Research Paper

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A Method for Cell Culture and RNA Extraction of Rabbit Bone Marrow Derived Mesenchymal Stem Cells

Bio-medical

KEYWORDS : Cell culture, Mesenchymal cells, RNA isolation

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ABSTRACT

Stem cells biology bagged a paramount portion of research studies of last decade. The most investigated adult stem cells were mesenchymal stem cells (MSCs). Bone marrow stroma represents the major source of MSCs. Bone marrow mesenchymal stem cells (MSCs) are self-renewing and are potent in differentiating into multiple cells and tissues. The objective of study was to develop a protocol for efficient collection, isolation, culture and purification of the MSCs from bone marrow of rabbit and to standardize a protocol RNA extraction from this isolated cell line.

INTRODUCTION

Stem cells have been considered as a powerful tool for cell- or tissue-based engineering in human and veterinary medicine because of their unique potential to develop into various specific cell lineages upon being cultured in appropriated conditions. Adult stem cells are derived from adult body organs. It can be classified as, mesenchymal stem cells, hematopoeitic stem cells, neural stem cells etc. They have unlimited self-renewal potential to replenish other cells as long as the animal is alive. During embryonic development, mesenchyme or the embryonic mesoderm contains precursor cells that differentiate into virtually all connective tissue phenotypes (Alberts et al., 2002). Upon the completion of prenatal morphogenesis, clusters of mesenchymal cells continue to reside in various tissues and are the logical sources of adult (somatic) MSCs. The adult MSCs are derived from a large variety of tissues including bone marrow (Pountos and Giannoudis, 2005), fat tissue (Lin et al., 2006), muscle (Sun et al., 2005), amniotic fluid, placenta (Portmann-Lanz et al., 2006), and umbilical cord (Moise, 2005). Bone marrow derived MSCs are the most studied MSCs (Prockop, 1997).

Rabbit MSCs can be obtained from healthy animals by taking aspirates of bone marrow from the iliac crest and expanding on tissue-culture plates. MSCs represent minor fraction (0.001-0.01%) of the total population of nucleated cells in bone marrow (Pittenger et al., 1999). It can be isolated and enriched using standard cell culture techniques. According to Luria et al., (1971), MSCs selectively adhere to plastic surfaces, whereas hematopoietic cells do not and can be removed while media change. The remaining heterogeneity of the culture progressively decreases by serial passaging and after some passages the culture is enriched with self-renewing fraction called stem cells. MSCs were initially isolated as the plastic adherent fraction of bone marrow (Friedenstein et al., 1976). Friedenstein went on to pioneer in vitro culture methods for the isolation and differentiation of MSCs (Friedenstein et al., 1987). Dazzi et al., (2006) reported another isolation protocol. In which isolation is based on the density of cell kind, that involves centrifugation over a percoll gradient and allows the enrichment of nucleated cells. Both this methods can be combined to increase the efficiency of isolation.

There are few criteria defined by International Society for Cellular Therapy (ISCT) for characterization of MSCs (Dominici et al., 2006). According to ISCT in addition to plastic adherence property, these MSCs should express some pluripotency genes and particular cell surface markers, and must differentiate into osteoblasts, chondroblasts and adipocytes, when they are cultured under standard differentiating conditions. Inorder to check the mRNA expression of the above mentioned pluripotency genes and cell surface markers total RNA should be extracted from the isolated cells.

Objective of the study was to isolate mesenchymal cells from rabbit bone marrow, to propagate and purify these cells over several passages and to isolate RNA from the this purified cell sample for further characterization studies.

MATERIALS AND METHODS

Collection of Bone marrow from rabbit

Adult healthy rabbits of either sex were used for bone marrow collection . Procedure was done under general anesthesia using xylazine hydrochloride at 6mg/kg and ketamine hydrochloride at 60 mg/kg bodyweight (Amarpal et al., 2006). Site of collection, the iliac crest was prepared for asceptic collection by clipping, shaving, scrubbing and painting with povidone iodine. Bone marrow was aspirated with bone marrow biopsy needle of 16 G. A little force was applied to penetrate cortex of the bone. After fixing the needle in marrow cavity stylet was removed and 5ml of bone marrow aspirate was collected in a 10 ml syringe containing heparin. All the procedures were in compliance with the guidelines of animal ethical committee of the institute. Further processing of bone marrow aspirate was done under laminar air flow cabinet to avoid microbial contamination.

Isolation and Culture of Mesenchymal Cells

The collected bone marrow samples were diluted with equal amount of HBSS. This diluted bone marrow samples were loaded carefully on histopaque (4:3 ratio) in pre-sterilized 15 ml centrifuge tube and centrifuged at 1800 rpm for 30 min at room temperature. The bone marrow was separated into different phases, plasma, polynuclear cells, and a buffy coat. Buffy coat was our area of interest which contains the target progenitor cells. The buffy coat was collected carefully and washed with 2-3 ml of PBS at 1800 rpm for 8 minutes. The supernatant was discarded and the cell pellet was again washed with PBS and re-suspended in 5 ml of complete growth media consisted of Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% fetal Bovine Serum (FBS) (Sigma, USA), antimycotic antibiotic (100 IU/ml). The re-suspended cells were seeded in to a T-25 culture flask and incubated at 37°C in humidified atmosphere with 5% CO_2. The non adherent cells were removed by the 3rd day of incubation and the media was changed with fresh growth media in order to propagate the plastic adherent MSCs. The medium was refreshed every 3 days. Passaging of the cells were done using By 12th day of primary culture 90 % confluency was attained. This phase of cells were used for 1st passaging. For this detached out the adherent cells from flask by enzymatic method using 0.25% Trypsin- EDTA (Hyclone) and repeated the plating of this enhanced number of

cells in larger surface area flask. Third passage cells were used for RNA isolation. We maintained the cells up to 10th passage. After that cells were freeze preserved.

