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In Vitro and *In Vivo* Biocompatibility Analysis of a New Transparent Collagen-based Wound Membrane for Tissue Regeneration in Different Clinical Indications

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Abstract. *Background/Aim: For the treatment of different tissue defects such as jawbone defects, open wound defect, chronic ulcers, dura mater defects and corneal defects, different biomaterials are available. The use of collagen-based materials for these applications has been significantly increased over the past decades due to its excellent biocompatibility and degradability. However, no transparent collagen-based biomaterial is available until now. Thus, a newly developed transparent collagen membrane (TCM) based on natural derived porcine pericardium, which offers numerous application possibilities, was developed. The present study aimed to analyze the in vitro and in vivo biocompatibility using established methods. Materials and Methods: The new TCM membrane and a commercially available collagen membrane (CM, Jason membrane, botiss biomaterials GmbH, Zossen, Germany) were tested for its in vitro cytocompatibility. Furthermore, the in vivo biocompatibility was analyzed using sham operations as control group. In vitro, cytocompatibility was tested in accordance with EN ISO 10993-5/-12 regulations and Live-Dead-stainings. In vivo, a subcutaneous implantation model in BALB/c mice was used and explants were prepared for analyses by established histological, immunohistochemical and histomorphometrical methods. Results: In vitro, both membranes showed promising cytocompatibility with a slightly better direct cell response in the Live-Dead staining assay for the TCM. In vivo, TCM induced a comparable inflammatory immune response after 10 and 30 days with comparable numbers of M1- and M2-macrophages as also found in the control group without biomaterial insertion. Conclusion: The newly transparent collagen membrane is fully biocompatible and is supporting safe clinical application in tissue repair and surgery.*

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Tissue defects as a result of various causalities such as surgical interventions, infections or poorly healing wounds often require the use of regenerative materials. To this day, the use of autologous grafts is considered to be the gold standard (1-3). However, the removal of autologous tissue is associated with co-morbidities at the extraction site as well as a restrictive overall availability (1-3). Moreover, the success rates are dependent on the applied method, the donor with possible comorbidities (*e.g.* diabetes) as well as the skill of the surgeon (4).

Medical devices based on collagen have manifoldly been identified as a reliable treatment option guiding wound healing processes due to the fact that collagen is the most abundant protein of the extracellular matrix guiding cell-matrix-interactions and creating a micro-environment for

wound healing (5, 6). Thus, it has been revealed in a broad variety of preclinical and clinical studies that collagen-based biomaterials provide an excellent biocompatibility (7-9). For example, it has been shown that collagen promotes bone tissue healing, epithelial or skin and cornea regeneration and can also function as a barrier structure for guided bone regeneration (GBR) during jaw bone reconstruction (5, 9, 10).

In this context, different medical indications require for transparent biomaterials. On the one hand, a transparent material could allow for visual inspections such as in case of skin wounds or jawbone reconstructions to check the course of wound healing. On the other side, indications such as cornea regeneration or fixation of a material *via* laser tissue soldering (LTS), which enables for laser-mediated adhesion due to utilization of blood serum proteins such as albumin as bio-adhesives, require for a new class of transparent biomaterials (11).

In the present study, the *in vitro* and *in vivo* cyto- and biocompatibility of this completely new and innovative transparent collagen membrane was analyzed. *In vitro*, direct and indirect cytocompatibility analyses were conducted in accordance with EN ISO 10993-5/-12 and in combination with Live-Dead staining assays. Additionally, a preclinical *in vivo* study up to 30 days *post implantationem* using a subcutaneous implantation model in BALB/c mice with subsequent histological preparation has been conducted (12).

Materials and Methods

Transparent pericardium-derived collagen membrane. The transparent collagen membrane (TCM, test group) was developed on basis of a bovine pericardium membrane (Jason membrane, botiss biomaterials GmbH, Zossen, Germany; further used as control group CM) using a new chemical treatment step integrating formic acid in the decellularization process. During the development process both dermis- and pericardium-derived membranes were tested for its suitability to receive transparency (data not shown). In the end, only the pericardium showed the desired degree of transparency (Figure 1). Moreover, the resistance against collagenases was tested for both the dermis and pericardium derived membranes with the result that only the pericardium-derived materials showed the desired degree of resistance (data not shown).

Ex vivo (ultra-) structure analysis. The (ultra-) structure of the membrane was imaged by scanning electron microscopy (SEM) using a XL30 CP SEM (Philips, Amsterdam, the Netherlands).

In vitro cytocompatibility test. Cytocompatibility analyses were carried out in accordance with the ISO 10993-5/-12 as already described in our previous studies (13-16). The following paragraphs briefly describe the conduct.

