Original article

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IN VITRO FIBROBLASTS' RESPONSE TO THE TWO COLLAGEN MEMBRANES OF DIFFERENT ORIGIN

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Collagen-based biomaterials are largely used in tissue engineering and regenerative medicine. The sources of collagen for design of those biomaterials are numerous. Although most of collagens are highly biocompatible, the origin can influence the physicochemical and biological properties and guide the final outcome after implantation *in vivo*. Large number of collagen membranes are used in oral and maxillofacial surgery as barrier membranes to cover the tissue defects in order to prevent connective tissue infiltration, and that is why interaction with fibroblasts is crucial to be examined. In this study, we examined the fibroblasts' response to the two commercially available collagen membranes of different origin: porcine vs. equine, in cell culture *in vitro*. The effect of collagen membrane on the proliferation of L929 fibroblasts was examined in a direct cell culture system. Cells were seeded on the collagen membranes and incubated for seven days. Proliferation rate was assessment by MTT test. There was a significant decrease in cell proliferation rate in the presence of both membranes with pronounced anti-proliferative effect of tested porcine membrane. This result speaks in favor of the application of both examined membranes as barrier membranes. Differences in examined collagen membranes may be due to the different origin of collagen although different manufacturing processes may significantly influence cell behavior *in vitro* as well. Further studies with more collagen membranes of various origin should be conducted in order to make final conclusions about the effect of collagen origin on cell behavior *in vitro*.

Key words: collagen, collagen membranes, fibroblasts, L929, *in vitro*, cell proliferation

INTRODUCTION

In order to restore cells, organs, or tissues that have been lost or damaged due to illness or trauma, regenerative medicine and regenerative dentistry aims to develop methods for creating new ones. Regenerative medicine and dentistry include techniques like tissue engineering, the construction of prosthetic organs, and the application of therapeutic stem cells (1). In scaffold-based approaches, cells, signals, biodegradable, and mechanically stable polymeric scaffolds are used to meet specific therapeutic needs and attain excellent cell survival and retention rates (2). In the field of regenerative dentistry, both soft and hard tissues can be restored and regenerated using tissue engineering methods (3, 4). It is an interdisciplinary field that integrates engineering and medical science ideas to produce biological replacements that maintain, repair, or improve tissue function. To treat a variety of tissue defects, tissue engineering combines cells, scaffolds and bioactive substances. Scaffold-based and scaffold-free treatment techniques have dramatically advanced thanks to design of novel functionalized dental biomaterials and regenerative engineering techniques (5).

Collagen is the most important polymer in bone and soft tissue engineering (6). It is the most prevalent protein of the extracellular matrix (ECM) in the mammalian body and it makes up one third of all proteins found in different tissues. Collagen is biocompatible, biodegradable and is neither cytotoxic nor immunogenic (4, 6). Those properties make collagen a gold standard for use in regenerative medicine and tissue engineering. There are different forms of collagen found in mammals, but the most abundant is collagen type I. Collagen can be utilized as a scaffold, membrane, gel or hydrogel, in liposomes, etc (2, 6, 7). The literature describes many resorbable collagen membrane types (2). In tissue engineering, collagen-based membranes are primarily categorized by species: porcine, bovine, equine; and tissue origin: dermis, peritoneum, pericardium, etc (8, 9). The clinician chooses the most

appropriate membrane depending on their characteristics and desired outcome. In addition to supporting wound healing for soft tissue augmentation, collagen-based membranes can serve as a physical barrier to stop connective and epithelial tissue ingrowth into the defect site so that defects can heal properly without forming a scarring tissue (10). The foundation of guided tissue regeneration (GTR) is the idea that placing physical barriers inhibits the flap's epithelium and connective tissue cells ingrowth and creates an isolated area for the inward migration of periodontal ligament cells and resist bacterial contamination (11).

