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Evaluation of cellular reactions to magnesium as implant material in comparison to titanium and to glyconate using the mouse tail model

Cellular reactions to magnesium as implant material
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1 Abstract

Purpose: Nowadays, research in magnesium alloys as a biodegradable implant material has increased. The aim of the present study was to examine osteoinductive properties and tissue responses to pure magnesium in comparison to conventional permanent (titanium) and to degradable (glyconate) implant materials.

Methods: Magnesium wires (0.4 mm in diameter, 10 mm length) were implanted into tail veins of mice and examined after 2, 4, 8, 16 and 32 weeks. Titanium and glyconate as controls were evaluated after 2, 4, 8 and 24 weeks. µ-computed tomography, histology and SEM examinations were performed.

Results: Magnesium implants showed increasing structural losses over time with fragmentation after an observation period of 32 weeks. Glyconate was fully degraded and titanium remained almost unaffected after 24 weeks. In contrast to some titanium and glyconate implants, first calcium and phosphat precipitations could be observed around magnesium implants after 2 weeks. However, ossification could not be observed even after 32 weeks, whereas enchondral ossification was found partially in the surrounding of glyconate and titanium implants after 8 weeks. Nevertheless, magnesium implants showed less inflammatory responses and fibrosing properties than the conventional implant materials.

Conclusion: Although the assumed osteoinductive properties could not be detected, magnesium seems to be a promising degradable implant material due to the low sensitizing and inflammatory potential.
2 Introduction

In recent years, magnesium alloys received a lot of interest as prospective degradable implant materials for orthopaedic applications \cite{1, 2, 3}, because they excel other degradable implant materials (e.g. polymers) in tensile and compressive strength \cite{4, 5, 6}. Magnesium ions are a natural component of the body \cite{7}. Furthermore, magnesium alloys were tested as non-allergic \cite{8} and have a lower immunogenic potential than surgical steel or titanium \cite{9, 10}. Magnesium alloys, having a low corrosion rate, are preferable for applications in the bone \cite{11}. A high corrosion rate results in gas formation and a rapid loss of mechanical strength \cite{12}. In vivo models are essential for evaluating magnesium alloys as an implant material, because in vivo and in vitro corrosion rates can differ to a large extent \cite{3, 13}. Used in trauma surgery, low inflammatory potential and osteoinductive properties of the implant would be desirable to accelerate bone healing.

In the available literature, osteoinduction has not been demonstrated for magnesium implants, but the in vitro formation of a bone-like matrix at the surface of magnesium alloys was described \cite{14}. In vivo studies also exist which report new bone formation at an orthotopic location \cite{1, 15}. The aim of the following study was to verify the assumed osteoinductive properties of magnesium implants and to examine inflammatory reactions in the neighbourhood in comparison to the clinically established materials titanium and glyconate. Therefor, intravenous location in a mouse tail model was chosen because other authors recommended examination of osteoinductive properties in different types of tissues \cite{16, 17}. This model permits a detailed characterisation of the implant material and host reactions using a wide spectrum of methods.
3 Materials and Methods

3.1 Implant material

High purity magnesium wires (0.4 mm Ø, 10 mm length, Goodfellow GmbH, Bad Nauheim, Germany) were used as an implant material after incubation in 1 M NaOH (24 h) and air drying. As control materials, titanium (Ara-T Advance GmbH, Dinslaken, Germany) and glyconate (Monosyn violet 70 cm lot 1-6255 2011-6, B.Braun Aesculap, Tuttlingen, Germany) wires with the same dimensions were used.

3.2 Animal model, study design and surgical procedures

The study was conducted with approval of the statutory animal welfare committee (33.14.42502-07/10.05).

Adult female Balb/c mice (Harlan-Winkelmann, Borchen Germany) were anaesthetised by an intraperitoneal injection of ketamine (10 mg/kg) and xylazine (4 mg/kg) and the materials were implanted into a tail vein. After 2, 4, 8, 16 and 32 weeks for the magnesium group and 2, 4, 8 and 24 weeks for the control groups, the animals were euthanized. Tissue-implant-composite were examined μ-computed-tomographically and histologically, SEM and EDS-analyses was performed for extracted implant material. The exact number of implants in each material and time group is shown in Table I.

