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Biokompatibilität und Immunantwort einer neuentwickelten volumenstabilen Barrieremembran auf Magnesiumbasis mit PVD-Beschichtung für die gesteuerte Knochenregeneration

Dissertation

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Zusammenfassung

Für die gesteuerte Knochen- oder Geweberegeneration (GBR/GTR) gibt es bislang in der Zahnmedizin keine bioresorbierbaren Alternativen zu nicht-resorbierbaren und zugleich volumenstabilen Membranen. Magnesium (Mg) hat sich als ein gutes Basismaterial für die Entwicklung stabilisierender Strukturen erwiesen. Eine vorzeitige Degradation von Magnesiummaterialien muss jedoch verhindert werden, um sowohl die Funktionalität als auch die Biokompatibilität solcher Magnesium-Implantate zu gewährleisten. Es wurden diverse Beschichtungsstrategien entwickelt, um diesem Problem entgegenzuwirken, von denen die meisten jedoch nicht die gewünschte Funktionalität erbringen. In der vorliegenden Studie wurde ein neuer Ansatz auf Grundlage der Ionenimplantation mittels einer *Physical Vapor Deposition* (PVD)-Beschichtung im Rahmen der Entwicklung einer Magnesium-Membran für GBR/GTR-Verfahren untersucht. Zum Nachweis einer umfassenden Biokompatibilität und erfolgreichen Passivierung der Mg-Membranen wurden unbehandeltes Mg (MG) und beschichtetes Mg (MG-Co) *in vitro* und *in vivo* untersucht. Dabei wurde eine Kollagenmembran mit bereits nachgewiesener Biokompatibilität als Kontrollmaterial verwendet.

Die *in vitro*-Ergebnisse zeigten, dass sowohl die unbehandelten als auch die PVD-beschichteten Membranen nicht zytokompatibel waren. *In vivo* andererseits erfüllen beide Membrantypen jedoch die Biokompatibiliätsanforderungen. Die PVD-Beschichtung hatte im Vergleich zur unbeschichteten Membran keinen Einfluss auf die Bildung von Gaskavitäten, induzierte aber im Vergleich zur reinen Mg-Membran und zur Kollagenmembran eine geringere Anzahl entzündungshemmender Makrophagen. Die reine Mg-Membran induzierte eine Immunreaktion, die mit der Kollagenmembran vergleichbar war.

Insgesamt zeigt diese Studie, dass reine Magnesiummembranen im Vergleich zu den nicht-resorbierbaren volumenstabilen Materialien eine vielversprechende Alternative für die GBR/GTR-Therapie darstellen.

Summary

To date, there are no bioresorbable alternatives to non-resorbable and volume-stable membranes in the field of dentistry for Guided Bone or Tissue Regeneration (GBR/GTR) procedures. Even magnesium (Mg) has been shown to constitute a favorable biomaterial for the development of stabilizing structures. However, it has been described that it is necessary to prevent premature degradation to ensure both the functionality and the biocompatibility of such Mg-based implants. Different coating strategies have already been developed, but most of them did not provide the desired functionality. The present study analyses a new approach based on ion implantation with a Physical Vapor Deposition (PVD) coating for the passivation of a newly developed Mg membrane for GBR/GTR procedures. To demonstrate comprehensive biocompatibility and successful passivation of the Mg membranes, untreated Mg (MG) and coated Mg (MG-Co) were investigated *in vitro* and *in vivo*. Thereby a collagen membrane with an already shown biocompatibility was used as control material. All investigations were performed according to EN ISO 10993 regulations. The *in vitro* results showed that both the untreated and PVD-coated membranes were not cytocompatible. However, both membrane types fulfilled the requirements for *in vivo* biocompatibility. Interestingly, the PVD coating did not have an influence on the gas cavity formation compared to the uncoated membrane, but it induced lower numbers of anti-inflammatory macrophages in comparison to the pure Mg membrane and the collagen membrane. In contrast, the pure Mg membrane provoked an immune response that was fully comparable to the collagen membrane. Altogether, this study shows that pure magnesium membranes represent a promising alternative compared to the non-resorbable volume-stable materials for GBR/GTR therapy.

Abkürzungsverzeichnis

GBR	*Guided Bone Regeneration*
GTR	*Guided Tissue Regeneration*
PGA	Polyglykolsäure (engl. *polyglycolic acid*)
PLA	Polymilchsäure (engl. *polylactic acid*)
PVD	*Physical Vapor Deposition*
MG	unbehandelte Magnesiummembran
MG-Co	PVD-beschichtete Magnesiummembran
PTFE	Polytetrafluorethylen
EZM	Extrazelluläre Matrix
$Mg[OH]_2$	Magnesiumhydroxid
NF-κB	Nuclear factor kappa B

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1 Einleitung

Aufgrund von Erkrankung oder fehlender Funktion von Knochenstrukturen in der Mundhöhle leiden Patienten unter Atrophie des Kieferknochens. Dementsprechend wurden über die Jahrzehnte Methoden entwickelt die zur gesteuerten Geweberegeneration (*Guided Tissue Regeneration*, GTR) dienen (1-3). In den 70er Jahren wurden die Theorien zur Kompartimentierung vorgestellt (4): Zur erfolgreichen Regeneration von dentalen Strukturen (z.B. Zement-, Knochen-, Gingivastrukturen) wurde die Invasion von schnell proliferierenden Zellen (v.a. Epithelzellen) von langsamer proliferierenden Zellen räumlich getrennt. Traditionelle Studien empfahlen hierfür nicht-resorbierbare Membranen (5, 6). Darauf aufbauend wurde das Konzept der gesteuerten Knochenregeneration (Guided Bone Regeneration, GBR) im zahnlosen Kieferkamm etabliert (7).

Für eine erfolgreiche Osseointegration von Dentalimplantaten muss eine gewisse Knochenbreite gegeben sein, die eine stabile und erfolgreiche knöcherne Integration der Implantate erlaubt. In atrophierten Kieferkämmen muss der Knochen wieder regeneriert werden, um die Osseointegration von Implantaten zu gewährleisten. Hierfür wird, ähnlich dem Konzept der gesteuerten Geweberegenation, den Knochenzellen (Osteoblasten) der Raum zur Knochenregeneration geschaffen und zeitgleich soll das Eindringen von schnell wachsenden Gewebezellen (Epithelzellen bzw. Fibroblasten) verhindert werden (8). Durch diesen Barriere-Effekt soll dem inserierten Knochenersatzmaterial innerhalb des knöchernen Implantationslagers, welches den Osteoblasten eine Leitstruktur zur Knochenapposition ermöglicht, ausreichend Zeit gewährt werden, um den Defekt zu regenerieren. Weiterhin ist eine Stabilität des Knochenersatzmaterials durch die Fixierung der Membranen ausschlaggebend. Nur so kann sich das initiale Blutkoagulum bilden, welches im Verlauf der Zeit in regenerierten Knochen umgebildet werden kann (9). Neben der Fixierung durch Schrauben, Pins oder Nähte, verleiht die Rigidität der Membran selbst die notwendige Volumenstabilität für das Knochenmaterial.

Dem Grundprinzip der Zellokklusion und den biologischen Prinzipien der Knochenregeneration entsprechend, spielt die Auswahl einer adäquaten Membran eine zentrale Rolle im Feld der gesteuerten Knochenregeneration. Hierbei wird zwischen synthetischen nicht-resorbierbaren, synthetischen resorbierbaren und

natürlichen resorbierbaren Membranmaterialien unterschieden (10). In klassischen Studien der gesteuerten Knochenregeneration wurden vor allem nicht-resorbierbare Membranen angewandt (5). Dabei werden vor allem Membranen auf Basis des Polytetrafluorethylens (PTFE) bzw. auf Basis des expandierten Polytetrafluorethylens (e-PTFE) als Barrieremembran verwendet (11-13). Auch im Feld der GBR-Therapie werden diese synthetischen und nicht-resorbierbaren Materialien von vielen Klinikern bevorzugt, da sie die Volumenstabilität des Knochenmaterials vorhersagbar beibehalten. Der Nachteil dieser Membranen liegt in einer hohen Membranexpositionsrate von 30% bis 40% (14, 15) sowie der Notwendigkeit eines erneuten chirurgischen Eingriffs, um die Membranen wieder zu entfernen. Basierend auf diesen Nachteilen der nicht-resorbierbaren Membranen wurde nach alternativen Biomaterialien gesucht, deren Einsatz diesen zweiten Eingriff überflüssig machen, was zu der erweiterten Entwicklung resorbierbaren Materialien führte. Zunächst wurden synthetische resorbierbare Membranen populär. Materialien dieser Klasse basieren vornehmlich auf Polymilchsäure (*polylactic acid*, PLA) oder Polyglykolsäure (*polyglycolic acid,* PGA) und deren Co-Polymeren (16). Aufgrund des strukturellen Grundbausteines dieser synthetischen Membranen führt ihr biologischer Abbau zu einem lokalen Anstieg des Säuregehalts (17). Dies schafft ein hinderliches Umfeld für die gesteuerte Knochenregeneration (18, 19). Dementsprechend können synthetisch bioresorbierbare Membranen eine leichte bis schwere Entzündungsreaktion hervorrufen, die Lymphozyten und mehrkernige Riesenzellen anlockt (20). Simion *et al.* hat resorbierbare synthetische Membranen (PLA/PGA) im Vergleich zu nicht-resorbierbaren e-PTFE-basierten Membranen histologisch verglichen (21). Die nicht resorbierbaren e-PTFE-Membranen erwiesen sich als wirksamer, da sie eine größere Menge regenerierten Knochens ermöglichten und dieses Knochengewebe war auch dichter im Vergleich zu dem Gewebe in der Gruppe der PLA/PGA-Membranen. Die Ergebnisse dieser Studie belegten, dass das Ziel der Knochenregeneration durch die nicht-resorbierbaren Membranen vorhersagbarer erzielt werden konnten.