Collagen-based membranes can differ by added additives and manufacturing procedures in addition to variances in indication and origin. Collagen, as part of the ECM, is naturally degraded by the group of endopeptidases, specifically matrix- metalloproteinases (12). Various pathogens, especially periodontal bacteria such as *Porphyromonas gingivalis* and *Treponema denticola* also produce collagenases and may affect degradation time of collagen membranes when implanted in oral region (13, 14). That is important in periodontal, oral and maxillofacial surgery because pathogens can jeopardise the treatment by premature degradation of the membrane. Many cross-linking methods are used to improve the physicochemical properties of collagen and to achieve control of collagen biodegradability time. Chemical cross-linking with agents such as aldehydes improves the mechanical strength and prolongs the time of degradation while physical cross-linking treatment with irradiation or biological using biological agents (transglutaminase and horseradish peroxidase) are nonchemical manufacturing techniques that lead to the control of biodegradability (2, 10, 15). However, it has been shown that modification of collagen by cross-linking techniques can lead to partial cytotoxicity (16-18). Additionally, that the origin of the collagen membrane was reported to influence the physicochemical behaviour of collagen membrane (19).

The aim of this study was to analyse and compare the *in vitro* biocompatibility and fibroblasts' response to the two collagen membranes of different species origin, porcine and equine.

MATERIALS AND METHODS

Collagen membranes

In this study, two commercially available collagen membranes of different species origin, porcine and equine, were analyzed:

- 4BONE RCM (MIS Implants Technologies Ltd., Israel) (membrane labeled as PM in the study) is a resorbable collagen membrane made from porcine skin collagen type I and III. According to manufacturer this membrane has prolonged time of resorption achieved by chemical cross-linking technique using formaldehyde and can be used in GTR as an effective barrier for 4-6 months period.
- PARASORB RESODONT® (RESORBA Medical GmbH, Germany) (membrane labeled as EM in the study) is collagen membrane of equine origin, which contains 2.8 mg of collagen fibrils per 1 cm^2. The production procedure involves cross-linking technique without chemical additives. Membrane is completely absorbable with no need for secondary intervention for removal, according to manufacturer.

Cell culture

L929 mouse fibroblasts were used in this study. The cells were cultured in complete DMEM (low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM stable glutamine and 1% antibiotic-antimycotic solution, all purchased from Capricorn, Germany), in standard cell culture conditions, in a humidified atmosphere at 37°C with 5% CO_2.

Proliferation assay

Prior to cell seeding, membranes were trimmed under sterile conditions to a square with dimensions 1x1 cm and one square membrane was placed per each well of the 24 well culture plate (Greiner Bio-One, Germany). Confluent culture of L929 cells was harvested using Trypsin-EDTA solution (Capricorn, Germany), centrifuged, washed in buffer solution and the number of cells was determined by Trypan blue dye exclusion method. Cells were plated out at density 10^4/well/mL and were directly seeded on examined collagen membranes in 24 well plates in complete DMEM. The cells were incubated on the membranes in standard cell culture conditions for seven days. Cells seeded in wells without membranes, in complete DMEM, incubated for seven days under the same conditions, served as a control culture. Each membrane, as well as control culture, were examined in triplicates. Cells were microscopically analyzed under phase contrast and images were acquired on inverted light microscope Axio Observer.Z1 equipped with the Axio Cam HRc camera and ZEN software, blue edition (Carl Zeiss, Germany). Cell proliferation was assessed by MTT test. Cell medium was removed, cells were washed with phosphate buffered saline and 500 µL of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)) in concentration 1 mg/mL was added per each well. The cells were incubated with MTT solution for the next three hours. MTT is reduced by the mitochondrial dehydrogenases of the living cells and as a product purple formazan is formed. The amount of formed formazan is in a direct correlation with the number of viable cells. Formazan was dissolved with 100% 2-propanol, and absorbance of the resulting solution was measured at 540 and 650 nm wavelengths on multichannel spectrophotometer (Multiskan Ascent plate reader, ThermoLab Systems, Helsinski, Finland). The mean absorbance values were calculated for each tested membrane, as well as for the control. The cell proliferation rate was calculated according to the following

formula: % cell proliferation = (absorbance value of cells incubated with membrane/absorbance value of control cell culture) × 100.

Statistical analysis

The results of MTT test were statistically processed and the mean percentage values were calculated according to above mentioned formula and presented with relative standard deviations. To determine the statistically significant differences between membranes and control culture, one way analysis of variance (ANOVA) test was performed. As statistically significant values were considered those for which $p<0.05$.

