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In-vitro-studies of the tissue compatibility of magnesium-based implants

THESIS

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Meinen Eltern
In Liebe und Dankbarkeit

Wer immer tut, was er schon kann,

bleibt immer das, was er schon ist.

- Henry Ford

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List of abbreviations

ADP	Adenosine diphosphate
AECGM	Airway epithelial cell growth medium
ALI	Air-liquid interface
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BrdU	Bromodeoxyuridine
Ca	Calcium
Cl	Chloride
CPM	Counts per minute
CRC	Collaborative Research Centre
CRS	Chronic rhinosinusitis
DIN	Deutsche Industrie Norm
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
ELISA	Enzyme-linked immunosorbent assay
Erk1/2	Extracellular signal-regulated kinase 1/2
FCS	Fetal calf serum
FITC	Fluorescin isothiocyanate
hBMSC	human bone marrow-derived stromal cells
HBSS	Hank’s balanced salt solution
HE	Haematoxylin-eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP	Horseradish peroxidase
IL	Interleukin
ICAM	Intercellular adhesion molecule
ISO	International Organization for Standardization
K	Potassium
kD	Kilodalton
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MagT1	Magnesium transporter 1
Mg	Magnesium
MPa	Megapascal
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
OD	Optical density
PEP	Phosphoenolpyruvic acid
PGE_2	Prostaglandin E_2
PBS	Phosphate buffered saline
PNEC	Porcine nasal epithelial cells
PK	Pyruvate kinase
RDA	Recommended daily allowance
RM ANOVA	Repeated measures analysis of variance

RPMI	Roswell Park Memorial Institue medium
SBF	Simulated body fluid
SD	Standard deviation
SDH	Succinate dehydrogenase
SEM	Standard error of the mean
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TGF	Transforming growth factor
Ti	Titanium
TNF	Tumor necrosis factor
TRPM	Transient receptor potential melastatin
TUNEL	Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling

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1. Introduction

The research on magnesium and its alloys as biomedical materials has gained a lot of attention since the beginning of the millennium. Although current permanent implants have already reached a very high medical standard there still exist some difficulties which remain to be resolved. Bioresorbable magnesium-based implants have some unique properties which make them attractive for certain applications.

Surely, their main advantage is the fact that they are supposed to degrade in a biological environment over a certain and potentially adjustable period of time. Thus, after having fulfilled their temporary function of supporting the healing and remodelling of a diseased tissue, they do not need to be removed in a second surgery which always poses the inherent risks of general anesthesia in addition to the risks of the surgical procedure itself. Degradable implants likewise do not act as foreign bodies for prolonged periods. Apart from that, there are some additional benefits depending on the application. Regarding the utilization of magnesium alloys for the production of osteosynthesis materials the mechanical characteristics of magnesium are particularly advantageous as they are very similar to those of cortical bone. Stress shielding effects often exerted by more rigid titanium alloy or stainless steel implants are thereby avoided. Magnesium implants are furthermore promising for a use in the cardiovascular system, e.g. for the production of coronary stents. Compared with permanent implant materials, magnesium is little thrombogenic and its degradability allows for a recovery of vascular contractility in the stented segments, and, even more intriguing, for retaining the growth adaptability of the treated arteries. The latter is an overwhelming advantage with respect to pediatric patients.

Despite all these favorable properties one of the main obstacles in the realization of magnesium-based implants is the fact that many of the basic processes which occur at the interface of implant and tissue are still unknown. As long as these mechanisms are not completely understood it remains very difficult to create the ideal implants for the various fields of application, regarding material composition and manufacturing as well as implant design, because these parameters in turn

critically affect the degradation behavior. Moreover, it is also largely unclear how the release of supraphysiological amounts of magnesium ions and the formation of other degradation products will impact on the biocompatibility performance of the corroding biomaterial as for the cells in direct contact with it. It is the endeavor of the present PhD project to make a contribution in understanding these basic mechanisms, thus bringing forth the development of biodegradable magnesium implants.

The interest in innovative new implants also led to the foundation of the Collaborative Research Centre 599 (SFB 599) “Sustainable bioresorbable and permanent implants of metallic and ceramic materials” by the German Research Foundation (DFG) a couple of years ago. The project division R is especially dedicated to the investigation of resorbable magnesium-based implants and it is divided into a number of sub-projects each focussing on the research for a certain field of application. Due to the complexity of the development process this is only possible by means of an interdisciplinary cooperation of material scientists, life science researchers and clinicians. The present PhD project is part of the sub-project R1 “Development of biocompatible magnesium alloys and investigations into their degradation behavior” which addresses the basic mechanisms of magnesium degradation and biocompatibility. The desired future field of application for the resorbable implants developed within this project is the treatment of chronic rhinosinusitis. Paranasal sinus stents made from magnesium alloys shall slowly degrade over the course of several weeks thus providing enough time for the tissue to heal and remodel stably. If this could be accomplished it would be a major advancement in the health care of patients who still quite often experience unsatisfying results from current therapies.

2. Literature review

2.1. Physiology of Magnesium

2.1.1. Basic aspects and functions of magnesium

In the mammalian organism Mg^{2+} represents the fourth most abundant cation, and it is even the second most common in the intracellular compartment (WOLF and CITTADINI 2003). The body of an adult human contains a total amount of approximately 24 g which equals 1 mol (MCCARTHY and KUMAR 1999). Of this about two thirds are stored in the bones, one third can be found in soft tissue like muscle and only 1 % of the total magnesium is present in the extracellular room (VORMANN 2003). The normal plasma concentration ranges from 0.7 – 1.1 mmol/l of which ca. 45 % are complex- or protein-bound (VORMANN 2003), thus around 0.5 mmol/l of magnesium is present as free form ions (COWAN 1995). As mentioned above the intracellular concentration of magnesium is way higher reaching 14 – 20 mmol/l (ROMANI 2007).

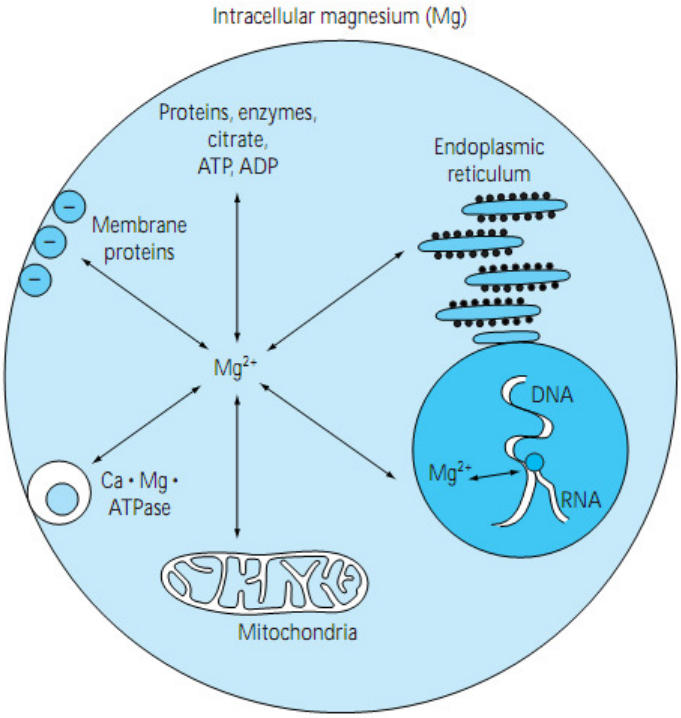

Fig. 1: Intracellular magnesium distribution (from MCCARTHY and KUMAR 1999)

However, the majority of this is again bound in several ways to nucleic acids, negatively charged phospholipids in cell membranes, structure proteins and enzymes as well as ATP (MCCARTHY and KUMAR 1999)(Fig. 1), so the concentration of free Mg ions of again about 0.5 mmol/l is only slightly different from the extracellular one (WOLF et al. 2003). This situation also leads to a certain compartimentalization on a sub-cellular level and it seems that compared with the cytoplasm a larger fraction of magnesium is stored in the ribosomes, endoplasmic reticulum, nucleus and mitochondria (HEATON 1993).

The RDA of magnesium for an adult is between 300 – 420 mg (VORMANN 2003). As opposed to other major cations in the body like sodium or calcium, there is no specific hormonal regulation of magnesium homeostasis. The latter is mainly but very efficiently regulated via absorption in the gastro-intestinal tract and excretion via the kidneys (BEYENBACH 1990). Moreover, in case of a deficiency magnesium will be released from the stores in the bone system, which thus acts as a kind of reserve pool to a certain extent (VORMANN 2003). Many hormones like calcitonin or parathyroid hormone which are involved in the regulation of other electrolytes as well as hormones like glucagon and vasopressin, however, have been reported to also exert a secondary effect on magnesium balance, mostly again via the kidney (SARIS et al. 2000).

If the homeostasis of magnesium is markedly disturbed clinical symptoms may occur. This is mainly true for a hypomagnesemia, which manifests in neuromuscular symptoms like headache, excitations and muscle cramping, depression or cardiac arrhythmias (IANNELLO and BELFIORE 2001). Severe hypomagnesemia is usually secondary to other conditions like diabetes, strong use of diuretics, chronic bowel inflammation, alcohol abuse, severe diarrhea etc. (VORMANN 2003). Hypermagnesemia on the other hand is a relatively rare condition primarily because of the highly efficient renal excretion of any surplus of magnesium. In case of yet elevated plasma levels the consequences include vomiting, hypotension, bradyarrhythmias, paralysis and, ultimately, respiratory and cardiac arrest (MCCARTHY and KUMAR 1999). Apart from the above described,

there are also reports about chronic dietary magnesium deficiency being associated with a number of diseases, especially eclampsia, stroke, heart attack, diabetes, hypertension, asthma bronchiale and others (reviewed in SARIS et al. 2000). However, more research is needed to confirm these hypotheses.

Besides its general importance for stimulus conduction and muscle contraction magnesium also has manifold essential roles on a cellular level (WILLIAMS 1993; WOLF and CITTADINI 2003; WOLF et al. 2003):

- Regulation of ion channels (Ca^{2+}, Na^{+})
- Stabilization of DNA, membranes and proteins
- Cofactor or catalytic modulator of more than 300 enzymes (e.g. Ribonuclease H, Exonuclease etc.)
- Formation of MgATP

Particularly the formation of MgATP is of utmost importance as this complex, and not free form ATP, is the actual substrate for ATP-consuming reactions comprising the action of kinases, ATPases and adenylate cyclase (RUBIN 2005).

However, despite the abundance of essential functions magnesium does not seem to be present in excess concentrations in the cell but rather in a concentration range where even slight alterations may exert a regulatory effect on several cellular processes (TERASAKI and RUBIN 1985). During the last years a lot of studies have been published which suggested a critical role for magnesium in the regulation of cell proliferation (WOLF and TRAPANI 2008). The total cell magnesium content was described to increase several-fold during the log-phase of proliferation (WOLF et al. 2003) while on the other hand an extracellular deficiency leads to a growth arrest (WOLF and TRAPANI 2008). In experiments with HC11 mammary epithelial cells an acute raise of extracellular magnesium was reported to stimulate cell proliferation (SGAMBATO et al. 2001), in contrast to a chronically increased concentration which yielded no effect on proliferation or cellular magnesium content (WOLF et al. 2004). However, the exact mechanisms

underlying the involvement of magnesium in cell growth are not completely resolved but different explanations have been proposed including MAP kinases (TOUYZ and YAO 2003), cell cycle regulatory proteins, mTOR, secondary actions via K^+, Ca^{2+} or Na^+ fluctuations or oxidative stress (reviewed in WOLF and TRAPANI 2008). RUBIN (2007) described an ingenious model linking magnesium and cell proliferation – the "Membrane, Magnesium, Mitosis" (MMM) model. He suggested the binding of growth hormones to their receptor or other non-specific signals will perturbate the cell membrane thus releasing bound magnesium ions from the inner membrane and increasing the concentration of free Mg^{2+} (RUBIN 2005). This in turn increases ribosomal loading and the formation of $MgATP^{2-}$, the latter stimulating the phosphorylation of mTOR, which is a key modulator of protein synthesis. The resulting enhancement of protein synthesis ultimately causes DNA replication and cell division (Fig.2).

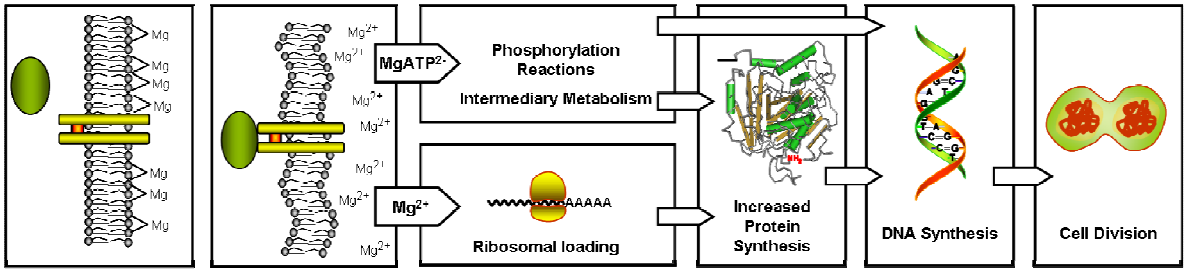

Fig. 2: Model for the involvement of Mg^{2+} in cell proliferation control (according to RUBIN 2005)

The mere rise in extracellular magnesium concentration is also rapidly followed by an increase in free intracellular Mg^{2+} (ZHANG et al. 1997) which may increase protein synthesis in the same way, but it has also been shown that too high concentrations will indeed inhibit protein synthesis transiently (RUBIN 2007). This can be explained by the bell-shaped curve of protein synthesis as a function of intracellular magnesium concentration (RUBIN et al. 1979). More recently, magnesium has been speculated to be crucial to tumor growth in a diverse fashion so the induction of hypomagnesemia may be an important feature of the anti-tumor efficacy of some chemotherapeutics (WOLF et al. 2009).

Apart from cell growth magnesium has also been suggested to affect energy metabolism, possibly via the numerous enzymes involved in glycolysis, citric acid cycle and respiratory chain because most of them are Mg-dependent (WOLF and TRAPANI 2008). This assumption is further underlined by the high magnesium content of mitochondria and endoplasmic reticulum and the importance of MgATP to the cell metabolism as depicted above.

Furthermore, an increase in magnesium concentration has been observed in the early phase of apoptosis for both the intrinsic (PATEL et al. 1994) and the extrinsic (CHIEN et al. 1999) pathway. This may lead to certain (de-)phosphorylations and/or the activation of endonucleases thus promoting the apoptotic process (WOLF and TRAPANI 2008). In contrast, magnesium has antioxidative properties as well which might also protect cells from apoptosis (WOLF and TRAPANI 2008). This may be an explanation for reports that magnesium deprivation can act both as a pro- or antiapoptotic stimulus depending on the cell type (COVACCI et al. 2000; MARTIN et al. 2003). The exact nature of the influence of magnesium on this pathophysiological process therefore remains unclear as yet.

2.1.2. Cellular homeostasis of magnesium

Because of its vital importance to so many cellular functions magnesium homeostasis needs to be extremely well controlled. Fig. 3 shows the various transport mechanisms which have been reported to influence intracellular magnesium concentration (reviewed in ROMANI 2007). Regarding Mg extrusion Na^+-dependent and Na^+-independent pathways have been described, but the Na-dependent one, being represented by a Na^+/Mg^{2+} exchanger, was suggested to be not only the main mechanism of Mg extrusion but indeed the major regulator of cell magnesium content (GUNTHER et al. 1984). As this exchanger depends on the activity of the Na^+/K^+ ATPase it is considered secondary active (BEYENBACH 1990). As for Mg influx a large variety of different mechanisms has been indicated. A protein called paracellin-1 which is exclusively expressed in the kidney is one of the main routes of renal magnesium reabsorption (SIMON et al. 1999). MagT1 is a Mg^{2+}-selective transporter expressed in many epithelial tissues and is regulated by

extracellular Mg concentration (GOYTAIN and QUAMME 2005). Latest publications suggest a critical role of the channels TRPM6 and TRPM7 for cellular magnesium homeostasis. These channels are also referred to as “chanzymes” because their C-terminus contains α-kinase activity (MONTELL 2003). TRPM6 was originally believed to be specific to intestinal and kidney epithelia, thus controlling systemic magnesium balance, while TRPM7 is ubiquitously distributed (WOLF and TRAPANI 2010). However, WOLF et al. (2010) demonstrated that in HC11 mammary epithelial cells TRPM6 and the Na^+/Mg^{2+} exchanger were the actual regulators of cell magnesium content in response to varying Mg availability. As opposed to this, results by other authors indicate that Mg entry into lymphocytes (SAHNI et al. 2010) and rumen epithelial cells (SCHWEIGEL et al. 2008) is mediated by TRPM7. It seems TRPM6/7 are key players in cellular magnesium homeostasis, particularly because they are also capable of forming homo- and heteromers (M. LI et al. 2006) and their exact interaction is likely to depend on the tissue (WOLF and TRAPANI 2010).

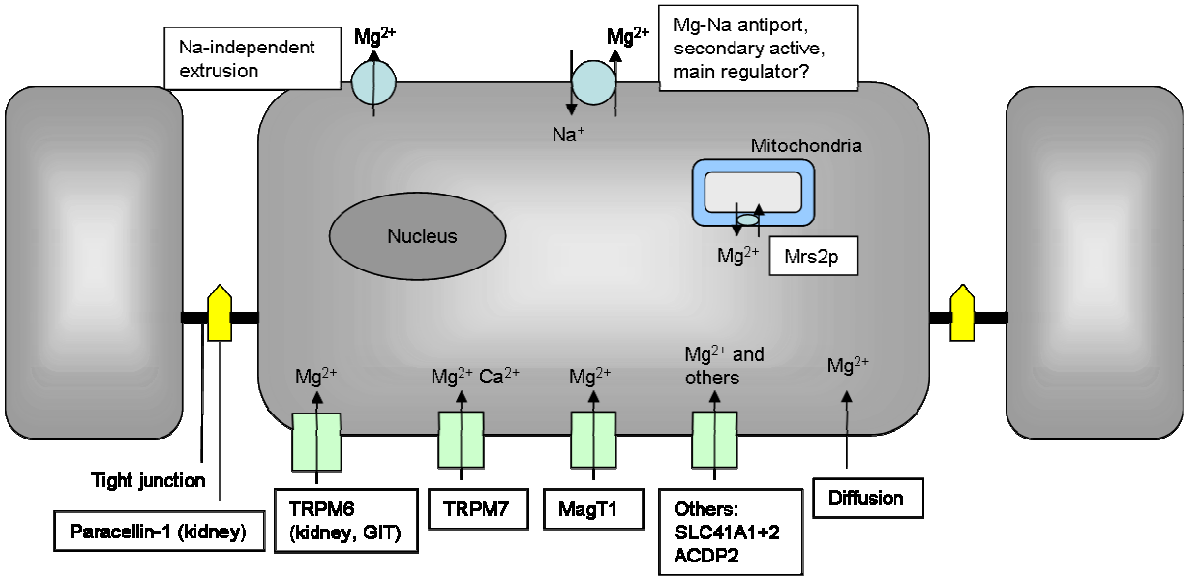

Fig. 3: Regulators of cellular magnesium homeostasis

How powerful the mechanisms of magnesium regulation can become was impressively shown by SGAMBATO et al. (2001) who succeeded in progressively adapting a mammary epithelial cell line to grow in media containing 25 µmol/l or 45

mmol/l magnesium, respectively. Although differences in magnesium content were observed the cells were able to maintain it in a physiological range and they exhibited comparable growth properties.

