

SUBCUTANEOUS TISSUE RESPONSE TO THE TWO IMPLANTED COLLAGEN-BASED MEMBRANES OF DIFFERENT ORIGIN

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Abstract

Collagen, as the main structural protein in mammals, fulfils the fundamental requirements to be suitable biomaterial component used in tissue engineering. Due to its biocompatibility and biodegradability, collagen can be utilized in various forms for guided soft and bone tissue regeneration. Collagen-based membranes, frequently used for both soft and hard tissue regeneration, can differ in their origin (porcine, bovine, equine), physicochemical characteristics such as architecture, porosity, absorption ability, and manufacturing processes which may influence tissue response and final outcome. In this study, we examined and compared tissue response to the two implanted collagen membranes of different origin: porcine vs. equine. The subcutaneous implantation model in BALB/c mice was used, and tissue response was evaluated 3, 10 and 30 days after implantation. Tissue was analyzed by histological and histomorphometrical methods. Our study revealed variations in subcutaneous tissue response, patterns of cell infiltration into collagen membranes, changes in membrane thickness and resorption that may be attributed to the differences in membrane origin but also to the differences in the manufacturing process. We can conclude that both membranes are suitable for application in guided tissue regeneration.

Key words: collagen membranes, tissue response, *in vivo*, subcutaneous implantation

ODGOVOR POTKOŽNOG TKIVA NA DVE IMPLANTIRANE MEMBRANE NA BAZI KOLAGENA RAZLIČITOG POREKLA

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Sažetak

Kolagen, kao glavni strukturni protein kod sisara, ispunjava osnovne zahteve da bude odgovarajuća komponenta biomaterijala koji se koriste u tkivnom inženjerstvu. Zbog svoje biokompatibilnosti i biorazgradivosti, kolagen se može koristiti u različitim oblicima za vođenu regeneraciju mekog i koštanog tkiva. Membrane zasnovane na kolagenu, koje se često koriste za regeneraciju mekih i tvrdih tkiva, mogu se razlikovati po svom poreklu (svinjske, goveđe, konjske), fizičko-hemijskim karakteristikama kao što su arhitektura, poroznost, sposobnost apsorpcije, kao i proizvodnim procesima koji mogu uticati na odgovor tkiva i konačni ishod. U ovom istraživanju smo ispitali i uporedili odgovor tkiva na dve implantirane kolagenske membrane različitog porekla: svinjskog vs. konjskog. Koristili smo model potkožne implantacije kod BALB/c miševa, a odgovor tkiva je analiziran 3, 10 i 30 dana nakon implantacije. Tkivo je analizirano histološkim i histomorfometrijskim metodama. Dobijeni rezultati su pokazali da postoje varijacije u odgovoru potkožnog tkiva, obrascima ćelijske infiltracije, kao i promene u debljini membrane i brzini resorpcije što se može pripisati razlikama u poreklu membrane, ali i razlikama u procesu proizvodnje. Možemo zaključiti da su obe membrane pogodne za primenu u vođenoj regeneraciji tkiva.

Ključne reči: kolagenske membrane, odgovor tkiva, *in vivo*, potkožna implantacija

INTRODUCTION

Collagen is one of the most frequently used components of biomaterials in bone and soft tissue engineering due to its biocompatibility and biodegradability [1, 2]. Collagen is a major structural protein in animals and the most abundant protein in the human body [3, 4]. Due to that, collagen-based biomaterials are widely used in hard tissue engineering: for bone, cartilage, and osteochondral defects, as well as in soft tissue for regeneration of cornea, skin regeneration and repair of the blood vessels [5]. Collagen can be used as a membrane, scaffold, gel or hydrogel, in the form of liposome, nanosphere, or as a delivery system of cells, drugs, organic molecules or growth factors [1, 2, 5]. In guided bone regeneration (GBR) collagen-based biomaterials are often used in combination with bone substitute materials based on hydroxyapatite and calcium-phosphate [6-8].

Collagen-based membranes can serve as a physical barrier to impede the ingrowth of connective and epithelial tissue into the defect site, while also promoting wound healing and providing support for soft tissue augmentation [9, 10]. The specific use of each membrane depends on their own characteristics. Various types of resorbable membranes are described in the literature. Collagen-based membranes in tissue engineering are mostly distinguished by species and tissue origin: porcine, bovine, or equine; derived from dermis, peritoneum, or pericardium [10, 11]. Additionally, collagen-based membranes from alternative sources such as some fishes or jellyfish, or human originated were shown as a promising tool for GBR [12, 13].