3.3 μ-computed tomography

Implant location in the tail and implant morphology were evaluated by μ-computed tomography (XtremeCT, Scanco Medical, Switzerland), with 41 μm slice thickness and 200 ms integration time. Different thresholds were used for the different material groups in order to optimise the visualisation of the implanted material.
Table I: Number of implant materials examined after different observation periods.

<table>
<thead>
<tr>
<th>Material</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>32 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>magnesium</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>titanium</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>glyconate</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Implants in each material group were examined histologically (Histo) and by using scanning electron microscopy (SEM) and energy-dispersive x-ray spectroscopy (EDS) after varying post-operative time periods.
3.4 Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS)

The explanted wires were examined by scanning electron microscopy (LEO 1455VO, Carl Zeiss AG, Oberkochen, Germany) to evaluate the implant morphology as well as precipitations and corrosion products on the implants’ surface. The analysis of the elements was carried out using EDAXGenesis (V5.11, EDAX Business Unit, Wiesbaden, Germany).

3.5 Histology

All implant-tissue composites in the magnesium groups and in the glyconate groups were embedded in Technovit 9100neu (Hereaus Kulzer, Germany). 4 µm thin sections were cut. Since it was not possible to section the titanium implants, these implants, except one in each time group, were removed prior to embedding. Additionally, the selected implant-tissue composites were used to produce slices according to Donath's cutting and grinding technique \[18\].

In the magnesium group, one implant (32 wk) was divided and additionally sectioned by the cutting and grinding technique.

Toluidine blue, Von-Kossa and Masson-Goldner were used as histological stains.
4 Results

4.1 Different implant material in vivo behaviours during the implantation period

The implanted magnesium wires could be located μ-computed tomographically in all investigated tails. Following 2 (Fig 1a), 4 and 8 weeks, the implants displayed no visible loss of material. After 16 weeks (Fig. 2a), fragmentation of the magnesium implants was found and this increased up to 32 weeks (Fig. 2b).

Glyconate implants could be detected after 2 (Fig 1b), 4 and, in part, after 8 weeks. However, no further material could be found after a time period of 24 weeks.

Titanium could be observed at all points in time and demonstrated no apparent structural losses (Fig. 1c, Fig. 2c).

Fig. 1: Magnesium implant (a), glyconate implant (b), titanium implant (c) in mouse tails 2 wk post operatively (μ-computed tomography) without evident structural losses. Differences in bone imaging due to different x-ray densities of implant material and thresholds; white bar = 5 mm.

Fig. 2: Magnesium implants in the mouse tail after 16 wk (a) and 32 wk (b) implantation period (μ-computed tomography). Following 16 wk, implant fragmentation was visible and continued after 32 wk (arrows); titanium implant following 24 wk implantation period (c) without changes; white bar = 5 mm.
SEM examination confirmed a continuing degradation process of the magnesium implants. Following 2 and 4 weeks, the explanted implants’ surfaces showed pits and microcracks (Fig. 3a). In the EDS-analysis, magnesium, oxygen and, in the analysed bright areas, also high amounts of calcium and phosphorus were detected as main elements. Deeper crevices and spalling of the implant material from the surface were visible following 8 and 16 weeks. After 32 weeks, the implant material separated into pieces with deep crevices on the surface (Fig. 3b, c). Besides magnesium, carbon and oxygen small amounts of chloride and sulphur were found in the EDS-analyses of the dark areas. In the bright areas, calcium and phosphorus could be detected.

![Fig. 3: Degradation process of magnesium implants in SEM examination. Implants (Ø 0.4 mm, 10 mm length) following 4 (a) and 32 wk (b, c) implantation period. After 4 wk, the implant surface appeared rough (a). After 32 wk, implant material is still detectable (b) but as pieces with deep cracks (c). a and b: white bar = 200 µm, c: higher magnification, white bar = 100 µm.](image)

Glyconate showed no loss of shape (Fig. 4) after 4 and 8 weeks in the SEM examination. In the EDS-analysis, mostly carbon and oxide could be detected on the implant surface. Following 8 weeks implantation duration, single small surface cracks were visible on the implant. In one of the examined implants, a crystalline structure with calcium and phosphorus as main elements was detected (Fig 4c). Following 24 weeks, the implant could no longer be detected.
In the titanium group, no structural losses could be observed in SEM examination; even after a post operative time period of 24 weeks (Fig. 5). The surface remained smooth with dark and bright areas. Titanium was the main element in every EDS-analysis performed in this group. In the dark areas, adjacent to the titanium, small amounts of sodium, chloride, phosphorous, potassium and sulphur were detectable.