2.2. Biomedical Magnesium – Degradation

The idea of using magnesium as a biodegradable implant material dates back to the beginning of the 20th century. A number of researchers used different magnesium alloys as orthopedic implants in pilot studies on humans. Back then a too rapid degradation with the consequential accumulation of (hydrogen) gas in the adjacent tissue became apparent as the major problem in spite of nevertheless mostly satisfying clinical biocompatibility (LAMBOTTE 1932; VERBURGGE 1934; MCBRIDE 1938; TROITSKII and TSITRIN 1944). This was the reason why magnesium was soon abandoned as implant material, the more so as stainless steel was introduced as a viable alternative at just about the same time (WITTE et al. 2005). Only a few years ago magnesium was rediscovered for the construction of biomedical implants as it may offer special advantages for certain applications (WILLIAMS 2006) and remarkable successes in the control of its biodegradation behavior have been achieved since then (SONG 2007).

Controlling the biodegradation of magnesium-based implants is a crucial prerequisite for their clinical utilization. Various methods have been used to achieve this:

- Alloying with other elements, usually aluminium, lithium, calcium, zinc, manganese, zirconium and/or rare earth elements (BACH et al. 2005b; STAIGER et al. 2006; SONG 2007; WITTE et al. 2008b; GU et al. 2009a)
- Ultrapure magnesium as impurities are extremely detrimental to the corrosion resistance because of galvanic effects (SONG and ATRENS 1999; REN et al. 2007; SONG 2007; WITTE et al. 2008b)
- Coating with e.g. fluoride, calcium phosphates, polymers, oxides and others in order to delay the contact between corrosion environment and magnesium (BACH and HASSEL 2005; BACH et al. 2005a; CHIU et al. 2007; GENG et al.

2009; WONG et al. 2009; XU et al. 2009; ZHANG et al. 2009c; GU et al. 2010a; LI et al. 2010; THOMANN et al. 2010)

- Special manufacturing procedures like extrusion or hot rolling which lead to grain refinement and thus slower degradation (WANG et al. 2007; LI et al. 2008)
- Surface treatments such as alkaline heat treatment or pre-incubation in NaOH or SBF which deliver a passivated, more corrosion resistant surface (LI et al. 2004; GU et al. 2009b; LORENZ et al. 2009)
- Combinations of the aforementioned

Among these methods the alloying with other elements in order to manipulate the mechanical and corrosion properties of magnesium is by far the most commonly used. The most important drawback in this respect is the fact that the toxicological potential of most of these elements when introduced into the body in larger quantities is unknown (YUEN and IP 2010). Aluminium was reported to be correlated with neurological disorders like Alzheimer's disease (EL-RAHMAN 2003). Lithium is potentially nephrotoxic and teratogenic (BICHET 2006; GILES and BANNIGAN 2006). Zirconium was reported to be associated with liver, lung, breast and nasopharyngeal cancer (SONG 2007). The long-term toxicity of rare earth elements is unresolved and may comprise hemolysis, chromosomal aberrations and hepatotoxicity (GU et al. 2009a). Whether these observations might pose limitations to the choice of alloying material remains to be figured out.

Apart from the composition of the implant itself the corrosion milieu has a critical influence on the degradation process. Metallic magnesium corrodes in an aqueous environment according to the following reaction (MUELLER et al. 2010):

$$Mg + 2H_2O \longrightarrow Mg(OH)_2 + H_2 \uparrow$$

Thus, magnesium degradation results in the formation of hydrogen gas and magnesium hydroxide, the latter leading to an alkalinization of the corrosion milieu.

In case the corrosion medium contains chloride a second reaction occurs in which water soluble magnesium chloride is generated (MUELLER et al. 2010):

$$Mg(OH)_2 + 2Cl^- \longrightarrow MgCl_2 + 2OH^-$$

These processes are illustrated in Fig. 4a and b.

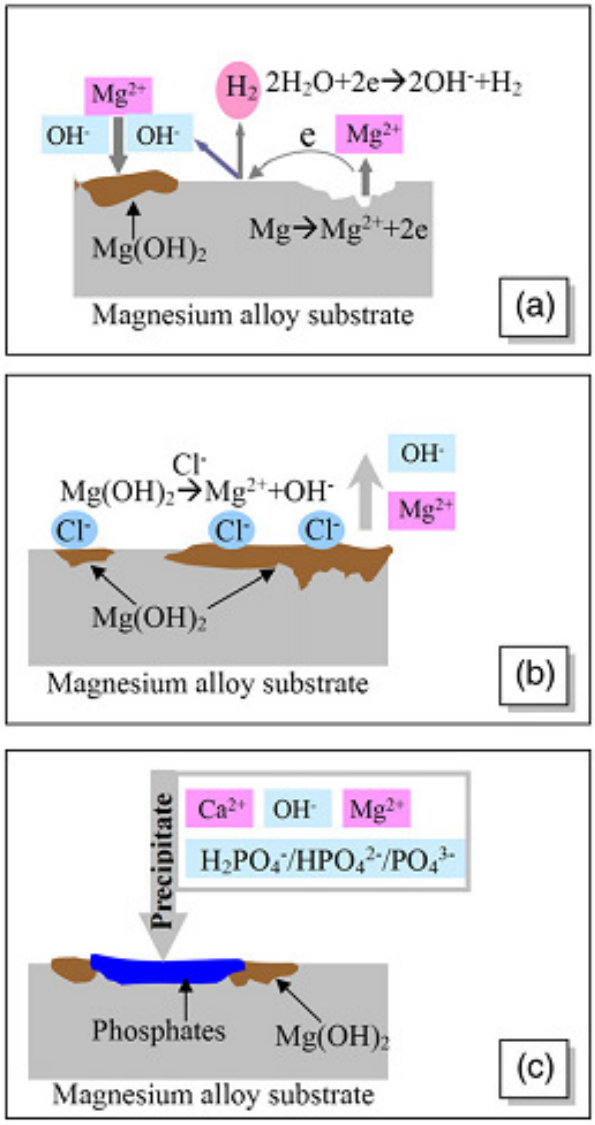

Fig. 4: Reactions between magnesium alloys and SBF; a) formation of $Mg(OH)_2$, b) formation and solution of $MgCl_2$, c) formation of phosphate-containing precipitates (from ZHANG et al. 2009b)

This is why chloride anions are considered “aggressive ions” which induce a rapid, localized “pitting” corrosion attack when the concentration exceeds 30 mmol/l e.g in blood plasma where the chloride concentration is 103 mmol/l (OYANE et al. 2003; SHAW 2003). This leads to an inhomogenous degradation of the implant material. The deposition of magnesium hydroxide on the implant surface itself is supposed to protect the underlying material from further corrosion (SHAW 2003). Unfortunately, it is only stable in a strongly alkaline environment and degradation progresses comparatively faster when the pH milieu is kept constant (WITTE et al. 2005). In a tissue a neutral pH is probably maintained despite magnesium degradation due to the presence of buffer substances in body fluids and the removal of corrosion products via circulation (LORENZ et al. 2009). It may therefore be expected that magnesium implants degrade more rapidly *in vivo* than *in vitro*. However, this does not seem to be the case. WITTE et al. (2006) reported an *in vitro* degradation rate several orders of magnitude higher than observed *in vivo* for the same alloys. This may be explained by differences in the composition of the corrosion environment, especially if standardized *in vitro* corrosion tests are performed using technical solutions (X% NaCl representing the simplest versions)(MUELLER et al. 2009; YAMAMOTO and HIROMOTO 2009). Those lack a large number of constituents of body fluids including various anorganic ions in different concentrations, buffer substances, amino acids and proteins (RETTIG and VIRTANEN 2008; XIN et al. 2008; MUELLER et al. 2009). Especially proteins seem to be of major relevance as they were shown to retard magnesium degradation (YAMAMOTO and HIROMOTO 2009; KIRKLAND et al. 2010). Moreover, most *in vitro* corrosion tests are performed in a static setup which does not take circulation into account (LEVESQUE et al. 2008; XIN et al. 2010) and the calculation of a corrosion rate from *in vitro* experiments is very difficult if pitting corrosion occurs (MUELLER et al. 2009) which makes comparisons even more complicated.

As far as the *in vivo* application of magnesium-based implants is concerned a further factor comes into play regarding the suitability of the material. This factor is the mechanical integrity of the implant during the period of degradation because

implants need to be able to resist severe cyclic loading (GU et al. 2010b). Thus, the corrosion of the magnesium alloys must be controlled not only to assure their biocompatibility by delaying the formation of degradation products but also to maintain their load-bearing capacity for the necessary duration. The length of this period depends on the respective use. GU et al. (2010b) recently reported that cyclic loading in turn increases the corrosion rate of magnesium alloys.

In general, magnesium alloys corrode very rapidly after the initial contact with the corrosive medium and large amounts of hydrogen and magnesium ions are released. Then, within 24 to 72 hours the material matrix is covered with degradation products which passivate the surface and the corrosion rate decreases significantly (WANG et al. 2008; XU et al. 2008; GU et al. 2009b; YAMAMOTO and HIROMOTO 2009). In this respect, a biphasic degradation kinetic seems to be the usual pattern of magnesium corrosion although some authors also suggested a more linear behavior (RETTIG and VIRTANEN 2008). In any case these kinetics were gathered *in vitro* and whether they are also true for the *in vivo* situation is unknown.

The assumption that the milieu composition is of vital importance to the degradation process is also reflected by the compounds found in the corrosion layers on magnesium alloys. When these materials were incubated in solutions designed to mimic body fluids calcium phosphates, apatites and/or carbonates were found instead of or in addition to magnesium hydroxide (LI et al. 2004; CORTES et al. 2007; LI et al. 2008; XU et al. 2008; RETTIG and VIRTANEN 2009; XIN et al. 2010)(Fig. 4c). Interestingly, these are the same degradation products also observed after implantation in animal experiments (WITTE et al. 2005; XU et al. 2007; ERDMANN et al. 2011; THOMANN et al. 2010). Therefore the deposition of such complex corrosion products on magnesium-based implants most likely offers some corrosion protection thus slowing down further degradation (XIN et al. 2010). Besides, the degradation products, calcium phosphates in particular, were suggested to promote osteoconductive effects (LI et al. 2004; WITTE et al. 2005; XU et al. 2007; LI et al. 2008).

For these reasons, some authors hypothesize that *in vitro* tests of degradation behavior would be far more predictive if the test conditions and media compositions would simulate the *in vivo* situation more closely (LEVESQUE et al. 2008; YAMAMOTO and HIROMOTO 2009; GU et al. 2010b; MUELLER et al. 2010). Unfortunately, a reliable system is not available yet.

2.3. Biomedical Magnesium – Biocompatibility

2.3.1. *In vitro* biocompatibility

The biocompatibility of magnesium and magnesium alloys has already been tested in a variety of cells, mainly fibroblasts cell lines or primary cells or cell lines of bony or vascular origin with respect to the desired field of application. Unfortunately, no definite conclusion can be drawn because of disparities in the experimental protocols, assessment of different parameters (cell adhesion, morphology, proliferation, metabolic activity) and, generally, the use of various materials regarding composition, geometry and manufacturing.

Regarding bone cells favorable biocompatibility was reported several times for magnesium salts (FESER et al. 2010; FEYERABEND et al. 2010) and different magnesium alloys (LI et al. 2004; WITTE et al. 2007a; PIETAK et al. 2008; ZHANG et al. 2009b; FEYERABEND et al. 2010; LI et al. 2010; YANG et al. 2010). Zreiqat et al. showed that Mg can indeed enhance bone cell adhesion on biomaterials via integrin expression (ZREIQAT et al. 2002) and the MAP kinase pathway (ZREIQAT et al. 2005). On the other hand, results by other authors indicate cytotoxicity of magnesium and its alloys (SERRE et al. 1998; GENG et al. 2009; GU et al. 2010a; WONG et al. 2010).

As magnesium-based implants may also be used in the cardiovascular system, biocompatibility was assessed in this respect, too. Human endothelial cells and vascular smooth muscle cells revealed no toxic effects of high concentrations of magnesium salts (PEUSTER et al. 2006) and living cells of the same kind were observed in the vicinity of MgCa alloys though there was no colonization of the materials themselves (DRYNDA et al. 2010). Moreover, hemocompatibility of

magnesium alloys was already investigated, again with inconsistent results depending on the type of alloy (GU et al. 2009a; HANSI et al. 2009; ZHANG et al. 2009b; DRYNDA et al. 2010; LU et al. 2011).

Further studies showed good biocompatibility of MgCa extracts for L929 fibroblasts (LI et al. 2008; GU et al. 2009a; HANSI et al. 2009; ZHANG et al. 2009b; DRYNDA et al. 2010; ZHENG et al. 2010) and murine dendritic cells (FESER et al. 2010) and results by YU et al. (2010) yielded no induction of DNA damage, chromosomal aberrations or gene mutations by magnesium phosphate bone cement extracts.

In the light of this non-uniformity of experimental protocols and results GU et al. (2009a) conducted an elegant systematic study of several binary magnesium alloys in order to provide comparable data for different materials and cell types. Binary Mg alloys with 1% Al, Ag, In, Mn, Si, Sn, Y, Zn or Zr were manufactured (pure magnesium served as control) and tested for mechanical properties, corrosion behavior in Hank's solution and SBF as well as for hemocompatibility and cytotoxicity. For the latter, two types of murine fibroblasts (L929 and NIH3T3), murine preosteoblasts MC3T3-E1, human endothelial cells ECV304 and rodent vascular smooth muscle cells (VSMC) were incubated with medium degradation extracts for different periods of time before viability was assessed (Fig. 5). The extracts of aluminium, tin and zinc alloys exerted no significant negative effect on fibroblast and osteoblast viability, while for ECV304 and VSMC this was only true for aluminium and zinc alloys. Pure magnesium extracts were found to be slightly (but statistically significant) detrimental to all cell types with the exception of L929 cells where this effect was more pronounced, and pure magnesium also yielded the worst results in terms of hemolysis and platelet adhesion. The researchers attributed this outcome to the very high pH caused by pure magnesium corrosion rather than to Mg^{2+} ion content (a hypothesis which was recently confirmed by YANG et al. (2010) using hBMSC). According to their results the authors recommended Al and Y for the production of magnesium alloy stents and Ca, Zn, Sn, Si, Mn and Al were considered suitable for orthopedic implants.

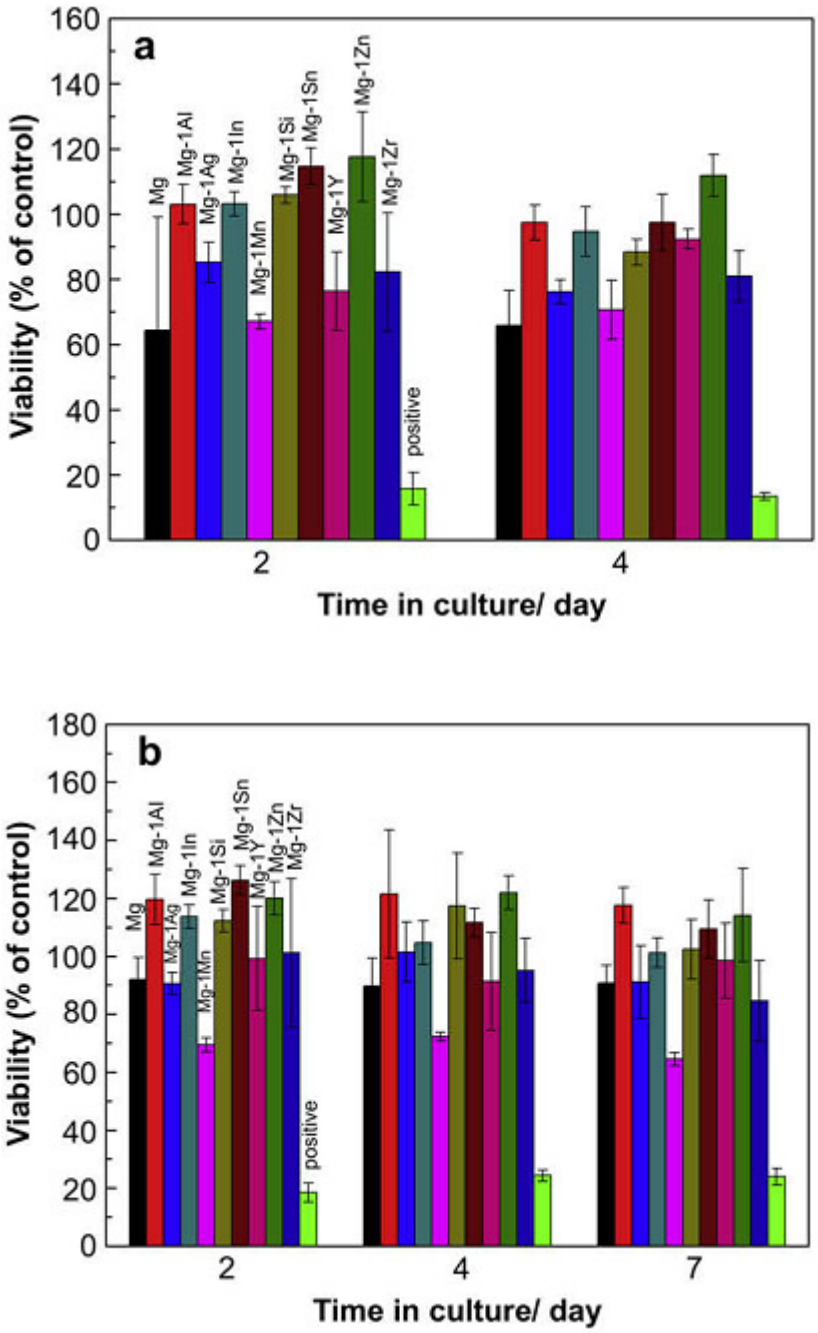

Fig. 5: Cell viability of different cell types after 2, 4 and 7 days of incubation with pure magnesium or Mg alloy extracts as published by GU et al. (2009a); a) L929, b) NIH3T3 (from GU et al. 2009a)

It should be remarked that many of the above biocompatibility evaluations were done utilizing an MTT assay. This tetrazolium salt-based test measures viability

and proliferation via the metabolic activity of the cells which leads to a conversion of the tetrazolium salt to formazan. The resulting color change is directly proportional to the number of metabolically intact cells. Unfortunately, it could recently be shown by FISCHER et al. (2010) that the products of magnesium corrosion also cause tetrazolium salt conversion leading to false high results thus rendering tetrazolium assays unsuitable in this context. The authors proposed the BrdU assay as an alternative and observed severe toxicity of pure magnesium extracts on MG63 osteoblast-like cells.