Medical grade commercially available titanium grade 4 (negative control) and RM-A (Hatano Research Institute, Food and Drug Safety Center, Japan; positive control) were used as reference materials. The materials were prepared with the same surface areas as the material specimens and sterilized by immersion in isopropanol for 5 min with subsequent drying in a laminar flow hood before use. Blind controls (wells without materials) served as a standard reference.

Figure 1. *Image of the newly developed transparent collagen membrane (upper row) in contrast to a traditional non-transparent collagen membrane.*

L-929 cells from the European Collection of Cell Culture, ECACC (Salisbury, UK) were cultured in cell culture medium [minimum essential medium (MEM)] supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml each) (all from Life Technologies, Carlsbad, CA, USA) and L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 4 mM) under cell culture conditions (37˚C, 5% CO_2, and 95% humidity). Cells were passaged at about 80% confluency.

All test samples were extracted for 72 h at a surface to volume ratio of 3 cm^2/ml under cell culture conditions in cell culture medium. For the blind control, cell culture medium alone was extracted. After 72 h, test samples were removed and extract medium was centrifuged for 10 min at 14,000 rpm.

For the colorimetric assays, 96 well plates were seeded with 1×10^4 L929 cells/well in 100 µl cell culture medium and incubated under cell culture conditions. After 24 h, cell culture medium was removed and extracts were added to each well and further incubated 24 h. Thereafter, BrdU (Bromodeoxyuridine/5-Bromo-2'-Deoxyuridine)-, XTT (Sodium 3,3'-[1(Phenylamino)Carbonyl]-3,4-Tetrazolium]-3is(4-Methoxy-6-Nitro) Benzene Sulfonic acid Hydrate, both: Roche Diagnostics, Mannheim, Germany)- and LDH (Lactate Dehydrogenase; BioVision, Milpitas, CA, USA)-assays were carried out according to the manufacturer´s instructions.

For Live-Dead staining, all test samples were placed in 12-well plates and seeded with 2.4×10^5 L929-cells in cell culture medium under cell culture conditions. After 24 h, 60 µl per ml medium propidium iodide (PI) stock solution (50 µg/ml in PBS) and 500 µl per ml medium fresh fluorescein diacetate (FDA) working solution (20 µg/ml in PBS from 5 mg/ml FDA in acetone stock solution) were added to each well. After 3 min incubation time at room temperature, all test materials were rinsed with prewarmed PBS and immediately examined with an upright fluorescence microscope (Nikon ECLIPSE Ti-S/L100, Nikon GmbH,

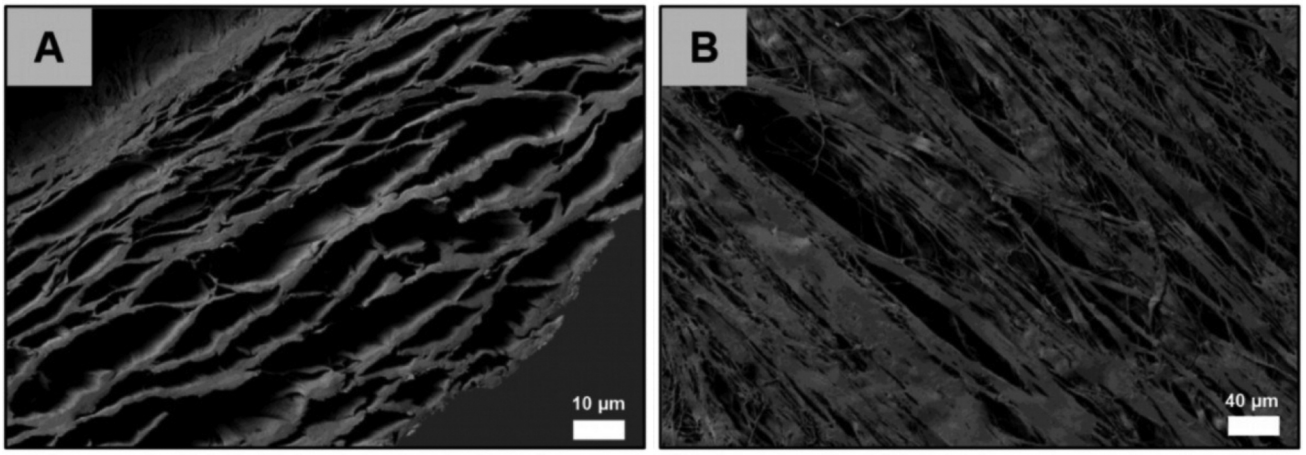

Figure 2. *SEM images of the newly developed transparent collagen membrane. (A) shows a cross section of the transparent collagen membrane that revealed its collagenous nature (200× magnification, scale bar=10 µm). (B) shows the fibrillar surface of the membrane (500× magnification, scale bar=40 µm).*

Düsseldorf, Germany) equipped with a filter for parallel detection of red and green fluorescence.