Auf Grund dieser Nachteile der synthetischen resorbierbaren Materialien haben sich die in der Gruppe der resorbieren Membranen mittlerweile vor allem Kollagen-basierte Membranen auf Grund ihrer guten Einheilung durchgesetzt (22, 23).

Bislang wurden etwa 28 Kollagenarten identifiziert, von denen der Typ I in der extrazellulären Matrix (EZM), insbesondere in Geweben wie Sehnen und Knochen,

am häufigsten vorkommt (24). Als Strukturprotein dieser Gewebe ist das Kollagen an diversen (molekular-) biologischen Vorgängen beteiligt und kann als Gerüstmaterial verwendet werden, das die Zellmigration, die Wundheilung und die Geweberegeneration fördert (25). Dies führte zu der Annahme, dass vor allem das Kollagen als Grundlage für Biomaterialien optimal geeignet ist, da es eine geringe Immunreaktivität und gute Verträglichkeit aufweist (26-28). Kollagen kann durch die Interaktion funktioneller Gruppen, durch Einbringen von Vernetzungen oder durch Kopplung mit diversen Moleküle leicht modifiziert werden, um eine Vielzahl von Materialien mit maßgeschneiderten mechanischen oder biologischen Eigenschaften zu generieren (29). Einen der Vorteile der Kollagenmembran stellt das "*turn-over*" des Kollagens dar, welches zu einem Ersatz der implantierten Membranen durch vitales Gewebe führt (30). Die Nachteile von Kollagenmembranen für GTR- und GBR-Anwendungen sind: (i) der Verlust der Fähigkeit zur Raumhaltung (v.a. unter liquiden Bedingungen) und (ii) die Implantation von Kollagen tierischen Ursprungs, welches ein potenzielles Risiko der Krankheitsübertragung vom Tier auf den Menschen birgt (31, 32). Alle Gewebe weisen immunologisch wirksame Bestandteile (z.B. Zellen oder Proteine) auf, welche eine Abstoßungsreaktion induzieren und damit die erfolgreiche klinische Applikation gefährden können (33-35). Dementsprechend müssen die aus tierischen Quellen gewonnenen Gewebe aufgereinigt werden, bevor sie als Biomaterial am Menschen angewendet werden können (36). Die Verwendung von Produkten aus Schweine- oder Rinderkollagen vom Typ I und III ist weit verbreitet.

Kollagenmembranen werden überwiegend durch enzymatische Aktivität (mittels Proteasen und Kollagenasen) von phagozytierenden Zellen (vornehmlich Makrophagen und auch multinukleäre Riesenzellen) degradiert (37). Um den Abbau zu verringern, wurden verschiedene physikalische und chemische Vernetzungsverfahren zur Herstellung quervernetzter Kollagenmembranen eingesetzt (38). Diese Techniken haben jedoch eine zytotoxische und auch proinflammatorische Tendenz und können demenentsprechend eine übermäßige Entzündungsreaktion hervorrufen und zu einer vorzeitigen Membranfragmentierung oder Membranexposition führen (39). Nichtsdestotrotz ist die Vernetzung von Kollagen heutzutage einviel untersuchtes Verfahren in der zahnmedizinischen aber auch in der medizinischen Therapie (40). Beispielsweise verglichen Becker *et al.* in einer prospektiven randomisierten klinischen Studie quervernetzte Kollagenmembranen mit

nativen Kollagenmembranen (41). Die Knochenzunahme war in beiden Studiengruppen vergleichbar, jedoch zeigte die Gruppe der quervernetzten Membranen eine signifikant erhöhte Rate an Membranexpositionen und zudem eine Inflammation des benachbarten Weichgewebes. Dies zeigt, dass die Entwicklung im Bereich der Barrieremembranen speziell für großvolumige Knochendefekte noch nicht abgeschlossen ist.

Ein neuer Ansatz, um die die Limitationen von Kollagenmembranen zu überwinden, ist die Entwicklung von resorbierbaren Barrieremembranen auf Grundlage weiterer im menschlichen Körper existierender Moleküle bzw. Elemente. Magnesiumionen sind ein natürlich vorkommender Bestandteil des menschlichen Organismus und für viele physiologische Prozesse ausschlaggebend (42). Magnesium ist ein biologisch abbaubares Metall, das vom menschlichen Körper ohne toxische Rückstände resorbiert wird (43). Aufgrund des natürlichen Vorkommens von Magnesium im Körper wird es physiologisch über die Nieren und den Darm ausgeschieden (44). Entsprechend den Eigenschaften des Magnesiums, wird die Anwendung von Magnesiummembranen zurzeit in der regenerativen Zahnmedizin verstärkt untersucht (45, 46). Jedoch liegt der Nachteil Magnesium-basierter Biomaterialien bisher in dem unzureichend steuerbaren Degradationsverhalten sowie der damit einhergehenden sog. Pitkorrosion, bei der Magnesiumhydroxid ($Mg[OH]_2$) und Hydrogengas aufgrund der elektrochemischen Reaktion entstehen, und der assoziierten Gastaschenbildung im Gewebe bei deren Abbau im Organismus (47). Im dentalen Bereich und insbesondere beim Einsatz im Rahmen der GBR sollte auf Grund der räumlichen Situation daher eine zu hohe Gasbildung vermieden werden, um nicht das Risiko einer (Material-assoziierten) Dehiszenz zu verstärken.

Um Magnesium-basierte Biomaterialien zu modifizieren und dabei vor allem die Abbaurate und dadurch die Biokompatibilität zu adaptieren, werden neben Legierungsveränderungen vornehmlich verschiedene Beschichtungs-prozesse genutzt (48). Verschiedene Verfahren werden dazu eingesetzt, wobei eines davon die sogenannte *Physcial Vapor Deposition* (PVD), auch Dünnschichtbeschichtung genannt, darstellt. Das Verfahren basiert auf der Freisetzung von metallischen Schichtkomponenten, wobei die abzuscheidenden Metalle im Hochvakuum zunächst in den dampfförmigen Zustand gebracht werden, um deren Ionen an der Oberfläche eines Materials abzuscheiden (49, 50).

So werden sehr dünne und stark haftende Metall- oder Metall-Keramik-Oberflächenschichten ausgebildet, die das Aussehen, die Haltbarkeit und/oder die Funktion eines Materials erheblich verbessern können (51). Diese Technik hat seit den 90er Jahren auch Anwendung in der Zahnmedizin gefunden(49).

Da hierbei die physikochemischen Materialeigenschaften verändert werden, ist jedoch die Biokompatibilität eines solchen Biomaterial-Werkstoffs zunächst unbekannt. In diesem Zusammenhang spielt vor allem die sogenannte materialinduzierte Fremdkörperreaktion, welche vornehmlich Makrophagen neben anderen regulierenden Zelltypen beinhaltet, von großer Bedeutung (52). Diese Kaskade, welche über die Einheilung und auch über die Degradation eines Biomaterials in Abhängigkeit von den Materialeigenschaften und der Biomaterial-induzierten Zellreaktionen entscheidet, stellt eine große Herausforderung bei der Entwicklung von Medizinprodukten dar(53). Ein angepasstes Ausmaß dieser Reaktionskaskade muss durch das Material induziertwerden, um die Funktionalität des finalen Medizinprodukts sicherzustellen (54). Dementsprechend sind präklinische Untersuchungsmethoden in diesem Zusammenhang von großer Bedeutung.

1.1 Ziele dieser Arbeit

Das Ziel der vorliegenden Arbeit lag darin, die vorangegangenen Limitationen von resorbierbaren Membranen zur gesteuerten Knochenregeneration zu überwinden und neuentwickelte Magnesium-basierte Membranen, welche auf der Grundlage der Ionenimplantation mittels PVD-Beschichtung verändert wurden, zu untersuchen. Die Biokompatibilität, das Gewebe-Integrationsverhalten und die Immunantwort dieser neuartigen Membran wurden dabei mittels etablierter *in vitro* und *in vivo* Methoden analysiert (55-58). Hierbei wurde eine Kollagenmembran mit bereits nachgewiesener Biokompatibilität als Kontrollmaterial verwendet (Das Aktenzeichen des Tierversuchs lautet 21 7566/6 21).

2. Publizierte Originalarbeit

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Article

Biocompatibility and Immune Response of a Newly Developed Volume-Stable Magnesium-Based Barrier Membrane in Combination with a PVD Coating for Guided Bone Regeneration (GBR)

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Abstract: To date, there are no bioresorbable alternatives to non-resorbable and volume-stable membranes in the field of dentistry for guided bone or tissue regeneration (GBR/GTR). Even magnesium (Mg) has been shown to constitute a favorable biomaterial for the development of stabilizing structures. However, it has been described that it is necessary to prevent premature degradation to ensure both the functionality and the biocompatibility of such Mg implants. Different coating strategies have already been developed, but most of them did not provide the desired functionality. The present study analyses a new approach based on ion implantation (II) with PVD coating for the passivation of a newly developed Mg membrane for GBR/GTR procedures. To demonstrate comprehensive biocompatibility and successful passivation of the Mg membranes, untreated Mg (MG) and coated Mg (MG-Co) were investigated in vitro and in vivo. Thereby a collagen membrane with an already shown biocompatibility was used as control material. All investigations were performed according to EN ISO 10993 regulations. The in vitro results showed that both the untreated and PVD-coated membranes were not cytocompatible. However, both membrane types fulfilled the requirements for in vivo biocompatibility. Interestingly, the PVD coating did not have an influence on the gas cavity formation compared to the uncoated membrane, but it induced lower numbers of anti-inflammatory macrophages in comparison to the pure Mg membrane and the collagen membrane. In contrast, the pure Mg membrane provoked an immune

response that was fully comparable to the collagen membrane. Altogether, this study shows that pure magnesium membranes represent a promising alternative compared to the nonresorbable volume-stable materials for GBR/GTR therapy.