According to standardized protocols (ISO10993-5:2009) most biocompatibility studies were performed via indirect contact using degradation extracts. One reason this is usually done is because the corrosion of magnesium leads to the evolution of hydrogen which hinders the attachment of cells onto the implant material (LORENZ et al. 2009; XIN et al. 2009). Nevertheless, this method too has some disadvantages because it does not consider all sorts of degradation products. Theoretically, four types of products can result from magnesium corrosion (GU et al. 2009a):

1. Metal ion release (magnesium and alloying elements)
2. OH^- ions
3. evolved hydrogen
4. discharged particles

Hydrogen evaporates during extract preparation and particles are removed via centrifugation or perhaps due to precipitation (WITTE et al. 2008b; GU et al. 2009a). The biocompatibility implications of these products can hence not be evaluated. On the other hand, the extracts have to be acutely supplemented to the cells. This may lead to osmotic shock damage due to high ion content and pH thus overestimating cytotoxicity (WITTE et al. 2008b). That these considerations may indeed matter can be illustrated using the example of pure magnesium. While acceptable compatibility of its extracts could be shown for different cells (GU et al. 2009a; FESER et al. 2010; YANG et al. 2010) the results of recent studies

indicated considerable cytotoxic effects of a direct contact between cells and magnesium (GENG et al. 2009; LORENZ et al. 2009). LORENZ et al. (2009) made a further point that another problem of *in vitro* biocompatibility testing is the low medium volume/surface area ratio in a non-dynamic setup which may overrate deleterious effects of corroding magnesium alloys. PURNAMA et al. (2010) even suggested that biocompatibility tests which were created for assessing corrosion-resistant materials are generally unsuitable for the evaluation of biodegradable metals. They named markers of genetic regulations like antioxidant enzymes, tumor suppressor gene products like p53, cytokines etc. as being more appropriate endpoints and proposed short duration biocompatibility testing in dynamic systems in order to reduce the probability of false positive results.

2.3.2. *In vivo* biocompatibility

2.3.2.1. Orthopedics

In theory, degradable magnesium-based implants would be near ideal devices for osteosynthesis purposes as the facilitation of fracture healing is by definition a temporary function and the respective implants should be removed afterwards anyway to assure they do not cause issues like stress-shielding effects themselves (STAIGER et al. 2006; ZHANG et al. 2009a). On top of that, magnesium has very appealing mechanical characteristics with regard to cortical bone (STAIGER et al. 2006; XU et al. 2007) and may even exert osteoinductive effects (WITTE et al. 2005; WITTE et al. 2007b; JANNING et al. 2010; CASTELLANI et al. 2011). As mentioned above, the first studies on orthopedic magnesium alloy implants date back almost one hundred years (MCBRIDE 1938; TROITSKII and TSITRIN 1944). Though clinical results had been decent the major side effect of gas accumulating in surrounding tissues led to the abandonment of this approach. Nevertheless, during the last couple of years an increasing number of experimental studies using novel magnesium alloys have been published and the results are predominantly promising.

Among the most cited ones is a pioneering study by WITTE et al. (2005). The authors investigated four magnesium alloys (the aluminium-zinc alloys AZ31 and

AZ91 and the rare earth-containing ones WE43 and LAE442) in comparison with the degradable polymer polylactide. Rods of 20 mm in length and 1.5 mm in diameter were implanted into the femura of guinea pigs and the degradation process was evaluated after six and eighteen weeks. They detected increased bone formation around the magnesium alloys (Fig. 6) and a corrosion layer consisting of amorphous calcium phosphate. The corrosion layer around the respective implants also contained rare earth elements which in contrast could not be found in the surrounding bone. The authors also observed subcutaneous gas pockets in the implantation area which however did not cause adverse clinical effects and which did not reappear after having been removed by aspiration. LAE442 was shown to be the most slowly corroding alloy although it was later reported to degrade more rapidly than AZ91D under *in vitro* conditions (WITTE et al. 2006). In a later study a further reduction of LAE442 corrosion could be achieved via extrusion and fluoride coating which again yielded an acceptable host response (WITTE et al. 2010).

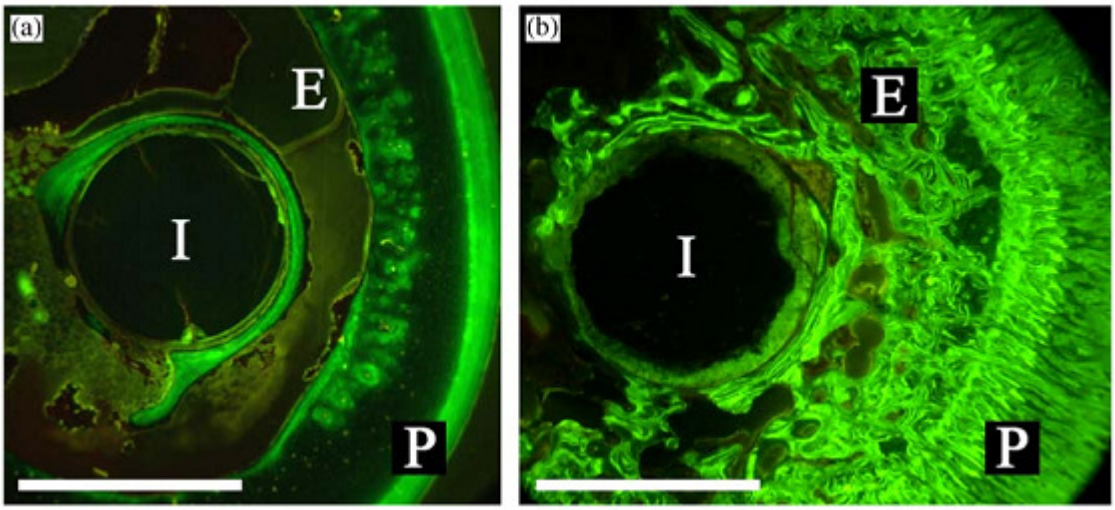

Fig. 6: Newly formed bone in guinea pig femura implanted with (a) degradable polymer or (b) magnesium alloy. Bar = 1.5 mm, I = implant, E = endosteal bone formation, P = periosteal bone formation (from WITTE et al. 2005)

Since the initial findings a lot of studies exploring the effects of different magnesium alloys, treatments and coatings in rabbits, guinea pigs or rats have

been published. New bone formation was confirmed several times (WITTE et al. 2007b; XU et al. 2007; LI et al. 2008; BOSIERS et al. 2009; ZHANG et al. 2009a; THOMANN et al. 2010; WONG et al. 2010) and results by JANNING et al. (2010) indicate that it might be induced by magnesium hydroxide though it remains unknown whether this effect is depends on increased Mg concentration or local alkalosis or both. Despite all efforts to slow down the *in vivo* corrosion rate of the alloys gas accumulation is still quite often observed if the implants are not additionally coated (WITTE et al. 2007c; LI et al. 2008; ERDMANN et al. 2011; ZHANG et al. 2010). Nevertheless, no study reported clinical adverse effects or abnormal animal behavior. Furthermore, no elevations of serum magnesium concentration (XU et al. 2007; LI et al. 2008; WONG et al. 2010) or indications of impaired organ function (neither histologically (ZHANG et al. 2010) nor by alterations in blood chemistry parameters (ZHANG et al. 2009a; WITTE et al. 2010; ZHANG et al. 2010)) were found. An inflammatory response to the degradation process could either not be detected at all (XU et al. 2007; WONG et al. 2010; CASTELLANI et al. 2011) or merely in form of an appropriate mild foreign body reaction (WITTE et al. 2007c) or a transient lymphoinfiltration (XU et al. 2009). In a recent study Erdmann et al. (2010) implanted MgCa0.8 screws in rabbit tibiae and examined the surrounding muscle tissue histologically. Inflammation of the periimplant tissue was found to be minimal to moderate, however the authors expressed concern at the fact that it reincreased after six to eight weeks at the end of the study, so they suggested longer-term investigations. Finally, WITTE et al. (2008a) observed no skin sensitizing potential of the alloys AZ31, AZ91, WE43 and LAE442 in guinea pigs. Therefore, in general a good biocompatibility of magnesium and its alloys can be assumed. However, it has to be noted that studies assessing possible long-term health effects of critical alloying elements released during degradation have not been performed so far.

Fracture healing takes six to twelve weeks for upper limbs and 12 to 24 weeks for lower limbs (GU et al. 2010a) and it was suggested that degradable orthopedic implants should maintain their integrity for at least twelve weeks (STAIGER et al. 2006). Not surprisingly, some authors reported faster degradation of magnesium-

based implants in the bone marrow cavity than in cortical bone (XU et al. 2007; ZHANG et al. 2009a; ERDMANN et al. 2011) which was attributed to the larger amounts of body fluid present there. ERDMANN et al. (2011) observed significantly higher pull-out forces of stainless steel screws in comparison with MgCa0.8 screws after 4 weeks implantation. In contrast to that, CASTELLANI et al. (2011) reported better osseointegration and higher push-out forces of MgYNdHRE rods as opposed to titanium alloy rods. So, apart from matters of biocompatibility, the question remains whether the degradation performance of currently available magnesium alloys is sufficient for fracture fixation over the necessary period of time.

2.3.2.2. Cardiology

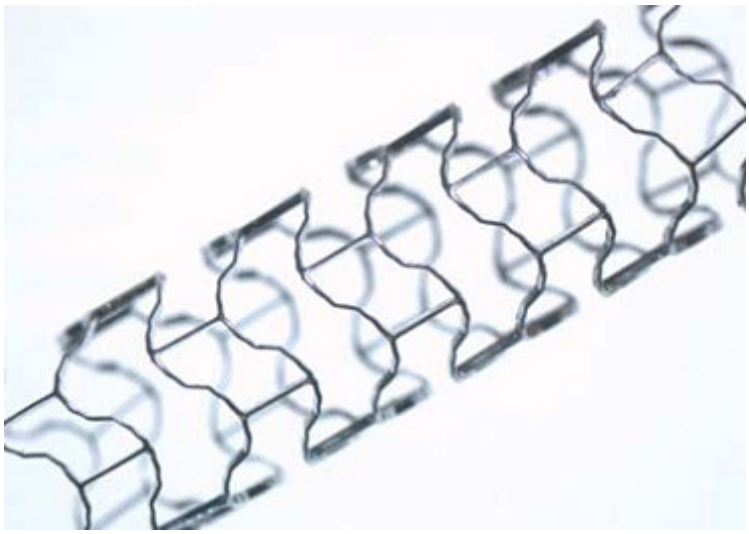

Fig. 7: Expanded magnesium stent (MAGIC, Biotronik) for coronary arteries (from PEUSTER et al. 2006)

Magnesium alloys are promising candidates for the manufacturing of degradable vascular stents (Fig. 7). Current permanent stent materials are stainless steel and cobalt-chromium alloys. These stents are well established and effective for the surgical intervention in arterial stenosis where they are inserted after the vessel has been dilated to prevent recoil and early restenosis (PEUSTER et al. 2006). But their use is also often accompanied by late in-stent restonosis because the prolonged irritation of the arterial wall causes intimal hyperplasia (DRACHMAN and

SIMON 2005; D. WILLIAMS 2006). Further problems are the limited growth adaptability of non-resorbable implants especially concerning pediatric surgery (PEUSTER et al. 2006; HEHRLEIN 2007) and the relatively high incidence of thrombembolic events which demands a continuous anti-coagulative therapy (ONG et al. 2005; WAKSMAN 2006). This is when magnesium alloy stents come into play. On one hand, they keep an artery open until remodelling has occurred (HERMAWAN et al. 2010) and on the other hand their complete degradability removes the irritating stimulus thereafter and may allow the arteries to regain their vasomotor functions (GHIMIRE et al. 2009). Adding even more benefit magnesium was also suggested to have antithrombogenic and antiarrhythmic properties (ADAMS and MITCHELL 1979; DI MARIO et al. 2004; PEUSTER et al. 2006).

The potential of degradable magnesium stents was assessed in a number of animal studies and clinical trials. HEUBLEIN et al. (2003) implanted AE21 alloy stents in the coronary arteries of domestic pigs. They observed no major problems but a transient loss of lumen area because of neointima formation. The stents had lost their integrity after 56 days, so it was concluded that the degradation should happen more slowly. In another study using pigs a commercial magnesium alloy stent was compared with a stainless steel stent and the Mg stent was considered safe and exhibited less neointima formation although this did not result in larger vessel lumina (WAKSMAN et al. 2006).

A preliminary trial in 20 human patients with lower limb ischemia revealed no adverse effects and clinical patency after three months was achieved in 17 patients (PEETERS et al. 2005). Moreover, there are some promising case reports on the successful and partly live-saving application of a commercial absorbable metal stent (AMS) in babies even though restenosis was observed in two cases (MCMAHON et al. 2000; ZARTNER et al. 2005; SCHRANZ et al. 2006). The results of a multicentre trial with 63 coronary artery lesion patients treated with the AMS showed safe degradation within four months but a higher incidence of restenosis compared with bare metal stents due to neointima formation and negative remodelling (ERBEL et al. 2007). Finally, there are two more recent

studies confirming this outcome in porcine coronary arteries (MAENG et al. 2009) as well as human patients with below-the-knee critical limb ischemia (BOSIERS et al. 2009).

In summary, the available studies indicate good biocompatibility and safety of biodegradable magnesium alloy stents but the anticipated efficacy seems to be not yet achieved because degradation and loss of integrity may still occur to early for the stented vessels to remodel stably (which was suggested to require a period of six to twelve months (HERMAWAN et al. 2010)).

2.4. Chronic rhinosinusitis

Rhinosinusitis is a highly prevalent medical condition which millions of people come down with every year (DYKEWICZ and HAMILOS 2010) and which leads to remarkable physical symptoms and negative affections of the quality of life (MELTZER et al. 2004). Should the disorder persist for more than twelve weeks it is defined as a chronic rhinosinusitis (CRS) (MELTZER et al. 2004). CRS is commonly treated with corticosteroid nasal sprays perhaps in combination with antihistamines, antibiotics or antifungals depending of the etiology of the disease (DYKEWICZ and HAMILOS 2010). However, if the condition turns out to be refractory to medical therapy (endoscopic) sinus surgery is indicated in order to restore patency of the sinus ostia and thus drainage (DYKEWICZ and HAMILOS 2010). Surgery of the frontal sinus can be especially challenging and approximately 30 percent of the patients experience restenosis of the outflow tract due to reactive scarring processes during the wound healing period (HUNTER et al. 2010). In order to prevent this simple silicon stents (FREEMAN and BLOM 2000) or their drug-eluting variants (HERRMANN et al. 2004; BEULE et al. 2008; BEULE et al. 2009) can be inserted after surgery but the effectiveness of this approach remains a matter of controversy (HOSEMANN et al. 2003). Short-term stenting may be inefficacious (BANHIRAN et al. 2006). Long-term stenting seems to yield better results in terms of preventing restenosis (WEBER et al. 2000; LIN and WITTERICK 2008) but it can be accompanied by other complications including stent dislocation or infection, nasal congestion, unpleasant odour and fresh trauma to the mucosal

tissue when the material is removed at last (PERLOFF and PALMER 2004; WEBER 2009; HUNTER et al. 2010).

A resorbable magnesium-based stent may be a viable option to overcome these limitations. As it is supposed to degrade after local wound healing has occurred a removal trauma is avoided, thus circumventing an insult which might again result in scar tissue formation and stenosis. Beyond that, a magnesium stent may be additionally conducive to the treatment of CRS as magnesium was reported to benefit healing processes (BANAI et al. 1990; GRZESIAK and PIERSCHBACHER 1995; DENDA et al. 1999). However, to date there have been no studies investigating this potential field of application.

2.5. Objectives of the project

This PhD project is dedicated to establishing a predictive *in vitro* test system for the biocompatibility screening of new magnesium alloys. As the alloys concerned will be designed for a use as stents in the nasal cavity for the treatment of chronic sinusitis the system comprises models of porcine nasal mucosa because nasal mucosa is the target tissue and the pig is regarded as an appropriate animal model for future preclinical experiments. It also comprises an isolated organ model enabling the investigation of degradation processes in a more complex environment.

The *in vitro* test system is established using pure magnesium test implants in order to exclude possible effects of alloying elements. In doing so the following objectives shall be accomplished:

- Determination of parameters suitable for the biologic evaluation of magnesium alloys
- Assessment of the biocompatibility of pure magnesium
- Deepening the insights into the processes, mechanisms and responses at the tissue-implant interface in the course of magnesium degradation

Thus, a knowledge base shall be provided for comparison purposes when magnesium alloys are tested using this system in the future. In offering the opportunity of evaluating the biocompatibility of magnesium alloys in *in vitro models* at an early stage of development this project shall furthermore contribute to a reduction of animal experiments.

3. *Ex vivo* examination of the biocompatibility of biodegradable magnesium via microdialysis in the isolated perfused bovine udder model

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Abstract

Purpose: Being biodegradable magnesium is considered a promising future implant material but very little is known about the biocompatibility for the tissues in direct contact with it. In this study the degradation of pure magnesium implants in the skin of an isolated bovine udder was examined over a period of five hours.

Methods: Microdialysis technique was used in order to investigate the reactions at the interface of implant and tissue. Pure titanium implants served as control. Degradation behavior and biocompatibility were evaluated via extracellular magnesium ion concentration and PGE_2 and TNF alpha served as indicators of inflammation.

Results: Concentrations of 5.5 mmol/l Mg^{2+} were detected at the beginning which decreased to a plateau of about 3.5 mmol/l after approximately two and a half hours. PGE_2 and TNF alpha concentrations indicated no major inflammatory tissue response to the degradation.

Conclusions: These results give an idea of the ion burden at the implantation site of degrading magnesium and suggest good biocompatibility even at the tissue-implant interface.

4. Diverse effects of pure magnesium on parameters of primary porcine nasal epithelial cell biocompatibility and metabolism

Stephan Schumacher, Jessica Stahl, Wolfgang Bäumer, Manfred Kietzmann

Manuscript in preparation of submission

Abstract

Resorbable magnesium-based implants offer a new therapeutic option in the treatment of chronic rhinosinusitis (CRS). To account for this field of application a model of primary porcine nasal epithelial cells (PNEC) was used for assessing the biocompatibility of degrading pure magnesium, while simultaneously investigating whether the degradation products may affect certain processes of cellular metabolism. In comparison with pure titanium or supraphysiological concentrations of magnesium ions, magnesium test implants did not induce apoptosis or a general inhibition of metabolic activity in PNEC. However, a large number of cells were found necrotic after 48 hours and an elevated IL-8 secretion indicates some proinflammatory reactions. Thus, the biocompatibility of magnesium itself appears less ideal than often supposed. Furthermore, pyruvate kinase and succinate dehydrogenase activities were also affected but in an almost contrary fashion compared with magnesium ions. An influence on cell protein synthesis was not detected. The exact mechanism behind the observed alterations is unclear though and remains to be elucidated.