In vivo biocompatibility test. The *in vivo* study was performed on 20 female 6-8 weeks old BALB/c mice obtained from Military Medical Academy (Belgrade, Serbia) after the approval of the Local Ethical Committee (Faculty of Medicine, University of Niš, Serbia). Thus, n=5 animals per time point were allocated for subcutaneous implantation for each of the two study time points (10 and 30 days). Additionally, 10 experimental animals were allocated for the control group and underwent the surgical procedure without biomaterial insertion. Animals were kept under standard conditions with regular mouse pellets, access to water *ad libitum* and an artificial light-dark cycle of 12 h each.

The implantations were conducted according to a previously established protocol (12, 17). Briefly, an initial intraperitoneal anesthesia [10 ml ketamine (50 mg/ml) with 1.6 ml xylazine (2%)] was applied and the membranes were implanted under sterile conditions in a preformed subcutaneous pocket within the animal's subscapular region. Finally, the implantation wound was stitched with 5.0 Prolene (Ethicon, NJ, USA).

Histological workup and staining methods. After euthanasia of the animals by an overdose of a mixture of ketamine and xylazin, tissue preparation was conducted according to a previously described method (12, 17). Briefly, the implanted collagen membranes were explanted, fixed in 4% formalin for 24 h and subsequently dehydrated in a series of increasing alcohol concentrations and xylol. After that, paraffin embedding and the preparation of histological sections with a thickness of 3-5 µm *via* a rotation microtome (Leica, Wetzlar, Germany) were performed. One slide of each of the three tissue blocks from each animal was stained with hematoxylin and eosin (H&E) for general (histological) evaluation of the material-tissue-interactions.

Histopathological analysis. Qualitative histopathological evaluation of the biomaterial-tissue interactions was conducted using a light microscope (Eclipse 80i, Nikon, Tokyo, Japan). The outcome of the tissue-biomaterial interaction in the specimens was evaluated by examination of the total implantation bed and its peri-implant tissue. In this context, the tissue reactions were described following an established research protocol to access the following characteristics (12): fibrosis, hemorrhage, necrosis, vascularization, presence of granulocytes, lymphocytes, plasma cells, monocytes/macrophages, and multinucleated giant cells. Microphotographs were taken using a DS-Fi1 digital camera and a digital sight control unit (Nikon, Tokyo, Japan).

Histomorphometrical analysis. The histomorphometrical measurements of the extents of M1- and M2-macrophages within the implantation beds of the collagen membrane as well as in the control group without biomaterial insertion were conducted following a previously published method (12). In brief, "total scans" were generated with the aid of a specialized scanning microscope (M8, precipoint, Munich, Germany) connected to a computer system running the respective software. These images contained the complete implant area, as well as the peri-implant tissue. The slides stained by the immunohistochemical methods were digitized. To measure the extent of the cells, the amounts of these cells were manually counted using the "count tool" of the ImageJ software and related to the total implant area (cells/mm^2).

Statistical analysis. Quantitative data are shown as mean±standard deviation after an analysis of variance (ANOVA) and a Tukey's post-hoc test using the GraphPad Prism 7.0d software (GraphPad Software Inc., La Jolla, CA, USA). Statistical differences were designated as significant if p-values were less than 0.05 ($*p \leq 0.05$), and highly significant if P-values were less than 0.01 ($**p \leq 0.01$) or less than 0.001 ($***p \leq 0.001$).

Results

Results of the ex vitro analysis. The analysis of the membrane properties showed that the newly developed transparent collagen membrane provided a material structure

comparable to a native barrier membrane also based on porcine pericardium (Figure 2). The membrane showed a layered structure, which is comparable to the collagen fiber bundles of the original tissue (Figure 2). Also, the material surfaces still showed a fibrillar ultrastructure (Figure 2). No cells or remaining cell remnants were observable (Figure 2).

In vitro results. According to ISO 10993-5:2009, values >70% of the negative control in the XTT and BrdU assays and values <130% of the negative control in the LDH assay represent the non-toxic area (14). All materials, except for the positive control, were found to be non-toxic. In all assays, titanium grade 4 was not significantly different compared to the blind control ($p>0.05$, not displayed), whereas the positive control significantly differed ($p\leq0.001$, not displayed) (Figure 3). Furthermore, the positive control showed highly significantly lower (BrdU, XTT) and higher (LDH) values compared to both test samples ($p\leq0.001$) (Figure 3). Only in the XTT assay, the values of the test samples significantly differed in comparison with titanium grade 4 ($p\leq0.001$) (Figure 3). Both test samples showed no significant differences compared to each other.