Fig. 4: SEM images of glyconate implants (Ø 0.4mm x 10mm length) following 4 wk (a) and 8 wk (b, c) implantation period. After 4 wk, the material showed no structural losses. After 8 wk, initial cracks (arrow) and crystalline structures were visible. a and b: white bar = 200 µm, c: higher magnification (area in square), white bar = 100 µm.

Fig. 5: SEM images of titanium implants (Ø 0.4 mm, 10 mm length): no structural losses of the material after 4 wk (a) and 24 wk (b, c) implantation period. a and b: white bar = 200 µm, c: higher magnification, white bar = 100 µm.

4.2 Magnesium implants induced minor tissue responses in contrast to glyconate and titanium, but demonstrated no assumed osteoinductive properties

In all examined material groups, vein thrombosis and acute and chronic thrombophlebitis were found with a low to moderate level of non-specific inflammation. The inflammatory infiltrates predominantly consisted of small amounts
of neutrophils, macrophages and mast cells (Fig 6e). Physiological organisation, epithelisation and vascularisation of the thrombi occurred. In cases of total vein occlusion, paravenular collaterals developed. Perivascular fibrosis could be found as well as peri-implant infiltration of macrophages (Fig. 6).

During longer implantation of all materials, organisation and revascularisation of the thrombi proceeded and inflammation was regressive.

Deposition of phosphates around the magnesium implants could be found following all the time periods examined (Fig. 7a-c). However, even after 32 weeks, the predominant parts of the von-kossa-stained areas around the magnesium implant remained without cell infiltration (Fig. 6b) and a small organised fibrous capsule and collateral veins could be found after 16 and 32 weeks of implantation (Fig. 6c).

In the titanium group, sporadic chondromatosis was found in 1 implant after 2 weeks. After 8 weeks implantation period chondromatosis progressed (Fig. 6d). The von-kossa staining confirmed an enchondral ossification around the titanium implant (Fig 7g). Periosteal reactions of the vertebral bodies at the site of implant material could be found in all samples after 8 weeks and in 1 of 3 samples after 24 weeks. After 24 weeks, only in 1 of 3 implants, phosphate depositions were found in the implant neighbourhood (Fig. 7h, i). Moderate chronic neutrophilic infiltration was found adjacent to tissue regenerates and decreased from 2 weeks to 24 weeks. Infiltration of basophils was higher than in the other material groups. Numerous apoptotic bodies could be found around the titanium implants. In contrast to the magnesium implants, the fibrosis around the implant material increased over time (Fig. 6f, 8).

Deposition of yellow and brown pigment was observed intercellularly and in macrophages after 4 and 8 weeks as well as after 24 weeks post operative time period (Fig. 6e).
Fig. 6: Cellular reactions to magnesium (a-c), titanium (d-f) and glyconate implants (g- i) after 8 wk (a, d, e, g, h, i), 24 wk (f) and 32 wk (c) implantation period.

Magnesium: the phosphate rich corrosion layer remained acellular (b, black star); Titanium and glyconate: chondromatosis was found in the implant neighbourhood (red arrow). Titanium: implant particles could be observed (yellow arrows); all groups: collaterals (white arrow); macrophages (green arrow) and mast cells (purple arrow) in the implant neighbourhood; fibrous capsule around the implant material (black arrow); staining Toluidine blue (a, b, d, e, g, h); Masson-Goldner (c, f, i).

In the glyconate group, the tissue reactions to intravenous implants were characterised as non-specific inflammation which led to a moderate fibrosis around the implant material. After an implantation period of 8 weeks, calcifications of the implant neighbourhood could be found in 3 of 4 implants (Fig. 7e). In the toluidine blue staining, chondromatosis, which is a sign of enchondral ossification, could be observed (Fig. 6g, h). After an observation period of 24 weeks, vein obliteration and
sporadic particles of implant material but no phosphate depositions could be detected.

Fig. 7: Phosphate deposition (black) around the implant materials: magnesium after 4 (a), 8 (b) and 32 (c) wk of implantation period, glyconate after 4 (d) and 8 (e) wk of implantation period and titanium after 4 (f), 8 (g) and 24 (h, i) wk of implantation period. Note the different individual reactions to titanium (h, j), staining: Von-Kossa.