Keywords: biocompatibility, CRS (chronic rhinosinusitis), degradation, epithelial cells, magnesium, porcine tissue

Introduction

The research on magnesium alloys as biomaterials for the development of resorbable implants has become a major interest in recent years. These implants are intended to slowly degrade after having fulfilled their temporary function of supporting tissue during healing and remodelling thus obviating the need for an implant removal surgery [1]. Because of the biocompatibility demands of implant materials this must happen without detrimental effects on any part of the organism. Magnesium itself, representing the main constituent of the alloys, is considered uncritical because it is a macromineral in the body. It is suggested that any surplus of magnesium released during implant corrosion can be easily excreted via the kidneys. Plasma magnesium levels and organ functions hence remain unchanged [2]. However, a significant increase in extracellular magnesium concentration is

anticipated in the immediate vicinity of an implant which is likely to be accompanied by an alkalinization and a hydrogen evolution on the biomaterial's surface [3]. These milieu changes may affect the cells at the tissue-implant interface reducing their viability and/or triggering an inflammatory reaction. Therefore, the local tissue tolerance of corroding magnesium is not as self-evident as it may seem which led us to investigate the effects of pure magnesium in more detail.

Biomedical magnesium alloys are predominantly designed for orthopaedic and cardiovascular applications but the nasal cavity has recently been proposed as another potential target [4]. Chronic rhinosinusitis (CRS) patients often suffer from stenosis of the paranasal sinus apertures and the formation of scar tissue is a common complication after surgical intervention. Silicon stents are frequently used to maintain the ventilation of the sinuses during the wound healing period but the outcome may be unsatisfactory. Depending on the duration of stenting typical problems include restenosis, stent dislocation or infection, nasal congestion, unpleasant odour and renewed tissue trauma when the silicon material is finally removed [5-7]. A resorbable magnesium-based stent may overcome some of these troubles thus presenting a therapeutic option in the treatment of CRS, but this field of application has not been investigated as yet.

The airway epithelial cells represent the cell type in direct contact with an implant in the nasal cavity. In order to simulate this situation we decided to assess the biocompatibility of magnesium utilising a model of *in vitro* differentiated primary porcine nasal epithelial cells (PNEC), because the pig is considered a suitable animal model for future *in vivo* studies of degradable paranasal sinus stents. Apart from biocompatibility aspects the local surplus of magnesium might also exert some effects on metabolic processes. Magnesium is an essential element in the body with diverse cellular functions as a regulator of ion channels, a stabilizer of membranes and nucleic acids, a co-factor of hundreds of enzymes and, in the form of $MgATP^{2-}$, the substrate for energy-consuming reactions [8]. Moreover, magnesium ions have been described as a regulator of protein synthesis and

proliferation [9]. Whether these parameters experience any changes due to the presence of degrading magnesium has not yet been studied in any detail.

Material and methods

Test implant preparation

Pure magnesium (99.66 % magnesium) was processed by gravity die-casting and extrusion, resulting in test specimen with a diameter of 8 mm and a height of 2 mm. These test implants were degreased with ethanol and heat-sterilized at 180 °C for two hours before use in the experiments.

Cell culture

Primary porcine nasal epithelial cells (PNEC) were isolated using a modified protocol according to Mao et al. [10]. In short, nasal septum and ventral turbinates were taken from pigs euthanized due to reasons not related to this study, and transported in Hank's balanced salt solution (HBSS; PAA Laboratories, Pasching) on ice. Nasal mucosa was then removed from underlying tissue, washed five times in M199 medium supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (all PAA Laboratories, Pasching), hereafter referred to as M199+, cut into small pieces and incubated in M199+ containing 0.6 mg/ml protease type XIV (Sigma-Aldrich, Steinheim) at 4 °C overnight. Cells were collected by gentle agitation in M199+ containing 10 % FCS (PAA Laboratories, Pasching) and preincubated in a Petri dish for 1 hour to reduce fibroblast contamination. Afterwards, non-adherent cells were collected with the supernatant, washed three times in M199+ and resuspended in airway epithelial cell growth medium (AECGM; Provitro, Berlin) supplemented with antibiotics as above, 5 % FCS and 10^{-7} M retinoic acid (AppliChem GmbH, Darmstadt). They were seeded on polycarbonate membranes (Nunc, Langenselbold) coated with rat-tail collagen (Roche, Mannheim) at a density of approx. 160000/cm² and leftover cells were cryopreserved in liquid nitrogen for later use. The PNEC were cultured for six days with a medium change every other day before the medium was removed from the upper compartment to create an air-liquid interface (ALI). Medium was changed to serum-free conditions and cultivation was continued for 14 days to allow for a

differentiation of the cells before experiments were started. Epithelial origin and differentiation of the cells were verified by immunocytological staining using antibodies against cytokeratin (clone C-11), vimentin (clone V9) or β-tubulin (clone TUB 2.1; all Sigma-Aldrich GmbH, Steinheim), respectively and a FITC-conjugated goat anti-mouse IgG secondary antibody (AbD Serotec, Düsseldorf).

For the experiments test implants were placed on the insert membranes and incubated with the PNEC for 48 hours. Unless indicated otherwise the experimental groups were as follows:

1. magnesium implant (Mg)

2. pure titanium implant as treatment control since titanium is supposed to be an inert biocompatible material (Ti)

3. titanium implant plus addition of 15 mmol/l $MgCl_2$ to the medium in order to be able to distinguish between effects exerted by magnesium ions and effects of other degradation products (Ti + $MgCl_2$). The concentration of 15 mmol/l was chosen as it did not yet result in a decrease of metabolic activity of L929 murine fibroblasts in preliminary experiments (data not shown).

4. cells without implant as untreated control.

After the incubation period the test implants were removed and cells and supernatants were used for the measurement of the respective parameters.

In addition to the experiments with differentiated cells, freshly isolated PNEC were also seeded in collagen-coated 96 well plates, cultured to confluence and then incubated with increasing concentrations of magnesium chloride for 24 hours before metabolic activity was measured. This was done to examine the dose-effect relationship of magnesium ions.

Parameters

Magnesium concentration: The supernatant magnesium concentration was measured colorimetrically utilising a commercial assay kit (Nanocolor Härte 20, Macherey-Nagel, Düren).

Metabolic activity: Metabolic activity was determined as an indicator of cell viability. Supernatants were removed and 20 % CellTiter96 AQueous One Solution (Promega, Mannheim) in culture medium was added to the well and incubated for one hour at 37 °C at 5 % CO_2. A color change indicated metabolic activity. The optical density of the mixture was then read at 490 nm by a MRX microplate reader (Dynatech, Denkendorf) and the optical density of blanks was subtracted. The viability results are expressed as percentages of control.

Inflammatory response: The culture media were collected, centrifuged at 3000 g at 4 °C for 5 minutes and the resulting supernatants were stored at -80 °C until further analysis. Prostaglandin E_2 (PGE_2; Cayman Chemical, Ann Arbor), porcine interleukin 6 (IL-6) and porcine interleukin 8 (IL-8; both R&D Systems, Minneapolis) concentrations were determined by commercially available ELISA kits according to the manufacturer's instructions. Stimulation of the PNEC with 100 µg/ml lipopolysaccharide (LPS) 0111:B4 (Sigma-Aldrich GmbH, Steinheim) served as a positive control for inflammatory response.

Apoptosis: Membranes with the cells were cut out of the inserts and stained for apoptosis and necrosis using the Annexin-V-Fluos Staining Kit (Roche, Mannheim) according to the manufacturer's instructions. The membranes were then analysed under a fluorescence microscope at 400x magnification and the number of positive cells in five randomly chosen fields were counted by an examiner blinded to the treatment.

Pyruvate kinase (PK) and succinate dehydrogenase (SDH) activity: PNEC were collected by scraping in 500 µl ice-cold STE buffer (250 mmol/l sucrose, 10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 7.4) supplemented with protease inhibitor cocktail (Complete EDTA-free, Roche, Mannheim) and lysed by sonication. Membranes

and debris were removed by a centrifugation at 300 g, 4°C for 5 minutes, followed by a centrifugation of the resulting supernatant at 14000 g, 4°C for 15 minutes. The final supernatant contained the cytosolic fraction and was used for the determination of PK activity. The pellet containing the mitochondrial fraction was resuspended with 500 µl ice-cold STE buffer with protease inhibitors and used for the determination of SDH activity. Both fractions were stored at -80°C until analysis. Their protein content was measured using a Bradford-based assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, München).

PK activity was determined using a modified method according to Gutmann and Bernt [11]. The reaction medium contained 97.5 mmol/l triethanolamine, 13 mmol/l $MgSO_4$, 74 mmol/l KCl, 185 µmol/l NADH, 1 mmol/l PEP, 2.5 U/ml LDH and 3 mmol/l ADP and the results are expressed as change in optical density (ΔOD) per µg protein.

SDH activity was measured using a reaction medium containing 200 mmol/l succinate as substrate and 1.34 mmol/l nitroblue tetrazolium (NBT) in a phosphate buffer (200 mmol/l Na_2HPO_4, 200 mmol/l KH_2PO_4, pH 7.4). 100 µl sample was mixed with 100 µl reaction medium and optical density at 570 nm was determined using a microplate reader. Readings without succinate in the reaction medium served as the respective blanks. The mixtures were then incubated at 37 °C for four hours in the dark before optical density was measured again. SDH activity results are expressed as ΔOD per mg protein.

^{14}C amino acid incorporation: In order to assess cellular protein synthesis the culture medium was supplemented with 3.7 KBq/ml ^{14}C-labeled amino acid mixture (GE Healthcare, Buckinghamshire) for the duration of incubation with the test implants. The suppression of protein synthesis by addition of 20 µg/ml cycloheximide served as negative control. After the incubation period the cells were washed three times with ice-cold TRIS-buffered saline (TBS). Then the cells were scraped in 500 µl TBS and transferred to glass vials before the protein was precipitated using 5% ice-cold trichloroacetic acid (TCA) for ten minutes. This was followed by two washing steps with TCA and solubilization with 0.2M NaOH for five

minutes. 100 µl of the resulting solution were mixed with 5 ml AquaSafe 300 plus scintillation fluid (Zinsser Analytic, Frankfurt) and counts per minute (CPM) were detected. Results are expressed as CPM per µg protein as determined by Bio-Rad Protein Assay.

Statistical analysis

Values are means ± SD. A randomized block design was used in all of the experiments. Therefore comparisons with the titanium treatment control were made using Friedman test or RM ANOVA followed by post hoc tests (GraphPad Prism 5.03, GraphPad Software Inc., La Jolla). P values < 0.05 were considered statistically significant.

Results

Biocompatibility

In order to assess the biocompatibility of degrading magnesium, the reactions of PNEC incubated with corroding pure magnesium were compared with the reactions of PNEC in contact with corrosion-resistant titanium or titanium plus a supraphysiological concentration (15 mmol/l) of magnesium ions, respectively. After the magnesium test implants had been incubated for 48 hours the magnesium concentration of the culture medium had increased by 41 ± 3 mmol/l (n = 7) accompanied by an alkalization (pH 8.33 ± 0.04, n = 7; treatment control pH 7.47 ± 0.14, n = 7), while these parameters remained unchanged in the other treatment groups.

Despite the remarkable changes in the environment of the degrading magnesium the metabolic activity of the PNEC was not significantly affected (Fig.1). The dose-effect experiments with $MgCl_2$ revealed a high tolerance of the PNEC for magnesium ions. In fact, the metabolic activity was found to be increased up to 173 % for a concentration of 30 mmol/l magnesium after 24 hours, while it was almost completely inhibited by concentrations of 100 mmol/l upwards (Fig. 2).

In order to assess a potential inflammatory response PGE_2, IL-6 and IL-8 were measured in the culture medium. IL-6 concentrations turned out to be too low to

detect. When PNEC were stimulated with 100 µg/ml LPS as a positive control the secretion of both PGE_2 and IL-8 increased significantly in comparison to the treatment control. In contrast to this, the contact with corroding magnesium resulted in a mere tendency towards higher PGE_2 values, which was not statistically significant either (Fig 3A). On the other hand, a significant rise in IL-8 production was found. Interestingly, such a response could not be observed when only the magnesium ion concentration had been raised (Fig. 3B). In a further set of experiments we discerned that neither a pH increase to 8.3 alone nor a combination of elevated pH and increased magnesium concentration could explain the strong IL-8 secretion as such an effect remained absent (data not shown).

When the PNEC were examined for positive Annexin-V labelling as a marker of apoptosis, no indication of a higher incidence due to magnesium degradation could be observed. However, starvation of the cells by replacing the medium by phosphate buffered saline (PBS) for 24 hours led to a significant increase in apoptosis, thus serving as a positive control (Fig. 4A). Surprisingly, as the cells were counterstained using propidium iodide to discriminate between apoptosis and necrosis, the assessment revealed a very strong increase in the number of necrotic cells in the magnesium implant group, which could not be noted in any of the other groups (Fig. 4B).

Effects on cell physiology

In order to clarify whether the surplus of magnesium resulting from implant degradation might exert an influence on the activity of magnesium-dependent enzymes, pyruvate kinase and succinate dehydrogenase were chosen as representative enzymes for the cytoplasmic or mitochondrial compartment, respectively. It could be observed that PK activity is indeed slightly upregulated by supraphysiological magnesium concentration of 15 mmol/l. However, cells incubated with degrading magnesium did not show this effect, but their PK activity seemed to have a tendency of being even slightly reduced instead (Fig. 5A). As opposed to this, SDH activity of PNEC which had been in contact with a

magnesium implant was significantly reduced, while that of cells incubated with an increased ion concentration remained unaltered (Fig. 5B).

Cell protein synthesis as another potential target, which might be affected by magnesium degradation products, was examined by means of ^{14}C amino acid incorporation. In doing so, neither the magnesium implant nor the magnesium ions were found to induce any change. Protein synthesis could however be almost completely inhibited by cycloheximide (Fig. 6).

Discussion

The study presented here has been conducted in order to investigate the effects of pure magnesium by simulating the prospective *in vivo* tissue-implant-interface in the nasal cavity. This was done using primary porcine nasal epithelial cells (PNEC) because the pig is considered an appropriate animal model for future *in vivo* studies in the course of implant development which allows for the best possible comparability of *in vitro* and *in vivo* results and, hopefully, contributes to the reduction of animal experiments. It was our endeavor to challenge the assumptions that magnesium itself is perfectly biocompatible, not only systemically but also locally, while simultaneously delivering basic knowledge on the cellular effects of degrading magnesium for later comparison when application-specifically more appropriate magnesium alloys will be tested using the same model in the future.

The biomaterial magnesium is thought to be non-toxic both locally and systemically [12], a supposition which has been confirmed by a number of *in vitro* studies using different cell types, although some of these pointed to the possibility that there may be a detrimental effect due to a pH increase [13-15]. Such an influence may be avoided *in vivo* by using slower degrading magnesium alloys which would also slow down the formation of hydroxide anions. However, it should be noted that most of these experiments were performed as an indirect contact test using degradation extracts. Extracts have the disadvantage of not taking into account the effects of some degradation products. Apart from the release of magnesium ions and the occurrence of hydroxides, hydrogen and possibly some particles will also

be formed during magnesium corrosion. Hydrogen escapes when the extracts are prepared and particles are removed via centrifugation although both of them may be the cause of certain (adverse) effects in an *in vivo* environment. Therefore, a direct contact setup may be more appropriate when the biocompatibility of magnesium-based biomaterials is assessed. Geng et al. [16] incubated MG63 human osteoblasts-like cells with pure magnesium and they observed reduced cell viability and proliferation. Similar results are also shown by Lorenz et al. [17] who reported a decrease in cell adhesion and spreading of human HeLa cells and GSP-C12 mouse fibroblasts on pure magnesium substrates.

In our own experiments with degrading magnesium test implants we found no indication of a decrease in overall cellular metabolic activity as a parameter of cell viability, despite a very strong increase in magnesium concentration and pH. This is in accordance with the dose-effect experiments and reports of mammalian cells having a very high adaptability regarding extreme magnesium ion availability [18]. Furthermore, a higher incidence of apoptosis could not be observed either although a linkage between magnesium and apoptosis has been suggested previously (reviewed in [19]). These findings, however, are counterpointed by a significant increase in necrosis after the contact with degrading magnesium. This points to a differential effect on metabolic activity and cell integrity which seemingly is not mediated by the surplus of magnesium ions. The reasons for this behavior as well as the responsible factor is yet unknown.

The contact between cells/tissues and biomaterials always carries a potential for inflammatory reactions which may exert detrimental effects on the biocompatibility [1]. Airway epithelium is able to respond to an inflammatory stimulus by secreting IL-8 and IL-6 [20] which have already been described as parameters in the context of biomaterials [21, 22]. While IL-6 was not detectable in the PNEC supernatants, IL-8 and PGE_2 were found significantly elevated in cells stimulated with LPS. In comparison to that, PGE_2 production in cells incubated with magnesium test implants experienced hardly any change but IL-8 reacted very strongly. Interestingly, the latter result cannot be explained as a magnesium ion or pH

mediated effect so again the exact mechanism is unclear and might involve other degradation products like hydrogen and/or released particles. In a previous *in vitro* study using whole porcine nasal mucosa the same parameters of an inflammatory response were evaluated, but none was found increased after contact with degrading magnesium (Schumacher et al., submitted). It might therefore be hypothesized that the airway epithelial cells, being the first line of defence as for biomaterials in the nasal cavity, may be especially susceptible to foreign material while an inflammatory response is not of pronounced importance on a larger scale when several different cell types are concerned.

Besides their manifold structural and electrophysiological functions magnesium ions are also actively involved in the cellular energy metabolism, for the most part by influencing many enzymes participating in glycolysis, the citric acid cycle and the respiratory chain [19, 23]. In order to examine whether this may also be of any relevance when magnesium is released from resorbable implants pyruvate kinase and succinate dehydrogenase were chosen as marker enzymes for cytosol and mitochondria. Again, we observed differences between the reactions elicited by degrading magnesium and magnesium ions. PK activity was enhanced by magnesium ions unlike the SDH activity which appeared unaltered, notwithstanding the fact that the latter was reported to be influenced by magnesium [24]. This could be interpreted in a way that the prolonged contact with supraphysiological extracellular magnesium ion concentrations (here 15 mmol/l) may have changed the cytosolic magnesium content but did not affect the mitochondrial one. As opposed to this, magnesium degradation resulted in an almost inverse behavior. Despite an even higher magnesium concentration in the culture medium PK activity was found to be on treatment control level while SDH activity was significantly reduced, so an effect of other degradation products seems to have overwhelmed the mere ion effect. On the other hand, a concentration-dependent ambiguous effect of magnesium ions, similar to what has been described for cell protein synthesis [9, 25], is also conceivable regarding enzyme activities. Interestingly, this outcome is contrasted by previous results using a model of whole porcine nasal mucosa in which degrading magnesium led to an

increase in SDH activity (assessed histochemically) in comparison with treatment control (Schumacher et al., submitted). The reason for this discrepancy is unclear, but it might be due to inherent differences in the models, i.e. the magnesium test implants were in direct contact with the whole tissue area while covering only about ten percent of the PNEC layer. Thus, the majority of the PNEC had no physical contact with the test implants and their reaction may have superimposed that of the cells underneath the implant. Apart from that, SDH activity also turned out to be dissimilar regarding the different cell types which the mucosal tissue is composed of.