Isolation of total RNA from MSCs

Total RNA was isolated from third passage ex vivo expanded rabbit MSCs using RNAgents according to manufacturer's recommendations (Ribozol™,RNA extraction reagent, Amresco). To minimize the effects of RNase activity, properly sterilized and DEPC treated laboratory wares were used. Sterile gloves were worn during the whole procedure of RNA isolation. Rabbit bone marrow derived mesenchymal cells grown in tissue culture flask lysed directly in the culture dish, after completing about 90% confluency. In brief, discarded the culture media in the flask and flask waswashed with PBS. Added 1 mL of RiboZol™ per 10 cm^2 of culture dish area and incubated it for 2 minute. Lysed the cells by flicking the solution several times through the tip of a pipette. Transferred the cells to a RNase-free tube. Sample was incubated at room temperature for 10 minutes to ensure the complete dissociation of nucleoprotein complex. In to this chloroform was added at the rate of 200µl per ml of Ribozol™. Shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. Sample was centrifuged at 12000x g for 15 minutes at 4^0C. Upper aqueous part was transferred to a fresh tube and to this isopropanol was added at the rate of 0.5ml per ml of RiboZol™. Incubated the sample at room temperature for 10 minutes and centrifuged at 12000xg for 10 minutes at 4^0C. Supernatant was removed and the pellet was washed with 1ml of ice-cold 75% ethanol and then centrifuged at 12000×g for 10min at 4°C. The pellet was air dried for 15min and reconstituted in appropriate volume of Nuclease Free Water and stored at -20°C for further use.

Checking purity and concentration of RNA

The absorbance of RNA was measured at 260nm/280nm in Nanodrop spectrophotometer (ND1000 Thermo USA). The OD260 /OD280 ratio was calculated to check purity.

Agarose gel electrophoresis

Agarose gel (1.5%) was prepared in TAE buffer containing ethidium bromide to a final concentration of 0.5µg/ml. The RNA sample (5µl) was mixed with 1µl of 6X loading dye and electrophoresed at 50 volts for 1 hr. After the electrophoresis, the RNA profiles were visualized in a UV transilluminator.

RESULTS

The plated live cells could be detected as glistening round bodies immediately after primary culture (Fig.1). The non-adherent cells were removed on 3rd day of cell culture and adherent cells started growing as fibroblast-like cells by this time. Which further formed symmetric colonies and attained about 90% confluent monolayer by day 12 of primary culture (Fig.2). RNA isolation from adherent cells were carried out and concentration of the isolated total RNA was found to be 602.2ng/ul and absorbance 260/280 was found to be 1.9 in nanodrop. RNA agarose gel electrophoresis showed two bands representing 18S and 28S rRNA showing the integrity of RNA (Fig.3).

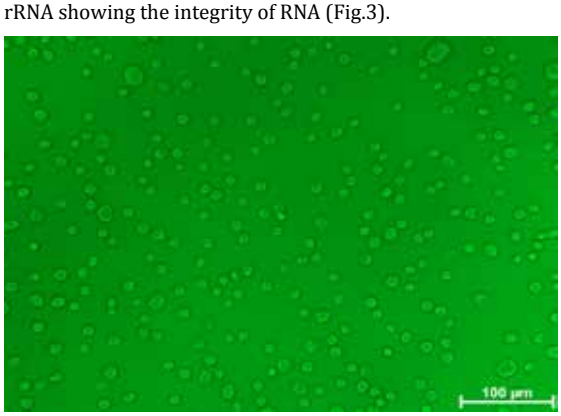


Fig.1: Live MSCs as round glicening bodies immediately after plating in tissue culture flask

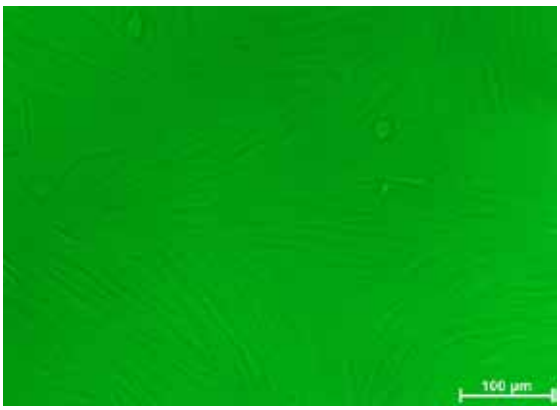


Fig.2: Symmetric fibroblastoid colonies at 90% confluency level by day 12 of primary culture.



Fig.3: 1.5% Agarose gel electrophoresis showing integrity of total RNA isolated from adherent cells

DISCUSSION

The method used for isolation and culture of mesenchymal cells was found suitable. Bone marrow mononuclear cell fraction contains heterogeneous cell population and mesenchymal cells were isolated on the basis of plastic adherence property. Isolated RNA can used for cDNA synthesis and hence for characterization of MSCs. Two important challenges associated with MSCs would be the topics of their safety and efficacy in use as therapeutic tool. These subjects need sufficient investigation in animal models, which in turn requires MSCs isolation and characterization, as the first step from the interest animal (Eslaminejad et al., 2009). Animal models are being used for experimental studies in various branches of medical and dental sciences, because certain of the research areas obviously cannot be done on human beings for practical and ethical reasons. And, rabbit being an easily available and less aggressive animal is a promising model if the guidelines are followed (Mapara et al., 2012).

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