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Comparative analysis of different methods for isolation of mesenchymal stem cells in mice

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Abstract
The present study was based on isolation and quantification of umbilical cord-derived mesenchymal stem cells (UC-MSCs) and adipose derived mesenchymal stem cells (AD-MSCs) from Swiss Albino mice. Umbilical cord and adipose tissues were collected from foetus and abdomen of mice respectively. Isolation of AD-MSCs and UC-MSCs from the collected tissues was done by collagenase type-I and trypsin-EDTA digestion. After processing the isolated cell pellet was seeded in a six well plate with fresh media containing DMEM Nutrient Mix F12, 10% FBS and 1% antibiotic antimycotic solution and incubated in humidified CO_2 (5%) incubator at 37 °C till 70-80% confluence. Then the cells were collected for quantification and viability assessment by Neubauer's chamber under microscope using trypan blue stain. Trypsinization of both tissue yielded less number of cells than collagenase treatment. But trypsinization provided higher viable cells as compared to collagenase treatment. Hence, trypsin-EDTA can be used for analytical study, where the cell yield is of lesser importance.

Keywords: Mesenchymal stem cells, mice, collagenase type-I, trypsin-EDTA

Introduction
In the emerging field of regenerative medicine, stem cell therapies hold high therapeutic promise based on the possibility of ex vivo/*in vivo* stimulation of stem cell expansion and differentiation into functional progeny that may repair and even replace damaged tissues or organs (Mizuno *et al.*, 2012) [18]. Stem cells include embryonic stem cells, induced pluripotent stem cells and adult stem cells. Although embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been studied, their research and development are limited owing to ethical issues and clinical applications. Adult stem cells are favoured for many reasons due to their immune compatibility and freedom from ethical constraints (Bendavid and Benvenisty, 2011) [4]. Among them mesenchymal stem cells (MSCs) are heterogeneous cell population capable of self-renewal and multilineage differentiation and are resident in different organs and tissues such as adipose tissue, bone marrow, umbilical cord, amniotic membrane, kidneys, liver, spleen, lungs, pancreas, tendons, synovial membranes, placenta, amniotic fluid and dental pulp (Han *et al.*, 2012; Vidor and Contesini, 2018) [12, 27]. Recently, many plastic surgeons have studied the potential clinical application of adipose-derived stem cells (ASCs), which represent a readily available adult stem cell population that has gathered a lot of attention in the field of regenerative medicine (Gould *et al.*, 2015) [11]. Adipose-derived stem cells are plastic-adherent, multipotent stem cell population having a differentiation potential to other MSCs, and have the ability to differentiate into cells of several lineages from all three germinal layers (Daher *et al.*, 2008) [6]. Similarly the solid tissues of umbilical cord were of no use and were treated as a valueless medical waste. Now umbilical cord-derived mesenchymal stem cells (UC-MSCs), which can be obtained from total umbilical cord or its dissected compartments (perivascular, intervascular, subamniotic zones of Wharton's jelly and subendothelial layer of blood vessels) have importance due to intensive development of biomedical products. These UC-MSCs have high proliferative potential, karyotype and phenotype stability, differentiation plasticity, paracrine activity, and immunomodulatory properties, hence it has now a new therapeutic direction in the field of regenerative medicine (Arutyunyan *et al.*, 2016; Ding *et al.*, 2015) [1, 7]. The viability, yield, proliferative efficacy and stemness of mesenchymal stem cells can be influenced by the type of tissue involved and harvesting procedure. There are different harvesting and processing procedures for both adipose and umbilical tissue derived stem cells by various scientists, but a standardized protocol is still in debate. In this study two digestion methods for isolation of stem cells were compared for their efficacy.