In 2005 Rubin [9] proposed an elegant model of the regulation of cell proliferation and protein synthesis by magnesium as a key factor. If magnesium ions released during implant degradation were able to increase protein synthesis this may be one possible explanation for reports of enhanced tissue remodelling *in vivo* [26]. This prompted us to test the protein synthesis in PNEC after contact with degrading magnesium. However, the fact that the results showed no obvious difference in amino acid incorporation suggest that neither magnesium ions nor degradation products from corroding pure magnesium exert a significant effect on cell protein synthesis over prolonged periods of time. This confirms previous findings assessing the same parameter on the tissue level (Schumacher et al., submitted).

Conclusion

Porcine nasal epithelial cells in contact with degrading magnesium exhibited signs of necrosis, inflammatory response and altered enzyme activities, which seem to be attributable to factors other than the release of magnesium ions. What exactly these factors and their mechanisms of action are remains to be determined. Beyond that, general metabolic activity and protein synthesis performance were examined, however, no influence was indicated in respect thereof. Yet, it cannot be confirmed that pure magnesium is perfectly biocompatible locally. Regarding the nasal cavity as a target area for resorbable implants these new data about the impact of pure magnesium on airway epithelial cells may prove useful for the future evaluation of magnesium alloys.

Acknowledgements

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References

1. Purnama A, Hermawan H, Couet J, Mantovani D. Assessing the biocompatibility of degradable metallic materials: state-of-the-art and focus on the potential of genetic regulation. Acta Biomater 2010; 6(5):1800-1807.

2. Zhang S, Zhang X, Zhao C, Li J, Song Y, Xie C, et al. Research on an Mg-Zn alloy as a degradable biomaterial. Acta Biomater 2009 doi:10.1016/j.actbio.2009.06.028.

3. Song GL. Control of biodegradation of biocompatable magnesium alloys. Corros Sci 2007;49(4):1696-1701.

4. Kramer S, Schwab B, Seitz JM, Lenarz T. [Insertion of biodegradable stents via balloon catheter into paranasal sinus ostia]. Biomaterialien 2010;11:118.

5. Hunter B, Silva S, Youngs R, Saeed A, Varadarajan V. Long-term stenting for chronic frontal sinus disease: case series and literature review. J Laryngol Otol 2010;124(11):1216-1222.

6. Perloff JR, Palmer JN. Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis. Am J Rhinol 2004;18(6):377-380.

7. Weber R. [Endonasal frontal sinus surgery. Part 2: Frontal sinus drainage type III (median drainage), tips and tricks, postoperative care]. HNO 2009;57(8):751-762.

8. Saris NE, Mervaala E, Karppanen H, Khawaja JA, Lewenstam A. Magnesium. An update on physiological, clinical and analytical aspects. Clin Chim Acta 2000;294(1-2):1-26.

9. Rubin H. Magnesium: The missing element in molecular views of cell proliferation control. Bioessays 2005;27(3):311-320.

10. Mao H, Wang Y, Yuan W, Wong LB. Ciliogenesis in cryopreserved mammalian tracheal epithelial cells cultured at the air-liquid interface. Cryobiology 2009;59(3):250-257.

11. Gutmann I, Bernt E. Pyruvate Kinase. In: Bergmeyer HU, editor. Methods of enzymatic analysis. Weinheim: Verlag Chemie, 1974. p. 774-777.

12. Williams D. New interests in magnesium. Med Device Technol 2006;17(3):9-10.

13. Gu X, Zheng Y, Cheng Y, Zhong S, Xi T. In vitro corrosion and biocompatibility of binary magnesium alloys. Biomaterials 2009;30(4):484-498.

14. Li L, Gao J, Wang Y. Evaluation of cyto-toxicity and corrosion behavior of alkali-heat-treated magnesium in simulated body fluid. Surf Coat Tech 2004;185(1):92-98.

15. Yang C, Yuan G, Zhang J, Tang Z, Zhang X, Dai K. Effects of magnesium alloys extracts on adult human bone marrow-derived stromal cell viability and osteogenic differentiation. Biomed Mater 2010;5(4):045005.

16. Geng F, Tan LL, Jin XX, Yang JY, Yang K. The preparation, cytocompatibility, and in vitro biodegradation study of pure beta-TCP on magnesium. J Mater Sci Mater Med 2009;20(5):1149-1157.

17. Lorenz C, Brunner JG, Kollmannsberger P, Jaafar L, Fabry B, Virtanen S. Effect of surface pre-treatments on biocompatibility of magnesium. Acta Biomater 2009;5(7):2783-2789.

18. Sgambato A, Faraglia B, Ardito R, Torsello A, Boninsegna A, Cittadini A, et al. Isolation of normal epithelial cells adapted to grow at nonphysiological concentration of magnesium. Biochem Biophys Res Com 2001;286(4):752-757.

19. Wolf FI, Trapani V. Cell (patho)physiology of magnesium. Clin Sci 2008;114(1):27-35.

20. Palmberg L, Larsson BM, Malmberg P, Larsson K. Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells in vitro by swine dust. Thorax 1998;53(4):260-264.

21. Drynda A, Deinet N, Braun N, Peuster M. Rare earth metals used in biodegradable magnesium-based stents do not interfere with proliferation of smooth muscle cells but do induce the upregulation of inflammatory genes. J Biomed Mater Res A 2009;91(2):360-369.

22. Hallab NJ, Vermes C, Messina C, Roebuck KA, Glant TT, Jacobs JJ. Concentration- and composition-dependent effects of metal ions on human MG-63 osteoblasts. Journal Biomed Mater Res 2002;60(3):420-433.

23. Cowan JA. Structural and catalytic chemistry of magnesium-dependent enzymes. Biometals 2002;15(3):225-235.

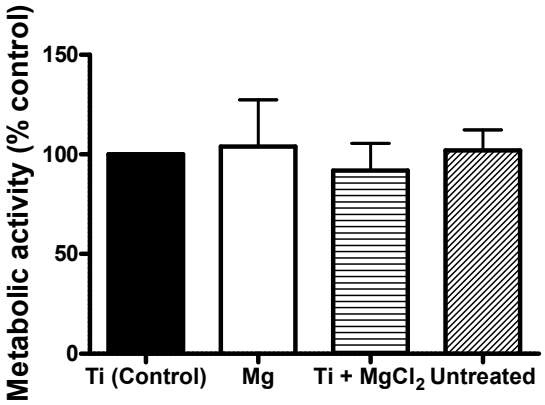
24. Panov A, Scarpa A. Mg2+ control of respiration in isolated rat liver mitochondria. Biochemistry 1996;35(39):12849-12856.

25. Schreier MH, Staehelin T. Initiation of mammalian protein synthesis: the importance of ribosome and initiation factor quality for the efficiency of in vitro systems. Journal Mol Biol 1973;73(3):329-349.

26. Witte F, Kaese V, Haferkamp H, Switzer E, Meyer-Lindenberg A, Wirth CJ, et al. In vivo corrosion of four magnesium alloys and the associated bone response. Biomaterials 2005;26(17):3557-3563.

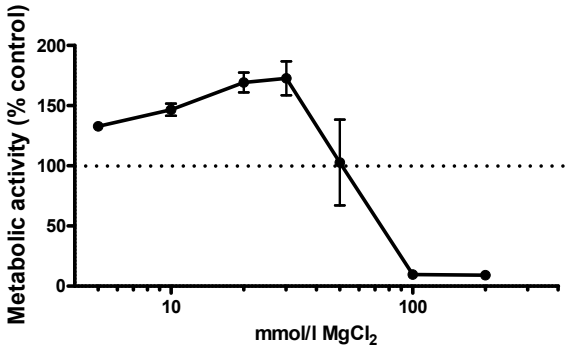
Figures

Figure 1



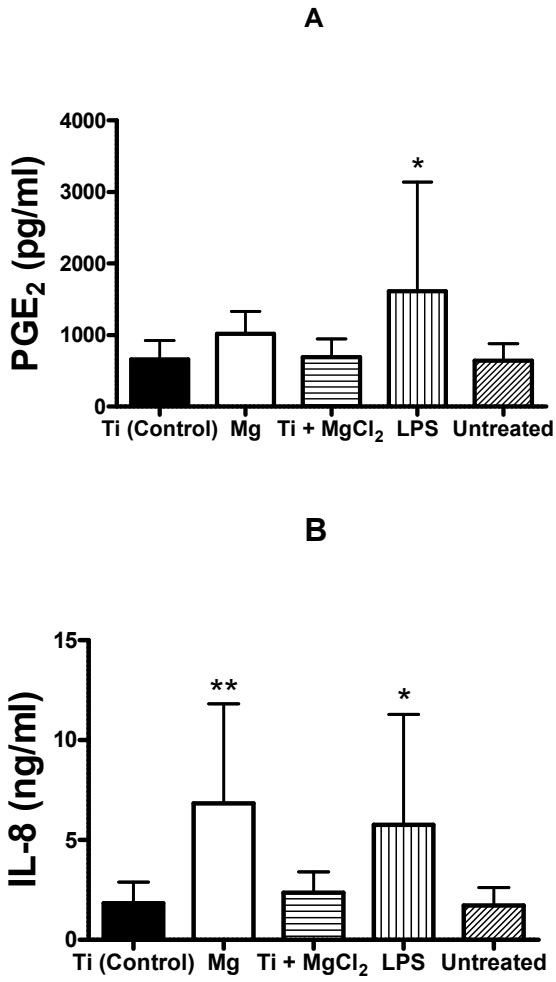
Metabolic activity of PNEC after 48 hours of incubation with test implants was determined colorimetrically using the CellTiter96 AQueous One Solution test and is expressed in % control. Ti = Titanium (treatment control), Mg = Magnesium, Ti + $MgCl_2$ = Titanium plus 15 mmol/l $MgCl_2$. Mean ± SD of n = 7; Friedman test, not significant (comparison with treatment control).

Figure 2



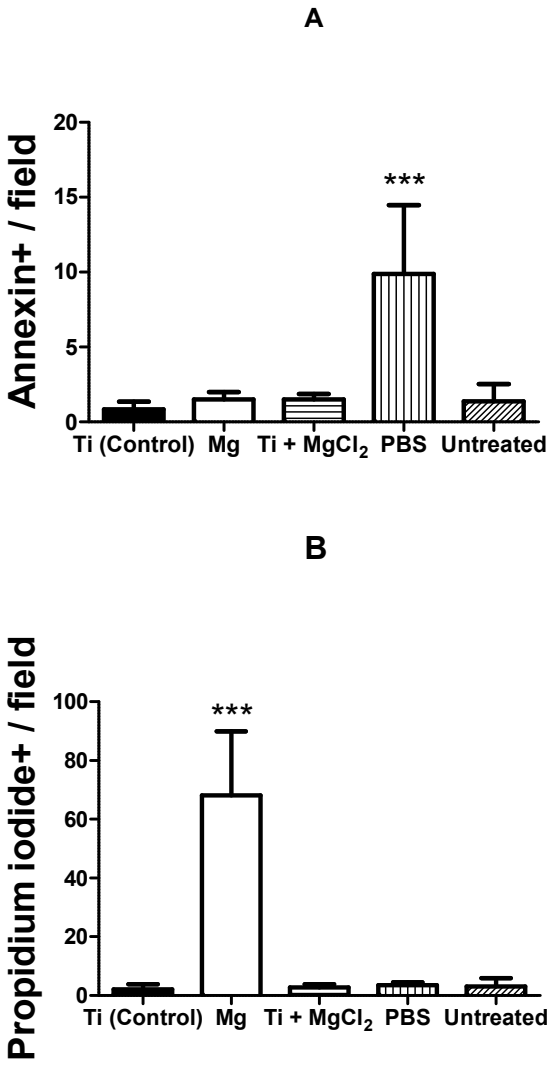
Metabolic activity of PNEC after 24 hours of incubation with different concentrations of magnesium chloride in culture medium was determined colorimetrically using the CellTiter96 AQueous One Solution test and is expressed in % control (0 mmol/l). Mean ± SD of n = 6.

Figure 3



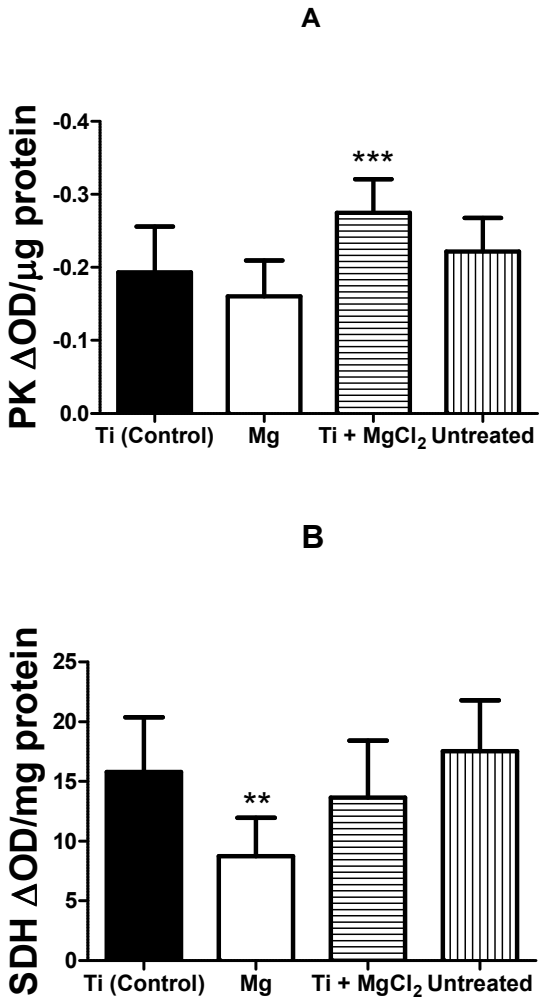
Cytokine concentrations in the supernatants of PNEC after 48 hours of incubation with test implants were determined using ELISA test kits. A: PGE_2 concentrations. B: IL-8 concentrations. Ti = Titanium (treatment control), Mg = Magnesium, Ti + $MgCl_2$ = Titanium plus 15 mmol/l $MgCl_2$, LPS = Titanium plus 100 µg/ml lipopolysaccharide. Mean ± SD of n = 7; RM ANOVA, * $p < 0.05$, ** $p < 0.01$ (comparison with treatment control).

Figure 4



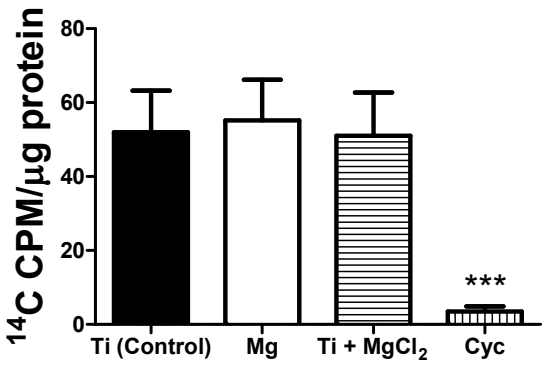
A: Apoptotic PNEC per microscopic field after 48 hours of incubation with test implants were determined via Annexin V-labelling. B: Necrotic PNEC per microscopic field after 48 hours of incubation with test implants were determined via propidium iodide staining. Ti = Titanium (treatment control), Mg = Magnesium, Ti + $MgCl_2$ = Titanium plus 15 mmol/l $MgCl_2$, PBS = Starvation of the cells by replacing medium by PBS for 24 hours. Mean ± SD of n = 4; RM ANOVA, *** $p < 0.001$(comparison with treatment control).

Figure 5



After 48 hours of incubation with test implants PNEC lysates were tested colorimetrically for enzyme activity. A: Pyruvate kinase activity. B: Succinate dehydrogenase activity. Ti = Titanium (treatment control), Mg = Magnesium, Ti + $MgCl_2$ = Titanium plus 15 mmol/l $MgCl_2$. Mean ± SD of n = 5; RM ANOVA, ** $p < 0.01$, *** $p < 0.001$ (comparison with treatment control).

Figure 6



^{14}C amino acid incorporation in PNEC after 48 hours of incubation with test implants was determined by scintillation counting (CPM = counts per minute). Ti = Titanium (treatment control), Mg = Magnesium, Ti + $MgCl_2$ = Titanium plus 15 mmol/l $MgCl_2$, Cyc = Titanium plus 20 µg/ml cycloheximide. Mean ± SD of n = 6; RM ANOVA, *** $p < 0.001$ (comparison with treatment control).

5. *In vitro* cultivated porcine nasal mucosa as a model for the biocompatibility assessment of biodegradable magnesium

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Summary

A model of *in vitro* cultivated porcine nasal mucosa is introduced for the biocompatibility testing of resorbable magnesium-based implants which are intended to be used in the nasal cavity of patients with chronic rhinosinusitis (CRS). Test specimen made from pure magnesium and titanium were incubated with the mucosal tissue for 48 hours. Afterwards, tissue viability, PGE_2, IL-6 and IL-8 release, magnesium ion release, histochemical succinate dehydrogenase activity, apoptosis and ^{14}C amino acid incorporation were determined. The results suggest a favorable biocompatibility even in the case of quickly degrading pure magnesium, while presumed effects on protein synthesis and apoptosis could not be confirmed.

6. General discussion

It was the aim of this PhD project to create a multistage *in vitro* test system for the biocompatibility assessment of newly developed magnesium alloys which are intended to be used in the nasal cavity. The system was established using pure magnesium test implants thus having enabled the identification of several parameters for the compatibility testing of magnesium-based biomaterials. In this way a knowledge base is provided which allows a differentiated evaluation of magnesium alloys in the future and the early detection of materials which are likely to be unsuitable in a biomedical context. This in turn shall make a contribution in reducing the number of animals used in preclinical experiments. The test system presented here consists of three distinct models of increasing complexity each of which was designed to mimic a tissue-implant interface. The first model is represented by primary PNEC which are differentiated *in vitro* utilizing an air-liquid interface culture. In doing so, these cells may deliver more relevant results than simple cell lines in immersion culture because their morphology is closer to the *in vivo* situation, while still being comparably easy to handle and allowing for the investigation of a large variety of parameters (GRAY et al. 1996; REED 1997). The second model simulates the implant environment by means of *in vitro* cultivated whole porcine nasal mucosa which takes the reactions and interactions of different tissues into account in contrast to a cell monoculture. Finally, the isolated perfused bovine udder was utilized. Although it deviates from the prospective area of application it offers the opportunity of examining the processes at the contact zone of implant and tissue under the dynamic conditions of a complex organ. These models differ in their sensitivity, limitations and predictive efficiency and hence complete each other, which, hopefully, leads to a reliable indication of whether a material is biocompatible or not. In addition to this, challenging the assumption that pure magnesium itself is innoxious both systemically and locally, as well as elucidating some of the processes and reactions occurring at the tissue-implant interface in the course of magnesium degradation were further objectives of this study, because they have largely remained a matter of speculation so far. All experiments were performed with pure titanium test implants as a treatment control

in order to ensure the best possible vehicle treatment in terms of physical/mechanical impacts because pure titanium was reported to be both biocompatible and almost biologically inert (STEINEMANN 1998; TSARYK et al. 2007).

