A NOVEL MANUFACTURED SYSTEM DESIGNED TO ENSURE CONSISTENT CONDITIONS IN MECHANICAL PRECONDITIONING OF OVINE ADIPOSE DERIVED STEM CELLS DIFFERENTIATION TOWARDS ENDOTHELIAL CELL LINEAGE

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Abstract: *Introduction: Present valvular substitutes are imperfect prostheses. Regenerative medicine imagines a viable prototype using stem cells, scaffolds and bioreactors. In our study we aimed to differentiate sheep adipose derived stem cells (ADSCs) towards endothelial cells using chemical and mechanical stimuli. For mechanical stimulation, a cooling system was imagined, adapting to the incubator's characteristics. Materials and methods: Six ADSCs cultures were isolated, frozen and stored to-140^{0}C. Following thawing, cells underwent an endothelial differentiation protocol composed of a static and a dynamic phase, the latter requiring a water recirculation system created by us. Results: Over 100 million ADSCs were cultured and cryopreserved. Thawing tests reported over 80% viability. Five endothelial cell cultures were obtained, confirmed by immunohistochemistry. During the dynamic differentiation the incubator's environment was constant and stable. Conclusions: The system represents a viable option for dynamic conditioning in a normal cell culture incubator environment. We certificated it in ADSCs endothelial differentiation, replacing an expensive acquisition.*

INTRODUCTION

By designing a novel heart valve substitute, using Regenerative Medicine and Tissue Engineering fundaments, researchers aspire to overcome at the moment valvular substitute's performances and drawbacks (the need for surgical (1) or interventional (2) procedures of replacement due to biological valve degeneration respectively lifelong anticoagulation for the mechanical valves (3)). Stem cells represent the central piece of regenerative medicine. They are defined by their capacity to proliferate and differentiate towards various cell lines, and are classified as embryonic or adult (postnatal) in regard to their origins.(4) ADSCs representing a group of adult stem cells were firstly isolated in 2002 by Zuk.(5) ADSCs were used in studies as cell therapies (6-8) or seeded on a scaffold in order to manufacture anatomical components of the cardiovascular system such as blood vessels (9) or heart valves.(10,11) Studies reveal no impact in ADSCs isolation in patients with cardiovascular risk factors or with vascular disease (12) and taking into consideration the facile and relatively painless procurement make the ADSCs an ideal resource for Cardiovascular Regenerative Medicine.

The vascular endothelium, formed by tightly aligned endothelial cells (EC), has important homeostatic role, responding to chemical and mechanical stimuli.(13,14) Shear stress is a well know vasoactive factor.(15) Differentiation of ADSCs towards endothelial cells was described in human using endothelial cell grow supplement and shear stress.(16)

AIM

In our study, we planned to differentiate sheep ADSCs toward heart valve specific cells – endothelial cells. In order to cultivate cells in the lab, an incubator is needed along with a CO_2 system to obtain the 37^{0}C, humidified and 5% CO_2 proprieties of ideal cell grow environment. Although laboratory equipment offers are extensive, there is a lack of items when aiming for consistency of all these characteristics meanwhile recreating the mechanical conditions of shear stress. The dynamic stimuli of ADSCs differentiation towards EC involve the activity of a small engine that while functioning increases incubator's temperature. For this purpose we imagined a cooling system that using water, allows that the dynamic phase of the protocol to take place in a perfectly controlled environment.

MATERIALS AND METHODS

This work is part of a research grant approved by the Ethics Committee of University of Medicine and Pharmacy Science and Technology from Târgu-Mureş, no 131/2016.

1. *Adipose tissue harvesting, ADSCs isolation and endothelial differentiation*

Under sedation, in aseptic conditions, adipose tissue was harvested from six sheep using a minimally invasive surgical technique.(17) Due to facile surgical approach it was opted for sub dermal fat tissue. Anatomically the paravertebral area was chosen in order to avoid infectious complications. The tissue fragments were immediately transported to the laboratory in sterile culture media (Dulbecco's Modified Eagles Medium - DMEM, 10% Fetal Bovine Serum - FBS and 2% antifungal/antibiotic - AA) where ADSCs were isolated using Zuk's protocol (18), by using chemical, biological and mechanical cell isolation factors in an aseptic, temperature

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controlled environment. ADSCs were cultured in a humidified atmosphere at 37^0C, with 5% CO_2. At the end of the protocol ADSCs were frozen in a previously prepared solution of 70% DMEM, 20% FBS and 10% DMSO (dimethylsulfoxide). The obtained cell suspension was placed in cryotubes at a ratio of 2 millions/cryotube. A slow freezing protocol was followed overnight at a rate of 3-4^0C/hour down to a temperature point of about -80^0C degrees. The next day cryotubes were transferred to a -140 ^{0}C freezer.