Keywords: magnesium implant; cytocompatibility; biocompatibility; barrier membrane; dentistry; guided bone regeneration; macrophage

1. Introduction

The regenerative principle of guided bone and tissue regeneration (GBR/GTR) is based on cell exclusivity through barrier membranes [1]. Until now, only nonresorbable membranes mostly based on compounds like dense polytetrafluoroethylene (dPTFE) or expanded polytetrafluoroethylene (e-PTFE) provide volume stability in combination with titanium meshes as so-called titanium-reinforced PTFE membranes [2,3]. However, such materials require a second surgical intervention for extraction and may lead to wound opening and bacterial infiltration with subsequent infection [4]. Up to date, this feature is exclusively reserved for this membrane type making its application indispensable even for multidimensional bony defects.

To overcome the limitations of the currently used non-resorbable barrier membranes, a new generation of membranes has been introduced, combining the advantages of resorbability with volume stability. An initial step in this direction was reported in a publication by Barbeck et al. that showed the good biocompatibility of a new bioresorbable barrier membrane composed of a hydrofluoric acid (HF)-treated magnesium (Mg) mesh embedded in a native collagen membrane for volume stable indications [5,6]. In this study, the HF-treatment was applied for passivation of the implanted magnesium meshes to slow down its biodegradation as it is known that the application of pure magnesium implants may lead to their rapid uncontrolled degradation along with hydrogen gas evolution and related tissue incompatibilities [5]. In this context, a variety of coatings have been analyzed for their suitability to enable the safe application of magnesium implants [5,7,8]. Interestingly, most of the coatings are based on (calcium) phosphate compounds and are thus not suitable for combination with a magnesium-based barrier membrane due to their brittleness and inflexibility [7,9,10]. An appropriate alternative method is coatings made via physical vapor deposition (PVD), which produces thin films on the surface of biomaterials [7,11,12]. This coating technique is mainly used to improve the optical, mechanical or tribological characteristics of different materials such as surgical instruments [7,11,12]. The technique ion implantation (II) includes the bombardment of ionized species and their implantation into the first atomic layers of a solid and can be combined with different other coating techniques [13,14]. Thus, both techniques allow for surface modifications using different implanted ions and an effect on material properties such as mechanical characteristics, antibacterial properties, allergenicity or also corrosion resistance. In this context, it has been used to produce different coatings on the surfaces of magnesium-based implants in order to improve their corrosion resistance [15].

Additionally, it has been suggested that the next generation of biomaterials used for (bone) tissue regeneration should also provide immunoregulative functionalities [16]. Thus, a biomaterial should provide a special composition of physicochemical material characteristics that allow to modify specific immune responses and support tissue regeneration on a molecular level. In this context, it has been revealed that especially macrophages, but also cell types such as resident stem cells are key players in the inflammatory tissue responses to a biomaterial and the regeneration [17–19]. These cell types should be influenced by the biomaterial to act as anti-inflammatory and immunomodulatory elements in the local environment with the aim to trigger bone replacement and optimally processes osteoinduction or osteogenesis [16,20].

The present study was conducted to test the functionality of the PVD coating method to improve the biocompatibility of a newly developed volume-stable Mg barrier membrane. After cytocompatibility assessment, the subcutaneous implantation model in BALB/c mice was used to

analyze the tissue reactions to this new membrane in comparison to an uncoated magnesium membrane and an already available and manifoldly examined collagen-based barrier membrane as already published [2,6,21,22]. Moreover, the immune responses were comparatively analyzed based on the detection of M1- and M2-macrophages based on a previously described protocol [2,23,24]. Established histological (immuno-) histochemical staining methods as well as histopathological and histomorphometric procedures were used for the execution of the present in vivo study [24–28].

2. Experimental Section

2.1. Ion implantation and Physical Vapor Deposition (PVD)

The Mg membranes were passivated by means of ion implantation under argon atmosphere followed by PVD treatment using a specially constructed coating system from the company Ts TriboSystems (Weingarten, Germany).

The deposition process steps were as follows: (a) vacuum level before starting the evaporation of Cr: 1×10^{-5} mbar, (b) heating up to 400–450 °C, (c) argon etching for 30 min, (d) deposition of a thin chromium interfacial layer and (e) deposition of CrN layer. After the deposition process, the treated and untreated Mg-membranes were cleaned in an ultrasonic bath and then dried and packaged under sterile conditions within a laminar flow chamber.

2.2. Cytocompatibility Analysis

Cytocompatibility analysis was conducted in accordance with the ISO 10993-5/-12 as described in our previous publications [5,29–31]. Therefore, the process is briefly summarized:

2.2.1. Reference Materials (Positive and Negative Controls)

RM-A (Hatano Research Institute, Food and Drug Safety Center, Japan) was used as positive control. This reference material induces a given level of cytotoxicity, and it is composed of a polyurethane film containing 0.1% zinc diethyldithiocarbamate [29]. Cell wells and titanium grades 4 and 5 were used as negative controls. All reference materials were prepared and treated in the same way as the experimental samples.

2.2.2. Cell Culture and Conditions

L-929 mouse fibroblasts were obtained from the European Collection of Cell Culture, ECACC (Salisbury, UK). Cells were cultured in cell culture medium under standard cell-cultured conditions. Cells were passaged at around 80% confluency. As 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 4 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) was used at 37 °C, 5% CO_2 and 95% humidity (further referred as cell culture conditions).

2.2.3. Extract Analysis

Extraction of the Biomaterials and the Reference Materials

For the cytotoxicity tests, the extract dilution method was used according to the respective DIN ISO 10993-5/-12 norms. Test and control samples were extracted for 72 h at a surface to volume ratio of 3 cm^2/mL in cell culture medium under cell culture conditions. After extraction, extracts were centrifuged and further utilized in the viability and toxicity assays.

Assay Procedure

A total of 100 µL of the extract was given to 96-well plates seeded with 1×10^4 L929 cells/well in 100 µL cell culture medium. The wells were incubated under cell culture conditions for 24 h. After 24 h, sodium 3,3′-[1(phenylamino)carbonyl]-3,4-tetrazolium]-3is(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT, Roche Diagnostics, Mannheim, Germany) assay, bromodeoxyuridine (BrdU,

Roche Diagnostics, Mannheim, Germany) assay and lactate dehydrogenase (LDH, BioVision, Milpitas, CA, USA) assay were conducted according to the manufacturer's instructions.

2.2.4. Live-Dead Staining

For direct cell viability, all materials were seeded with 2.4×10^5 cells in 1 mL cell culture medium in 12-well plates (surface-area/medium ratio: 5.65 cm^2/mL) for 24 h under cell culture conditions. Staining was achieved by adding 50 µL of the propidium iodide (PI) stock solution (2 mg PI in 1 mL PBS) and 8 µL of the fluorescein diacetate (FDA) stock solution (5 mg FDA in 1 mL acetone) to each well. After 3 min incubation time at room temperature, the test samples were rinsed with prewarmed PBS and examined with an upright fluorescence microscope (Nikon ECLIPSE Ti-S/L100, Nikon GmbH, Düsseldorf, Germany).

2.3. In Vivo Study

The in vivo study was conducted at the Faculty of Medicine (University of Niš, Niš, Serbia) following a previously described protocol following authorization by the local ethical committee (Faculty of Medicine, University of Niš, Niš, Serbia) on the basis of which the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia issued the decision number 323-07-00278/2017-05/6 (Date: 13/07/2017) [2,5,6,21,22]. Thereby, the experimental animals were housed using standard conditions, i.e., water ad libitum, artificial light and regular rat pellets, in combination with standard pre- and postoperative care.

Briefly, the in vivo study was conducted using 30 female, 6–8 week-old BALB/c mice that were obtained from the Military Medical Academy (Belgrade, Serbia). These experimental animals were divided into three study groups with 10 animals per group and 5 animals per time point ($n = 5$), i.e., 10 and 30 days. In brief, after anesthesia of the experimental animals by means of intraperitoneal injection with 10 mL ketamine (50 mg/mL) combined with 1.6 mL xylazine (2%), the subscapular region of the animals was initially shaved and disinfected. Subsequently, an incision was made, followed by blunt preparation of a subcutaneous pocket in which the biomaterials were inserted. Finally, suturing using a standard suture material was conducted.

The explantation included the initial euthanasia of the experimental animals by means of an overdose of the previously described anesthetics. Afterward, the tissue within the implantation area, including the (remnants of the) biomaterials and the peri-implant tissue, were removed and directly inserted into fixation solution (4% formalin solution) for 24 h to be ready for further histological preparation.