Metabolic activity as tested by the conversion of MTS served as a parameter of viability in the porcine *in vitro* models. MTS is a water-soluble variant of MTT which has frequently been used when the cell compatibility of magnesium alloys was tested, notwithstanding the fact that its conversion was reported to be interfered with by magnesium degradation products (cf. 2.3.1.). In order to preclude such artefacts, in the experiments presented here degradation supernatants were removed after the incubation period and fresh MTS-containing medium was added to the cells/tissue. There was no indication of pure magnesium degradation impairing metabolic activity in either mucosa model. The dose-effect experiments with increasing extracellular magnesium chloride concentration even showed an enhanced MTS conversion for up to 30 mmol/l which is in accordance with reports by Sgambato et al. (SGAMBATO et al. 2001) who reported growth stimulating effects of the acute supplementation of up to 40 mmol/l of magnesium sulphate to HC11 mammary epithelial cells. It is not clear why such impact could not be observed in the experiments with differentiated PNEC (neither in the pure magnesium implant nor in the magnesium chloride treatment group). It might simply be due to the differences in the physiology between proliferating cells in immersion culture (which were used in the dose-effect experiments) and differentiated cells which are not proliferating anymore. In pseudostratified airway epithelium basal cells are believed to retain the capability of cell division whereas ciliated cells are considered terminally differentiated (PUCHELLE and PEAULT 2000) and in a study on *in vitro* differentiated human respiratory epithelial cells WENTGES (2003) described that the cells of the upper layer were negative for basal cell markers suggesting they lost their proliferation potential. It is rational to believe that this is also applicable to differentiated PNEC. This explanation attempt is further supported by indications that Mg efflux may be stimulated during the process of cell differentiation (WOLF and CITTADINI 1999). A transient nature of

the metabolism-stimulating effect of elevated magnesium ion concentrations is another possible explanation for the differing results as the immersed PNEC were incubated with magnesium chloride for 24 hours while the incubation period for PNEC at the ALI lasted 48 hours. The adaption to chronically high extracellular Mg concentrations was observed to exert no influence on proliferation (WOLF et al. 2004) and cells were reported to have the ability to efficiently lower their Mg content to physiological levels (RUBIN 2007; WOLF et al. 2010).

Quite surprisingly, in the *ex vivo* experiments using the isolated udder the extracellular magnesium ion concentrations in the direct vicinity of a degrading pure magnesium implant were remarkably lower than anticipated. The highest concentration right after implantation was 5.5 mmol/l and it rapidly decreased to a plateau of around 3.5 mmol/l presumably because of the formation of a magnesium hydroxide-containing corrosion layer (detected by EDX) which protected the implant surface thus retarding the release of magnesium ions (WANG et al. 2008). These values are much smaller than the concentrations measured in the cell and tissue culture experiments (41 or 28 mmol/l, respectively) and they are also well below the critical magnesium concentrations reported in the literature (HALLAB et al. 2002; FEYERABEND et al. 2010). This should definitely be taken into account when the other results of the *in vitro* models are evaluated. As mentioned before, the major limitation of the isolated udder is its short lifespan so longer term reactions remain subject to speculation. However, degradation rates of magnesium alloys have been reported to decrease over time *in vitro* (own results, (WANG et al. 2008; XIN et al. 2008; ZHANG et al. 2010)) as well as *in vivo* (WITTE et al. 2010) and as the magnesium concentrations in the subcutaneous udder tissue had already reached a steady state during the experimental period it seems reasonable to assume that they are not very likely to increase again. Moreover, this also stresses the importance of utilizing experimental setups for biocompatibility testing which simulate a dynamic corrosion environment as suggested by PURNAMA et al. (2010) and LORENZ et al. (2009).

Regarding the biomarkers of inflammatory reactions the results obtained from the different models were again heterogenous. While neither porcine nasal mucosa explants nor isolated udders showed enhanced production of cytokines or PGE_2 in reaction to the degradation of pure magnesium, the latter elicited a very strong release of IL-8 from PNEC. PGE_2 on the other hand remained relatively unchanged in PNEC, too. It must be noted that bovine TNF alpha was the only cytokine available for measurement in samples from the isolated udder and, unfortunately, its microdialysate concentrations turned out to be too low to determine in most cases. Perhaps, the duration of the experiment might not have sufficed to trigger detectable TNF alpha production. On the other hand, TNF alpha could be determined in the samples from one udder but no difference between the treatments or a change over time were observed, so this may be ascribed to a unrecognized preexisting inflammation in that cow's mamma. PGE_2 was present in the microdialysates in measurable amounts but it underwent no change in any of the models. The increased IL-8 secretion by PNEC may indicate a localized inflammatory reaction of the cells in direct contact with the corroding implant. In this case the response may have been superimposed in the mucosa explants by their overall higher cytokine production. As IL-8 acts as a chemokine attracting immune cells (PALMBERG et al. 1998; STRIETER 2002), this explanation is in good accordance with results obtained by ERDMANN et al. (2010). After implantation of MgCa0.8 screws into rabbit tibiae they observed a fibrous layer populated with macrophages, giant cells and heterophil granulocytes in the direct vicinity of the screw head, whereas the surrounding tibialis cranialis muscle maintained physiologic morphology. Apart from these considerations, it is also yet unclear which product of magnesium corrosion triggered the IL-8 increase. There were no alloying elements present and neither an elevated magnesium ion concentration nor an alkaline pH could be identified as being responsible. Thus, theoretically, only hydrogen evolution and peeled-off particles remain as potential causes. To the best of our knowledge there are no reports about molecular hydrogen triggering cytokine release from cells but micro- and nanoparticles of cobalt-chromium-molybdenum alloys (CAICEDO et al. 2009; HALLAB and JACOBS 2009; KANAJI

et al. 2009; CAICEDO et al. 2010) and titanium (FRITZ et al. 2002; KAUFMAN et al. 2008) have been shown to stimulate the production of several proinflammatory cytokines in macrophages, monocytes and bone cells. Further experiments would be required to investigate whether particulate magnesium corrosion products may cause enhanced cytokine secretion from PNEC. Taking together the results from the different models so far, particularly the absence of increased inflammation marker release from the mucosa explants, the degradation of pure magnesium does, however, not seem to provoke an extensive inflammatory response.

A further point may be addressed considering that magnesium-based stents shall ultimately be applied in patients with CRS. While acute rhinosinusitis is predominantly infectious in nature the chronic form is generally accepted to have a major inflammatory component (MELTZER et al. 2004). This means that the preexisting conditions in the nasal cavity of a CRS patient are already markedly altered compared with the presumably healthy mucosa used for the porcine *in vitro* models. It may appear worthwhile trying to adapt these models to simulate the CRS situation in the future but this was beyond the scope of this project.

A number of studies revealed evidence that magnesium availability is involved in the process of apoptosis although the exact nature of this interplay could not be elucidated as yet (PATEL et al. 1994; CHIEN et al. 1999; COVACCI et al. 2000; MARTIN et al. 2003). The corrosion of metallic magnesium causes substantial release of magnesium ions so it was interesting to investigate if these would alter the apoptosis pattern of the surrounding cells and tissues. However, neither porcine model showed differences between the treatments as for apoptosis but a remarkable percentage of PNEC was found to having become necrotic during the incubation with degrading pure magnesium. Unfortunately, techniques for the detection of necrosis in the explant sections were not available so comparison is not possible. The result is all the more striking as significant necrosis occurred in the cells although metabolic activity was not affected which indicates differential effects on metabolism and cellular integrity. Once more this finding is comparable to ERDMANN et al. (2010) who found increased necrosis restricted to the

periimplant fibrous tissue around a MgCa0.8 screw. On the other hand, FESER et al. (FESER et al. 2010) reported no augmented necrosis in murine dendritic cells incubated with pure magnesium or MgCa alloy extract media. The PNEC results moreover raise the question which degradation product they should be attributed to because again increased magnesium ion concentration did not seem to be the cause. Titanium dioxide particles were shown to induce apoptotic and necrotic cell death (LAI et al. 2008) therefore a particle effect seems possible in this respect.

The incorporation of ^{14}C-labelled amino acids was investigated as an indicator of protein synthesis since RUBIN (2007) proposed an essential role of magnesium ions in its regulation. The porcine nasal mucosa model allowed for the examination of protein synthesis in relation to the distance from the implant. No effect could be observed but the variation of the results turned out to be quite large so minor effects would have remained undetected. However, the results from the PNEC model did not suggest an influence either. In these experiments the variation was much smaller so it may be concluded that the degradation of pure magnesium is unlikely to continuously affect protein synthesis in the surrounding cells. This confirms reports in the literature that chronically elevated extracellular magnesium ion concentrations did not exert an effect on the growth characteristics of cells probably because of an activation of adaptation mechanisms (SGAMBATO et al. 2001; WOLF et al. 2004). Nevertheless, it may be of interest for future studies to investigate proteins involved in proliferation signalling pathways, which were demonstrated to be influenced by magnesium, e.g. Erk1/2 (TOUYZ and YAO 2003; ZREIQAT et al. 2005), cell cycle proteins (SGAMBATO et al. 2001; TOUYZ and YAO 2003) or mTOR (RUBIN 2007) at different time-points in order to obtain more information about the cellular responses in the course of magnesium degradation.

Finally, the porcine models were also used for experiments in view of potential effects on magnesium-dependent enzymes. In PNEC corroding pure magnesium did not affect PK activity, but it decreased SDH-dependent substrate conversion. In contrast to this, solely increased magnesium ion concentration showed the anticipated stimulation of PK whereas an alteration of SDH activity was not

observed. As already discussed for the other parameters, this raises the same issue, which agent may be responsible and again it remains unanswered, unfortunately. Interestingly, the results were completely different for the mucosa explants where histochemical SDH activity was much higher after pure magnesium treatment in comparison with treatment controls but still lower as opposed to untreated tissue. The implications and drawbacks of histochemical SDH activity determination in the explant model have been discussed in detail in chapter 5 and may be inherent to the experimental setup rendering comparison with the PNEC result difficult. Taking into account the loss of tissue from the explant during the preparation of the histochemical assay and the relatively large variation of the results, the PNEC model may be considered the more sensitive and thus more relevant system for the evaluation of enzyme activity changes. Still, definite conclusion cannot be drawn at this point.

Summarizing the results obtained using the different models the biocompatibility of the rapidly degrading pure magnesium seems to be acceptable although there are some concerns regarding the necrotic cell damage and cytokine release observed in PNEC. However, the experiments with the isolated bovine udder indicated that degradation in a dynamic environment occurs much slower than in the static porcine models. Nevertheless, the biocompatibility will probably be improved if the corrosion process of magnesium is slowed down. For the construction of paranasal sinus stents the more corrosion-resistant neodymium-containing alloy MgNd2 is currently in development. A notable indication arising from the project presented here is that whenever degrading pure magnesium elicited an effect on any parameter, it could not be attributed to the presence of supraphysiological concentrations of magnesium ions.

As an outlook, the most intriguing question refers to the identification of the degradation product responsible for the observed changes with abraded corrosion particles being the most promising candidate. Moreover, it would be interesting to investigate possible effects of corroding magnesium on cell signalling and cell cycle proteins and to assess the tissue response under preexisting

proinflammatory conditions. If the required technical equipment for confocal live imaging was available it would furthermore be fascinating to monitor realtime changes in cellular Mg distribution possibly occurring during magnesium degradation. Finally, *in vivo* microdialysis experiments may reveal valuable information about whether *in vivo* and *ex vivo* degradation kinetics are comparable as well as the longer-term degradation behavior.

7. Summary

Stephan Schumacher

In-vitro-studies of the tissue compatibility of magnesium based implants

This PhD project was dedicated to establishing of an *in vitro* test system for the biocompatibility assessment of degradable magnesium alloys intended to be used in the nasal cavity of patients with CRS. For this purpose, three *in vitro* models were adapted in order to simulate a tissue-implant interface using pure magnesium test implants. In doing so, not only the biocompatibility of pure magnesium could be evaluated but also some light was shed on how the tissue responds to the presence of degrading magnesium.

In the model of differentiated primary PNEC pure magnesium exhibited acceptable biocompatibility although a certain amount of necrotic cell damage and an increase in the proinflammatory cytokine IL-8 was observed. A potential influence of the degrading material on cell apoptosis and protein synthesis could not be identified but an effect on the activity of cytosolic and mitochondrial marker enzymes (PK or SDH, respectively) was detected.

In vitro cultivated porcine nasal mucosa explants were utilized as the second model to allow for the interaction of different cell types thus creating a more complex experimental setting. Again the results suggested favorable tissue compatibility of pure magnesium. None of the proinflammatory substances was found to be significantly elevated and the outcomes regarding apoptosis and protein synthesis could also be confirmed. SDH activity was altered after incubation with degrading magnesium but in a contrary fashion compared with the PNEC.

Interestingly, in both models none of the observed changes could be explained by the action of magnesium ions released from the test implants, but the question which degradation product is indeed responsible remains unanswered.

Being a complete organ the isolated perfused bovine udder is closest to the *in vivo* situation of an intact organism. Its use enabled the insertion of test implants into the dynamic environment of the subcutaneous tissue and the investigation of reactions at the tissue-implant interface via microdialysis without causing animal suffering. In comparison with other *in vitro* experiments the release of magnesium ions from the degrading implants was substantially lower so the extracellular concentration decreased from 5.5 mmol/l to 3.5 mmol/l within two hours. Besides, an increased production of inflammation markers was not observed in this model either.

In summary, the *in vitro* test system for magnesium alloys was successfully established. The results indicate an overall acceptable biocompatibility of pure magnesium and noteworthy insights into its actions at the tissue implant-interface could be gained. However, the exact mechanisms behind these reactions still remain unclear and require further research in order to be elucidated. In rendering an early biologic evaluation of new implant materials possible, the test system presented here will hopefully contribute to the reduction of animal experiments.

8. Zusammenfassung

Stephan Schumacher

In-vitro-Studien zur Gewebeverträglichkeit von magnesiumbasierten Implantaten

Dieses PhD-Projekt war dazu bestimmt, ein *In-vitro*-Testsystem für die Beurteilung der Biokompatibilität degradierbarer Magnesiumlegierungen zu etablieren, die in der Nasenhöhle von CRS-Patienten eingesetzt werden sollen. Zu diesem Zweck wurden drei *In Vitro*-Modelle dafür adaptiert, um am Beispiel von Reinmagnesium das Implantat-Gewebe-Interface zu simulieren. Auf diesem Wege konnte nicht nur die Biokompatibilität von Reinmagnesium bewertet werden, sondern es wurde auch etwas Aufschluss darüber gegeben, wie das Gewebe auf degradierendes Magnesium reagiert.

Im Modell differenzierter primärer PNEC zeigte Reinmagnesium eine akzeptable Verträglichkeit, obwohl eine gewisse Menge nekrotischer Zellschädigung und ein Anstieg des proinflammatorischen Zytokins IL-8 beobachtet wurden. Ein möglicher Einfluss des degradierenden Materials auf die Zellapoptose und –proteinsynthese konnte nicht ermittelt werden, aber es wurde ein Effekt auf die Aktivität der zytosolischen und mitochondrialen Markerenzyme (PK bzw. SDH) festgestellt.

In vitro kultivierte porcine Nasenschleimhautexplantate wurden als zweites Modell eingesetzt, um die Interaktion verschiedener Zellarten zuzulassen und so eine komplexere Versuchsumgebung zu schaffen. Wiederum wiesen die Resultate auf eine günstige Gewebverträglichkeit hin. Keine der proinflammatorischen Substanzen war signifikant erhöht und auch die Ergebnisse hinsichtlich Apoptose und Proteinsynthese konnten bestätigt werden. Nach der Inkubation mit degradierendem Magnesium hatte sich die SDH-Aktivität verändert, aber im Vergleich zu den PNEC in entgegengesetzter Weise.

Interessanterweise konnten in keinem der beiden Modelle die beobachteten Veränderungen über die Einwirkung der aus den Testimplantaten freigesetzten

Magnesiumionen erklärt werden, aber die Frage nach dem tatsächlich verantwortlichen Degradationsprodukt bleibt offen.

Als komplettes Organ ist das isoliert perfundierte Rindereuter der *In-vivo*-Situation eines intakten Organismus noch am Nächsten. Seine Verwendung machte es möglich, Testimplantate in das dynamische Milieu des Unterhautgewebes einzubringen und mittels Mikrodialyse die Reaktionen am Implantat-Gewebe-Interface zu untersuchen, ohne dabei einen Leidensdruck im Tier zu erzeugen. Verglichen mit anderen *In-vitro*-Experimenten war die Magnesiumionenfreisetzung aus den degradierenden Implantaten deutlich geringer und die extrazelluläre Konzentration fiel innerhalb von zwei Stunden von 5,5 mmol/l auf 3,5 mmol/l. Daneben konnte auch in diesem Modell keine vermehrte Bildung der Entzündungsmarker verzeichnet werden.

Zusammenfassend wurde das *In-vitro*-Testsystem für Magnesiumlegierungen erfolgreich etabliert. Die Ergebnisse deuten auf eine insgesamt akzeptable Biokompatibilität von Reinmagnesium hin und es konnten beachtenswerte Einblicke in dessen Wirkungen am Implantat-Gewebe-Interface gewonnen werden. Allerdings bleiben die genauen dahinter stehenden Mechanismen ungeklärt und es bedarf weiterer Forschung, um diese aufzudecken. Indem es die frühzeitige biologische Bewertung neuer Implantatmaterialien ermöglicht, wird das hier vorgestellte Testsystem hoffentlich einen Beitrag zur Einsparung von Tierversuchen leisten.