2. Endothelial differentiation static phase

Thawing of ADSCs was followed by endothelial differentiation. From ADSCs culture #2-#6 a cryotube as randomly thawed for this protocol. Static phase of differentiation took place in an incubator for an average of 30 days. Initially cells were cultured on a gelatin coated T75 cell culture flask for seven-eight days in non-differentiating media (Media 199, FBS 13%, AA 12 ml/L, L-glutamina 0.1 g/l, Heparine 7.5 U/ml) with culture media exchanged every three days. Since day eight culture media was enriched with endothelial Cell Growth Supplement (ECGS) 50 ug/ml with media exchange twice a week. ECGS is growing factor, known for its ability to stimulate proliferation of differentiated endothelial cells in culture.(19) Subsequently cells were placed on gelatin coated 6-well plates (50.000/cm^2) on an orbital shaker put in the incubator 37^0C, with 5% CO_2. The dynamic protocol begun only the next day (200 rotations per minute – rpm), allowing cells attachment.

3. Endothelial differentiation dynamic phase- the need

For the last seven days of the protocol, the gelatin coated 6-well plates, situated on an orbital shaker underwent the dynamic phase. Working with cell cultures, the use of an incubator with a constant temperature is mandatory. In the first experiments, due to shaker engine function, incubator's temperature oscillations were observed along with temperature increase with more than 1^0C. To meet this purpose and having in mind that none of the cell culture incubators have the ability to cool the inside environment we imagined and tested a cooling system, using water.

An orbital shaker (GFL®, type 3005, with a size of the rotating table of 330*330 mm, 10 mm movement amplitude, U=220 Volt, 50 Hz, Power = 0.0065 kW) was adapted in order that the engine temperature will not surpass 37^0C. A manufactured system based on an assembly of cooper pipes through which water was circulated repeatedly. The system consisted in 5.3 meters of cooper pipes, having 8 millimeters diameter obtained from a local hardware store; a ventilator; 3 meters of silicone tube with 10 mm diameter; 2 aluminum plates and 34, 4'' cable ties. The cooper pipes were arranged in 17 lines connected fixed on the two aluminum plates, 10 on one side respectively 7 on the other. These pipes were connected using 8 cm silicone tubes jointed with 4'' cable ties. This assembly was interposed in the structure of a common orbital shaker.

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The upper and the down side of the aluminium plate were connected also through a silicone tube fixated with 4'' cable ties (figure no. 2).

The pipe system was connected to an U1 Ultra Thermostatic Water Bath (figure no. 3), produced by Medingen. The mechanical characteristics of the Water Bath were: power: 650 W, U=220V/50 Hz, engine: 1450 rpm, thermostat.

By using this system, the excessive temperature form the incubator is transferred outside, so that consistency of temperature is achieved (figure no. 3).

Figure no. 1. Intercalation of the system in the orbital shaker structure – A – General aspect of orbital shaker (nonskid surface) – B – Metallic protection structure of the shaker – C – Aspect of the upper side of aluminium plate – D – Removal of the plate – E – core of the shaker

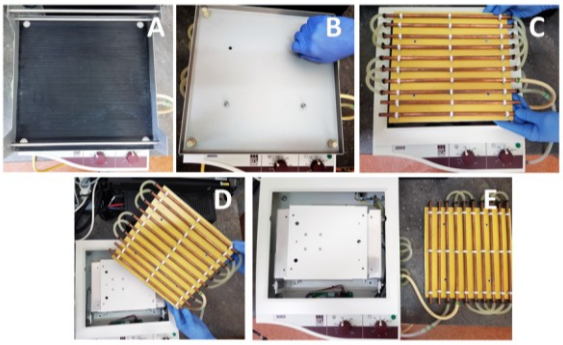


Figure no. 2. Connection between the sides of the aluminium plate – A – Upper side of the plate – B – Silicone tube connecting the two sides – C – Aspect of the down side of aluminium plate (cooper pipes and ventilator

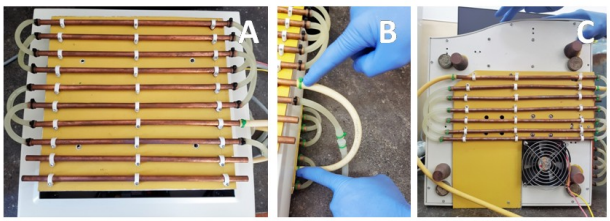


Figure no. 3. General aspect of engineered structure – A – the adapted orbital shaker placed in the incubator –B– the U1 Ultra Thermostatic Water Bath –C– the U1 Ultra Thermostatic Water Bath upper side view –D– Incubator + U1 Ultra Thermostatic Water Bath connected through the silicone tubes

4. Testing the results – immunohistochemistry

For the testing of endothelial cells using immunohistochemistry we started by fixing cells for 30 minutes with 4% paraformaldehyde, followed by rinse, permeabilization with Triton, and ending with 5% BSA block.

