 1970) and rat (Rich *et al.,* 1983) Sertoli cell. Pig Sertoli cell contain specific EGF binding sites (Bernier *et al.,* 1986) and this factor is mitogenic for Sertoli cell.

The present study demonstrates that Sertoli cell from testes of neonatal piglets can be cultured in a medium with serum and growth factors by use of enzymatic digestion that employs collagenase and pancreatin for two consecutive digestions. The cells cultured by this procedure are functionally active and provide an *in vitro* model for comparison of Sertoli cell function in active and inactive immature testis. This may provide valuable clues to identify and isolate factor (s) important for the regulation of spermatogenesis *in vitro.*

In conclusion, the present data indicate that FBS and EGF are the most potent mitogens of the factors studied for Sertoli cell culture. Research of this laboratory is underway to learn whether condition medium from neonatal piglet Sertoli cells cultured in DMEM + Ham's F-12 medium supplemented with FBS, EGF, ITS, and FSH have stimulatory effect for the transformation of spermatogenic cells *in vitro.*

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