9. Literature

ADAMS, J. H. and J. R. MITCHELL (1979):
The effect of agents which modify platelet behaviour and of magnesium ions on thrombus formation in vivo.
Thromb Haemost 42, 603-610

BACH, F.-W. and T. HASSEL (2005):
Herstellung und Deformationsanalyse zur Qualifizierung einer Schutzschicht aus Magnesiumfluorid zur gesteuerten Degradation von Implantatlegierungen auf Magnesiumbasis.
Biomaterialien 6, 163

BACH, F.-W., T. HASSEL, D. BORMANN and R. KUCHARSKI (2005a):
Elektrochemisch induzierte Abscheidung von Calciumphosphat auf der Oberfläche von kompakten und zellularen Strukturen aus Magnesiumlegierungen.
Biomaterialien 6, 162

BACH, F.-W., T. HASSEL, A. GOLOVKO, C. HACKENBROICH and A. MEYER-LINDENBERG (2005b):
Resorbierbare Implantate aus Magnesium durch Mikrolegieren mit Calcium, deren Verarbeitung und Eigenschaften.
Biomaterialien 6, 163

BANAI, S., L. HAGGROTH, S. E. EPSTEIN and W. CASSCELLS (1990):
Influence of extracellular magnesium on capillary endothelial cell proliferation and migration.
Circ Res 67, 645-650

BANHIRAN, W., Z. SARGI, W. COLLINS, S. KAZA and R. CASIANO (2006):
Long-term effect of stenting after an endoscopic modified Lothrop procedure.
Am J Rhinol 20, 595-599

BEULE, A. G., C. SCHARF, K. E. BIEBLER, A. GOPFERICH, E. STEINMEIER, E. WOLF, W. HOSEMANN and H. KAFTAN (2008):
Effects of topically applied dexamethasone on mucosal wound healing using a drug-releasing stent.
Laryngoscope 118, 2073-2077

BEULE, A. G., E. STEINMEIER, H. KAFTAN, K. E. BIEBLER, A. GOPFERICH, E. WOLF and W. HOSEMANN (2009):
Effects of a dexamethasone-releasing stent on osteoneogenesis in a rabbit model.
Am J Rhinol Allergy 23, 433-436

BEYENBACH, K. W. (1990):
Transport of magnesium across biological membranes.
Magnes Trace Elem 9, 233-254

BICHET, D. G. (2006):
Lithium, cyclic AMP signaling, A-kinase anchoring proteins, and aquaporin-2.
J Am Soc Nephrol 17, 920-922

BOSIERS, M., P. PEETERS, O. D'ARCHAMBEAU, J. HENDRIKS, E. PILGER, C. DUBER, T. ZELLER, A. GUSSMANN, P. N. LOHLE, E. MINAR, D. SCHEINERT, K. HAUSEGGER, K. L. SCHULTE, J. VERBIST, K. DELOOSE and J. LAMMER (2009):
AMS INSIGHT--absorbable metal stent implantation for treatment of below-the-knee critical limb ischemia: 6-month analysis.
Cardiovasc Interventional Radiol 32, 424-435

CAICEDO, M. S., R. DESAI, K. MCALLISTER, A. REDDY, J. J. JACOBS and N. J. HALLAB (2009):
Soluble and particulate Co-Cr-Mo alloy implant metals activate the inflammasome danger signaling pathway in human macrophages: a novel mechanism for implant debris reactivity.
J Orthop Res 27, 847-854

CAICEDO, M. S., P. H. PENNEKAMP, K. MCALLISTER, J. J. JACOBS and N. J. HALLAB (2010):
Soluble ions more than particulate cobalt-alloy implant debris induce monocyte costimulatory molecule expression and release of proinflammatory cytokines critical to metal-induced lymphocyte reactivity.
J Biomed Mater Res A 93, 1312-1321

CASTELLANI, C., R. A. LINDTNER, P. HAUSBRANDT, E. TSCHEGG, S. E. STANZL-TSCHEGG, G. ZANONI, S. BECK and A. M. WEINBERG (2011):
Bone-implant interface strength and osseointegration: Biodegradable magnesium alloy versus standard titanium control.
Acta Biomater 7, 432-440

CHIEN, M. M., K. E. ZAHRADKA, M. K. NEWELL and J. H. FREED (1999):
Fas-induced B cell apoptosis requires an increase in free cytosolic magnesium as an early event.
J Biol Chem 274, 7059-7066

CHIU, K. Y., M. H. WONG, F. T. CHENG and H. C. MAN (2007):
Characterization and corrosion studies of fluoride conversion coating on degradable Mg implants.
Surf Coat Technol 202, 590-598

CORTES, D. A., H. Y. LOPEZ and D. MANTOVANI (2007):
Spontaneous and biomimetic apaptite formation on pure magnesium.
Mater Sci Forum 539-543, 589-594

COVACCI, V., N. BRUZZESE, A. SGAMBATO, R. GANAPATHI, A. CITTADINI and F. I. WOLF (2000):
Effect of extracellular magnesium on topoisomerase II activity and expression in human leukemia HL-60 cells.
J Cell Biochem 78, 325-333

COWAN, J. A. (1995):
Introduction to the biological chemistry of magnesium ion.
In: Cowan, J.A. (Ed.): The Biological Chemistry of Magnesium
VCH, New York, S. 1-23

DENDA, M., C. KATAGIRI, T. HIRAO, N. MARUYAMA and M. TAKAHASHI (1999):
Some magnesium salts and a mixture of magnesium and calcium salts accelerate skin barrier recovery.
Arch Dermatol Res 291, 560-563

DI MARIO, C., H. GRIFFITHS, O. GOKTEKIN, N. PEETERS, J. VERBIST, M. BOSIERS, K. DELOOSE, B. HEUBLEIN, R. ROHDE, V. KASESE, C. ILSLEY and R. ERBEL (2004):
Drug-eluting bioabsorbable magnesium stent.
J Interv Cardiol 17, 391-395

DRACHMAN, D. E. and D. I. SIMON (2005):
Inflammation as a mechanism and therapeutic target for in-stent restenosis.
Curr Atheroscler Rep 7, 44-49

DRYNDA, A., T. HASSEL, R. HOEHN, A. PERZ, F. W. BACH and M. PEUSTER (2010):
Development and biocompatibility of a novel corrodible fluoride-coated magnesium-calcium alloy with improved degradation kinetics and adequate mechanical properties for cardiovascular applications.
J Biomed Mater Res A 93, 763-775

DYKEWICZ, M. S. and D. L. HAMILOS (2010):
Rhinitis and sinusitis.
J Allergy Clin Immunol 125, 103-115

EL-RAHMAN, S. S. (2003):
Neuropathology of aluminum toxicity in rats (glutamate and GABA impairment).
Pharmacol Res 47, 189-194

ERBEL, R., C. DI MARIO, J. BARTUNEK, J. BONNIER, B. DE BRUYNE, F. R. EBERLI, P. ERNE, M. HAUDE, B. HEUBLEIN, M. HORRIGAN, C. ILSLEY, D. BOSE, J. KOOLEN, T. F. LUSCHER, N. WEISSMAN and R. WAKSMAN (2007):
Temporary scaffolding of coronary arteries with bioabsorbable magnesium stents: a prospective, non-randomised multicentre trial.
Lancet 369, 1869-1875

ERDMANN, N., N. ANGRISANI, J. REIFENRATH, A. LUCAS, F. THOREY, D. BORMANN and A. MEYER-LINDENBERG (2011):
Biomechanical testing and degradation analysis of MgCa0.8 alloy screws: A comparative in vivo study in rabbits.
Acta Biomater 7, 1421-1428

ERDMANN, N., A. BONDARENKO, M. HEWICKER-TRAUTWEIN, N. ANGRISANI, J. REIFENRATH, A. LUCAS and A. MEYER-LINDENBERG (2010):
Evaluation of the soft tissue biocompatibility of MgCa0.8 and surgical steel 316L in vivo: a comparative study in rabbits.
Biomed Eng Onl 9

FESER, K., M. KIETZMANN, C. KRAUSE, F. W. BACH and W. BAUMER (2010):
Effects of Degradable Mg-Ca Alloys on Dendritic Cell Function.
J Biomater Appl (in press) doi:10.1177/0885328209360424

FEYERABEND, F., J. FISCHER, J. HOLTZ, F. WITTE, R. WILLUMEIT, H. DRUCKER, C. VOGT and N. HORT (2010):
Evaluation of short term effects of rare earth and other elements used in magnesium alloys on primary cells and cell lines.
Acta Biomater 6, 1834-1842

FISCHER, J., M. H. PROSENC, M. WOLFF, N. HORT, R. WILLUMEIT and F. FEYERABEND (2010):
Interference of magnesium corrosion with tetrazolium based cytotoxicity assays.
Acta Biomater 6, 1813-1823

FREEMAN, S. B. and E. D. BLOM (2000):
Frontal sinus stents.
Laryngoscope 110, 1179-1182

FRITZ, E. A., T. T. GLANT, C. VERMES, J. J. JACOBS and K. A. ROEBUCK (2002):
Titanium particles induce the immediate early stress responsive chemokines IL-8 and MCP-1 in osteoblasts.
J Orthop Res 20, 490-498

GENG, F., L. L. TAN, X. X. JIN, J. Y. YANG and K. YANG (2009):
The preparation, cytocompatibility, and in vitro biodegradation study of pure beta-TCP on magnesium.
J Mater Sci Mater Med 20, 1149-1157

GHIMIRE, G., J. SPIRO, R. KHARBANDA, M. ROUGHTON, P. BARLIS, M. MASON, C. ILSLEY, C. DI MARIO, R. ERBEL, R. WAKSMAN and M. DALBY (2009):
Initial evidence for the return of coronary vasoreactivity following the absorption of bioabsorbable magnesium alloy coronary stents.
EuroInterv 4, 481-484

GILES, J. J. and J. G. BANNIGAN (2006):
Teratogenic and developmental effects of lithium.
Curr Pharm Des 12, 1531-1541

GOYTAIN, A. and G. A. QUAMME (2005):
Identification and characterization of a novel mammalian Mg2+ transporter with channel-like properties.
BMC Genomics 6, 48

GRAY, T. E., K. GUZMAN, C. W. DAVIS, L. H. ABDULLAH and P. NETTESHEIM (1996):
Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells.
Am J Resp Cell Mol Biol 14, 104-112

GRZESIAK, J. J. and M. D. PIERSCHBACHER (1995):
Shifts in the concentrations of magnesium and calcium in early porcine and rat wound fluids activate the cell migratory response.
J Clin Invest 95, 227-233

GU, X. N., N. LI, W. R. ZHOU, Y. F. ZHENG, X. ZHAO, Q. Z. CAI and L. RUAN (2010a):
Corrosion resistance and surface biocompatibility of a microarc oxidation coating on a Mg-Ca alloy.
Acta Biomater (in press) doi:10.1016/j.actbio.2010.11.034

GU, X. N., W. R. ZHOU, Y. F. ZHENG, Y. CHENG, S. C. WEI, S. P. ZHONG, T. F. XI and L. J. CHEN (2010b):
Corrosion fatigue behaviors of two biomedical Mg alloys - AZ91D and WE43 - In simulated body fluid.
Acta Biomater 6, 4605-4613

GU, X., Y. ZHENG, Y. CHENG, S. ZHONG and T. XI (2009a):
In vitro corrosion and biocompatibility of binary magnesium alloys.
Biomaterials 30, 484-498

GU, X. N., W. ZHENG, Y. CHENG and Y. F. ZHENG (2009b):
A study on alkaline heat treated Mg-Ca alloy for the control of the biocorrosion rate.
Acta Biomater 5, 2790-2799

GUNTHER, T., J. VORMANN and R. FORSTER (1984):
Regulation of intracellular magnesium by Mg2+ efflux.
Biochem Biophys Res Commun 119, 124-131

HALLAB, N. J. and J. J. JACOBS (2009):
Biologic effects of implant debris.
Bull NYU Hosp Jt Dis 67, 182-188

HALLAB, N. J., C. VERMES, C. MESSINA, K. A. ROEBUCK, T. T. GLANT and J. J. JACOBS (2002):
Concentration- and composition-dependent effects of metal ions on human MG-63 osteoblasts.
J Biomed Mater Res 60, 420-433

HANSI, C., A. ARAB, A. RZANY, I. AHRENS, C. BODE and C. HEHRLEIN (2009):
Differences of platelet adhesion and thrombus activation on amorphous silicon carbide, magnesium alloy, stainless steel, and cobalt chromium stent surfaces.
Catheter Cardiovasc Interv 73, 488-496

HEATON, F. W. (1993):
Distribution and function of magnesium within the cell.
In: Birch, N.J. (Ed.): Magnesium and the Cell
Academic Press, London, 121-136

HEHRLEIN, C. (2007):
Promises of biodegradable stents.
Catheter Cardiovasc Interv 69, 739

HERMAWAN, H., D. DUBE and D. MANTOVANI (2010):
Developments in metallic biodegradable stents.
Acta Biomater 6, 1693-1697

HERRMANN, B. W., M. J. CITARDI, G. VOGLER, L. GARDNER, G. SMITH, A. R. JAVER, H. M. BURT, J. JACKSON and F. A. KUHN (2004):
A preliminary report on the effects of paclitaxel-impregnated stents on sheep nasal mucosa.
Am J Rhinol 18, 119-124

HEUBLEIN, B., R. ROHDE, V. KAESE, M. NIEMEYER, W. HARTUNG and A. HAVERICH (2003):
Biocorrosion of magnesium alloys: a new principle in cardiovascular implant technology?
Heart 89, 651-656

HOSEMANN, W., E. SCHINDLER, E. WIEGREBE and A. GOPFERICH (2003):
Innovative frontal sinus stent acting as a local drug-releasing system.
Eur Arch Otorhinolaryngol 260, 131-134

HUNTER, B., S. SILVA, R. YOUNGS, A. SAEED and V. VARADARAJAN (2010):
Long-term stenting for chronic frontal sinus disease: case series and literature review.
J Laryngol Otol 124, 1216-1222

IANNELLO, S. and F. BELFIORE (2001):
Hypomagnesemia. A review of pathophysiological, clinical and therapeutical aspects.
Panminerva Med 43, 177-209

ISO10993-5:2009 (2009):
Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity.
ANSI/AAMI

JANNING, C., E. WILLBOLD, C. VOGT, J. NELLESEN, A. MEYER-LINDENBERG, H. WINDHAGEN, F. THOREY and F. WITTE (2010):
Magnesium hydroxide temporarily enhancing osteoblast activity and decreasing the osteoclast number in peri-implant bone remodelling.
Acta Biomater 6, 1861-1868

KANAJI, A., M. S. CAICEDO, A. S. VIRDI, D. R. SUMNER, N. J. HALLAB and K. SENA (2009):
Co-Cr-Mo alloy particles induce tumor necrosis factor alpha production in MLO-Y4 osteocytes: a role for osteocytes in particle-induced inflammation.
Bone 45, 528-533

KAUFMAN, A. M., C. I. ALABRE, H. E. RUBASH and A. S. SHANBHAG (2008):
Human macrophage response to UHMWPE, TiAlV, CoCr, and alumina particles: analysis of multiple cytokines using protein arrays.
J Biomed Mater Res A 84, 464-474

KIRKLAND, N. T., N. BIRBILIS, J. WALKER, T. WOODFIELD, G. J. DIAS and M. P. STAIGER (2010):
In-vitro dissolution of magnesium-calcium binary alloys: clarifying the unique role of calcium additions in bioresorbable magnesium implant alloys.
J Biomed Mater Res B Appl Biomater 95, 91-100

LAI, J. C., M. B. LAI, S. JANDHYAM, V. V. DUKHANDE, A. BHUSHAN, C. K. DANIELS and S. W. LEUNG (2008):
Exposure to titanium dioxide and other metallic oxide nanoparticles induces cytotoxicity on human neural cells and fibroblasts.
Int J Nanomed 3, 533-545

LAMBOTTE, A. (1932):
L'utilisation du magnesium comme material perdu dans l'osteosynthese.
Bull Mem Soc Nat Chir 28, 1325-1334

LEVESQUE, J., H. HERMAWAN, D. DUBE and D. MANTOVANI (2008):
Design of a pseudo-physiological test bench specific to the development of biodegradable metallic biomaterials.
Acta Biomater 4, 284-295

LI, J., Y. SONG, S. ZHANG, C. ZHAO, F. ZHANG, X. ZHANG, L. CAO, Q. FAN and T. TANG (2010):
In vitro responses of human bone marrow stromal cells to a fluoridated hydroxyapatite coated biodegradable Mg-Zn alloy.
Biomaterials 31, 5782-5788

LI, L., J. GAO and Y. WANG (2004):
Evaluation of cyto-toxicity and corrosion behavior of alkali-heat-treated magnesium in simulated body fluid.
Surf Coat Tech 185, 92-98

LI, M., J. JIANG and L. YUE (2006):
Functional characterization of homo- and heteromeric channel kinases TRPM6 and TRPM7.
J Gen Physiol 127, 525-537

LI, Z., X. GU, S. LOU and Y. ZHENG (2008):
The development of binary Mg-Ca alloys for use as biodegradable materials within bone.
Biomaterials 29, 1329-1344

LIN, D. and I. J. WITTERICK (2008):
Frontal sinus stents: how long can they be kept in?
J Otolaryngol Head Neck Surg 37, 119-123

LORENZ, C., J. G. BRUNNER, P. KOLLMANNSBERGER, L. JAAFAR, B. FABRY and S. VIRTANEN (2009):
Effect of surface pre-treatments on biocompatibility of magnesium.
Acta Biomater 5, 2783-2789

LU, P., L. CAO, Y. LIU, X. XU and X. WU (2011):
Evaluation of magnesium ions release, biocorrosion, and hemocompatibility of MAO/PLLA-modified magnesium alloy WE42.
J Biomed Mater Res B Appl Biomater 96, 101-109

MAENG, M., L. O. JENSEN, E. FALK, H. R. ANDERSEN and L. THUESEN (2009):
Negative vascular remodelling after implantation of bioabsorbable magnesium alloy stents in porcine coronary arteries: a randomised comparison with bare-metal and sirolimus-eluting stents.
Heart 95, 241-246

MARTIN, H., L. RICHERT and A. BERTHELOT (2003):
Magnesium deficiency induces apoptosis in primary cultures of rat hepatocytes.
J Nutr 133, 2505-2511

MCBRIDE, E. D. (1938):
Absorbable metal in bone surgery.
J Am Med Assoc 111, 2464-2467

MCCARTHY, J. T. and R. KUMAR (1999):
Divalent Cation Metabolism: Magnesium.
In: SCHRIER, R.W. (Ed.): Atlas of dieseases of the kidney
Blackwell Science, Malden, 4.1 - 4.8

MCMAHON, C. J., C. S. SNYDER, S. M. RIVENES, C. J. SANG, JR. and C. D. FRASER, JR. (2000):
Neonatal arterial switch operation for transposition of the great arteries in a patient with mirror image dextrocardia and situs inversus totalis.
Tex Heart Inst J 27, 193-195

MELTZER, E. O., D. L. HAMILOS, J. A. HADLEY, D. C. LANZA, B. F. MARPLE, R. A. NICKLAS, C. BACHERT, J. BARANIUK, F. M. BARODY, M. S. BENNINGER, I. BROOK, B. A. CHOWDHURY, H. M. DRUCE, S. DURHAM, B. FERGUSON, J. M. GWALTNEY, M. KALINER, D. W. KENNEDY, V. LUND, R. NACLERIO, R. PAWANKAR, J. F. PICCIRILLO, P. ROHANE, R. SIMON, R. G. SLAVIN, A. TOGIAS, E. R. WALD and S. J. ZINREICH (2004):
Rhinosinusitis: establishing definitions for clinical research and patient care.
J Allergy Clin Immunol 114, 155-212

MONTELL, C. (2003):
Mg2+ homeostasis: the Mg2+nificent TRPM chanzymes.
Curr Biol 13, R799-801

MUELLER, W. D., M. F. DE MELE, M. L. NASCIMENTO and M. ZEDDIES (2009):
Degradation of magnesium and its alloys: dependence on the composition of the synthetic biological media.
J Biomed Mater Res A 90, 487-495