RESULTS

Fibroblasts' proliferation on both examined membranes, assessed by MTT test, is shown in Figure 1. There was noticeable difference in the cell proliferation rate among equine-derived (EM) and porcine-derived (PM) collagen membrane as well as compared to the control cell culture.

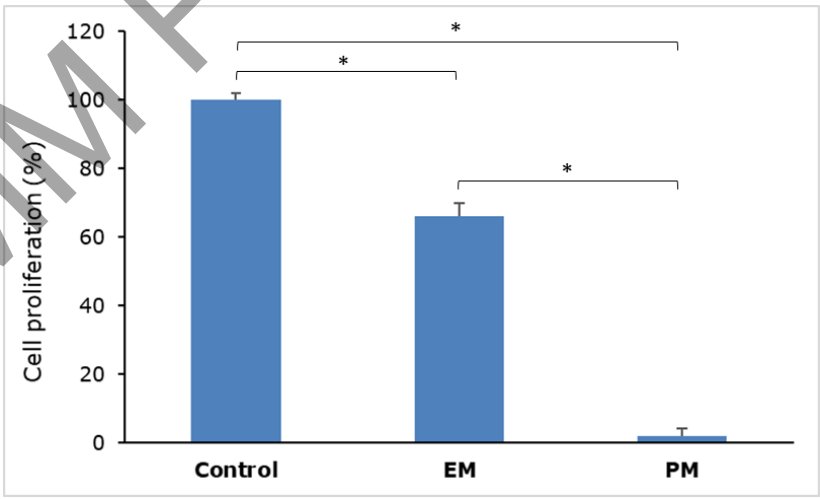

Figure 1. Proliferation of L929 cells in control cell culture, on EM and PM membrane; (*) $p<0.001$.

Interaction of cells with collagen membranes and proliferation pattern were monitored microscopically and images under the phase contrast were made at the end of incubation period, prior to MTT test, which is shown in Figure 2.

Figure 2. Morphological appearance of L929 cells in control cell culture (A), L929 cells cultured on EM collagen membrane of equine origin (B) and L929 cells cultured on PM collagen membrane of porcine origin (C); phase contrast, objective magnification 10x.

Both examined membranes showed anti-proliferative effect and significantly influenced cell proliferation compared to the control cell culture. In addition, PM membrane showed higher anti-proliferative effect than EM membrane. Rare, elongated cells close to, onto and below the PM membrane were noticed, while significantly higher number of cells, without significant morphological changes, were noticed in close proximity to, onto and bellow the EM membrane.

DISCUSSION

There are reports in the literature that origin of collagen may influence the physicochemical properties of collagen membranes and cell behaviour *in vitro*, but most of these studies were focused on comparing porcine and bovine membranes that are largely used in clinical practice. To the best of our knowledge, there are only a few studies with a comparative

analysis of collagen membranes of porcine and equine origin, with focus on defining the influence of the origin to their effects on cell culture *in vitro* (14, 19, 20).

Regarding species origin of collagen, there are some concerns and questions about risk of collagen use and expected tissue response. For instance, the implantation of collagen-based biomaterials of bovine origin carries a risk of transmission of zoonoses such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), or virus-associated diseases, while porcine originated collagen membranes can be rejected due to religious constraints (21, 22). Contrarily, the collagen of equine origin is not associated with virus disease transmission and there is no possibility of rejection due to ethical reasons (23). Furthermore, collagen originated from various species and tissue sources can differ in amino-acid sequence and consequently in its biostability (17, 24). Thus, in this study, we examined two types of collagen membranes of different species origin: porcine vs. equine. We conducted *in vitro* cytocompatibility testing on L929 cell line. *In vitro* testing of biomaterials is the first step when developing new biomaterials. It gives necessary guidance for the *in vivo* testing that comes afterwards. It is less expensive method, experimentally controllable, repeatable and it does not raise any legal nor ethical questions. The biggest disadvantages are that it cannot test chronic effects nor pharmacokinetics (8, 10). In our study, proliferation of L929 fibroblasts was tested after 7 day-cultivation period on two collagen membranes of different species origin. EM membrane demonstrated significantly higher proliferation potential than the PM membrane. Kasaj et al. (20) tested three collagen membranes and EM also showed the highest proliferation potential among tested collagen membranes. Compared to the positive control, proliferation of cells on the tested membranes in our study was significantly lower compared to the control, which is also in accordance with the results obtained in the above-mentioned study. Data from the literature, also, indicate that the membrane of equine origin can be more suitable for cell proliferation compared to