2.3.1. Histological and Immunohistochemical Staining Methods

Initially, dehydration by means of increasing alcohol concentrations and a final xylol treatment followed by paraffin embedding was conducted. For further histological processing, sections with a thickness of 3–5 µm were cut using a rotary microtome (SLEE, Mainz, Germany). Afterward, the histochemical staining methods hematoxylin and eosin (H&E), Masson Goldner, Movat's pentachrome and Giemsa were applied on three consecutively taken sections. Immunohistochemical detection of the M1- and M2-macrophage subforms was performed by means of antibodies against the hemoglobin scavenger receptor (CD163) and the transcription factor "nuclear factor kappa-light-chain-enhancer" (NF-kB). Workup and slide preparation were standardized in accordance with previously published methods [2,5,6,21,22]. As a preparatory step for antigen demasking, slides were treated with citrate buffer and Tris EDTA pH 8 buffer (Zytomed Systems, Berlin, Germany) for 20 min in a water bath at 96 °C, followed by equilibration and cooling down using TBS-T buffer. To avoid irregular antibody binding, which leads to unspecific background staining, incubation with blocking solution (Zytomed Systems, Berlin, Germany) was inserted before continuing with the respective first antibody for 30 min. Final chromogenic detection was effected by incubation with the secondary antibody (goat anti-rabbit IgG (H + L) secondary antibody, AP, Invitrogen, Carlsbad, California, USA) and subsequent chromogen exposure with permanent AP-red chromogen

(Zytomed Systems, Berlin, Germany) for 10 min at room temperature. Afterward, counterstaining with diluted hematoxylin (Merck KGaA, Darmstadt, Germany) and bluing was conducted.

2.3.2. Histopathological and Histomorphometric Analyses

The histopathological analysis was conducted based on a previously described protocol to investigate the tissue-biomaterial-interactions by means of a light microscope (Axio.Scope.A1, Zeiss, Oberkochen, Germany) [2,5,6,21,22]. These analyses focused on the evaluation of a variety of parameters within the framework of the early and the late tissue response based on the DIN ISO 10993-6 norm package. Microscopic images were made by means of a connected microscope camera (Axiocam 305 color, Zeiss, Oberkochen, Germany) in combination with a computer system running the ZEN Core 3.0 (Zeiss, Oberkochen, Germany) connected to the microscope.

The histomorphometric analyses were done to compare the occurrence of gas cavities within the implantation beds of the membranes and the induction of M1- and M2-macrophages by the three membranes as previously published [5]. In summary, the slides were initially digitized using a specialized scanning microscope combined with an Axio.Scope.A1 microscope, an Axiocam 305 color digital camera, an automatic scanning table (Maerzhaeuser, Wetzlar, Germany) and a PC system with the ZEN Core 3.0 software (all: Zeiss, Oberkochen, Germany). The measurement of the gas cavities included the marking of the total implant area (TIA, in mm^2) and the gas cavities (in mm^2), which were subsequently related to obtaining the percentage of the gas formation within the implant beds of the membranes. Additionally, the amounts of the immunohistochemically detected pro- and anti-inflammatory macrophages were related to the total implant area (cells/mm^2) after their manual counting.

2.3.3. Statistical Analyses

The statistical analysis included an analysis of variance (ANOVA) and a following LSD post hoc test for a comparison of the data from the different study groups by means of the GraphPad Prism 8.1 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 (*** $p \leq 0.001$). Finally, the data were displayed as mean ± standard deviation.

3. Results

3.1. In Vitro Results

In the cytotoxicity tests, values <130% of the medium control in the cytotoxicity assay (LDH) and values >70% of the medium control in the proliferation and cell differentiation assays (XTT, BrdU) are within the nontoxic range according to ISO 10993-5:2009 (Figure 1) [5,29–31]. Thereby, both treated and untreated magnesium membranes showed values outside these limits in all assays. The values of both membranes more closely approximated the positive control than the other test samples. In both the BrdU and XTT assay, the untreated membrane exhibited higher values than the passivated Mg membrane. In the XTT assay, this difference was highly significant.

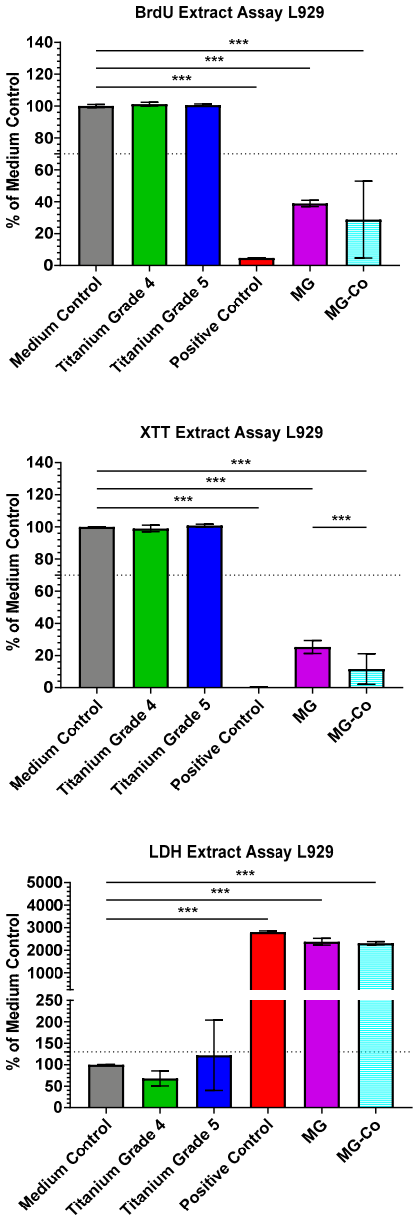

Figure 1. Cytocompatibility of both treated and untreated magnesium variants. Cytotoxicity was measured by LDH-assay; viability was measured by sodium 3,3′-[1(phenylamino)carbonyl]-3,4-tetrazolium]-3is(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay, and proliferation was measured by bromodeoxyuridine (BrdU) assay. Means with error bars indicating standard deviations. The dotted line indicates thresholds that should not be exceeded (lactate dehydrogenase (LDH)) or fall below (XTT; BrdU) (*** $p < 0.001$).

In concordance with the extract results, live–dead staining revealed similar results (Figure 2). Here, green-colored cells indicate living cells, whereas red-colored cells indicate dead cells. Both titanium controls showed green cells with spindle-shaped cell morphology. In contrast, cells on the positive control were red and rounded. The untreated Mg membrane showed mostly green, non-attached cells and gas bubbles as a sign of active corrosion. In contrast, the treated Mg membrane exhibited mostly dead cells, gas bubbles and dark discoloration.

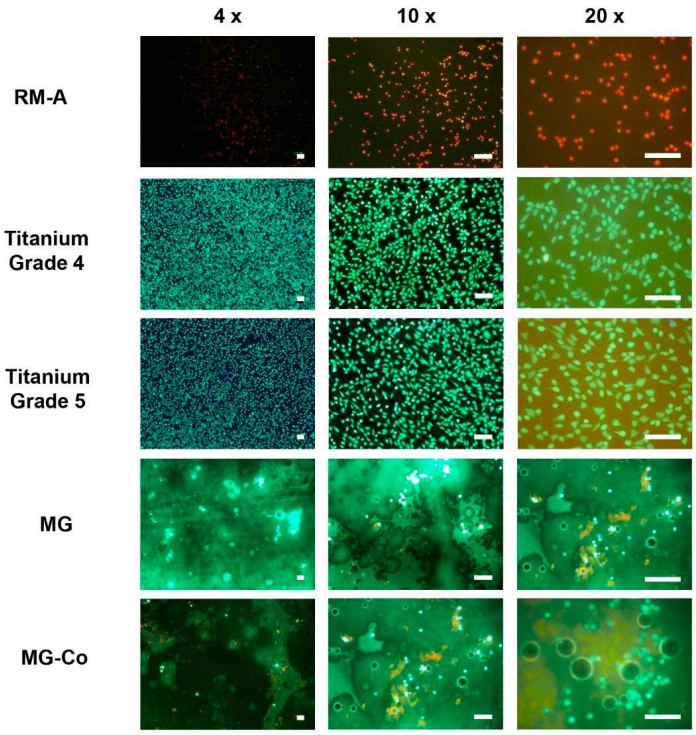

Figure 2. Live–dead staining of the test samples. Pure magnesium membrane = MG, coated magnesium membrane = MG-Co. Green: vital cells; red: dead cells (scale bars = 100 µm).

3.2. Histopathological Analyses

The histopathological analysis showed that all membranes induced an inflammatory tissue reaction at day 10 post implantation within their subcutaneous implantation beds (Figure 3). In addition to high numbers of macrophages, higher numbers of (mostly eosinophilic) granulocytes and fibroblasts were also detectable within the cell multilayers at the material–tissue interfaces (Figure 3). Especially in the study group of the coated Mg-membranes, an accumulation of macrophages was observed directly attached to the material surfaces at this early study time point (Figure 3). Moreover, single mast cells were observable within the reactive cell walls, especially in the groups of both magnesium-based barrier membranes (Figure 3). Furthermore, moderate numbers of blood vessels were especially findable within the surrounding tissue of the pure MG membranes (Figure 3).

