The effect of sterilization by irradiation of human pericardium and skin frozen tissues on their ability to influence the proliferation of endothelial cells *in vitro*

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Abstract

The delayed wound healing is considered as important medical, social and economical problem. The effective wound management is one of major goals in health system strategy. Various dressing materials were used for this purpose among them skin and pericardium. The aim of the present paper was to compare the effect of these two materials, frozen and radiation-sterilized or non-sterilized, on the proliferation of HECa10 endothelial cells (EC) in vitro.

The influence of tissue samples on EC proliferation was evaluated with the Alamar Blue (AB) reagent, allowing the evaluation of viability and metabolic activity of cells.

Stimulation of EC proliferation was obtained for all used materials except sterilized pericardium, in comparison to the control cultures containing 10% of FBS (fetal bovine serum). No difference was observed between sterilized and non-sterilized skin.

Key words: *biological dressings, pericardium, skin, endothelial cells, proliferation.*

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Introduction

The use of skin as dressing material has a long history. Utilization of skin allograft for wound care was firstly demonstrated in 19th century. In the late 1800s and early 1900s cadaver and animal skin was used to cover burn wounds. These early attempts failed because of immunologic rejection. Later, cadaver skin allografts were used as temporary dressings for burn wounds, removed before rejection and replaced by autografts. Gamma-irradiated human skin allografts were also used as temporary dressings for lower extremity ulcerations [1, 2]. Recently, skin allografts were recognized as an attractive source of multipotent stem cells which can differentiate into endothelial lineage [3-8]. It was also reported that fibroblast growth factor binding protein (FGF-BP) exerted a positive effect on the growth and migration of skin-derived precursors and wound healing [9].

Reduction of immune response and degradation processes may be obtain by various preservation methods. Earlier use of formaldehyde and glutaraldehyde was replaced by deep freezing, cryopreservation with cryoprotectants (glycerol, DMSO) freeze-drying and irradiation. It was postulated, that instead of viability, other aspects, such as antimicrobial safety of the preservation method and cost should be the primary criteria for the choice of method to be used for skin and other tissues allografts [10-13].

Preserved animal and human pericardium was used experimentally and clinically as a material in diaphragmatic

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herniorrhaphy, for urinary bladder muscular wall regeneration, and for abdominal wall reconstruction. In humans, tissue-engineered pericardium was used as wound dressing, total dura substitute in the spine made from polyactide layer and bovine pericardium, and for reconstruction of abdominal wall and diaphragmatic defects [14-20]. Bovine pericardium is commonly used in the fabrication of cardiovascular devices, after decellularization to remove cellular antigens. Pericardium is also identified as a novel source for an autologous scaffold for treating myocardial infarction [21-24].

An often problem with tissue-engineered skin is delayed vascularization. It is important to know, how biological dressings might influence angiogenesis, and how preservation of these materials may change this effect.

The aim of our study was to evaluate the effect of routinely used in Central Tissue Bank sterilization method (irradiation dose of 35 kGy) of human skin and pericardium, on the ability of these tissues to influence proliferation of endothelial cells in *in vitro* cell culture.

Material and methods

Skin and pericardium samples

Experiments were performed on already prepared, non-sterilized or radiation-sterilized skin and pericardium dressings, prepared routinely by the Department of Transplantology and Central Tissue Bank of Biostructure Center, the Medical University of Warsaw. The procedure for collection and preparation of dressings, which meet all the requirements for biological materials intended for clinical application, is covered by legislative regulations, together with appropriate approval of the local ethics committee. Sterilization of deep-frozen tissue samples was performed on dry ice, in a 10 MeV electron accelerator with the dose of 35 kGy. Afterwards, samples were stored at –70°C until their further use.

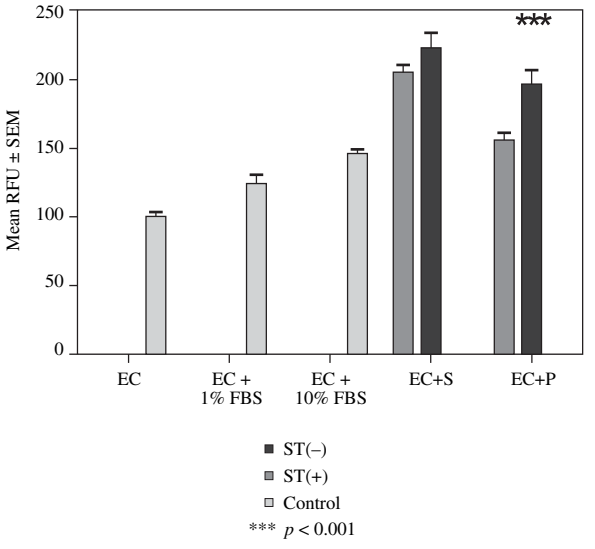

Fig. 1. The effect of sterilized (ST+) and non-sterilized (ST–) samples of human skin (S) or pericardium (P) on the proliferation of HECa10 endothelial cells (EC) *in vitro*