Besides differences in indication and origin, collagen-based membranes may differ by used additives and manufacturing processes. Many cross-linking methods are used to improve the physicochemical characteristics of collagen and to achieve controllable collagen biodegradability. Collagen-based materials are degraded over time by the enzymes, mostly matrix-metalloproteinases, thus avoidance of secondary intervention can be achieved [9]. Chemical cross-linking with agents such as aldehydes, improves the mechanical strength and prolongs the time of degradation. Physical cross-linking treatment with irradiation or biological using biological agents (transglutaminase and horseradish peroxidase) are non-chemical manufacturing techniques that lead to controllable biodegradability [1, 9, 14]. However, it has been shown that modification of collagen by chemical cross-linking techniques can lead to cytotoxicity and may impair biocompatibility [15-18].

Bearing in mind that collagen membranes of different origin are available on the market, and differences in a behavior regarding the origin described in the literature, our aim was to analyze and compare the tissue response to the two commercially available collagen membranes of different species origin, porcine and equine, in subcutaneous implantation model in mice.

MATERIAL AND METHODS

Collagen membranes

4BONE RCM (MIS Implants Technologies Ltd., Israel) is resorbable collagen-based membrane composed of collagen type I and III, originated from porcine skin (labeled as PM membrane in the study). The prolonged time of resorption for this collagen-based membrane is achieved by chemical cross-linking technique using formaldehyde and can be used in guided tissue regeneration as an effective barrier for 4-6 months period, based on manufacturers' guidance.

PARASORB RESODONT® (RESORBA Medical GmbH, Germany) is equine-derived collagen-based membrane, which contains 2.8 mg collagen fibrils per square centimeter (labeled as EM membrane in the study). This membrane is completely absorbable, produced without the use of chemical cross-linkers.

Animals

The study was performed on animals from the Vivarium of the Scientific Research Center for Biomedicine, Faculty of Medicine, University of Niš, Serbia. All animal procedures in the study were authorized by the local Ethical Commission of the Faculty of Medicine, University of Niš, Serbia based on the approval number 323-07-00278/2017-05/6 of the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (date of approval: July 13, 2017)

In this study, 20 syngeneic male BALB/c mice, age of 8 to 10 weeks, weighing 22 - 24 g, were used. Animals were kept in standard laboratory conditions with an artificial light-dark cycle 12 h each and access to water and food *ad libitum*.

Experimental design

Collagen membranes were implanted subcutaneously right below the scapular region of animals. Prior to implantations, the animals were anesthetized by a mixture of ketamine and xylazine according to standard protocols for mice anesthesia and surgical procedure was performed following described protocols [19, 20]. Animals were shaved and the area of implantation was disinfected with iodine solution. An incision was made on the back and a biomaterial was inserted in formed subcutaneous pockets below the scapulae. The animals were randomly divided into two experimental groups, with 10 animals per group. Experimental groups were: Group PM – implanted 4BONE RCM porcine-origin membrane and Group EM – implanted PARASORB RESODONT® equine-origin membrane. The animals were sacrificed and membranes with surrounding tissue were explanted 3, 10 and 30 days after implantation. Tissue explants were fixed in 10% neutral buffered formalin (NBF) and further processed.

Histology

After fixation in 10% neutral buffered formalin (NBF), explant tissue samples were processed in serial ascending ethanol concentrations, cleared in xylene and embedded in paraffin. Paraffin-embedded tissue blocks were cut on a microtome and tissue slides were stained with standard hematoxylin and eosin (H&E) technique, to visualize tissue structures, cells and implanted biomaterial, and Azan trichrome (AT) specific staining technique for collagen. The light microscope Leica DMR was used for histological analysis while micrographs were recorded with a microscope camera Leica DC 300.

Histomorphometric analysis

Histomorphometric measurements were performed on stained tissue slides micrographs made at 10x objective magnification. NIS Elements software version 2.0 (Nikon, Tokyo, Japan) was used to measure the thickness of both examined membranes. Thickness of membranes was measured at 15 different points, calculated and expressed in μm . Results of membrane thickness measurements are shown as mean \pm standard deviation (SD).

Statistical analysis

Results of histomorphometric measurements were statistically analyzed by performing one-way analysis of variance (ANOVA). The results were presented as mean \pm SD. The statistical significance was set to $p < 0.05$.

