

The influence of sterilized and non-sterilized amniotic dressings on the proliferation of endothelial cells *in vitro*

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Abstract

The amnion is considered as a rich source of factors beneficial for tissue regeneration. However, some studies revealed, that amniotic membrane also displays some anti-angiogenic properties.

The aim of the study was to compare the effect of radiation-sterilized and non-sterilized amnion dressings, 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.

Results: *Non-sterilized amnion tissue significantly stimulated ($p < 0.05$) and radiation-sterilized samples highly significantly suppressed ($p < 0.001$) EC proliferation, as compare to the control cultures.*

No difference was seen between the control fluorescence values (cultures with 10% of FBS) and cultures with non-sterilized amnion tissue homogenate. Significantly lower EC proliferation was observed in cell cultures containing homogenate of radiation-sterilized amnion membrane ($p < 0.001$).

Key words: *biological dressings, amnion, radio-sterilization, endothelial cells, proliferation.*

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Introduction

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. Extensive skin loss and chronic wounds present a significant problem to the clinician. Then, a number of approaches to use various natural and synthetic dressings have been developed [1-6]. An important component of tissue engineering (TE) is supporting scaffold (matrix upon which cells and tissues grow). The amniotic membrane (AM) is an ideal candidate for creating scaffolds used in tissue engineering [7-12].

The procedure of obtaining amniotic membrane even at the time of cesarean section does not guarantee a complete sterility and its proper sterilization is fundamental. Accordingly, various procedures of the preparation, sterilization and long-term storage of AM samples have been

developed and the effects of these procedures on the total protein and growth factors content were described. Even preserved human AM expresses mRNAs for a number of growth factors and contains several growth factors proteins enhancing epithelialization and suppressing inflammation, what is beneficial for wound healing [13-23]. However, it was also reported that human AM secretes and releases angiogenesis inhibitors: tissue inhibitor of metalloproteinases (TIMP 2), pigment epithelium-derived factor (PEDF) and endostatin [24-29].

Conditioned culture medium of AM suppresses neo-vascularization induced by basic fibroblast growth factor (bFGF), inhibiting the migration and growth of endothelial cells. This unwanted effect of AM may create some limitation of its use in tissue engineering, as vascularization in healing wound is essential for successful tissue repair.

On the other hand, this unwanted effect appears to be beneficial in some other clinical situations, for example in

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ophthalmology, where AM may be applicable for treatment of corneal diseases with neovascularization.

The aim of our study was to evaluate the effect of routinely used in Central Tissue Bank sterilization method (irradiation 35 kGy) of deeply frozen human amnion, on the ability of this tissue to influence proliferation of endothelial cells in *in vitro* cell culture.

Material and methods

Amnion samples

Experiments were performed on already prepared, non-sterilized or radiation-sterilized amnion dressings, prepared routinely by the Department of Transplantology and Central Tissue Bank of Biostructure Center, the Medical University of Warsaw [30]. The procedure for collection and preparation of dressings, which meet all the requirements of biological materials intended for clinical application, is covered by legislative regulations, together with appropriate approval of the local ethics committee. Sterilization of tissue samples was performed after their deep freezing, on dry ice, in a 10 MeV electron accelerator with the dose of 35 kGy. Samples were stored at -70°C until their further use.

Alamar Blue assay for HECa10 cell proliferation [31]

HECa10 mouse endothelial cell line was supplied 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 cm² (BD Bioscience) culture flasks under standard culture conditions of 5% CO₂ at 37°C with medium renewal every 2-3 days. Cells which in log phase growth stage were harvested (Accutase, PAA) and cell count determined, suspended (4×10^4 cells/ml) in culture medium (DMEM, 4.5 g/ml glucose (PAA) with L-glutamine and antibiotics), and then seeded into wells of a 24-well plates (1 ml per well). Next, plates were incubated at standard culture conditions of 5% CO₂ in air at 37°C.

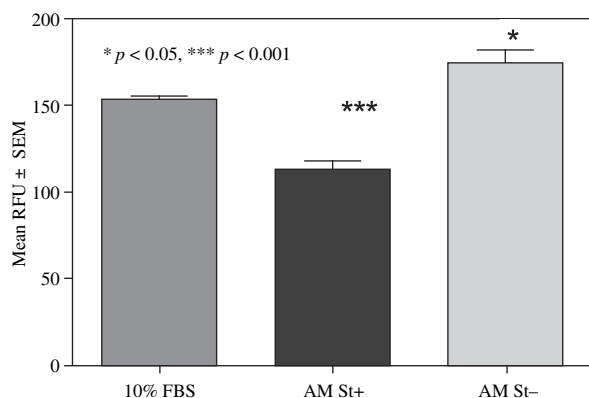


Fig. 1. The effect of human amniotic membrane sterilized (St+) or non-sterilized (St-) on the proliferation of endothelial cells (HECa10) in tissue culture

After an hour the inserts (0.4 μm, BD) were imposed on each well and 100 μl of FBS (PAA) or small pieces (0.01 ±0.002 g) of the radiation-sterilized or non-sterilized AM were added to the upper chamber (to the inserts). Also, 100 μl of the homogenate (0.02 g of the sterilized or non-sterilized AM in 1 ml of FBS free DMEM; mechanically homogenized, incubated 1 h under standard culture conditions and centrifuged at 1000 G) was added to inserts. As a negative control a cells suspended in the DMEM medium (without FBS) were used. Next the plates were returned to the incubator.