other applicable membranes for the concept of GTR. Kasaj et al. (20) demonstrated the highest rate of human gingival fibroblasts (HGF) proliferation on TutoDent® membrane of bovine origin followed by EM membrane examined in our study as well, at an observation period of one hour and 48 hours, compared to the resorbable membrane of porcine origin and three other non-resorbable membranes. In that study, the resorbable collagen membranes, including EM, induced significantly higher number of cells at all examined periods compared to the non-resorbable membranes in the periodontal ligament fibroblasts (PDLF) cell line (20). Authors suggested that different patterns of cell proliferation can be caused by a difference in surface topography and characteristics as well as in pore sizes (20). Abovementioned findings about the influence of surface topography were confirmed in the study of Willershausen et al. (25) as well, where it was shown that proliferation rate of HGF was higher on the compact layer of two examined native biomaterials of porcine origin, followed by different growth pattern, compared to spongy layer, during observation time of 48 hours. In the study of Toledano et al. (14) difference in biodegradation process analysed *in vitro* between membranes of different origin (porcine vs. equine) was shown, but also different results in degradation tests were obtained between two membranes of equine origin. Through the three different degradation tests, equine collagen membrane covered with equine bone particles was more susceptible to the degradation process in comparison with other membranes, derived from the porcine dermis and equine pericardium tissue (14). The authors assumed that different biodegradation findings in this study can be related not only to different species and tissue origin but also, with the manufacturing process, in this case, the lyophilizing treatment which influenced 3D architecture of collagen (14). Furthermore, scaffold based on native equine collagen (PARASORB Sombrero, RESORBA), the same collagen materials and producer as EM membrane examined in our study, was evaluated as

more suitable for Human-Periosteal Cells (hPCs) proliferation than inorganic scaffolds based on PLGA alone or in combination with Hydroxyapatite (HA) (26).

Based on a comparison of data from our studies and other mentioned findings, we can assume that equine-based collagen material may be a good basis and environment for cell growth but it depends on the type of cells which is going to be seeded, as well as its 3D architecture. Thus, Raimondi et al. (27) showed that native, non-crosslinked collagen type I from equine Achile tendon (commercially available sponge Antema®) is not suitable to support human chondrocyte survival *in vitro* during the observation period of two weeks, even newly synthesized collagen was detected (27). On the other hand, Masci et al. (28) reported that a collagen scaffold of the same origin, is convenient scaffold for proliferation, migration, and adhesion of murine fibroblasts (NIH 3T3), through extended filopodia and macrovesicles shedding (28). There was no literature data about previous PM membrane testing. In our study, cell proliferation rate on PM membrane was significantly lower than one on EM membrane. Previous studies of porcine collagen membranes (25) showed that they caused decreased cellular proliferation and higher cytotoxic effect compared to the collagen membranes of other origins. Also, the porcine membrane was shown to lead to an increased production of proinflammatory mediators by mononuclear cells at 4 and 12 h of incubation and decreased cell viability compared to the bovine membrane (29). Behring et al. (17) suggested that not only the origin of membranes is important, but also the manufacturing process. There are data in the literature about connections between the prolonged period of biodegradation caused by cross-linking modification, with a reduction in biocompatibility (30, 31). Chemical crosslinkers that are frequently used in production of natural polymer-based biomaterials are shown to significantly influence the biocompatibility of biomaterials, making the biomaterials to be cytotoxic for cells (32-34). Naturally-derived chemical crosslinkers are much better solution for the crosslinking process in polymer-based