RESULTS

Histological analysis

In Figures 1 to 3, histological images of explanted PM and EM membranes with surrounding tissue are presented. Three days after implantation, in PM group, compact membrane structure with randomly distributed pores of unequal size, can be noticed. Mononuclear cells with flattened morphology as well as mononuclear macrophages and different inflammatory cells were found on the material surface (Figs. 1a, 1c). On membrane periphery multinuclear phagocytes also were noticed. In some places mononuclear cells infiltrated PM membrane.

In EM group at 3 days numerous pores can be noticed through the whole membrane structure, evenly distributed, with a thin layer of mononuclear cells, mostly with flattened morphology, on membrane surface with cells started to infiltrate the membrane pores. Rarely presented inflammatory cells were noticed in some spots.

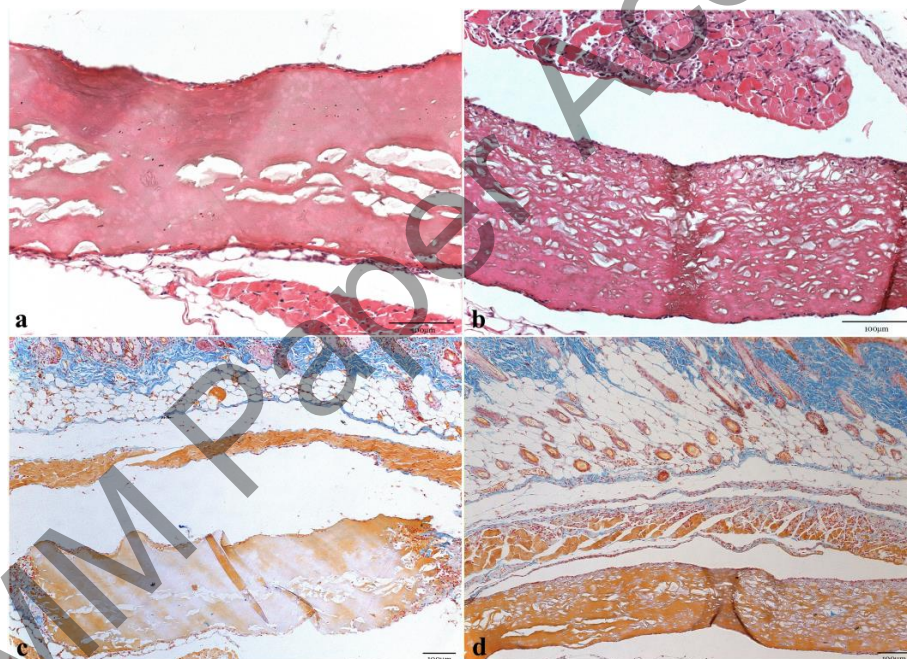


Figure 1. Tissue sections of PM (a, c) and EM (b, d) implants 3 days after implantation, stained with H&E (a, b), objective magnification 20x, 100 μ m scale bar, and AT technique (c, d), objective magnification 10x, 100 μ m scale bar.

Ten days after implantation, PM collagen membrane still looked as a stable barrier, but was less compact than earlier, with more pores than at 3 days. Mononuclear macrophages have been noticed on and within the membrane. A layer of fibroblast-like cells and inflammatory cells on membrane surface, as well as cells infiltrated into large pores through the whole membrane are observed (Figs. 2a and 2c).

Infiltration process was noticed in EM group as well, with cells mostly maintained in peripheral parts of the membrane with visible migration zones towards the inside of the membrane and sporadically cells infiltrated in the center of membrane (Figs. 2b, 2d). In some

spots on the periphery of the membrane, inflammatory cells of leukocyte type were seen. EM membrane was completely stained in blue with Azan staining method (blue color refers to the stained collagen) and the membrane structure closely resembles the structure of native collagen fibers (Fig. 2d).