In the Live-Dead staining assays, green stained cells represent living cells, while red stained cells represent dead cells (Figure 4). Good cell adherence is expressed by spindle-shaped cells, while rounded cells indicate poor attachment. The positive and negative controls showed the expected cell reaction with red and rounded cells on the positive control and green and spindle-shaped cells on the negative control. Both collagen membranes showed a similar negative control response, while the TCM membrane showed slightly more spindle-shaped cells on the surface.

Histopathological results. The histological analysis revealed that the transparent collagen membrane induced a tissue reaction mainly including macrophages and granulocytes as well as single fibroblasts und lymphocytes at day 10 *post implantationem* (Figure 5A). At this early study time point no multinucleated giant cells were found within the implantation beds of the collagen membranes.

At this time point an inflammatory tissue reaction was also observed in the control group without biomaterial insertion including high numbers of macrophages and granulocytes beside lower numbers of fibroblasts and lymphocytes (data not shown).

The collagen membranes still induced an inflammatory tissue response including mainly macrophages and single granulocytes and fibroblasts at day 30 *post implantationem* that was lower compared to day 10 (Figure 5B). No multinucleated cells have been observed at this study time point. Also, in the control group a moderate inflammatory tissue response was still visible including mainly macrophages and low numbers of granulocytes and fibroblasts (data not shown).

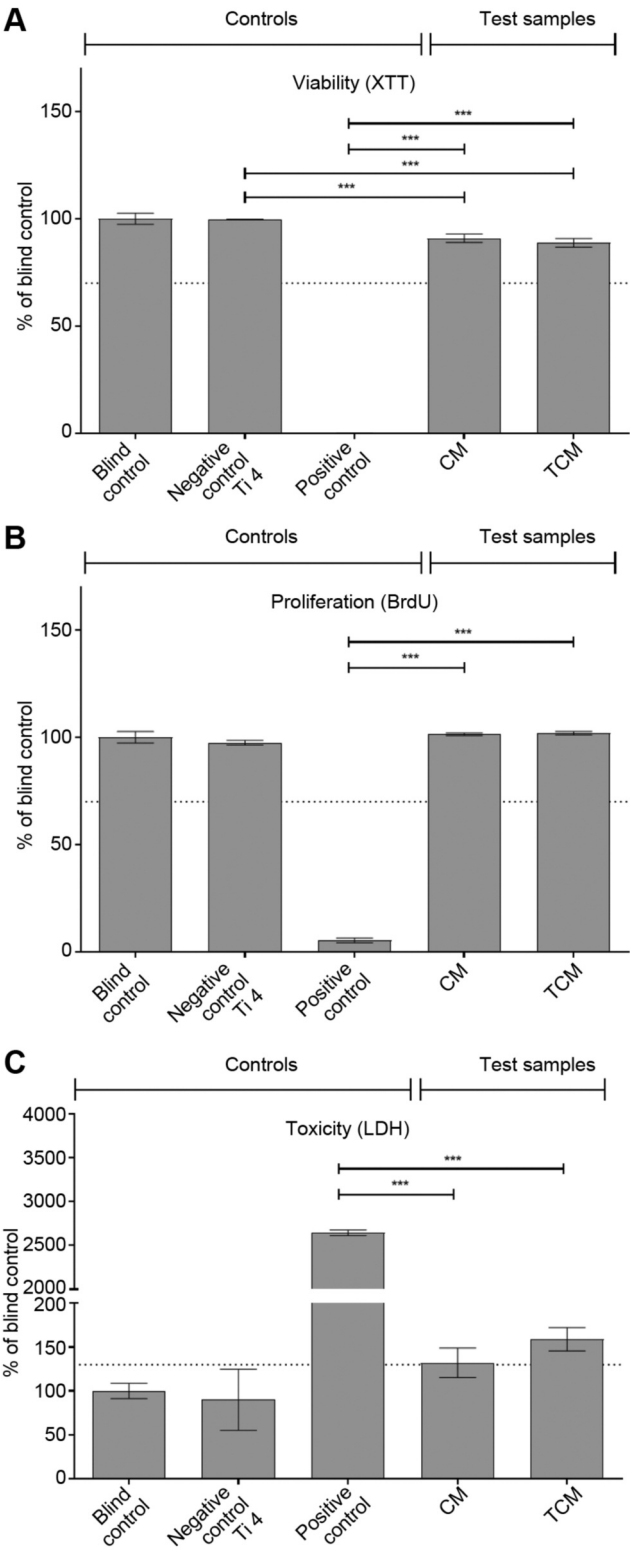

Figure 3. *Extract (colorimetric) assays with L-929 cells of the new transparent collagen membrane (TCM) and the conventional collagen membrane (CM) in comparison with the controls. (A) XTT viability assay. (B) BrdU proliferation assays. (**C**) LDH cytotoxicity assay. Asterixes (*) represent significant differences.*