biomaterials production which was shown in the case where EDC-NHS was compared with genipin for crosslinking of wound dressing material based on alginate and chitosan (35) In our study, the production of EM membrane involves cross-linking technique without chemical additives (information provided by manufacturer), while in the production process of PM membrane, chemical crosslinking method was used (information provided by manufacturer), which could cause pronounced anti-proliferative effect of PM membrane compared to EM membrane. Study by Schorn et al. (36) showed that not only origin, collagen type and modification process such as cross-linking can affect the proliferation rate, attachment, and cytotoxicity rates, but also adding other substrates on the membrane. Results from that study showed higher cell proliferation and cell viability of osteogenic cell lines on Bio-Gide® membrane of porcine origin and RESODONT® membrane of equine origin than the other membranes tested. On the other hand, GENTA-FOIL resorb® membrane of equine origin, with added gentamycin, showed the highest cytotoxicity rate (36). Authors of the same study assumed that the rough surfaces of the RESODONT® and Bio-Gide® membranes might be one of the reasons for their high rates of cell attachment (36).

We must mention the limitations of our study. It cannot provide us with precise information regarding the tissue response to these membranes because it was only carried out on one cell line under controlled *in vitro* cell culture conditions. It merely provides us with the appropriate direction regarding what ought to be anticipated while conducting *in vivo* study, which is the following stage in research of biomaterials intended for regenerative medicine and tissue engineering.

CONCLUSION

Our results show that there is a significant difference in the proliferation rates between cells cultured on examined membranes, in examined conditions. Proliferation of fibroblasts was

significantly reduced in the presence of PM membrane (membrane of porcine origin), while slightly reduced on EM membrane (membrane of equine origin). This suggest that both membranes, particularly PM, may be use as a good barrier membrane to prevent connective tissue ingrowth into the bone defect site. The difference in proliferation of fibroblasts on examined membranes could be due to the different origin of collagen membranes, but also observed differences and anti-proliferative effect could be due to the differences in manufacturing process that may significantly affect the cell growth *in vitro*.

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IN VITRO ODGOVOR FIBROBLASTA NA DVE KOLAGENSKE MEMBRANE RAZLIČITOG POREKLA

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Biomaterijali na bazi kolagena se u velikoj meri koriste u tkivnom inženjerstvu i regenerativnoj medicini. Izvori kolagena za proizvodnju ovih biomaterijala su brojni. Iako je većina kolagena izuzetno biokompatibilna, poreklo kolagena može uticati na fizičko-hemijska i biološka svojstva biomaterijala i usmeriti konačni ishod nakon implantacije *in vivo*. Veliki broj kolagenskih membrana se koristi u oralnoj i maksilofacijalnoj hirurgiji u svojstvu barijerne membrane za pokrivanje defekta koštanih tkiva, kako bi se sprečila infiltracija vezivnog tkiva, pa je zato interakcija ovih membrana sa fibroblastima ključan faktor. U ovoj studiji, ispitali smo odgovor fibroblasta na dve komercijalno dostupne kolagenske membrane različitog porekla, svinjskog i konjskog, u ćelijskoj kulturi *in vitro*. Uticaj kolagenskih membrana na proliferaciju L929 fibroblasta je ispitivan u sistemu direktne ćelijske kulture. Ćelije su zasađene na kolagenske membrane i inkubirane sa njima sedam dana. Proliferacija ćelija je procenjivana MTT testom. Došlo je do značajnog smanjenja proliferacije ćelija u prisustvu obe membrane sa izraženijim antiproliferativnim efektom membrane svinjskog porekla. Ovaj rezultat govori u prilog primeni obe ispitivane membrane kao barijerne membrane. Razlike u ispitivanim kolagenskim membranama mogu biti posledica različitog porekla kolagena, iako različiti primenjeni proizvodni procesi mogu značajno uticati i na ponašanje ćelija *in vitro*. Treba sprovesti dalja istraživanja sa više kolagenskih membrana različitog porekla kako bi se doneli konačni zaključci o uticaju porekla kolagena na ponašanje ćelija u prisustvu ovih biomaterijala *in vitro*.

Ključne reči: kolagen, kolagenske membrane, fibroblasti, L929, *in vitro*, proliferacija ćelija