Fig 8: Thick fibrous layer and cellular reactions around the titanium after 24 wk (a); thin fibrous layer around magnesium after 32 wk (b). Fewer areas with corrosion products in magnesium group (red arrows); staining: Toluidine blue.
5 Discussion

In the present study, a mouse tail model was chosen for the evaluation of tissue reactions to different implant materials at an ectopic location. The model turned out to be suitably applicable owing to the many different possible evaluation techniques. In particular, the assumed osteoinductive properties of magnesium implants \([1, 15]\) should be verified and compared with titanium and glyconate as permanent and degradable implant materials, respectively.

Osteoinductive properties, which were observed for magnesium implants in previous in vitro \([14]\) and in vivo studies with magnesium implant materials \([15, 11, 19]\), could not be confirmed in the present study. Although calcium phosphate precipitates were already found on the surface of magnesium after two weeks of implantation; which corresponds to an in vitro study, in which calcium phosphate precipitates were described on pure magnesium surface in synthetic biological media \([20]\), chondromatosis or cellular bone structures could not be observed even after an implantation period of 32 weeks. Nevertheless, the precipitated calcium and phosphorus on the implant surface may have a positive effect on the bone formation. Habibovic et al. assumed that calcium and phosphorous on the implant surface could be a physico-chemical trigger for local stem cells to differentiate into the osteogenic lineage \([21]\). Biemond et al. affirmed that calcium phosphates (hydroxyapatite and brushit) on the surface of titanium implants result in a higher degree of bone ingrowth in titanium implants \([22]\). Hence, calcium phosphate precipitates on the surface of orthopaedic implants are assessed as a positive factor for the formation of new bone.

The authors of this study additionally assume that these precipitations might be the reason for the lower cellular reactions and the minor fibrous encapsulation of the magnesium implants. This contrasts with the fibrous encapsulation of titanium
implants, which is described in the literature \cite{23, 24} and could also be observed in the present study.

Against all odds and in contrast to magnesium, titanium and glyconate partially induced enchondral bone formation in the neighbourhood of the implant material. These results contradict the statement of Habrovic et al. that biomaterials can only induce bone formation intramembraneously and that in rodents, bone is rarely induced at all \cite{21}. Even the macro- and microstructures, which were seen as an essential element of the osteoinduction by biomaterials \cite{21}, do not seem to be indispensable. However, additional periosteal reactions of the coccygeal vertebrae might have also influenced the bone formation since it always started at the medial site of the implant.

In addition to this, in the titanium group, particles and discolouration were found as a sign of implant corrosion in the implant’s neighbourhood. These findings are also described in other studies with titanium implants \cite{24}. Although the magnesium implant degraded, remaining corrosion particles were found less frequently in contrast to titanium, which is used as a permanent implant material. Presumably, macrophages eliminate the corrosion particles of the magnesium alloys \cite{31, 32}. Titanium particles however, seem to accumulate in the implant’s neighbourhood, although phagocytosis by macrophages is also described \cite{24}.

As expected, almost fully degraded implants were found in the glyconate group after an observation period of 24 weeks. Only small pieces could be observed histologically in the surrounding tissue. At the same time, calcifications could no longer be found and inflammation processes decreased. Moderate inflammation and fibrosis around different glycolide implants were also seen in other studies \cite{26}. Since calcification around the implant material could be observed after 8 weeks observation
period, it might be possible that the tissue was already remodelled subsequent to the missing stimulus of the foreign material. Inter individual differences in the reactions to the implant material, which were seen in titanium as well, may be another explanation.

One limitation of this study is the small number of examined implants and tissue-implant composites. However, the findings in the present study, especially in the magnesium group, are meaningful because the low cellular reactions to the implanted material in this group were consistent over all examination periods. Although magnesium implants seem to have a very good biocompatibility in comparison to conventionally used implant materials, the mechanical strength of pure magnesium is not sufficient for their use in load bearing bone. For orthopaedic applications, alloying elements, especially the rare earths, are predominantly used to enhance the primary strength and to slow down the degradation rate \cite{11}.

In conclusion, assumed osteoinductive properties of magnesium could not be found, but, nevertheless, magnesium seems to be a promising implant material due to its good biocompatibility. In contrast to titanium, which is widely accepted as biocompatible, magnesium demonstrated lower fibroblastic and sensitising properties. However, longer time periods have to be examined in vivo to evaluate the tissue reactions following the complete degradation of magnesium implants.
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