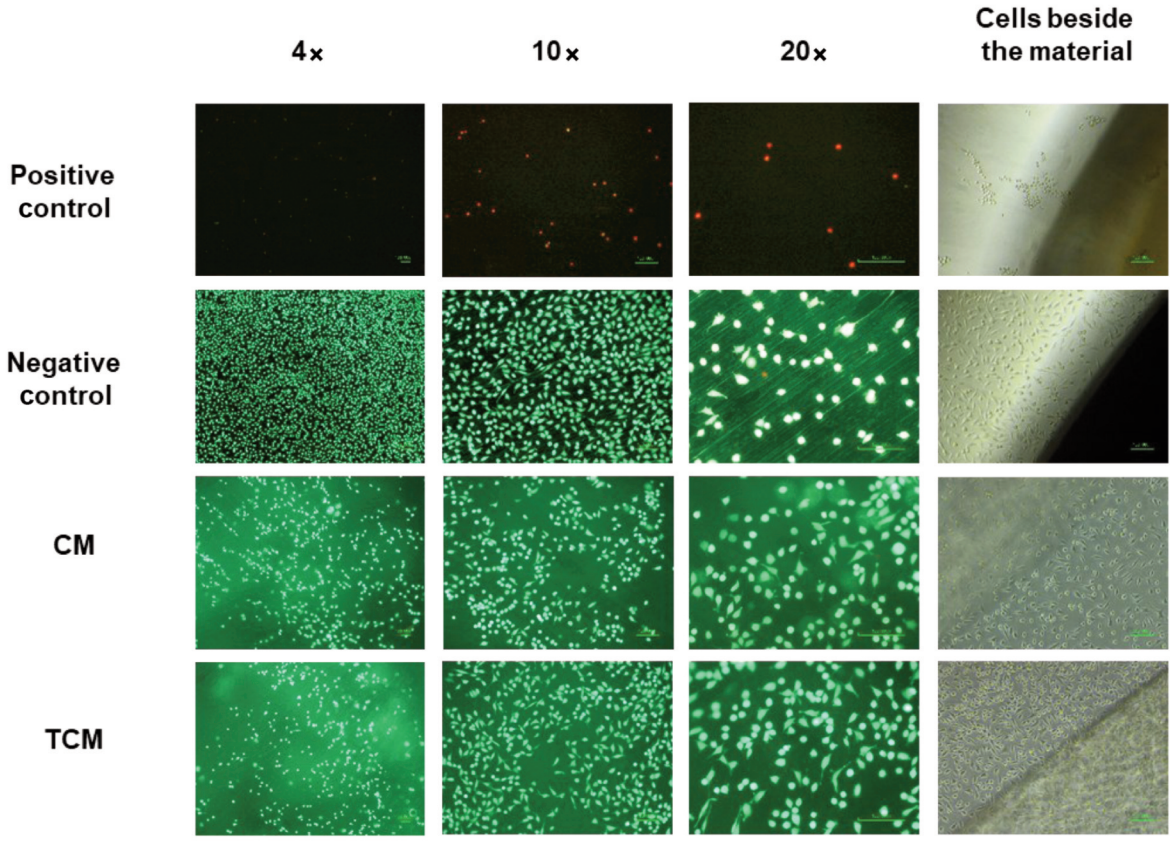

Figure 4. *Live-dead staining assays with L-929 cells of the new transparent collagen membrane (TCM) and the conventional collagen membrane (CM) in comparison with the controls. Green fluorescence represents living cells, red fluorescence represents dead cells.*

