

Cryopreservation Increases Availability and Usability of Ovine Adipose-tissue Derived Stem Cells for Regenerative Medicine Search

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Abstract: *Aim: Our long-term aim is to develop a living valvular substitute using Regenerative Medicine principles, by seeding decellularized porcine heart valve scaffolds with adult stem cells and conditioning them in bioreactors before implantation. In this study, adult stem cells were isolated from sheep adipose tissue (ADSCs). However, we found it impractical to use cells immediately after propagation and thus, in order to extend their availability in time, a preservation method was needed. Methods: Adipose tissue was harvested from 6 sheep. ADSCs were isolated using enzymatic agents and cultured. The cells were tested for plasticity using chondrogenic, adipogenic and osteogenic differentiation kits and then cryopreserved in DMSO at -140°C. Viability was tested after a 3 week storage using Trypan Blue Staining. Results: Ovine ADSCs exhibited excellent plasticity and differentiation potential. An average of 18 million ADSCs were obtained from each ovine, exhibiting more than 88% viability after a 3-week cryopreservation period followed by thawing. Conclusions: DMSO cryopreservation represents a suitable method for ovine ADSCs for regenerative medicine. This method expands the usage of stem cells in vitro before they are differentiated into more specialized cells, offering large numbers of usable ADSCs with minimal cell loss at any desired time point.*

Keywords: regenerative medicine, heart valve, stem cells, cryopreservation

1. Introduction

The shortcomings of present valvular substitutes-permanent anticoagulation for the mechanical one and limited lifespan for the biological ones - are well known [1]. These downsides create the premises on which valvular Regenerative Medicine and Tissue Engineering principles are established, aiming to obtain the perfect living valvular substitute, characterized by: high durability, identical hemodynamic characteristics to the native valve, growth capacity, non-immunogenicity, non-thrombogenicity, with minimal or absent regurgitation [2].

In our study we aim to obtain a Tissue Engineered Heart Valve (TEHV) using: biological scaffolds – prior decellularized porcine aortic valve, ADSCs harvested from sheep, which will undergo in vitro differentiation toward specific valvular cells (endothelial and fibroblast) and a bioreactor that will mimic the in vivo condition, in the lab.

The promising results in Regenerative Medicine and Tissue Engineering obtained in numerous and varied medical fields [3-6] inspired researchers to manufacture tissues and organs in order to substitute the failing and damaged ones. This can be fulfilled using stem cells, scaffolds (temporary supports for cells) and bioreactors. Scaffolds are three dimensional structures composed of natural or synthetic constituents. The natural ones are usually obtained through a decellularization [7-8] procedure whereas the synthetic ones are constituted of largely used biomaterials [9-10]. Stem cells, defined by their capacity for self-renewal and plasticity to differentiate towards various cell lineages, represent the center

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piece of tissue regeneration. The literature classifies the stem cells into two types: embryonic stem cells and adult stem cells [11]. Since 2007, after the first description of induced pluripotent stem cells in humans [12], this new cell category has been added to the classification. Due to ethic controversies and teratoma formation, interest in the embryonic stem cells is limited. Adult stem cells have the advantage of being able to be harvested from various tissues, being the resident cells living in the small germinal niches of most tissues [13], having the downside of a limited span of differentiation. The most widely spread adult stem cells are the mesenchymal stem cells (MSC). MSC have represented a point of interest since their first isolation in 1970 [14], culminating with the standardization and publishing in 2006 of the minimal requirements that define the MSC by the Society for Cell Therapy [15]. The most used sources of MSC in studies are: bone marrow, adipose tissue and umbilical cord [16]. Adipose derived stem cells (ADSCs) – firstly isolated in humans by Zuk [17] - have the advantageous presence in the sub dermal tissue with a facile procurement in large quantities. Following the procedures of cell isolation and culture, they are ready to use, requiring a well-organized schedule with no or minimal time adjustments. This makes it impractical to use cells immediately after propagation and thus, in order to extend their availability, a preservation method was needed. The literature presents miscellaneous approaches suitable for different types of stem cells [18-20] including sheep ADSCs isolation and culture [21,22] but with no or little information regarding their preservation.

2. Materials and methods

Ethics and study overview

This study is part of a research grant, which has the approval no 131/2016 of the Ethics Committee of University of Medicine and Pharmacy Science and Technology from Targu Mures. All animals received care according to “Guide for the care and use of laboratory animals” and “Principles of Laboratory Animal-Care” stated by the National Institutes of Health.

