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Comparison of different in vitro tests for biocompatibility screening of Mg alloys ☆

L. Scheideler [a,*], C. Füger [a], C. Schille [a], F. Rupp [a], H.-P. Wendel [b], N. Hort [c], H.P. Reichel [d], J. Geis-Gerstorfer [a]

[a] University of Tübingen, Section Medical Materials and Technology, Department of Prosthodontics, Center of Dentistry, Oral Medicine and Maxillofacial Surgery, Osianderstrasse 2-8, D-72076 Tübingen, Germany
[b] University of Tübingen, Division of Congenital and Paediatric Cardiac Surgery, University Children's Hospital, Calwerstrasse 7/1, D-72076 Tübingen, Germany
[c] GKSS Research Center, Institute of Materials Research, Max-Planck-Strasse 1, D-21502 Geesthacht, Germany
[d] Weisensee Company, D-36124 Eichenzell, Germany

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ABSTRACT

Standard cell culture tests according to ISO 10993 have only limited value for the biocompatibility screening of degradable biomaterials such as Mg alloys. The correlation between in vitro and in vivo results is poor. Standard cytotoxicity tests mimic the clinical situation to only a limited extent, since in vivo proteins and macromolecules in the blood and interstitial liquid will influence the corrosion behaviour and, hence, biocompatibility of Mg alloys to a significant extent. We therefore developed a modified cytotoxicity test simulating the in vivo conditions by use of bovine serum as the extraction vehicle instead of the cell culture medium routinely used in standard cytotoxicity testing according to ISO 10993-5. The modified extraction test was applied to eight experimental Mg alloys. Cytotoxicity was assayed by inhibition of cell metabolic activity (XTT test). When extraction of the alloy samples was performed in serum instead of cell culture medium the metabolic activity was significantly less inhibited for six of the eight alloys. The reduction in apparent cytotoxicity under serum extraction conditions was most pronounced for MgZn1 (109% relative metabolic activity with serum extracts vs. 26% in Dulbecco's modified Eagle's medium (DMEM)), for MgY4 (103% in serum vs. 32% in DMEM) and for MgAl3Zn1 (84% vs. 17%), resulting in a completely different cytotoxicity ranking of the tested materials when serum extraction was used. We suppose that this test system has the potential to enhance the predictability of in vivo corrosion behaviour and biocompatibility of Mg-based materials for biodegradable medical devices.

1. Introduction

In recent years Mg alloys have gained increasing interest as new materials for use in bioresorbable medical devices. Proposed applications include cardiovascular implants such as, for example, coronary stents, orthopaedic implants and bone replacement materials [1–5]. These alloys offer a fascinating clinical potential [6], combining the initial strength of a metallic material with the bioresorbability of a polymer like, for example, polylactide [7–9]. The biocompatibility and corrosion behaviour of these Mg alloys under clinical conditions in vivo are, however, difficult to predict.

Many alloys tested so far were initially developed for non-biomedical industrial purposes. Typical examples are the alloys of the AZ system, such as AZ31 and AZ91. These industrial alloys contain various additives besides their main alloy components, to improve different technical parameters such as yield strength and temperature stability [10]. Alloys of the AZ series contain, for example, small amounts of manganese as an alloying constituent, while others contain the rare earth elements, yttrium, zirconium and lithium (for reviews see Zeng et al. [4] and Witte et al. [11]). In order to separate the influences of the different alloying elements used a set of complementary binary and ternary alloys containing only the main constituents without any additives was manufactured and investigated for corrosion behaviour [12] and biocompatibility. The alloys investigated were MgZn1, MgAl3, MgAl9, MgNd2, MgY4, MgAl3Zn1, MgAl9Zn1, and MgY4Nd2.

Some authors [11,13–15] have reported problems in correlating the results of in vitro corrosion and cell culture tests obtained in various test systems with the corrosion behaviour and resulting biocompatibility under real conditions in vivo. Summing up the current state of the art, Witte, in his 2008 review on degradable

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* Corresponding author. Tel.: +49 7071 2985781; Fax: +49 7071 293982.
E-mail address: lutz.scheideler@med.uni-tuebingen.de (L. Scheideler).

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materials based on magnesium corrosion [11], stated that "a systematic approach to determine suitable in vitro methods is needed. This in vitro test system should be able to simulate the desired implantation site and its local environment."

The "local environment" for orthopaedic or maxillofacial implants, at least in the initial period following insertion, will in most cases be a more or less blood-filled cavity. Thus the biological "corrosion solution" for orthopaedic implants such as resorbable screws, fixation plates or bone replacement materials comprises a mixture of interstitial fluid and, initially, human blood. Likewise, the biological corrosion solution for stents, at least for several days before they eventually become covered by neointima, is human blood.

Blood is much more complex than the artificial salt solutions used in conventional, standardized corrosion testing. Even the relatively complex, fully reconstituted cell culture media used in standard cytotoxicity assays mimic the clinical corrosion conditions to only a limited extent. This results in differing rates of dissolution of potentially toxic corrosion products. A fully reconstituted cell culture medium usually contains, for example, between 5 and 10% serum. Thus the concentration of, for example, albumin is 10- to 20-fold lower than in blood.