On day 30 post implantation, still, the aforementioned cell types were found within the implantation beds of all three membrane types (Figure 3). Macrophages were still the dominating cell type within the surrounding tissue at this time point, while also lower numbers of fibroblasts as well as single (eosinophilic) granulocytes and mast cells were detectable (Figure 3). Moreover, moderate

numbers of vessels were especially found in the study groups of the magnesium-based membranes in the neighborhood of the material surfaces (Figure 3)

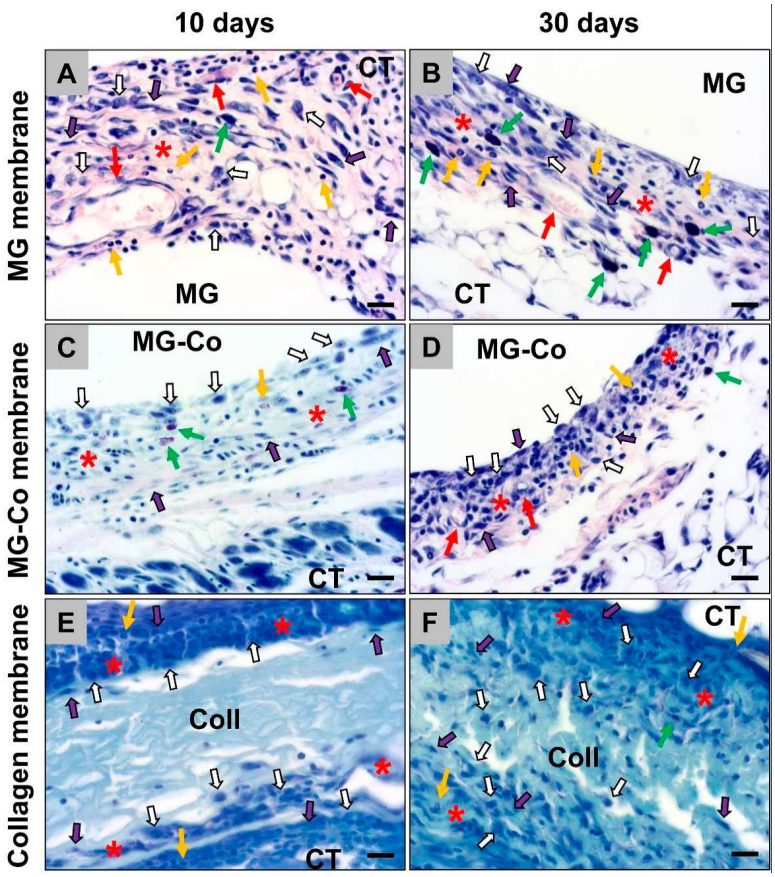

Figure 3. Exemplary histological images of the tissue reactions to the three different membrane types, i.e., the pure magnesium membrane (MG) (**A** and **B**), the coated magnesium membrane (MG-Co) (**C** and **D**) and the collagen membrane (Coll) (**E** and **F**), within the subcutaneous connective tissue (CT) at day 10 and 30 post implantation. Red asterisks = reactive cell multilayer at the material surfaces, white arrows = macrophages, yellow arrows = eosinophils, purple arrows = fibroblasts, green arrows = mast cells, red arrows = blood vessels (Giemsa staining, 400× magnification, scale bars = 10 µm).

The histopathological analysis of the occurrence of anti-inflammatory CD163-positive cells revealed that comparable numbers were detectable in the study groups of the pure MG membrane and the collagen membrane at day 10 post implantation (Figure 4). In the group of the coated MG membrane, lower numbers of this macrophage subtype were detectable at this early time point (Figure 4).

At day 30 post implantation, the histopathological analysis revealed that comparable numbers of CD163-positive macrophages were observable in all study groups (Figure 4). Thereby, tendentially lower numbers of anti-inflammatory macrophages were detected in the group of the collagen membrane (Figure 4).

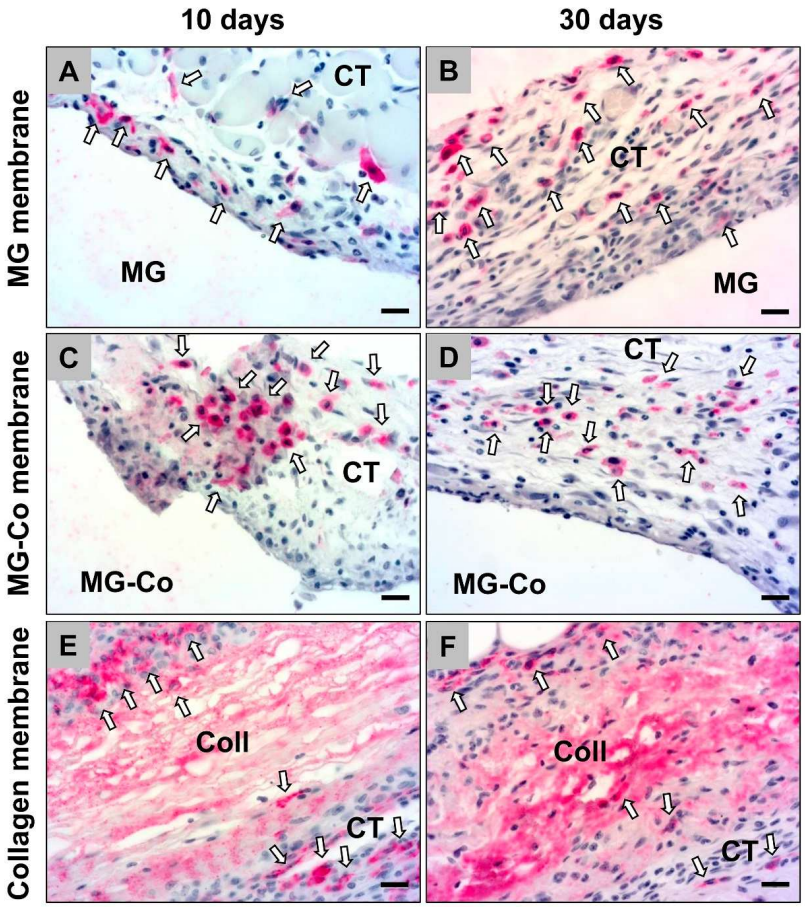

Figure 4. Exemplary histological images of anti-inflammatory (CD163-positive) macrophages (white arrows) within the implant beds of the different membranes, i.e., the pure magnesium membrane (MG) (**A** and **B**), the coated magnesium membrane (MG-Co) (**C** and **D**) and the collagen membrane (Coll) (**E** and **F**). CT = connective tissue (CD163 immunostainings, 400× magnification, scale bars = 10 µm).

The histopathological analysis of the occurrence of pro-inflammatory NF-κB-positive cells showed that comparable numbers of this macrophage subtype were observable in the study groups of the pure MG membrane and the collagen membrane at day 10 post implantation, while lower numbers were detectable in the group of the coated MG membrane were detectable at this study time point (Figure 5).

The histopathological analysis revealed that comparable numbers of CD163-positive macrophages were still observable in all study groups at day 30 post implantation (Figure 5). Thereby, tendentially lower numbers of anti-inflammatory macrophages were still observed in the collagen membrane group (Figure 5).

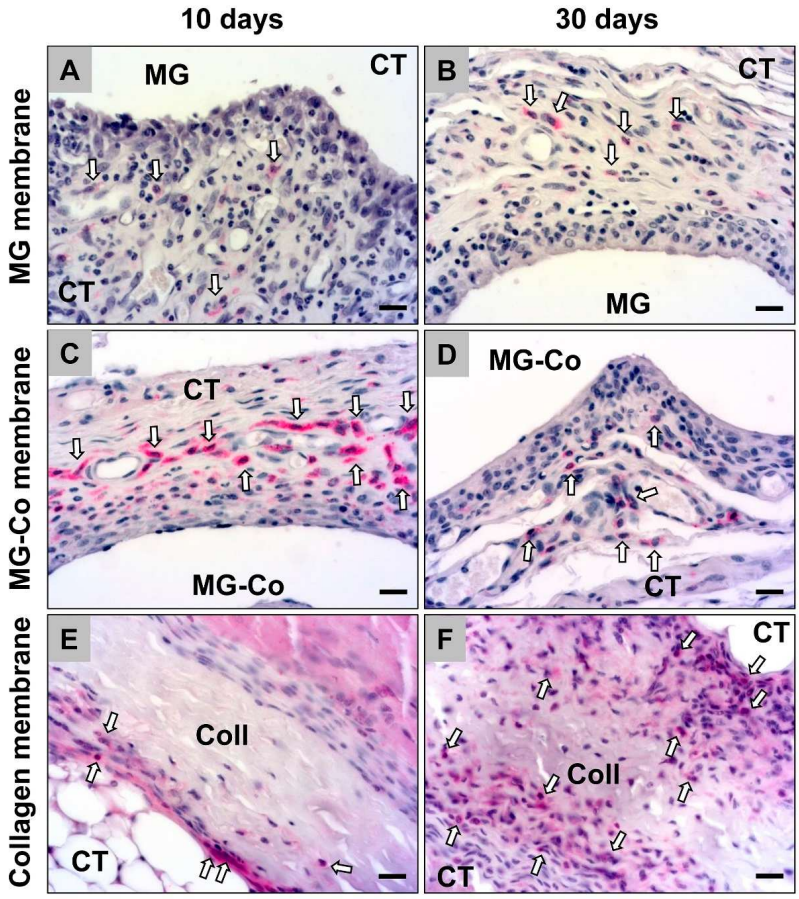

Figure 5. Exemplary histological images of pro-inflammatory (NF-κB-positive) macrophages (white arrows) within the implant beds of the different membranes, i.e., the pure magnesium membrane (MG) (**A** and **B**), the coated magnesium membrane (MG-Co) (**C** and **D**) and the collagen membrane (Coll) (**E** and **F**). CT = connective tissue (NF-κB immunostainings, 400 × magnification, scale bars = 10 µm).

3.3. Histomorphometric Analyses

The histomorphometric analysis of the formation of gas cavities within the implant beds of the pure (MG) and the coated magnesium membranes (MG-Co) showed that comparable values were found at day 10 post implantation (MG: 7.16 ± 2.57%, MG-Co: 12.79 ± 2.98%), while the values in the MG-Co group were tendentially higher (Figure 4). At day 30 post implantation, also comparable values were found in both study groups (MG: 7.37 ± 3.27%, MG-Co: 7.29 ± 4.43%) (Figure 6).