ADSCs harvesting and isolation

Under sedation and analgesia, using an aseptic minimally invasive surgical procedure, interscapular, subdermal adipose tissue was obtained from 6 female sheep (approx. 1 cm³) [23]. From each harvested tissue, a random fragment was picked for microbiological testing of sterility. Subsequently the adipose tissue was immediately placed in sterile cell culture media (Dulbecco's Modified Eagles Medium - DMEM, 10% Fetal Bovine Serum - FBS and 2% antifungal/antibiotic - AA) and transferred to the lab. ADSCs were isolated in a temperature controlled, mechanic and enzymatic manner, following the isolation protocol described by PA Zuk [17]. Tissue processing procedures took place in the lab in a time span of maximum 4 h.

ADSCs self-renewal and multipotency – culture and plasticity test

Isolated ADSCs were cultured in cell culture media (DMEM, 10% FBS, 2% AA) and incubated in a humidified atmosphere at 37°C, with 5% CO₂, in small cell culture flasks (T25) for 4 days until the first ADSCs appeared by inverted light phase microscopy. Cell culture media was changed every 3-4 days. When stem cells reached over >85% confluence, they were passaged in a sterile hood into 3 larger culture flasks (from 1 T25 flask into 3 T75, from 1 T75 to 3 T150). Cell detachment was obtained using an enzymatic agent - a solution of Trypsin (0.02g of 1 mg/mL Trypsin+ 0.2g of 1% Bovine Serum Albumin+ 20 ml PBS). Firstly, the flasks were washed with Phosphate-buffered saline – BPS for 1 minute at an appropriate ratio (5 mL/T25, 15 mL/T75, 30 mL/T150). Secondly, the Trypsin solution was added to the flask in flask size adapted quantities (1/2/3 mL for T25/T75/T150) and quickly placed at 37°C, with 5% CO₂, in the incubator for 5-6 minutes. After incubation cells detachment from the flask was appreciated under the microscope. If detachment seemed complete, neutralization of the Trypsin was performed using culture media, at a ratio of 8:1 (8/16/24 mL media for T25/T75/T150). If cell detachment was incomplete, supplementary incubation of the flasks for 1 min was performed, repeatedly until full cell detachment was achieved. The cell suspensions were transferred into sterile tubes and

centrifuged at 1000 rpm for 5 min. After the aspiration of the supernatant, the cells were suspended in culture media and placed in larger tissue culture flasks. When ADSCs expansion in the 3 T150 reached >80% coverage, cells underwent one last trypsinization, followed by cell counting using a Scepter™ cell counter. To test their plasticity, we used a differentiation kit (PromoCell™) in which the ADSCs underwent distinct protocols toward 3 different cell lineages (chondrocyte / adipocyte/ osteocyte) using mesenchymal stem cell differentiation media provided by the company. After incubation for 3-4 weeks, cells were stained Alcian Blue for chondrogenesis, Oil Red O for adipogenesis and Alizarin Red for osteogenesis (Figure 1).



Figure 1. Differentiation of ADSCs microscopy aspects – Left -Adipogenic differentiation– Oil Red O staining, Ob. 10x; Middle -Osteogenic differentiation– Alizarin Red S staining, Ob. 4x Right - Chondrogenic differentiation -Alcian Blue staining, Ob. 4x

ADSCs cryo-preservation, thawing and viability testing

In a sterile hood, the cryo-preservation solution was prepared and sterile filtered, containing 70% DMEM, 20% FBS and 10% DMSO (dimethylsulfoxide). After the last centrifugation of the trypsinization protocol the supernatant was aspirated and, cells were resuspended in the cryo-preservation solution at 2 million cells/mL. The mix was thoroughly homogenized, 1 mL placed in 2 mL cryotubes and tubes frozen down, overnight at a rate of 3-4°C/h down to a temperature point of about -80°C degrees and sequentially transferred to a -140 °C freezer.

Three weeks later, randomly selected cryotubes were thawed in warm water (37°C) with light shaking movements until a minuscule ice nucleus could still be observed. The cryotubes were placed in the sterile hood and their content transferred to a conical tube pre-filled with 10 mL of cell culture media for neutralization. From this cell suspension, 40 µL were subtracted and placed in 10 µl Trypan Blue (0.4% Trypan Blue in PBS, pH 7.2-7.4) and the result was charged in a Fuchs-Rosenthal Counting Chamber. Results were read and interpreted using a light inverted microscope in four 1mm² squares. The cell suspension was then centrifuged at 1000 rpm for 5 min. After supernatant removal, the ADSCs were resuspended in 5 mL media culture and placed in culture flasks in the incubator (37°C, with 5% CO₂).