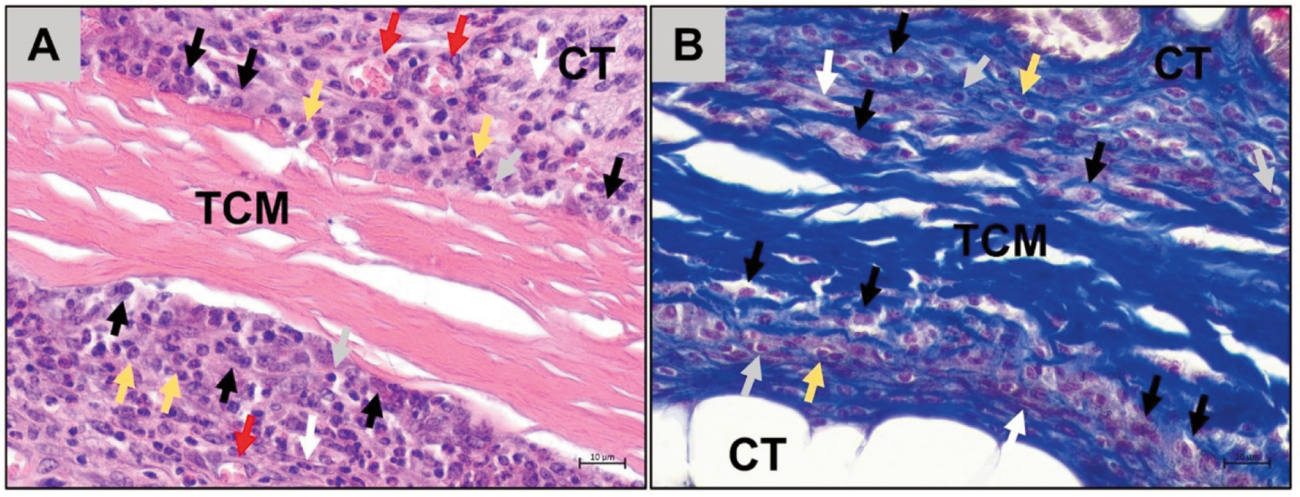

Figure 5. *Representative histological images of the tissue reaction to the transparent collagen membrane (TCM) at day 10 and 30 post implantationem. (A) At day 10 a mild inflammatory tissue reaction mainly including macrophages (black arrows), granulocytes (yellow arrows) as well as single fibroblasts (white arrows) and lymphocytes (grey arrows) combined with high numbers of local vessels (red arrows) was observed (HE-staining, 400× magnification, scale bar=10 µm). (B) At day 30 the severity of the inflammatory tissue reaction decreased. Mainly macrophages were visible (black arrows) beside single granulocytes (yellow arrows) and single fibroblasts (white arrows) and lymphocytes (grey arrows) within the implant beds of the membranes (Masson Trichrome-staining, 400× magnification, scale bar=10 µm).*