Materials and Methods

Swiss Albino mice were used for collection of adipose tissue and umbilical cord tissue for isolation of stem cells which was approved by the Institutional Animal Ethical Committee (IAEC) of College of Veterinary Science and Animal Husbandry. The mice were anaesthetised individually using an intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (80 mg/kg) cocktail before surgery (Tranquilli *et al.,* 2007) [25]. Under mid-ventral incision umbilical tissue and adipose tissues were collected and kept in phosphate buffer saline with 1% antibiotic-antimycotic solution (Gibco, Thermo Scientific, USA). Isolation of AD-MSCs and UC-MSCs from collected tissues was done by using two tissue disaggregation agents i.e. collagenase type I and trypsin-EDTA. The adipose tissue and umbilical cord tissue cell culture was done as per the standardized protocol of Reich *et al.* (2012) [21] with some modification.

About 10 gm of each adipose and umbilical cord tissue were minced, washed in PBS buffer and digested for 15-20 min with collagenase I solution containing 1 mg/ml collagenase I (Himedia). The resulting cell suspension was filtered through a 70 µm falcon strainer (SPL, Life Sciences, India) and collagenase I activity was stopped by foetal bovine serum (FBS, Gibco, Thermo Scientific, USA). The filtrate was centrifuged at 1200 rpm for 10 min. Pellet was re-suspended in 5 ml RBC lysis buffer (Himedia) and incubated at room temperature for 5 min and again centrifuged. The cell pellet was re-suspended in fresh media containing DMEM nutrient mix F12, 10% FBS and 1% antibiotic antimycotic solution (Gibco, Thermo Scientific, USA). Isolated cells were seeded in a six well cell culture plate for culture inside a humidified CO_2 (5%) incubator at 37 °C. Similarly in trypsin digestion method, the minced tissues were treated with 5 ml of 0.05% trypsin-EDTA (Gibco, Thermo Scientific, USA) instead of collagenase type I and the remaining steps were same. Media was changed in every 2 days interval and after the conluence was reaching 80%, cells were counted.

Viable cells from adipose and umbilical tissue by both, trypsinization and collagenase treatment were quantified with the help of the Neubauer's chamber under microscope using trypan blue stain. About 10µl of the single cell suspension was taken and mixed with 10µl of 0.4% trypan blue dye solution (1:1 ratio). After keeping for 5 min at room temperature, 10µl of the suspension was loaded into haemocytometer chamber. The cells were focused at 40x under microscope and cells in the four WBC squares were counted. The viable (unstained) and non-viable (blue stained) cells were counted separately. Number of the cells per ml = (Total no. of cells/4) x dilution factor x 10^4. Similarly % Viability = [Number of live cells/Total No. of Cells (Live+dead)]×100.

Results and Discussion

Collection of the umbilical cord tissue and adipose tissue were done from Swiss albino mice under general anaesthesia using intraperitoneal injection of xylazine hydrochloride and ketamine hydrochloride cocktail mixture. Other workers used different anaesthestics and various sites of adipose tissue for collection of stem cells. Fujimura *et al.* (2005) [9] used diethyl ether and Ikegame *et al.* (2011) [15] used sodium pentobarbital (90 mg/kg, intraperitoneal) for collection of adipose tissue form mice inguinal fat pads. Similarly, Jang *et al.* (2011) [16] used abdominal fat and Perez *et al*. (2015) [20] used subcutaneous adipose tissue for collection of adipose derived stem cells in mice. But literature was silent regarding collection of UC from mice. In the present study, the anaesthetic combination was found effective for collection of the tissues without any complication. It was observed that collection of umbilical tissue from mice require more care than adipose tissue.

Abdominal adipose tissue and umbilical cord tissues were subjected to two types of tissue disaggregation agents i.e. collagenase type-I and trypsin-EDTA for isolation of AD-MSCs and UC-MSCs. On analysis of the use of digestion agent, it was easier to use trypsin than collagenase owing to its readily available form. It was also observed that disaggregation of adipose tissue was easier and require less time in comparison to umbilical cord tissue. This might be due to loose connective tissue nature of adipose tissue. But the isolation of cell pellet sediment after tissue disaggregation was better in umbilical cord tissue digestion. There were fewer blood vessels found in adipose tissue than umbilical cord tissue during processing. Enzymatic digestion by trypsin and collagenase was first described by Rodbell (1964) [22]. It is a widely used method for digestion and degradation of the collagen network of tissue. This method has some disadvantages like relatively high costs of enzymes, purity and purification of the enzymes, time-consuming labour and inconsistent results (Hyder, 2005; Baptista *et al.,* 2009) [14, 3]. There is also deleterious effects of enzymes on the phenotype and behaviour of cells (Liu *et al.,* 2009; Patel *et al.,* 2009) [17, 19]. Previous studies have investigated other enzymatic methods for mesenchymal stem cell isolation, such as the use of liberase, trypsin and hyaluronidase, in order to achieve a reproducible and qualitatively improved tissue digestion and avoid damage to the isolated cells, as an alternative to crude collagenase digestion (Hyder, 2005; Semenov *et al.,* 2010; Tsagias *et al.*, 2011) [14, 23, 26].