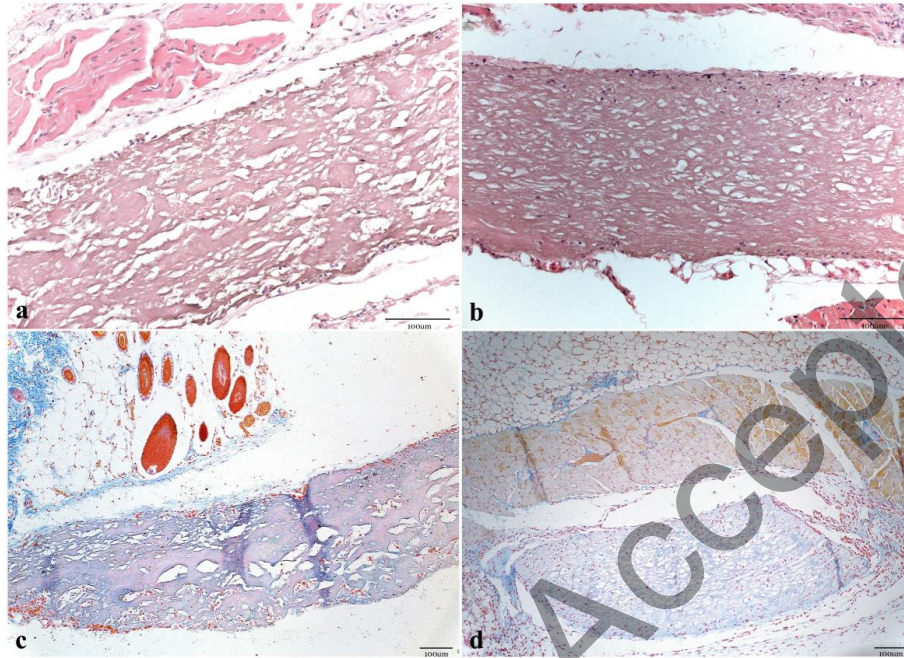


Figure 2. Tissue sections of PM (a, c) and EM (b, d) implants 10 days after implantation, stained with H&E (a, b) objective magnification 20x, 100 μm scale bar, and AT technique (c, d) objective magnification 10x, 100 μm scale bar.

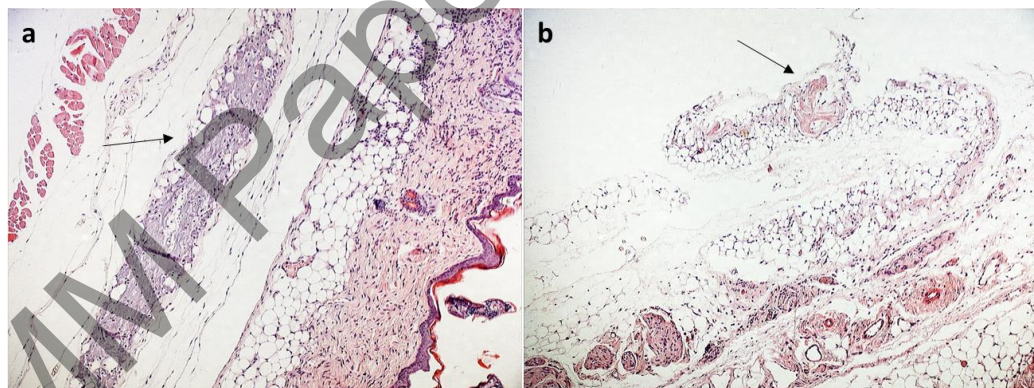


Figure 3. Tissue sections of PM (a) and EM (b) implants 30 days after implantation stained with H&E, objective magnification 10x, 100 μm scale bar, arrows indicate remnants of collagen membranes.

Thirty days after implantation, initial membrane structure was disturbed. Only remnants of examined collagen membranes were noticed in both groups, with larger parts of PM membrane presented compared to the EM membrane (Figure 3). This indicates that degradation process occurred which was more pronounced in the case of EM membrane. Greater stability, even at this time point, was observed in the case of PM collagen membrane compared to the EM collagen membrane. The remnants of both membranes are populated with cells. Resorption of both membranes is noticed over time, which was more pronounced in the case of EM membrane.

The results of histomorphometrical measurements

In both experimental groups statistically significant increase in membrane thickness was observed from 3 to 10 days (Fig. 4), but there was no statistically significant differences between PM and EM membrane at examined time points. The measurement of membrane thickness at day 30 was not performed due to the resorption of membranes and the presence of remnants of membranes only.