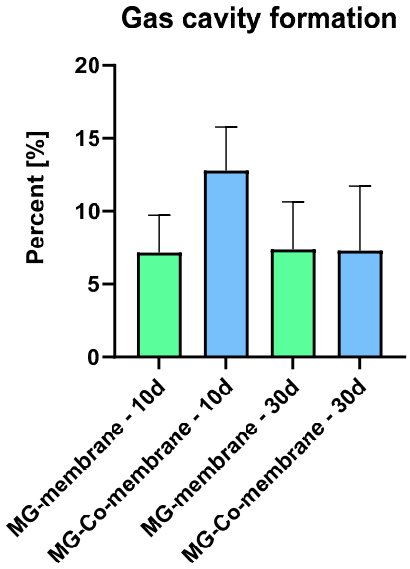

Figure 6. Histomorphometric results of the gas cavity formation in case of the analyzed magnesium-based membranes.

The histomorphometric analysis of the occurrence of the macrophage subtypes showed that comparable numbers of CD163-positive macrophages were found in the study groups of the pure MG membrane and the collagen membrane at day 10 post implantation, while lower numbers were found in the study group of the coated MG membrane at this early time point (Table 1 and Figure 7). Thereby, the numbers in the group of the coated MG membrane differed significantly compared to the numbers in the group of the collagen membrane (* $p < 0.05$), while no significant differences were found compared to the numbers in the group of the pure MG membrane (Table 1 and Figure 5). At this time point, the numbers of the NF-κB-positive cells did not significantly differ in all study groups (Table 1 and Figure 7). However, the lowest numbers were found in the group of the collagen membrane, while the numbers in the groups of both magnesium-based membranes were at a comparable level (Table 1 and Figure 7).

On day 30 post implantation, the histomorphometric analysis showed that the numbers of both the CD163-positive and the NF-κB-positive macrophages were comparable in all study groups, while tendentially lower numbers of pro- and anti-inflammatory macrophages were detected in the collagen membrane group (Table 1 and Figure 7).

Table 1. Occurrence of pro- and anti-inflammatory macrophages in the three study groups (cells/mm²).

Time point	MG Membrane		Coated MG Membrane		Collagen Membrane	
	CD163	NF-κB	CD163	NF-κB	CD163	NF-κB
Day 10	365.9 ± 67.75	396.6 ± 157.3	108.2 ± 32.84	386.6 ± 175.4	466.4 ± 190.3	137.9 ± 62.04
Day 30	351.3 ± 167.4	302.9 ± 45.54	310.1 ± 200.8	391.5 ± 159.4	186.5 ± 104.3	120.9 ± 85.64

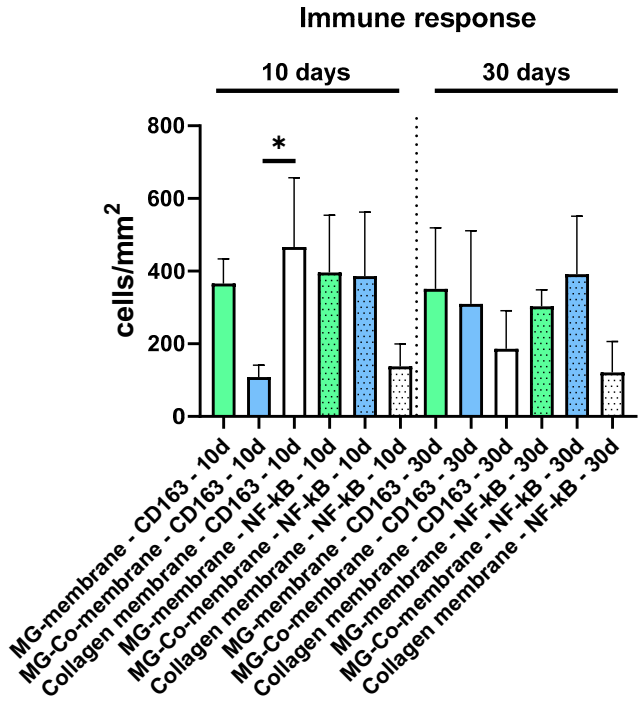

Figure 7. Histomorphometric results of the immune response, i.e., the detection of anti- (CD163 antigen) and pro-inflammatory macrophages (NF-κB antigen) at day 10 and day 30 post implantationem (separated by the dotted line) (* $p < 0.05$).

4. Discussion

Bioresorbable membranes are the emerging alternative to non-resorbable membranes in the field of dentistry, especially in periodontal regenerative procedures, including guided bone or tissue regeneration (GBR/GTR) cases. Thereby, this membrane entity could also be used for other surgical specialties. Clinical relevance of biocompatible materials has gained importance from the clinicians as well as patients' point of view in order to avoid additional surgical interventions as well as the reassurance of the usage of the human body's degradable materials. Even collagen-based barrier membranes are nowadays mainly used for most of the oral standard defects representing successful and biocompatible medical devices [2,5,6,32,33]. However, they do not present the degree of volume stability that is required for multidimensional jaw defects [2,5,6,32,33]. In these cases still, nonresorbable membranes based on compounds like dense polytetrafluoroethylene (dPTFE) or expanded polytetrafluoroethylene (e-PTFE) in combination with titanium meshes as so-called titanium-reinforced PTFE membranes are the materials of choice, although their application requires a second surgical intervention with a variety of related side effects [4]. Thus, the development of resorbable volume-stable membranes is of special interest.

Among different other chemical compounds, Mg has been shown to constitute a favorable biomaterial for the development of stabilizing structures due to its satisfying biocompatibility, biodegradability and mechanical properties [5]. Furthermore, the combination of a magnesium mesh embedded in a native collagen membrane allows volume-stable interventions and complete biodegradation. Interestingly, previous studies confirmed the high level of biocompatibility with minimal inflammation, as well as the ability to support bone regeneration.

In this context, it has already been revealed that the combination of a magnesium mesh embedded in a native collagen membrane allows volume-stable interventions and complete biodegradation [5]. In this study, a hydrofluoric acid (HF)-treatment was analyzed that should prevent premature Mg degradation. The in vitro results of this study showed that the HF-treated Mg showed higher cytocompatibility, and HF-Mg prevented in vivo the formation of gas cavities. Thus, the HF-Mg meshes embedded in native collagen membranes were classified as a volume stable and biocompatible alternative to the non-absorbable synthetic materials.

In the present study, a new approach called ion implantation (II) with PVD coating was used for the passivation of Mg. In order to demonstrate comprehensive biocompatibility and successful passivation of the Mg membranes, untreated Mg (MG) and coated Mg (MG-Co) were investigated in vitro and in vivo. Thereby, in vitro tests were accomplished in accordance with EN ISO 10993-5/-12 regulations. In vivo, both membrane types were implanted using a manifoldly described subcutaneous implantation model in BALB/c mice for up to 30 days post implantation [2,5,6,21,22]. Furthermore, established histological, histopathological and histomorphometric analysis methods were applied that included the investigation of the M1-/M2-macrophage response to the biomaterial [5,21,22]. Both an uncoated Mg-based membrane and a collagen membrane (Jason membrane) with a previously described excellent biocompatibility were used as controls [5]. It must be noted that such studies, including the different steps of biomaterial testing in accordance with the development process following the respective guidelines, are of great importance prior to clinical trials. Especially studies analyzing material characteristics and preclinical studies allow examining the influence of (new) biomaterials on disease progression [34,35]. In this context, they also help to clarify the molecular basis of biomaterial applications [36–38].

In vitro, both membrane entities showed poor cytocompatibility with signs of massive gas bubble formation. In comparison, MG-Co showed inferior values than MG. Gas bubbles can be interpreted as a sign of degradation, which has been shown in previous publications [30,31]. However, the passivation seems to be an even triggering factor for degradation and the PVD coating was visible as black discoloration in the live–dead staining. The black discoloration can be attributed to the coating method. It is reasonable to assume that the layer is not stable in an aqueous environment and does not have a stabilizing effect in the form of slowed down degradation.

The results of the histopathological analysis showed that all membranes induced an inflammatory tissue reaction. The cells were mainly macrophages in addition to lower numbers of granulocytes and fibroblasts within the subcutaneous implantation beds. Interestingly, the MG-Co induced the highest extent of inflammation, including mainly macrophages directly attached to the material surfaces at this time point. The uncoated Mg-membrane induced a moderate level of inflammation, while the implantation of the collagen membrane was associated with the lowest level of inflammatory tissue response. In this context, it is known that collagen-based membranes show excellent biocompatibility without induction of major inflammatory responses [2,5,32]. Furthermore, it has already been revealed in different preclinical and clinical studies that the pericardium-based membrane used in the present study as a control device induces a tissue response that leads to the integration of the material involving even the cells of the collagen metabolism and also to a balanced M1-/M2-macrophage ratio needed for tissue regeneration in contrast to an inflammation-driven degradation [2,6,39].

Interestingly, these findings of poor cytocompatibility, the higher inflammatory response and the macrophage accumulation at the material surfaces indicate the involvement of the applied PVD coating in this process. It can be concluded that the induced macrophages contribute to the phagocytosis-driven degradation of the surface coating, as also found in the case of the aforementioned HF-treated Mg-membrane [5]. In this study, also an accumulation of macrophages in the group of the coated membranes was detected, and it was concluded that the HF-induced magnesium fluoride MgF2-layer was also phagocyted by macrophages in contrast to the pure Mg-meshes that induced only slight fibrosis, which has shown not to interfere with regenerative bone growth in a new study, but to serve as an osteoconductive scaffold (publication submitted).