MUELLER, W. D., M. L. NASCIMENTO and M. F. L. DE MELE (2010):
Critical discussion of the results from different corrosion studies of Mg and Mg alloys for biomaterial applications.
Acta Biomater 6, 1749-1755

ONG, A. T., E. P. MCFADDEN, E. REGAR, P. P. DE JAEGERE, R. T. VAN DOMBURG and P. W. SERRUYS (2005):
Late angiographic stent thrombosis (LAST) events with drug-eluting stents.
J Am Coll Cardiol 45, 2088-2092

OYANE, A., K. ONUMA, A. ITO, H. M. KIM, T. KOKUBO and T. NAKAMURA (2003):
Formation and growth of clusters in conventional and new kinds of simulated body fluids.
J Biomed Mater Res A 64, 339-348

PALMBERG, L., B. M. LARSSON, P. MALMBERG and K. LARSSON (1998):
Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells in vitro by swine dust.
Thorax 53, 260-264

PATEL, T., S. F. BRONK and G. J. GORES (1994):
Increases of intracellular magnesium promote glycodeoxycholate-induced apoptosis in rat hepatocytes.
J Clin Invest 94, 2183-2192

PEETERS, P., M. BOSIERS, J. VERBIST, K. DELOOSE and B. HEUBLEIN (2005):
Preliminary results after application of absorbable metal stents in patients with critical limb ischemia.
J Endovasc Therap 12, 1-5

PERLOFF, J. R. and J. N. PALMER (2004):
Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis.
Am J Rhinol 18, 377-380

PEUSTER, M., P. BEERBAUM, F. W. BACH and H. HAUSER (2006):
Are resorbable implants about to become a reality?
Cardiol Young 16, 107-116

PIETAK, A., P. MAHONEY, G. J. DIAS and M. P. STAIGER (2008):
Bone-like matrix formation on magnesium and magnesium alloys.
J Mater Sci Mater Med 19, 407-415

PUCHELLE, E. and B. PEAULT (2000):
Human airway xenograft models of epithelial cell regeneration.
Respir Res 1, 125-128

PURNAMA, A., H. HERMAWAN, J. COUET and D. MANTOVANI (2010):
Assessing the biocompatibility of degradable metallic materials: state-of-the-art and focus on the potential of genetic regulation.
Acta Biomater 6, 1800-1807

REED, C. J. (1997):
In vitro models of nasal cavity toxicity.
Mut Res 380, 97-111

REN, Y., H. WANG, J. HUANG, B. ZHANG and K. YANG (2007):
Study of biodegradation of pure magnesium.
Key Eng Mater 342-343, 601-604

RETTIG, R. and S. VIRTANEN (2008):
Time-dependent electrochemical characterization of the corrosion of a magnesium rare-earth alloy in simulated body fluids.
J Biomed Mater Res A 85, 167-175

RETTIG, R. and S. VIRTANEN (2009):
Composition of corrosion layers on a magnesium rare-earth alloy in simulated body fluids.
J Biomed Mater Res A 88, 359-369

ROMANI, A. (2007):
Regulation of magnesium homeostasis and transport in mammalian cells.
Arch Biochem Biophys 458, 90-102

RUBIN, A. H., M. TERASAKI and H. SANUI (1979):
Major intracellular cations and growth control: correspondence among magnesium content, protein synthesis, and the onset of DNA synthesis in BALB/c3T3 cells.
Proc Natl Acad Sci USA 76, 3917-3921

RUBIN, H. (2005):
Magnesium: The missing element in molecular views of cell proliferation control.
Bioessays 27, 311-320

RUBIN, H. (2007):
The logic of the Membrane, Magnesium, Mitosis (MMM) model for the regulation of animal cell proliferation.
Arch Biochem Biophys 458, 16-23

SAHNI, J., R. TAMURA, I. R. SWEET and A. M. SCHARENBERG (2010):
TRPM7 regulates quiescent/proliferative metabolic transitions in lymphocytes.
Cell cycle 9, 3565-3574

SARIS, N. E., E. MERVAALA, H. KARPPANEN, J. A. KHAWAJA and A. LEWENSTAM (2000):
Magnesium. An update on physiological, clinical and analytical aspects.
Clin Chim Acta 294, 1-26

SCHRANZ, D., P. ZARTNER, I. MICHEL-BEHNKE and H. AKINTURK (2006):
Bioabsorbable metal stents for percutaneous treatment of critical recoarctation of the aorta in a newborn.
Catheter Cardiovasc Interv 67, 671-673

SCHWEIGEL, M., M. KOLISEK, Z. NIKOLIC and J. KUZINSKI (2008):
Expression and functional activity of the Na/Mg exchanger, TRPM7 and MagT1 are changed to regulate Mg homeostasis and transport in rumen epithelial cells.
Magnes Res 21, 118-123

SERRE, C. M., M. PAPILLARD, P. CHAVASSIEUX, J. C. VOEGEL and G. BOIVIN (1998):
Influence of magnesium substitution on a collagen-apatite biomaterial on the production of a calcifying matrix by human osteoblasts.
J Biomed Mater Res 42, 626-633

SGAMBATO, A., B. FARAGLIA, R. ARDITO, A. TORSELLO, A. BONINSEGNA, A. CITTADINI and F. I. WOLF (2001):
Isolation of normal epithelial cells adapted to grow at nonphysiological concentration of magnesium.
Biochem Biophys Res Commun 286, 752-757

SHAW, B. A. (2003):
Corrosion resistance of magnesium alloys.
In: STEPHEN, D. (Ed.): ASM Handbook Volume 13A: Corrosion: Fundamentals, Testing, and Protection
ASM International

SIMON, D. B., Y. LU, K. A. CHOATE, H. VELAZQUEZ, E. AL-SABBAN, M. PRAGA, G. CASARI, A. BETTINELLI, G. COLUSSI, J. RODRIGUEZ-SORIANO, D. MCCREDIE, D. MILFORD, S. SANJAD and R. P. LIFTON (1999):
Paracellin-1, a renal tight junction protein required for paracellular Mg2+ resorption.
Science 285, 103-106

SONG, G. L. (2007):
Control of biodegradation of biocompatable magnesium alloys.
Corros Sci 49, 1696-1701

SONG, G. L. and A. ATRENS (1999):
Corrosion mechanisms of magnesium alloys.
Adv Eng Mater 1, 11-33

STAIGER, M. P., A. M. PIETAK, J. HUADMAI and G. DIAS (2006):
Magnesium and its alloys as orthopedic biomaterials: a review.
Biomaterials 27, 1728-1734

STEINEMANN, S. G. (1998):
Titanium--the material of choice?
Periodontol 2000 17, 7-21

STRIETER, R. M. (2002):
Interleukin-8: a very important chemokine of the human airway epithelium.
Am J Physiol Lung Cell Mol Physiol 283, L688-689

TERASAKI, M. and H. RUBIN (1985):
Evidence that intracellular magnesium is present in cells at a regulatory concentration for protein synthesis.
Proc Natl Acad Sci USA 82, 7324-7326

THOMANN, M., C. KRAUSE, N. ANGRISANI, D. BORMANN, T. HASSEL, H. WINDHAGEN and A. MEYER-LINDENBERG (2010):
Influence of a magnesium-fluoride coating of magnesium-based implants (MgCa0.8) on degradation in a rabbit model.
J Biomed Mater Res A 93, 1609-1619

TOUYZ, R. M. and G. YAO (2003):
Modulation of vascular smooth muscle cell growth by magnesium-role of mitogen-activated protein kinases.
J Cell Physiol 197, 326-335

TROITSKII, V. V. and D. N. TSITRIN (1944):
The resorbing metallic alloy "Osteosynthezit" as a material for fastening broken bone.
Khirurgiia 8, 41-44

TSARYK, R., M. KALBACOVA, U. HEMPEL, D. SCHARNWEBER, R. E. UNGER, P. DIETER, C. J. KIRKPATRICK and K. PETERS (2007):
Response of human endothelial cells to oxidative stress on Ti6Al4V alloy.
Biomaterials 28, 806-813

VERBURGGE, J. (1934):
Le Material Metallique Resorbable En Chirurgie Osseuse.
Presse Med 23, 460-465

VORMANN, J. (2003):
Magnesium: nutrition and metabolism.
Mol Aspects Med 24, 27-37

WAKSMAN, R. (2006):
Update on bioabsorbable stents: from bench to clinical.
J Interv Cardiol 19, 414-421

WAKSMAN, R., R. PAKALA, P. K. KUCHULAKANTI, R. BAFFOUR, D. HELLINGA, R. SEABRON, F. O. TIO, E. WITTCHOW, S. HARTWIG, C. HARDER, R. ROHDE, B. HEUBLEIN, A. ANDREAE, K. H. WALDMANN and A. HAVERICH (2006):
Safety and efficacy of bioabsorbable magnesium alloy stents in porcine coronary arteries.
Catheter Cardiovasc Interv 68, 607-619

WANG, H., Y. ESTRIN, H. FU, G. SONG and Z. ZUBEROVA (2007):
The effect of pre-processing and grain structure on the bio-corrosion and fatigue resistance of magnesium alloy AZ31.
Adv Eng Mater 9, 967-972

WANG, Y., M. WEI, J. C. GAO, J. Z. HU and Y. ZHANG (2008):
Corrosion process of pure magnesium in simulated body fluid.
Mater Letters 62, 2185-2188

WEBER, R. (2009):
Die endonasale Chirurgie der Stirnhöhle Teil 2: Stirnhöhlendrainage Typ III (Mediandrainage), Tipps und Tricks, Nachbehandlung
HNO 57, 751-762

WEBER, R., R. MAI, W. HOSEMANN, W. DRAF and P. TOFFEL (2000):
The success of 6-month stenting in endonasal frontal sinus surgery.
Ear Nose Throat J 79, 930-932, 934, 937-938

WENTGES, M. (2003)
Isolierung, Kultivierung und magnetische Separation von Vorläuferzellen aus humanem respiratorischem Epithel.
Berlin, Charite-Universitätsmed., Med. Fak., Diss.

WILLIAMS, D. (2006):
New interests in magnesium.
Med Device Technol 17, 9-10

WILLIAMS, R. J. P. (1993):
Magnesium: an introduction to its biochemistry.
In: Birch, N.J. (Ed.): Magnesium and the Cell
Academic Press, New York, 15-30

WITTE, F., J. FISCHER, J. NELLESEN, C. VOGT, J. VOGT, T. DONATH and F. BECKMANN (2010):
In vivo corrosion and corrosion protection of magnesium alloy LAE442.
Acta Biomater 6, 1792-1799

WITTE, F., I. ABELN, E. SWITZER, V. KAESE, A. MEYER-LINDENBERG and H. WINDHAGEN (2008a):
Evaluation of the skin sensitizing potential of biodegradable magnesium alloys.
J Biomed Mater Res A 86, 1041-1047

WITTE, F., N. HORT, C. VOGT, S. COHEN, K. U. KAINER, R. WILLUMEIT and F. FEYERABEND (2008b):
Degradable biomaterials based on magnesium corrosion.
Curr Opin Solid State Mater Sci 12, 63-72

WITTE, F., F. FEYERABEND, P. MAIER, J. FISCHER, M. STORMER, C. BLAWERT, W. DIETZEL and N. HORT (2007a):
Biodegradable magnesium-hydroxyapatite metal matrix composites.
Biomaterials 28, 2163-2174

WITTE, F., H. ULRICH, C. PALM and E. WILLBOLD (2007b):
Biodegradable magnesium scaffolds: Part II: peri-implant bone remodeling.
J Biomed Mater Res A 81, 757-765

WITTE, F., H. ULRICH, M. RUDERT and E. WILLBOLD (2007c):
Biodegradable magnesium scaffolds: Part 1: appropriate inflammatory response.
J Biomed Mater Res A 81, 748-756

WITTE, F., J. FISCHER, J. NELLESEN, H. A. CROSTACK, V. KAESE, A. PISCH, F. BECKMANN and H. WINDHAGEN (2006):
In vitro and in vivo corrosion measurements of magnesium alloys.
Biomaterials 27, 1013-1018

WITTE, F., V. KAESE, H. HAFERKAMP, E. SWITZER, A. MEYER-LINDENBERG, C. J. WIRTH and H. WINDHAGEN (2005):
In vivo corrosion of four magnesium alloys and the associated bone response.
Biomaterials 26, 3557-3563

WOLF, F. I. and A. CITTADINI (1999):
Magnesium in cell proliferation and differentiation.
Front Biosci 4, D607-617

WOLF, F. I. and A. CITTADINI (2003):
Chemistry and biochemistry of magnesium.
Mol Aspects Med 24, 3-9

WOLF, F. I., A. R. CITTADINI and J. A. MAIER (2009):
Magnesium and tumors: ally or foe?
Cancer Treat Rev 35, 378-382

WOLF, F. I., S. FASANELLA, B. TEDESCO, A. TORSELLO, A. SGAMBATO, B. FARAGLIA, P. PALOZZA, A. BONINSEGNA and A. CITTADINI (2004):
Regulation of magnesium content during proliferation of mammary epithelial cells (HC-11).
Front Biosci 9, 2056-2062

WOLF, F. I., A. TORSELLO, S. FASANELLA and A. CITTADINI (2003):
Cell physiology of magnesium.
Mol Aspects Med 24, 11-26

WOLF, F. I. and V. TRAPANI (2008):
Cell (patho)physiology of magnesium.
Clin Sci 114, 27-35

WOLF, F. I. and V. TRAPANI (2010):
TRPM7 and magnesium, metabolism, mitosis: An old path with new pebbles.
Cell cycle 9, 3399

WOLF, F. I., V. TRAPANI, M. SIMONACCI, L. MASTROTOTARO, A. CITTADINI and M. SCHWEIGEL (2010):
Modulation of TRPM6 and Na(+)/Mg(2+) exchange in mammary epithelial cells in response to variations of magnesium availability.
J Cell Physiol 222, 374-381

WONG, H. M., K. W. YEUNG, K. O. LAM, V. TAM, P. K. CHU, K. D. LUK and K. M. CHEUNG (2010):
A biodegradable polymer-based coating to control the performance of magnesium alloy orthopaedic implants.
Biomaterials 31, 2084-2096

XIN, Y., T. HU and P. K. CHU (2010):
In vitro studies of biomedical magnesium alloys in a simulated physiological environment: A review.
Acta Biomater (in press) doi:10.1016/j.actbio.2010.12.004

XIN, Y., K. HUO, H. TAO, G. TANG and P. K. CHU (2008):
Influence of aggressive ions on the degradation behavior of biomedical magnesium alloy in physiological environment.
Acta Biomater 4, 2008-2015

XIN, Y., J. JIANG, K. HUO, G. TANG, X. TIAN and P. K. CHU (2009):
Corrosion resistance and cytocompatibility of biodegradable surgical magnesium alloy coated with hydrogenated amorphous silicon.
J Biomed Mater Res A 89, 717-726

XU, L., F. PAN, G. YU, L. YANG, E. ZHANG and K. YANG (2009):
In vitro and in vivo evaluation of the surface bioactivity of a calcium phosphate coated magnesium alloy.
Biomaterials 30, 1512-1523

XU, L., G. YU, E. ZHANG, F. PAN and K. YANG (2007):
In vivo corrosion behavior of Mg-Mn-Zn alloy for bone implant application.
J Biomed Mater Res A 83, 703-711

XU, L., E. ZHANG, D. YIN, S. ZENG and K. YANG (2008):
In vitro corrosion behaviour of Mg alloys in a phosphate buffered solution for bone implant application.
J Mater Sci Mater Med 19, 1017-1025

YAMAMOTO, A. and S. HIROMOTO (2009):
Effect of inorganic salts, amino acids and proteins on the degradation of pure magnesium in vitro.
Mater Sci Eng C Biomimetic Supramol Syst 29, 1559-1568

YANG, C., G. YUAN, J. ZHANG, Z. TANG, X. ZHANG and K. DAI (2010):
Effects of magnesium alloys extracts on adult human bone marrow-derived stromal cell viability and osteogenic differentiation.
Biomed Mater 5, 045005

YU, Y., J. WANG, C. LIU, B. ZHANG, H. CHEN, H. GUO, G. ZHONG, W. QU, S. JIANG and H. HUANG (2010):
Evaluation of inherent toxicology and biocompatibility of magnesium phosphate bone cement.
Colloids Surf B Biointerfaces 76, 496-504

YUEN, C. K. and W. Y. IP (2010):
Theoretical risk assessment of magnesium alloys as degradable biomedical implants.
Acta Biomater 6, 1808-1812

ZARTNER, P., R. CESNJEVAR, H. SINGER and M. WEYAND (2005):
First successful implantation of a biodegradable metal stent into the left pulmonary artery of a preterm baby.
Catheter Cardiovasc Interv 66, 590-594

ZHANG, A., B. T. ALTURA and B. M. ALTURA (1997):
Elevation of extracellular magnesium rapidly raises intracellular free Mg2+ in human aortic endothelial cells: is extracellular Mg2+ a regulatory cation?
Front Biosci 2, 13-17

ZHANG, E., L. XU, G. YU, F. PAN and K. YANG (2009a):
In vivo evaluation of biodegradable magnesium alloy bone implant in the first 6 months implantation.
J Biomed Mater Res A 90, 882-893

ZHANG, S., X. ZHANG, C. ZHAO, J. LI, Y. SONG, C. XIE, H. TAO, Y. ZHANG, Y. HE, Y. JIANG and Y. BIAN (2010):
Research on an Mg-Zn alloy as a degradable biomaterial.
Acta Biomater 6, 626-640

ZHANG, S. X., J. A. LI, Y. SONG, C. L. ZHAO, X. N. ZHANG, C. Y. XIE, Y. ZHANG, H. R. TAO, Y. H. HE, Y. JIANG and Y. J. BIAN (2009b):
In vitro degradation, hemolysis and MC3T3-E1 cell adhesion of biodegradable Mg-Zn alloy.
Mater Sci Eng C Mater Biol Appl 29, 1907-1912

ZHANG, Y., G. ZHANG and M. WEI (2009c):
Controlling the biodegradation rate of magnesium using biomimetic apatite coating.
J Biomed Mater Res B Appl Biomater 89, 408-414

ZHENG, Y. F., X. N. GU, Y. L. XI and D. L. CHAI (2010):
In vitro degradation and cytotoxicity of Mg/Ca composites produced by powder metallurgy.
Acta Biomater 6, 1783-1791

ZREIQAT, H., C. R. HOWLETT, A. ZANNETTINO, P. EVANS, G. SCHULZE-TANZIL, C. KNABE and M. SHAKIBAEI (2002):
Mechanisms of magnesium-stimulated adhesion of osteoblastic cells to commonly used orthopaedic implants.
J Biomed Mater Res 62, 175-184

ZREIQAT, H., S. M. VALENZUELA, B. B. NISSAN, R. ROEST, C. KNABE, R. J. RADLANSKI, H. RENZ and P. J. EVANS (2005):
The effect of surface chemistry modification of titanium alloy on signalling pathways in human osteoblasts.
Biomaterials 26, 7579-7586

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