The isolation methods of stem cells from different tissue can vary between researchers. These methods start with enzymatic and centrifugal separation of collected tissue from stromal cells and vascular and the resulting pellet is known as stromal vascular fraction (SVF). These pellets contain blood cells, fibroblast, pericytes, endothelial cells and preadipocytes (adipocytes progenitor) (Faust *et al.,* 1977) [8]. Stem cells can be isolated by digesting with collagenase and trypsin for several times, followed by exchange of medium for several time to clean the red blood cells instead of using chemical substance (Zhu *et al*., 2008) [28]. In the present study the isolation procedure was similar with Taha and Hedayati (2010) [24] and Reich *et al.* (2012) [21] with some modifications.

Viable cells from adipose tissue and umbilical tissue by both, trypsinization and collagenase treatment were quantified with the help of Neubauer's chamber under microscope using trypan blue stain. Viable cells stained colorless and non-viable cells stained blue. Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. Live cells or tissues with intact cell membranes are not coloured. In the present study, the viable cell had a clear cytoplasm whereas the nonviable cell had a blue cytoplasm. The mean ± SE values of no. of cell yielded per gram of tissue from adipose tissue on trypsin-EDTA and collagenage type-I treatment were $0.68x10^6$ ± 0.11 $x10^6$ and $1.44x10^6$ $\pm 0.13x10^6$ respectively. Similarly, the mean ± SE values of no. of cell yielded per gram of tissue from umbilical tissue on trypsin-EDTA and collagenage type-I treatment were

$0.73x10^6 \pm 0.21x10^6$ and $1.54x10^6 \pm 0.15x10^6$ respectively. It was observed that in both tissue, cell yield was significantly ($P<0.05$) higher in collagenage type-I digestion than trypsin digestion. The viability of the cells from adipose tissue on trypsin-EDTA and collagenage type-I treatment were found 92% and 74% respectively. Similarly, the viability of the cells from umbilical tissue on trypsin-EDTA and collagenage type-I treatment were found 95% and 76% respectively. On the basis of viability, trypsinization provided higher viability as compared to collagenase treatment. It was also observed that the MSCs derived from umbilical cord of mice were having faster multiplication potential than that were obtained from adipose tissue. Other scientist such as Black *et al.* (2008) [5] reported 23g and 15g subcutaneous tissue yield $4.2\text{-}5x10^6$ and $3\text{-}5x10^6$ viable cells respectively. Zhu *et al*. (2008) [28] isolated $1\text{-}2x10^7$ nucleated cells and $5x10^5$ stem cells from 400-600 mg subcutaneous tissue. Ganey *et al.* (2009) [10] reported average 12.11±2.93g subcutaneous tissue yielded $3.21x10^7 \pm 1.47x10^7$ viable cells. Balaji *et al*. (2010) [2] isolated a novel population of multipotent stem cells of approximately $2.5x10^6$ numbers from epidermal layer of human skin sample measuring about $4cm^2$ areas. Harsan *et al.* (2015) [13] isolated $2x10^6$ numbers of stem cells from adipose tissue after reaching 80% confluence.

Conclusion

The adipose and umbilical cord tissue of mice are very rich source of mesenchymal stem cells (MSCs) and the cells have clonal property with high multiplication capacity. Mesenchymal stem cells derived from the umbilical cord have the faster replication and multiplication capability when compared to the mesenchymal stem cells isolated from adipose tissue.

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