Moreover, these findings are in line with further results in the present study. Thus, the histomorphometric data of the macrophage distribution revealed that the collagen membrane induced the highest numbers of CD163-positive M2-macrophages and the lowest numbers of NF-κB-positive M1-macrophages at day 10 post implantation. In contrast, the lowest numbers of M2-macrophages were found in the group of MG-Co, while the pure Mg-membrane induced a tissue response including a balanced number of M1- and M2-macrophages at this early time point. Furthermore, the analysis showed a trend towards a more pronounced M2-response in the groups of the collagen membrane and the pure MG-membrane at day 30 post implantation, while a trend towards a pro-inflammatory response was still visible in the MG-Co group. Altogether, these results reveal that the decreased the biocompatibility of the MG-Co membrane. However, the results furthermore show the good biocompatibility of the MG membrane as it induces similar results as also found in the collagen membrane group.

Finally, the histomorphometric analysis of the gas formation showed that the gas cavities occupied around 13% of the implant bed areas in the group of the MG-Co in contrast to around 7% in the group of the untreated Mg-membrane without significant differences at day 10 post implantation. However, no further differences were found at day 30 post implantation. Thus, in concordance with the in vitro results, the PVD coating seems to increase the degradation of the Mg-membrane instead of preventing the (initial) gas formation based on its absorbability of the H_2 gas resulting from the membrane degradation. This observation may also be resulting from the observed higher immune response, including mainly pro-inflammatory macrophages, as this cell type is known for its phagocytic activity [6,22,23,33]. In this context, it is assumable that the higher macrophage accumulation, even in the case of MG-Co, led to increased degradation of the coating that had only a thickness of around 2 μm. Thus, the loss of the coating might have been led to the accelerated gas release.

Although the results of the present study show different tissue responses to the newly developed PVD coated Mg-membrane based on standardized research methods, it includes different limitations. First, the results of in vitro tests are limited as only standard methods based on ISO 10993-5/-12 were applied. In this context, the hydrogen release and the related (micro-) milieu conditions need to be applied, including special adaptions for the testing of Mg materials as already described by Jung et al. [29,30]. Furthermore, different other in vitro analysis approaches, such as innovative test setup, i.e., for example, CAD-CAM-based standardized sample models with equal shape and size, may provide deeper insights into cell-biomaterial interactions and improve the development of biomaterials already from this preclinical level [40].

Additionally, the implantation model, even in mice, is only used to show a general trend regarding the biocompatibility of a biomaterial. However, it cannot display the tissue reactions within the "target tissue", i.e., the soft tissue of the oral cavity and the neighborhood to the underlying bone defect. Thus, it is assumable, especially the gas cavity formation differs after implantation within the oral cavity of larger animals like dogs or even in humans. This assumption is based on the fact that (a) hydrogen molecules can easily penetrate all tissue structures and quickly spread throughout the body due to their small atomic mass [30,31,41–43]. Furthermore, hydrogen gas is soluble in both water and fat at the same time [30,31,41–43]. These properties may ensure that the molecules can also penetrate fat layers or cell membranes and thus into fluid-filled cells. Additionally, the vascularization and hemodynamic, but also the tissue perfusion is higher, which should also lead to faster removal of the hydrogen gas. Furthermore, the counting of the M1- and M2-macrophages does not allow for the exact quantification of the immune response to a biomaterial and is thus only an initial indicator to assess the general material-related tissue response. For this purpose, specialized analysis methods, such as laser-assisted cell microdissection that enables the measurement of cytokine release from single cells, are more adequate tools for biomaterial research and development [44].

Taken together, the results of the present study revealed that the coated magnesium membranes did not show a significant benefit over the pure magnesium membrane. Furthermore, the additional PVD coating did not prevent gas formation but rather shows an acceleration. The pure magnesium

membranes seem to represent a promising alternative compared to the actual generation of nonresorbable volume stable materials for GBR/GTR therapy. The present study showed that the pure magnesium membranes fulfill the requirements for biocompatibility even due to the comparability of the integration behavior of the collagen membrane.

5. Conclusions

Magnesium membranes represent a promising alternative compared to the nonresorbable volume of stable materials for GBR/GTR therapy. The present study showed that both the untreated and PVD-coated membranes were not cytocompatible, but the pure magnesium membranes fulfill the requirements for biocompatibility even due to the comparability of the integration behavior and the immune response of the collagen membrane. Interestingly, the PVD coating did not have a positive influence on the gas cavity formation or the improvement of the immune response compared to the uncoated membrane. Thus, the present study revealed that the pure magnesium membranes fulfill the requirements for biocompatibility and seem to represent a promising alternative compared to the actual generation of nonresorbable volume stable materials for GBR/GTR therapy.

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3. Diskussion

Die in vitro-Ergebnisse dieser Studie zeigten, dass sowohl die unbehandelten als auch die PVD-beschichteten Membranen nicht zytokompatibel waren. Jedoch erfüllten beide Membrantypen die Biokompatibilitätsanforderungen in-vivo. Die PVD-Beschichtung hatte im Vergleich zur unbeschichteten Membran keinen Einfluss auf die Bildung von Gaskavitäten. Im Vergleich zur reinen Mg-Membran und zur Kollagenmembran induzierte die PVD-Beschichtung jedoch eine geringere Anzahl entzündungshemmender Makrophagen. Die reine Mg-Membran löste eine Immunreaktion aus, die mit der Kollagenmembran vergleichbar war.
Die klinische Relevanz biokompatibler Materialien ist aus Sicht der Kliniker gefragt, um post-operative Komplikationen zu vermeiden, aber auch um intraoperative Praktiken zu vereinfachen. Auch aus Sicht der Patienten haben solche Materialien an Bedeutung gewonnen, da sie zusätzliche chirurgische Eingriffe vermeiden, da abbaubare Materialien durch den menschlichen Körper verstoffwechselt werden können. Barrieremembranen auf Kollagenbasis werden heutzutage für die meisten oralen Standarddefekte verwendet und sind dementsprechend auf Grund ihrer guten Biokompatibilität erfolgreiche Medizinprodukte.
Dennoch weisen sie nicht den Grad an Volumenstabilität auf, der für mehrdimensionale Kieferdefekte erforderlich ist. Demzufolge sind weitere physikalische und/oder chemische Fortschritte für volumenstabile resorbiere Membranen erforderlich. Studien zur Analyse von Materialeigenschaften und präklinische Studien ermöglichen die Untersuchung des Einflusses von (neuen) Biomaterialien auf die Physiologie des menschlichen Körpers und helfen dementsprechend den Zusammenhang der molekularen Grundlagen von Biomaterialanwendungen zu klären.

Neben anderen chemischen Verbindungen hat sich Magnesium als ein Biomaterial mit stabilisierender Struktur erwiesen. Dies basiert auf Abbaubarkeit und den mechanischen Eigenschaften des Magnesiums als Werkstoff (44). So wurde gezeigt, dass Magnesium vergleichbare mechanische Eigenschaften wie auch die Knochenmatrix aufweist (59, 60).

Als stützende Struktur wurden bereits Magnesiumnetze eingebettet in eine native Kollagenmembran getestet, welche eine erweiterte Stabilität des

Gesamtmaterials ermöglichten (61). Des Weiteren erlaubte diese Kombination vollständige biologische Abbaubarkeit mit minimaler Entzündung sowie die Fähigkeit die Knochenregeneration zu unterstützen (62).

In der vorliegenden Studie wurde ein neuer Ansatz der Ionenimplantation mittels PVD-Beschichtung zum Zwecke der Passivierung von Magnesiummembranen verwendet mit dem Ziel, die Abbaubarkeit durch die PVD-Beschichtung zu verzögern und damit insgesamt die Volumenstabilität der Barrieremembranen zu erhöhen. Mit einem ähnlichen Ansatz, untersuchten Barbeck *et al.*, inwiefern eine Flusssäure-Behandlung den vorzeitigen Magnesium-Abbau verhindern kann (62).

Die *in vitro*-Ergebnisse zeigten, dass die Flusssäure-behandelte Magnesiummembran, die in native Kollagenmembranen eingebettet waren, eine höhere Zytokompatibilität aufwies. Die *in vivo*-Ergebnisse zeigten, dass die Flussäure-Behandlung die Bildung von Gashohlräumen verhinderte. Somit zeigte diese Studie, dass die Flussäure-Magnesium-Netze als volumenstabile und biokompatible Alternative zu den nicht-resorbierbaren synthetischen Materialien dienen kann. Dies wurde weiterhin durch eine kombinierte *in vitro/in vivo*-Studie bezogen auf Flusssäure-behandelte Magnesiumschrauben nachgewiesen (63).

In der vorliegenden Studie wurden zum Nachweis der Biokompatibilität und der erfolgreichen Passivierung der Magnesium-Membranen, unbehandelte Magnesium-Membranen als auch eine Kollagenmembran untersucht. Die *in vitro*-Tests wurden gemäß den DIN EN ISO 10993-5/-12- Vorschriften durchgeführt. Die *in vivo*-Versuche beinhalteten die Implantation beider Membrantypen mit Hilfe des subkutanen Implantationsmodells in BALB/c-Mäusen. Zur Evaluierung wurden etablierte histologische, histopathologische und histomorphometrische Analysemethoden angewandt (62, 64-66).