After a 20-h exposure medium and inserts with tissues were discarded and fresh FBS free DMEM medium (1 ml) with Alamar Blue (AB, 100 μl, 1/10 v/v) was added directly to the wells. Cells were incubated for 5 h at 37°C and 5% CO₂. After this time, fluorescence was measured (FLUOstar Omega, BMG Labtech) at excitation 544 nm and emission 590 nm.

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

Statistical evaluation of the results

The results obtained from 36 cell cultures were analyzed. 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

Alamar Blue assay of cells proliferation has been shown to be an alternative to the ³H-thymidine incorporation assay [30]. Previously, in experiments with endothelial cells cultures exposed to 900 MHz electromagnetic field we obtained comparable results by these two methods [32].

The results of experiments with amniotic membrane fragments are presented on Fig. 1. Non-sterilized amnion

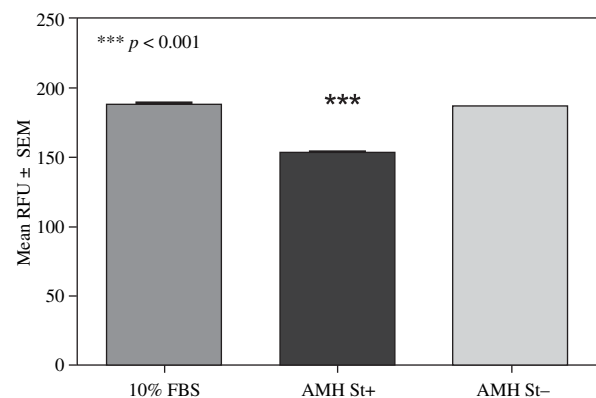


Fig. 2. The effect of sterilized (St+) or non-sterilized (St-) human amniotic membrane homogenate on the proliferation of endothelial cells (HECa10) in tissue culture

significantly stimulated ($p < 0.05$) endothelial cells proliferation. In contrast, sterilized amnion samples significantly diminished Alamar Blue reduction ($p < 0.001$).

The results of experiments performed with AM homogenate are presented on Fig. 2. No difference was seen between the control fluorescence values (cultures with 10% of FBS) and cultures with non-sterilized amnion tissue homogenate. Significantly lower EC proliferation was observed in cell cultures containing homogenate of radiation-sterilized AM ($p < 0.001$).

Then, the results of our present study showed the existence of some angiostimulatory activity in AM before its sterilization, probably connected with growth factors of epithelial origin [12, 18, 23]. Sterilization, however, recovers strong inhibitory activity visible both in cultures with sterilized AM fragments and in cultures with sterilized AM homogenate.

Such inhibitory activity might be dependent on the presence of previously described endogenous anti-angiogenic agents (TIMPs, PEDF, endostatin) as well as on the presence of hyaluronic acid. It has been reported that hyaluronic acid glycans and oligosaccharide exert different effects on the biological function of the vascular endothelial cell, resulting in altered regulation of angiogenesis [33].

Sterilized AM is often used in ophthalmological surgery. Amniotic membrane transplantation has been shown to be effective in the management of ocular surface pathologies, also of inflammatory origin [34, 35]. Amniotic membrane transplantation can be a first – step procedure for ocular surface reconstruction in ocular-cicatricial pemphigoid [36], can be used as an efficient and safe treatment for symptomatic bullous keratopathy, when penetrating keratoplasty is not available [37] and to repair of necrotizing scleritis [38]. Amniotic membrane implantation was described to reduce extraocular muscle adhesions to a titanium implant placed during prior surgery [39]. Amniotic cell culture supernatant, which contains potent inhibitors of angiogenesis, may be applicable for treatment of corneal diseases with neovascularization [40]. Finally, AM may be used as a drug reservoir, slowly releasing antibiotic, especially in infectious keratitis [41]. Anti-inflammatory effect of AM is connected with suppression of inflammatory cytokine production, inhibition of proliferation and induction of apoptosis in various cell populations, among them, peripheral blood mononuclear cells [42].

Other applications of amnion dressings comprise local treatment of chronic wounds and traumatic skin defects [43, 44], venous ulcers [45-47], burns [48, 49] and for preventing prolonged air leakage after thoracic surgery [50].

It is plausible, that in the applications performed from non-ophthalmic indications, the results would be even better by combining amniotic dressing with angio- and immunostimulators, (growth factors and their sources, stimulatory antibiotics and other antimicrobial substances,

immunostimulators and stimulators of angiogenesis of natural origin, and some physical methods [32, 51-59].

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