3. Results and discussions

Six adipose tissue fragments were isolated and six ADSCs cultures were obtained (Table 1). Sterility tests revealed absence of microbial contamination. Each of it underwent 2 passages, and an average of 3 feeding procedures, obtaining 3 T150 culture flasks at the end of the procedure, without culture media infections. For the ADSCs cultured after thawing, 5 T150 were obtained pointing to preserved propagation ability.

Table 1. ADSC cultures

ADSCs culture No.	No. of Cell feeding	No. of cell passage	Cell count	No. of cryotubes
#1	3	3	24.900.000	12
#2	2	2	14.590.000	7

#3	2	2	16.220.000	8
#4	2	2	19.130.000	9
#5	4	2	17.210.000	8
#6	3	2	16.636.000	8

On the average, approximately 5.4 millions of ADSCs/T150 were obtained. ADSC differentiation towards chondrocyte / adipocyte/ osteocyte lines was successful. For the adipocytes, the Red Oil O stained the fat deposits. Through the differentiation process the adipocytes gain specialized structures – vacuoles that were filled with lipids. Chondrogenic differentiation was highlighted using the Alcian Blue stain, revealing presence of extracellular matrix proteoglycans. Whereas for the osteocytes, the Alizarin red stain revealed the presence of bone tissue by binding to the calcium deposits. ADSC viability tests performed after the freezing / thawing procedures revealed that more than 88% of ADSCs were viable; values ranged from 88.3 to 88.7%.

A careful literature survey of papers based on ADSC preservation showed work done on human [24], murine or equine origin [25,26]. There is limited research reported on the ovine model, most attention being paid to ADSC plasticity proved in cartilage repair [27] and several characterization studies between different types of mesenchymal stem cells [28,29]. For the vivo testing and assessment of valvular bio-prosthesis, sheep are considered the gold standard animal model [30]. This is based on the calcification rates of these animal models - higher, compared to the one in humans. There is an increased interest in the plastic surgery field with a constant increase in the number of liposuctions procedures performed routinely using perpetually improving surgical techniques [31]. Following breast augmentation, liposuction is the second most frequently performed surgical procedure worldwide according to data from the 2017 International Society of Aesthetic Plastic Surgery (ISAPS). Regarding tissue harvesting, obtaining adipose tissue is less traumatic and less harmful for the patient than bone marrow. Having this perspective in mind, large quantities of fat tissue could be used tissue that is usually disposed of. Besides the isolation method described by Zuk [17], using collagenase, the one that is widely used for ADSCs procurement, there are published protocols with another enzymatic agent, Trypsin [32]. Comparative studies between these two enzymatic isolation agents reveal Trypsin to be a less expensive alternative with similar proliferation rates regarding ADSCs [33]. The same comparison applied for MSC harvested from the umbilical cord revealed absence of cell culture for cells isolated with Trypsin [34]. Regarding stem cell preservation solutions, there is a growing interest in the field, sustained by sparked huge public interest to collect stem cells from sources such as umbilical cord blood. At the same time, researchers are looking for feasible solutions for stem cell storage, solutions that are safe, easy to use and compatible with the clinical processing.

With reference to ADSC preservation and by default to mesenchymal stem cells, there is not standardized protocol for this procedure. One of the most common used agents for cryo-preservation is represented by DMSO, with a well-known downside in affecting cell viability [35]. For hematopoietic stem cells, the 10% concentration is reported to be the most commonly used [36]. In humans, DMSO is documented to cause a series of cardiovascular [37], renal [38], digestive [39] side effects and even death secondary to DMSO toxicity [40]. Studies regarding cell viability were performed also with a reduced DMSO concentration of 5% comparing it to the 10 % concentration solution, revealing an improved viability from 52% up to 74% [41]. In conclusion, now, most of the cryopreservation experience materializes from hematopoietic stem cell manipulation, derived from the growing numbers of bone marrow transplants.

4. Conclusions

Ovine ADSCs represent a feasible cell source for Regenerative Medicine, proving rapid multiplication and multipotent plasticity induced in vitro.

This study validates the 10% DMSO / 20% FBS in medium for cryopreservation of ADSCs derived from sheep, giving the opportunity of obtaining stem cell banks for research. By having them stored, ADSCs become ready to use in large quantities, with minimal loss.

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