Die *in vitro*-Ergebnisse zeigten, dass beide Membranen Anzeichen einer Gasblasenbildung aufwiesen. Im Vergleich zeigten die beschichteten Membranen jedoch keine Unterschiede in der Gasblasenbildung im Vergleich zu den reinen Magnesiummembranen. Gasblasen sind Indikatoren von Degradationsprozessen des Magnesiumwerkstoffs (56, 67) und dementsprechend zeigten beide Membranen niedrige Zytokompatibilitätswerte. Die Passivierung der Magnesiummembranen schien dabei ein auslösender Faktor für die Degradation zu sein. Die PVD-Beschichtung war als schwarze Verfärbung in der Lebend-/Tot-Färbung sichtbar. Es

kann also spekuliert werden, dass die schwarze Verfärbung auf die Beschichtungsmethode zurückzuführen ist. Es scheint, dass die Schicht in wässriger Umgebung nicht stabil ist und nicht die erwünschte stabilisierende Wirkung in Form eines verlangsamten Abbaus hat. Daher hat sich die gewünschte Degradationsstabilität *in vitro* nicht bewährt.

Die Ergebnisse der histopathologischen Analyse zeigten, dass alle Membranen eine entzündliche Gewebereaktion hervorgerufen haben. Dies wurde hauptsächlich belegt durch die Makrophaghenaggregation an der Materialoberfläche. Die eingesetzten Perikardmembranen zeigten in vorausgegangen präklinischen und klinischen Studien eine ausgezeichnete Biokompatibilität (68) und diente demzufolge als Kontrollmembranen. Diese Membran wird nicht nur in das umliegende Gewebe durch die Zellen des Kollagenstoffwechsels integriert, sondern induziert zudem auch ein ausgewogenes M1-/M2-Makrophagen-Verhältnis (68-70), was hervorragende Bedingungen für die Geweberegeneration schafft. Talebi *et al.* (71) kultivierte in seiner *in vitro*-Studie Humane gingivale Fibroblastenzellen der ersten Generation (HGF1-RT1) auf drei Membrantypen: 1. Schweineperikard, 2. Humanes Perikard (beide native Kollagenmembranen) und 3. mit Glutaraldehyd vernetzte Kollagenmembran. Das Überleben und die Adhäsion von Zahnfleischfibroblasten war auf der mittels Glutaraldehyd vernetzten Kollagenmembran geringer als im Falle der zwei anderen Perikardmembranen (Human- und Schweineperkard). Demzufolge unterstützen die natürlichen Kollagenmembranen (Human- und Schweineperkard) die Proliferation und Adhäsion von Zahnfleischfibroblasten.

Die schlechtere Zytokompatibilität, die höheren Entzündungsreaktion und die Makrophagenansammlung an den Materialoberflächen deuten darauf hin, dass die aufgebrachte PVD-Beschichtung keine Vorteile in Hinsicht auf die Biokompatibilität erbrachte. Die Material-induzierten Makrophagen führen zum Phagozytose-getriebenen Abbau der Oberflächenbeschichtung (62). In vorangegangen Studien mit Flussäure- behandelten Magnesiumoberflächen wurde ebenfalls eine Anhäufung von Makrophagen in der Gruppe der beschichteten Membranen festgestellt. Vergleichbar zu den hier präsentierten Studienergebnissen, wurde die Beschichtung auch von Makrophagen phagozytiert. Im Gegensatz dazu wurde im Falle der puren Magnesiummembranen nur eine leichte Fibrose induziert.

Die histomorphometrischen Daten wiesen ein Trend zu einer pro-inflammatorischen Reaktion in der Gruppe der PVD-behandelten Membranen auf. Die

Makrophagenverteilung zeigte, dass die Kollagenmembran die höchste Anzahl an CD163-positiven M2-Makrophagen und die niedrigste Anzahl an NF-κB-positiven M1-Makrophagen am Tag 10 nach der Implantation induzierte. Im Gegensatz dazu wurde die geringste Anzahl von M2-Makrophagen in der Gruppe der PVD-behandelten Membranen gefunden, während die reine Magnesium-Membran eine Gewebereaktion mit einer ausgewogenen Anzahl von M1- und M2-Makrophagen zu diesem frühen Zeitpunkt hervorrief. Darüber hinaus zeigte die Analyse einen Trend zu einer ausgeprägten M2-Antwort in den Gruppen der Kollagenmembran und der reinen Magnesiummembran (am Tag 30). Zusammengefasst zeigen diese Ergebnisse, dass die PVD-behandelten Membranen weniger biokompatibel ist. Sie belegen jedoch die gute Biokompatibilität der Magnesiummembran, welche ähnliche Ergebnisse zur Kollagenmembran-Gruppe hervorrief.

Weiterhin zeigte die histomorphometrische Analyse die Gasbildung der getesteten Membranen. Die Gashohlräume nahmen rund 13% der Implantatbettflächen in der Gruppe der Gruppe der PVD-behandelten Membranen im Gegensatz zu rund 7% in der Gruppe der unbehandelten Magnesiummembran ein. Weder am Tag 10 noch am Tag 30 nach Implantation gab es zu der Gruppe der behandelten Magnesiummembran signifikante Unterschiede. Die *in vivo*-Ergebnisse sind somit in Übereinstimmung mit den zuvor diskutierten *in vitro*-Ergebnissen. Die PVD-Beschichtung scheint die Degradation der Magnesiummembran eher zu verstärken, anstatt die (anfängliche) Gasbildung zu verhindern. Diese Beobachtung könnte auch ein Resultat von der beobachteten pro-inflammatorischen Immunreaktion sein, die hauptsächlich entzündungsfördernde Makrophagen beinhaltet. Die höhere Makrophagenakkumulation in der Gruppe der PVD-behandelten Membranen könnte zu einem verstärkten Abbau der Beschichtung (selbst bei einer Beschichtung von nur 2 µm) geführt haben. Weitergehend könnte der Verlust der Beschichtung nachfolgend zu einer beschleunigten Gasfreisetzung geführt haben.

Auf der Grundlage der eingesetzten standardisierten Untersuchungsmethoden zeigen die Ergebnisse unterschiedliche Zell- und Gewebereaktionen der neuartigen PVD-beschichteten Magnesiummembran auf, wobei einige Limitation dieser Studie anzumerken sind.

Zunächst sind ist die Aussagekraft der von *in vitro*-Tests begrenzt, da nur Standardmethoden (auf der Grundlage der ISO10993-5/-12) angewandt werden. In

diesem Zusammenhang müssen im Falle der Wasserstofffreisetzung durch die Magnesium-basierten Biomaterialien auch entsprechende (Mikro-) Milieubedingungen angewendet werden. Dabei sind spezielle Anpassungen für die Prüfung von Magnesium-Materialien zu beachten, wie sie bereits von Jung *et al.* beschrieben wurden (56). Darüber hinaus sind andere *in vitro*-Analyseansätze, wie z. B. innovative Testaufbauten, erforderlich, d.h. z.B. standardisierte Probenmodelle mit gleicher Form und Größe. Dies kann zukünftig tiefere Einblicke in die Zell-Biomaterial-Wechselwirkungen geben und die Entwicklung von Biomaterialien bereits auf dieser präklinischen Ebene verbessern (72).

Außerdem wird das angewendete Implantationsmodell in Kleintieren verwendet, um einen allgemeinen Trend hinsichtlich der Biokompatibilität eines Biomaterials zu zeigen. Es kann jedoch nicht die Gewebereaktionen des "Zielgewebes", d. h. des Weichgewebes der Mundhöhle und dem darunterliegenden Knochendefekt widerspiegeln. Dabei ist im Falle dieses Implantationsmodells anzunehmen, dass sich insbesondere die Gashöhlenbildung nach der Implantation in der Mundhöhle von größeren Tieren (z.B. Hunden) als auch beim Menschen selbst unterscheidet. Diese Vermutung beruht auf der Tatsache, dass Wasserstoffmoleküle aufgrund ihrer geringen Atomgröße alle Gewebestrukturen leicht durchdringen können und sich schnell im Körper ausbreiten können (73-75). Außerdem ist Wasserstoffgas gleichzeitig in Wasser und Fett löslich (67, 73). Diese Eigenschaft kann dafür sorgen, dass die Moleküle auch in Fettschichten oder Zellmembranen und damit in flüssigkeitsgefüllte Zellen eindringen können. Hinzu kommt die Vaskularisierung und Hämodynamik und die erhöhte Gewebedurchblutung, welche ebenfalls zu einem schnelleren Abtransport des Wasserstoffgases führen kann. Außerdem erlaubt die quantitative Auswertung der M1- und M2-Makrophagen keine genaue Quantifizierung der Immunantwort in Bezug auf ein Biomaterial und ist daher nur ein erster Indikator zur Beurteilung der allgemeinen materialbedingten Gewebe-/ Immunreaktion. Zu diesem Zweck können weitere spezialisierte Analysemethoden, z.B. die Laser-unterstützte Zellmikrodissektion zur Messung der Zytokinexpressionh angewandt werden (76).

Zusammengenommen zeigen die Ergebnisse der vorliegenden Studie, dass die beschichteten Magnesiummembranen keinen signifikanten Vorteil gegenüber der reinen Magnesiummembran aufweisen. Die zusätzliche PVD-Beschichtung hat die

Gasbildung nicht verhindert, sondern eher eine Beschleunigung hervorgerufen und zudem keine Verbesserung der Immunantwort induziert. Die reinen Magnesiummembranen hingegen erfüllen aufgrund der Vergleichbarkeit des Integrationsverhaltens und der Immunantwort zu der traditionellen Kollagenmembran die Anforderungen an die Biokompatibilität eines Biomaterials. Aufgrund dessen kann aus dieser Studie geschlossen werden, dass die reinen Magnesiummembranen eine vielversprechende Alternative zur aktuellen Generation nicht-resorbierbarer volumenstabiler Materialien für die GBR/GTR-Therapie sind.

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