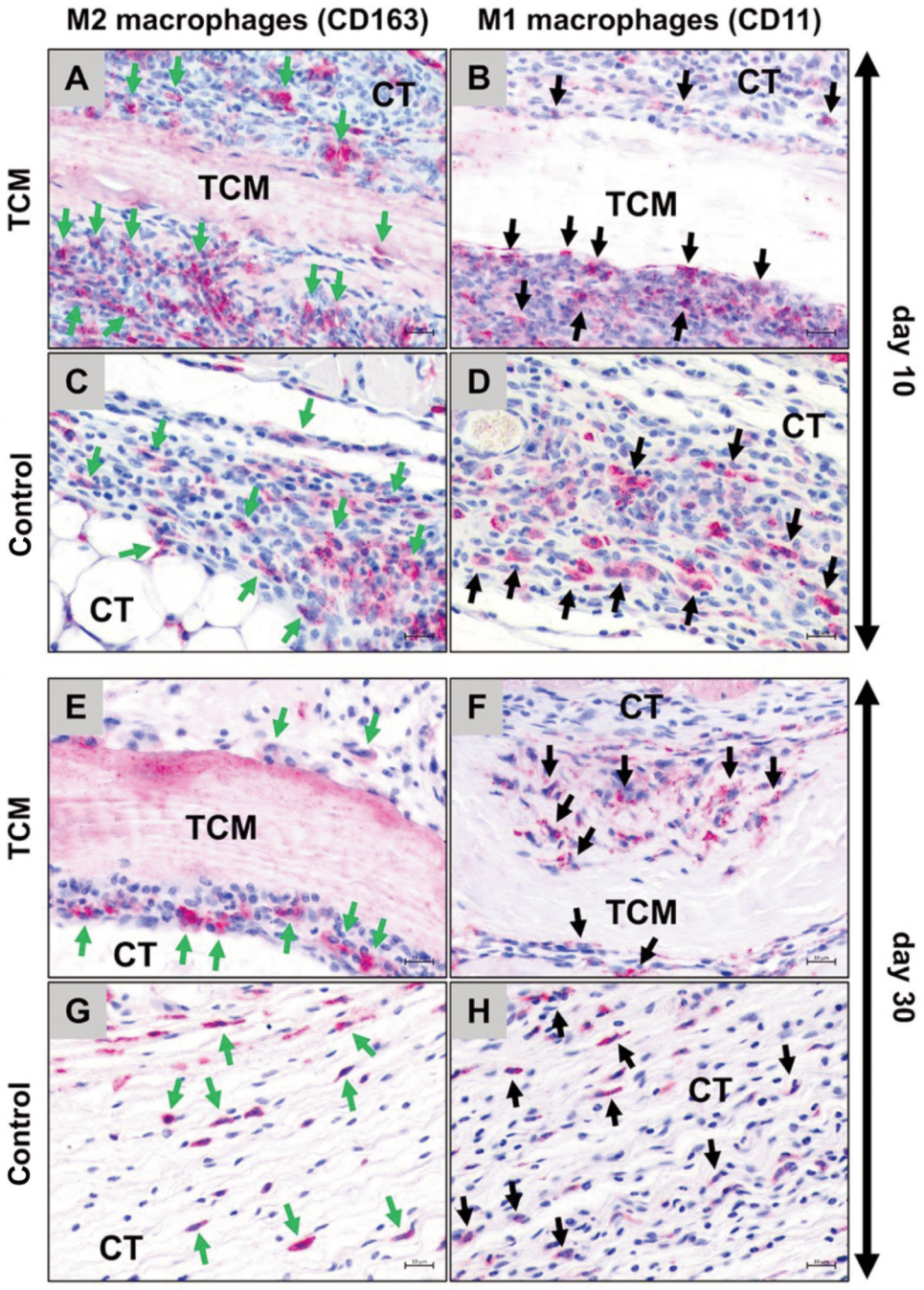

Figure 6. *Representative images of the anti-inflammatory M2- (left row) and pro-inflammatory M1-macrophages (right row) within the implant beds of the transparent collagen membrane (TCM) (A, B, E and F) and in the control group (C, D, G and H) at day 10 and 30 post implantationem. CD163-positive M2-macrophages=green arrows, CD11-positive M1-macrophages=black arrows (CD163- and CD11-immunostainings, 400× magnifications, scale bars=10 μm).*

The immunohistochemical detection of M1- and M2-macrophages showed that comparable numbers of both macrophage subtypes were observable within the implantation beds of the membranes and within the control tissue defect areas at both study time points (Figure 6).

Histomorphometrical results. The histomorphometrical analysis of the occurrence of pro- and anti-inflammatory macrophages showed that comparable high numbers of both subtypes (M2: 472.9±147.3 cells/mm^2; M1: 452.8±107.1 cells/mm^2) were found within the implantation beds of the

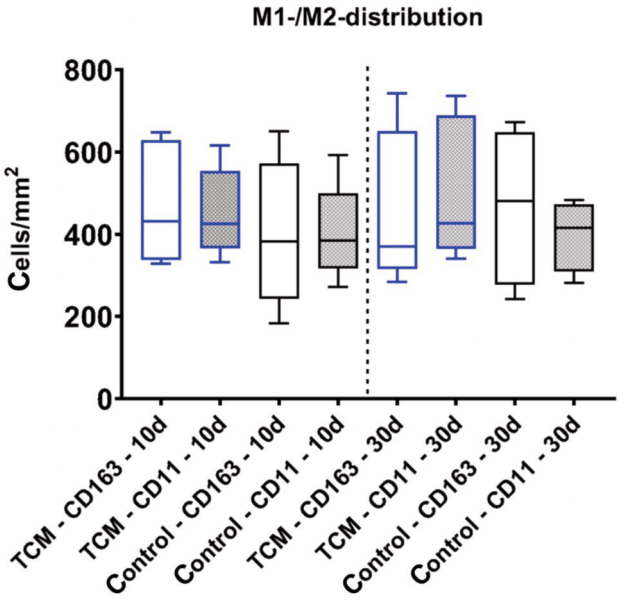

Figure 7. *Results of the histomorphometrical measurements of the occurrence of M1- and M2-macrophages. TCM, Transparent collagen membrane; CD163, marker for detection of anti-inflammatory macrophages; CD11, marker for detection of pro-inflammatory macrophages.*

collagen membranes at day 10 *post implantationem* (Figure 7). The numbers of the M1- and M2-macrophages in the membrane group were comparable to the respective macrophage subtypes in the control group (M2: 402.7±178.7 cells/mm^2; M1: 403.1±117.5 cells/mm^2) (Figure 5). Furthermore, the measurements showed that comparable numbers of both pro- and anti-inflammatory macrophages (M2: 460.3±187.8 cells/mm^2; M1: 506.9±172.0 cells/mm^2) were found within the implantation beds of the transparent collagen membranes at day 30 *post implantationem* (Figure 5). In addition, in the control group without biomaterial insertion comparable numbers of M1- and M2-macrophages (M2: 468.9±192.0 cells/mm^2; M1: 398.8±86.2 cells/mm^2) were found that did also not statistically differ from the respective numbers in the membrane group at day 30 *post implantationem* (Figure 5).