Alamar Blue assay for HECa10 cell proliferation [25]

HECa10 mouse endothelial cell line was kindly provided by Laboratory of Glycobiology and Cell Interactions, Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wrocław. Cells were maintained in 75 cm^2 (BD Bioscience) culture flasks under standard culture conditions of 5% CO_2 at 37°C with medium renewal every 2-3 days.

The cells (in log phase growth stage) were harvested (Accutase, PAA), suspended in FBS (fetal bovine serum)-free culture medium [DMEM, 4.5 g/ml glucose (PAA) with L-glutamine and antibiotics], to the final density 4×10^4 cells/ml and then seeded into 24-well plate (1 ml per well). Next the inserts (0.4 μm, BD) were imposed on each well. The plate (with inserts) was incubated at standard culture conditions of 5% CO_2 in air at 37°C for one hour.

Then, small pieces (0.01 ±0.002 g) of the radiation-sterilized or non-sterilized human skin (S) or pericardium (P) were added to the appropriate upper chamber (the inserts), followed by incubation for 20 hours in standard conditions. The cells cultured in DMEM culture medium alone were used as a reference control, whereas cells cultured in the presence of 1% or 10% FBS (PAA) served as positive control.

After a 20-h exposure the medium and inserts with tissues were discarded and fresh FBS free DMEM medium (1 ml) with Alamar Blue reagent (AB, 100 μl, 1/10 v/v) was added directly to the wells. Cells were incubated for 5 h at 37°C and 5% CO_2. Afterwards, fluorescence was measured (FLUOstar Omega, BMG Labtech) at excitation 544 nm and emission 590 nm.

The results were shown as the relative fluorescence units (RFU) of treated (with tissues) and control cells (without FBS, and with 1% or 10% FBS, respectively). Because the culture medium was not changed during last period, the results are a cumulative value.

Statistical evaluation of the results

Statistical analysis was done by 2-way ANOVA and the significance of differences between the groups was verified by Bonferroni post-test (Graph Pad Prism software).

Results and discussion

The Alamar Blue Assay (ABA) is designed to measure quantitatively the proliferation of various human and animal cell lines. The ABA incorporates a fluorometric/colorimetric growth indicator based on detection of metabol-

ic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. The assay is relatively simple to perform since the indicator is well soluble in water (cell medium).

The results of experiments are presented on Fig. 1. Both irradiated and non-irradiated skin and pericardium tissue fragments have stimulated proliferation of endothelial cells (in culture medium without FBS) as compared to the control cultures maintained without addition of FBS or with 1% of FBS. It is noteworthy, that the proliferation of HE Ca10 cells in experimental cultures with radiation-sterilized pericardium was similar to that of the control group, cultured in the presence of 10% FBS. Furthermore, the results obtained in all other cultures were significantly higher than in 10% FBS control. Interestingly, there was no statistical difference between the results of radiation sterilized and non-sterilized skin.

The results of the study may suggest that the radiation-sterilized skin possesses the endothelial cells proliferation – stimulatory potential similar to that of non sterilized one. This finding is of great practical value, since it confirms that the accelerated electron beam does not affect visibly the beneficial properties of irradiated skin allografts. In contrast to that, the radiation-sterilized pericardium dressing may require some additional processing, since the irradiation visibly decreases its pro-angiogenic potential. Therefore, it seems to be necessary the development of some additional mechanisms which could support the better growth of newly formed blood vessels.

Nowadays, various approaches in tissue engineering have been established, among them addition of various autologous cell types, pro-angiogenic growth factors, hypoxia, etc. [26-30]. Another solution might be a local use of synthetic or natural immunostimulatory and angiostimulatory substances displaying antimicrobial or anti-viral activity, blood sera, cytokine-stimulated stem cells [31-39]. However, no reports regarding such modification of pericardium tissue have been published till now. Therefore, this issue will require further studies.

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