ORIGINAL RESEARCH PAPER

Genetics

KEY WORDS:

ENRICHMENT OF RAT SPERMATOGONIAL STEM CELLS BASED ON BSA GRADIENT METHOD

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ABSTRACT

Introduction:- Spermatogonial stem cells (SSCs) consists a small population of germ cells that have self-renewal and differentiation potential may useful in area of regenerative medicine. However, there are no reliable and established procedures to enrich SSCs population from rat testes. Therefore, it is necessary to develop a method for SSC enrichment.

Aim & Objectives:- To study the potential of BSA gradient for enrichment of SSCs population from rat testes.

Materials and Methods:- Testes were isolated from 5 to 6-wk-old mature rats of Charles Foster (CF) stain. The seminiferous tubules were digested using two step enzymatic (collagenase Type IV; trypsin; DNase I, and hyaluronidase) digestion method. The contaminating somatic cells (myoid and sertoli cells) were eliminated by filtration of cell lysate. Finally, enrichment of SSCs was done by using different gradient solution of bovine serum albumin (BSA fraction V). The isolated SSCs were characterized by stem cell markers.

Results:- The 6% BSA solution showed maximum efficiency for enrichment of SSCs as compared to other concentration of BSA. Isolated SSCs were found positive for stem cell markers THY-1 and alkaline phosphatase strengthens the idea that these cells are stem cells.

Conclusion:- Development of a new technique for SSC isolation and enrichment from wild type testes using 6% BSA solution will facilitate to get maximum SSCs population for therapeutic and research purposes.

Introduction:-

Spermatogonial stem cells (SSCs) are unipotent adult stem cells, which are responsible to provide a continual supply of differentiating spermatogonia in the postnatal testes. However, population of SSCs in the testis are very low and it is reported to vary between 0.02%–0.03% of male germ cells[1-2]. Due to small number, it is difficult to perform biological experiments or therapy. The spermatogonial stem cells are valuable tool in modifying the male germ line to generate transgenic animals[3] besides their application in transplantation and infertility treatment. It is also believed that SSCs can serve as a model for better understanding of adult stem cell self renewal, differentiation mechanism.

Isolation and enrichment of SSCs population involves a multi-step enzymatic digestion of the testicular tissues[4]. Previously, SSCs have been isolated from different mammalian species including human[5], buffalo[6], cattle[7] goat[8], mice[9], rat[10] using different enzymatic methods. Several other strategies are also used for the enrichment of SSCs, including discontinuous Percoll density gradient method[11], differential plating[12], fluorescence activated cell sorting (FACS) [13] and magnetic cell sorting (MACS) [14]. The density gradient method is considered to be a time saving and cost-effective method for the enrichment of isolated SSCs and in this regard it is thought that BSA may be useful for enrichment of SSCs. However, different methods showed different efficiency for enrichment of SSCs therefore, a need to develop some other cost effective method for enrichment of SSCs. Though, enrichment of SSCs for establishment of long term culture system has not yet been exploited substantially in rat. Hence, in the present study, we demonstrated enrichment of SSCs by using BSA density gradient centrifugation technique.

Materials and Methods:-

Isolation and enrichment of SSCs

Spermatogonial stem cells from rat testes were isolated by 2-step enzymatic digestion procedure. In brief, seminiferous tissue was dissociated and digested by an enzymatic solution of collagenase (5mg/ml; Type IV, Sigma Chemical Co., St Louis, MO) and trypsin (1 mg/ml, Sigma) and was incubated at 37°C for 30 minutes with continuous shaking in water bath. After three washes in DMEM/F-12 medium and removal of most of the interstitial cells, seminiferous cord fragments were further incubated in medium containing 10mg/ml DNase I and 1 mg/ml hyaluronidase (Sigma-Aldrich) and trypsin (1 mg/ml, Sigma) at 37 ^{0}C for 20 min. The somatic cells (myoid and sertoli cells) contamination was removed by cut-off filter (100 and 40 µm) and enrichment of SSCs was done by BSA (2%, 4%, 6%, 8% and 10%) gradient centrifugation. A population of SSCs was collected from cell suspension and cultured in DMEM supplemented with 10% FBS, 10mM 2-mercaptoethanol, 1% non-essential amino acids, 10 ng/ml recombinant human basic fibroblast growth factor, 100 IU/ml penicillin streptomycin and 40g/ml gentamicin at 37 ^{0}C and 5% CO_2 in air. The viability of isolated SSCs was checked by 0.4% trypan blue and cell viability percentage was calculated as per standard procedures. The process of isolation and enrichment were illustrated in Fig. 1.