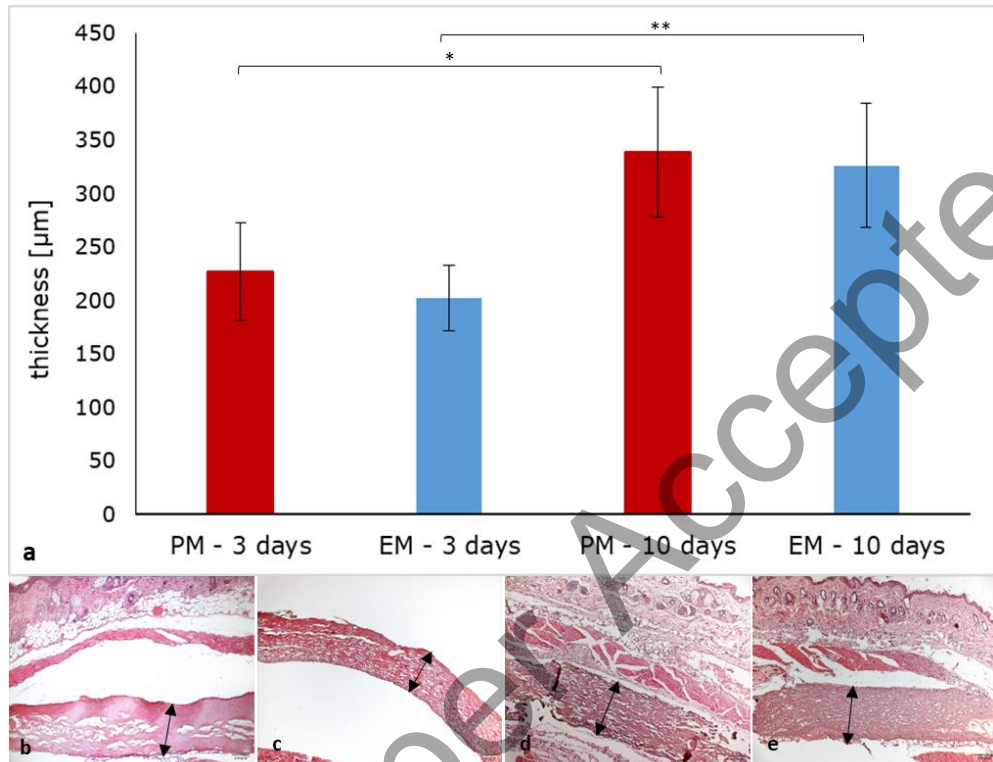


Figure 4. The results of histomorphometrical measurements of membrane thickness (a), results are presented as mean \pm standard deviation. Black arrows on histological images (b-e) indicate measured membrane thickness, H&E staining, objective magnification 10x, scale bar 100 μ m; (*) $p < 0.05$, (**) $p < 0.01$

DISCUSSION

Tissue regeneration guided by biomaterials requires good integration with surrounding peri-implant tissue and induction of regeneration process. Collagen-based membranes have a great application in both bone and soft tissue regeneration, in maxillofacial and oral surgery. Biomaterials based on collagen can induce chemotaxis, adhesion, and angiogenic process but also can stop peri-implant tissue ingrowth into the bone defect when serve as a barrier [9, 10, 21]. Degradation of collagen biomaterials is caused by enzymes, mostly matrix metalloproteinases (MMPs), which can be released by activated polymorphonuclear leukocytes, fibroblasts, and mononuclear phagocytes [4, 9, 15]. There are a lot of commercially available membranes of heterologous origin, with differences in physicochemical characteristics, examined in different studies *in vitro* and on various animal models of implantation [9, 10]. Collagen of different species and tissue origin can diverge in amino-acid sequence which can affect the biostability and resorption time of collagen-based biomaterials [22]. Bozkurt et al. [23] states that it is difficult to define unique conclusions about biodegradability and biocompatibility of collagen membranes from numerous studies, citing that different degradation period of the same collagen membrane of porcine origin, is reported in the literature from different studies. They indicated the importance of a direct

comparison of different collagen membranes, in the same study, in the same animal model as well as applying the same surgical procedure [23]. Thus, in this study, we examined and compared two collagen membranes of different species origin: porcine vs. equine in subcutaneous implantation model in mice, implanted by the same researchers in the same time.

The histological findings showed different membrane architecture, which may be the basis for diverse cell behavior, and expected tissue response. Despite differences in the porosity of these membranes, they looked as a stable barrier at earlier examined time points. PM membrane seemed to be more compact in structure with a lower number of unequal sized pores compared to EM membrane, where numerous pores, more equal in size and evenly distributed are noticed. It is known that the number, architecture, and distribution of pores are important factors that affect cell infiltration and resorption of implanted biomaterial. Different membrane architecture in examined groups was followed by infiltration of the same type cells, but with the difference in infiltration pattern, number, and period of appearance.