Discussion

Up to date, the gold standard treatment of a variety of tissue defects are autologous grafts (1-3). However, these treatment options are associated with different side effects and varying success rates (1-3). A broad number of biomaterials has already been developed to overcome these issues and most of them are based on collagen. Due to the excellent biocompatibility and degradability, collagen-based materials are most often the first option as substitute materials for many indications (4). In this context, it has been shown that collagen-based biomaterials enable to support tissue healing including bone, epithelial, skin and cornea regeneration and can also function as a barrier structure for Guided Bone Regeneration (GBR) during jaw bone reconstruction (5, 9, 10, 16).

However, different medical indications require for transparent biomaterials. Thus, transparent materials could be applied for external or necessary visible indications such as skin defects, jawbone reconstructions or for cornea regeneration.

Altogether, the aim of the present study was to test the cyto- and biocompatibility of an innovative new collagen-based and transparent wound healing membrane.

In vitro, the control membrane and the new transparent membrane showed promising cytocompatibility in the colorimetric extract and direct Live-Dead staining tests. In accordance with EN ISO 10993-5, the values of both membranes were above the 70% threshold in the BrdU- and XTT assays. In the LDH assay, the newly developed transparent membrane slightly exceeded the 130% threshold. In conjunction with the other *in vitro* test results and because of the highly sensitive LDH assays, this can be regarded as non-critical (14, 16). In the extract tests, both membranes showed similar and insignificant differences in all assays. While both membranes always showed significantly different values in comparison with the positive control, only the XTT assays exhibited significant different values between the negative control and both membranes. However, according to EN ISO 10993-5, both membranes showed cytocompatible test results in the colorimetric assays. In the Live-Dead staining assays, both membranes and the negative control showed similar results with mostly green adherent cells on the surfaces. However, the newly transparent membrane exhibited slightly more adherent, spindle-shapes cells on the surface, indicating improved tissue adherence. Taken together, the innovative transparent membrane showed similar results in comparison with the conventional membrane with slightly improved cell adherence properties.

The *in vivo* study showed that the transparent collagen membrane induced a slightly increased inflammatory response at day 10 *post implantationem*, while the tissue reaction decreased until day 30 *post implantationem*. Additionally, the analysis of the induction of pro- and anti-inflammatory macrophages showed that no differences in their induction were measured at both study time points and also no differences were found between the induction of both macrophage subtypes. Interestingly, no differences of their numbers were found compared to the control group without biomaterial insertion over the complete study time span. Furthermore, no multinucleated giant cells have been observed within the implantation beds of the collagen membranes.

These results indicate the exceptional biocompatibility of the newly developed transparent collagen membrane.

Thereby, it has already been described that optimally decellularized collagen materials should induce a tissue reaction only including mononuclear cells such as macrophages, eosinophils and fibroblasts as a sign for the physiologic collagen turnover (18). Thus, it can be concluded that the new collagen membrane is fully biocompatible. Moreover, the material did not induce an inflammatory microenvironment that differs from the control group and can thus be traced to the implantation process *per se*. In this context, comparable tissue reactions including comparable numbers of M1- and M2-macrophages have also been described in a study conducted by Korzinskas *et al*. that analyzed a non-resorbable polytetrafluoroethylene (PTFE)-based barrier membrane and a manifoldly studied collagen membrane for Guided Bone Regeneration (GBR) (17).

The results further showed that no multinucleated giant cells have been found within their implantation beds up to day 30 *post implantationem*. This cell type, which arises through fusion of monocytes and macrophages, is induced by foreign bodies and a variety of biomaterials and also by different collagen membranes (19, 20). Interestingly, it has been shown in further studies that their induction by collagen membranes may lead to their premature fragmentation and a loss of their barrier functionality (21, 22). Thus, the present results indicate that the newly developed collagen material can provide the desired standing time that is needed for oral wound healing.

In conclusion, the newly developed transparent collagen wound membrane seems to be a promising option for larger tissue defects. In different surgical fields, the new wound patch may allow for quick, easy and precisely fixed wound closure, protecting the wound from bacteria and facilitate the healing process. The possible combination of the transparent collagen membrane and a laser-assisted wound closure may further allow optimal healing conditions.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization: M.B. and J.P.; methodology: OJ, M.B., J.P. and S.N.; validation: O.J. M.B., M.R. and S.N.; formal analysis: M.R.; investigation: M.R. and C.L.; resources: M.B., O.J. and S.N.; data curation: M.B., M.B. and S.S; writing-original draft preparation, M.B., O.J., M.R., C.L. and A.P.; writing-review and editing: S.N. and O.G..; visualization, M.B., M.R. and A.P.; supervision, M.B., O.J. and S.N.; project administration: M.B. and S.N.

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