Characterization of SSCs

Immunocytochemistry

For immunocytochemistry, undifferentiated spermatogonial stem cells were fixed on gelatin coated (0.1%, to increase adherence of the cells) glass slides. After PBS wash, cells were fixed in 4% paraformaldehyde for 10 min. Nonspecific background were blocked by 10% normal mouse serum. Cells were then incubated with mouse anti-THY-1 antibodies (1:200, Santacruze Biotechnology) for 1 hr at 37 ^{0}C. Cells were also stained with alkaline phasphatase. For negative control the first antibody was omitted with the same amount of mouse IgG. Detection of the primary antibody was performed by incubation of cells with FITC linked secondary antibodies (Santacruze Biotechnology). Immunostaining were analysed using fluorescence microscopy (EVOS 4300 colour imaging system; life technologies).

Results:-

We have isolated the rat SSCs using a protocol involving two step enzymatic digestion methods and enriched the SSCs by different BSA gradient solution. Our results showed that 6% BSA density gradient centrifugation enriched maximum fractions of SSCs as compared to other BSA concentration. This fraction contained 1.5 fold ($p<0.05$) more THY-1 positive cells than those in other fraction (Fig. 2A) and also efficiently formed colonies under in vitro culture condition (Fig. 2B). Trypan blue staining of the isolated cells showed maximum viability (>90 %) in all fraction of SSCs. As shown in Fig. 2D, immunofluorescence staining revealed that isolated SSCs express spermatogonial stem cell marker THY1. The immunofluroscence results showed the presence of THY-1 positive

SSCs in both the enriched and non-enriched fractions. However, following fractionation, 6% BSA gradient showed maximum THY-1 positive SSCs population and high reactivity for alkaline phosphatase (Fig. 3C). This observations support the hypothesis that the undifferentiated spermatogonial cell expressing THY-1 belongs to the spermatogonial stem cells.

Discussion:-

It is well known that mammalian testes possess very limited number of SSCs constituting only 0.03 % of total germ cells[15]. Due to limited number of SSCs in the testes there is a need to develop efficient and newer methods for isolation and subsequent enrichment of SSCs. In this study we tried to introduce new method for the enrichment of SSCs and showed that we have successfully enriched maximum population of SSCs (1.5 fold) using 6% BSA solution for gradient centrifugation. Many other authors have used Percoll gradients (32, 36 and 40 %) centrifugation method and showed it yielded 3.65 folds more abundance of 6 integrin positive cells in the enriched fractions. However, the enrichment yield has also depends on what kinds of enzymes and isolation procedure adopted for digestion and enrichment respectively. Although, similar level of purity was obtained by Percoll density gradient (30–32 %) centrifugation in rat and cattle, but it required four enzymes for testicular disaggregation[11-12]. On the other hand, when numbers of enzyme were reduced to two, it required an additional enrichment step of differential surface-attachment after Percoll density gradient to obtain a purity nearing to 86.7 % [16].

THY1, is a conserved surface marker for SSCs[17]. In the present study, using anti-mouse THY1 antibodies, we demonstrated the expression of SSC specific markers by almost all the enriched SSCs. These observations strengthen our data that undifferentiated spermatogonial cells from mature rat testes are spermatogonial stem cells. Morphological, immnocytochemistry and alkaline phosphatase staining analysis revealed that the enriched SSCs are putative stem cells which are able to maintain potency of self renewal and differentiation.

In conclusion, our results demonstrate that THY1 is a marker of undifferentiated spermatogonia in rat testes, and enrichment of THY1+ cells can be done using 6% BSA. The characterization and understanding of SSCs will have immense practical implications considering the major genetic gain comes through male.

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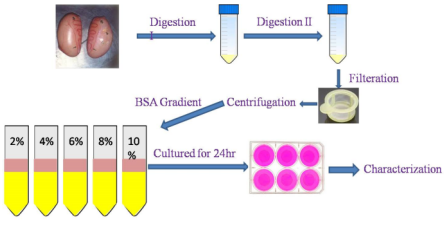

Figure 1. Figure shows procedure for isolation and enrichment of SSCs from rat testes using different concentration of BSA solution.

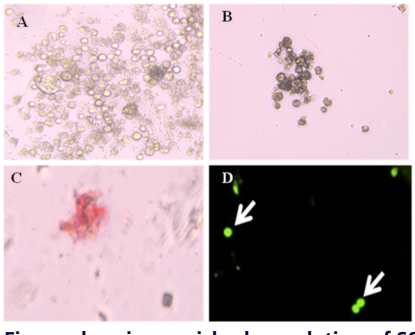

Figure 2. Figure showing enriched population of SSCs using BSA (A), SSCs colonies (B), alkaline phosphatase positive SSCs colonies (C) and THY1 positive SSCs.

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