The primary antibodies were then added and cells incubated for 2 hours at room temperature. Primary antibodies were all from Abcam: rabbit anti CD31 (diluted 1:200), anti

eNOS (diluted 1:1000), anti vWF (diluted 1:400). After incubation cells were rinsed and incubated with 5 ug/ml secondary antibody goat anti-rabbit IgG labeled with AlexaFluor 488 (Abcam).

After rinsing, cell nuclei were stained with DAPI and images on an Olympus inverted fluorescent microscope. Acetylated, fluorescently labeled low-density lipoproteins (DiI-Ac-LDL) from Cell Applications Inc was added to cells and incubated for 2 hours.

Cell nuclei were stained with DAPI and uptake of the tracer was imaged using the TRITC filter.

The extent of staining was judged as: +++ intense (more than 75% of cells), ++ moderate (40-60% of cells), + weak (10-30%) , +/- very few stained cells (<10%).

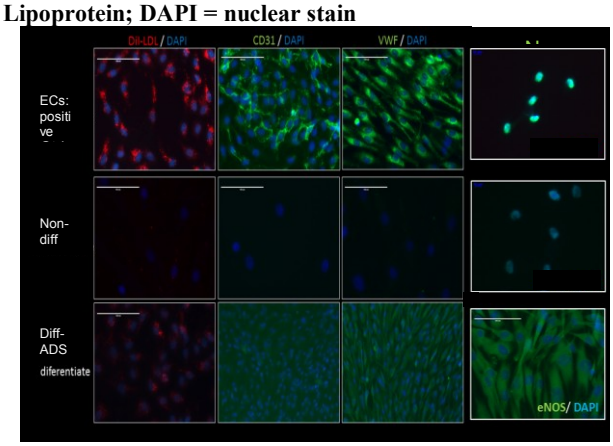
RESULTS

Six ADSCs cultures were obtained, with more than 100 millions cells. ADSCs cultures were frozen and cryopreserved at minus 140^0C, resulting in 52 cryotubes.

Thawing tests reported more than 80% viability.

Five endothelial cell cultures were obtained. Immunohistochemistry testing was positive for specific markers (figure no. 4).

Figure no. 4. ADSCs = adipose derived stem cells; Dif = differentiated; vWF = von Willebrand factor; CD31 = PECAM (Platelet EC Adhesion Molecule); eNOS = EC-derived NO synthase; Ac-LDL = acetylated Low Density Lipoprotein; DAPI = nuclear stain



When making a comparison between cells that followed different protocols, endothelial cells revealed intense expression of eNOS, vWF and Ac-LDL uptake respectively moderate expression of CD31 (table no. 1).

Table no. 1. Summary of differentiation results

Cells	eNOS	vWF	CD31	Ac-LDL uptake
EC controls	+++	+++	++	+++
Non-Diff-ADSCs	+	+	+/-	-
Dif-ADSCs, **STATIC**	+++	+++	+/-	+
Dif-ADSCs, **SHEAR**	+++	+++	++	++

During differentiation protocols incubator's temperature was constant, at 37^0C, without registered variations. Also the usage of this novel system didn't interfere with the CO_2

DISCUSSIONS

This paper describes an option through which researchers can easily adapt basic cell grow laboratory equipments. In order to differentiate ADSCs towards endothelial cells, dynamic preconditioning is mandatory in order to recreate the sheer stress. It was proven that the action of ECGS alone is insufficient for ADSCs differentiation, having a role in just "priming" ADSCs for the final step of the differentiation that takes place in the dynamic phase.(16)

Although various laboratory equipment providers offers in recent years present options with cells grow incubators with orbital shakers incorporated (20-22) or specially designed shaker for cells incubators (23) but they have the downside of a picky price.

This research article comes as a solution when in need for this kind of system whilst having limited financials or time span.

For our study, the shaker was needed in order to recreate the sheer stress, but its utility could be extended for situation in which cell media cultures are more heterogeneous, requiring permanent shaking in order to provide optimal growth environment.

CONCLUSIONS

The cooling system that we imagined represents a viable option when researchers may be in need for dynamic conditions in an environment that requires constant temperature and CO_2 concentration.

By using it in our experiment, on obtaining endothelial cells for ADSCs we certificated it as suitable and practical. With minimal resources this engineered laboratory system replaced the expensive acquisition of brand new shaker incorporated cell incubator.

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Conflict of interest:

Chertes Alexandru is also part of the research team.

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