A greater number of inflammatory cells was noticed in PM group at a 3-day term compared to the EM group. Time point of 3 days refers to the inflammation phase after tissue incision and biomaterial implantation, followed by infiltration of mononuclear cells, polymorphonuclears, leukocytes, macrophages and others [24]. The noticeable greater infiltration of mononuclear cells was observed in both examined membranes after 10 days compared to 3-day time point. Cells penetration was noticed in bigger pores mainly in PM membrane, while cells were evenly distributed in EM group. On the other hand, mononuclear cells were noticed in a greater number in EM group than PM, at 10 days.

At 10 days, a thin layer of fibrous tissue within boundary of both materials appeared, which showed the beginning of tissue integration.

Histomorphometrical analysis showed a statistically significant increase in membrane thickness at 10 days compared to 3 days. An increase in the thickness of both examined membranes, change in color when stained with AT method from yellowish to blue which is the color of collagen stained by AT method, went parallelly with the phase of cell infiltration. We assumed that increase in cell density together with produced collagen by infiltrated fibroblasts and exposure to body fluids, is the main reason for increased thickness of membranes and observed change in AT staining. It is known that exposure to the body fluids can also affect membrane thickness. In the study of Willershausen et al. [4], was shown that thickness of collagen biomaterials of porcine origin can be changed significantly after swelling in NaCl, and was different in dry, wet, and *in vivo* conditions [4].

There are literature data showing no statistically significant differences between membrane thickness of porcine origin between 3 and 10 days, with a sign of degradation beginning after 10 to 15 days [25, 26]. In the study where subcutaneous rat implantation performed, it was shown that equine-derived collagen hemostatic sponges, which contains twice more content of native non-crosslinked equine collagen fibrils compared to the EM membrane, decreased in thickness at 3 and 15 days, followed by cell infiltration, new vascularization formation, and degradation process up to 30 days after implantation [27]. Hence, it is expected that PM and EM membranes go through the process of degradation under the influence of the collagenase enzymes in longer observation periods.

Literature data suggest that desirable time of membrane degradation *in vivo* should be between four weeks and a few months depending on the clinical outcome that needs to be accomplished [9]. Different biodegradation period was reported for Bio-Gide® collagen membrane of porcine origin, between four weeks [18, 28] and three months [29]. In mice and rat subcutaneous implantation models this membrane was shown to be a stable barrier in a period of two months [15, 23]. In our study, PM membrane is more stable than EM membrane, from the beginning, and 30 days after implantation this membrane has retained its

structure, was completely populated with connective tissue cells and more membrane remnants were presented compared to EM membrane.

In addition, in the study of subcutaneous implantation in rats, it was shown that modification of equine collagen-based sponge to flatted shape by pressing, led to different patterns of cell infiltration, the degradation rate of implanted material, as well as alternation of the inflammatory response [27]. These findings indicated that physical modification of material, may affect tissue response to biomaterial and rate of collagen biodegradability [27, 30].

Overall, the results of our study showed that both examined membranes are suitable for guided tissue regeneration.

EM membrane is more suitable for the cellular environment than PM during examined period. The intensity of blue color after AT staining is higher in EM than in PM group, which is consistent with more fibroblast-like cells observed that may indicate higher rate of collagen production in EM compared to PM, or better recovery of membrane collagen fibers after implantation, up to 10 days. This can also be related to the manufacturing process of examined collagen membranes, since PM membrane was chemically cross-linked while EM membrane was produced without the use of chemical cross-linkers, which are known to prolong the degradability time of collagen-based materials. In the context of these observations, we can assume that these membranes can be applied for different indications in guided tissue regeneration. According to obtained results, EM membrane is better to be used as a collagen matrix for soft tissue engineering, supporting the initial phase of wound healing, due to a high level of cell penetration and faster degradability rate. On the other hand, PM membrane is better to be used as a barrier membrane, due to its lower rate of cell infiltration, and greater stability over time.

CONCLUSION

The results of our study revealed that both examined collagen membranes are suitable for guided tissue regeneration. Examined membranes are biocompatible, with differences in the pattern of cell infiltration and degradation rate, probably due to their different origin, physicochemical characteristics, and different manufacturing process. Nevertheless, further preclinical studies with longer observation periods and other models of implantation, as well as clinical studies, are required to clarify all observed differences in the behavior of these collagen membranes and impact on tissue regeneration in various clinical conditions.

Acknowledgements

This study was supported by the Ministry of Science, Technological Development and Innovations of the Republic of Serbia (Contracts No. 451-03-68/2020-14/200113, 451-039/2021-14/200113, 451-03-68/2022-14/200113, 451-03-47/2023-01/200113 and 451-03-47/2023-01/200113).

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