The concentration of albumin, as well as of other blood proteins, has, however, a significant influence on the corrosion rate. Anodic polarization measurements in our group have already shown that the corrosion current i_{corr} for MgZn1 determined in artificial plasma was increased from 7 to 24 $\mu A\ cm^{-2}$ when 0.1 $g\ l^{-1}$ bovine serum albumin (BSA) was added. The corrosion current measured for MgAl3 increased 4-fold, from 45 to 195 $\mu A\ cm^{-2}$.

Depending on the albumin concentration, enhancement of corrosion as well as passivation effects due to the development of a corrosion-resistant albumin layer have been described [4,13]. Therefore, it seemed sensible that any in vitro biocompatibility test system, in particular when it is based on extracts of the materials to be tested, should have concentrations of albumin and other biomolecules in the extraction medium as close as possible to the in vivo situation. The logical consequence for blood contacting materials would be that tests for such materials are most suitably performed with blood in a cell culture incubator at 37 °C, with a suitable CO_2 concentration to maintain buffer capacity, since pH and temperature also exert a significant influence on the corrosion rate [16].

To investigate this we performed corrosion tests in freshly collected whole human blood in cooperation with the GLP Laboratory of the Division of Congenital and Paediatric Cardiac Surgery, University Children's Hospital. These experiments revealed that the rate of Mg release for certain alloys can change by up to a factor of 10 in comparison with corrosion tests performed in, for example, phosphate-buffered saline [17]. Unfortunately, the use of this whole blood test system for extended periods of time, as suggested in the ISO standard (between 24 and 72 h at 37 °C), is not applicable due to deterioration of the blood cells.

We therefore set out to establish an alternative in vitro cell culture toxicity test system for initial screening of Mg-based implant materials, which is able to simulate the in vivo corrosion conditions as far as possible. In order to mimic the clinical conditions for orthopaedic or maxillofacial implants, which are frequently inserted into a blood-filled cavity, we chose bovine serum instead of cell culture medium as the extraction medium. This test system may also be especially useful for further development of intracoronary stents. Magnesium stents have already undergone animal and clinical testing [18–20]. However, to the authors' knowledge, so far no magnesium stent has been launched on the market, although development of these implants began several years ago [2,21]. Obviously, there is still a demand for improvements in the various Mg alloys proposed for different biomedical applications. For ethical as well as economical reasons in vivo testing of all possible material modifications is not possible. Therefore, there is an urgent demand for predictable in vitro screening methods.

As a first screening of test sensitivity we started with a comparison of two common standardized in vitro tests for cytotoxicity, namely the XTT test for metabolic cell activity measuring metabolization of the tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium (XTT), and the bromodeoxyuridine (BrdU) test for cell proliferation, which quantifies the amount of BrdU incorporated into the DNA of replicating cells.

2. Methods

2.1. Sample preparation

Alloys were die cast in rectangular moulds with the dimensions $125 \times 280 \times 60$ mm. Round sample discs of 10 mm diameter and 1 mm height were turned from the resulting bars. Samples were wet ground with 1200 grit SiC paper using ethanol as lubricant, and subsequently cleaned and sterilized by three 10 min washes in pure ethanol under sonication. The alloys tested were MgZn1, MgAl3, MgAl9, MgNd2, MgY4, MgAl3Zn1, MgAl9Zn1, and MgY4Nd2.

2.2. Cytotoxicity tests

Cytotoxicity was tested with L929 fibroblasts using colorimetric assays for inhibition of metabolic activity (Roche Cell Proliferation Kit II, XTT) and cell proliferation (Roche cell proliferation ELISA, BrdU).

For extract tests under standard conditions samples were extracted in DMEM with 10% foetal calf serum (FCS) with a surface/volume ratio of 3 $cm^2\ ml^{-1}$ for 24 h at 37 °C. Parallel to the onset of extraction, L929 fibroblasts were inoculated in 96-well plates (10,000 cells per well in DMEM/10% FCS) and pre-cultivated for 24 h at 37 °C. After 24 h the medium was replaced by DMEM extract (DMEM-E) at the respective concentrations. Standard extraction tests were routinely performed with three extract concentrations, namely 0.07-fold (10 µl extract plus 140 µl extraction medium, dilution factor 1:15), 0.33-fold (50 µl extract and 100 µl extraction medium, dilution 1:3) and 1-fold (150 µl extract).

For proliferation testing BrdU labelling reagent was added and the amount of BrdU incorporated into cells was determined after 24 h incubation with the extracts, using substrate conversion by the enzyme-conjugated anti-BrdU antibody provided in the test kit.

To determine metabolic activity XTT reagent was added after 24 h incubation with the extracts and formazan formation was determined photometrically. Additionally, sample extracts without cells were used as background controls for non-specific interaction of Mg extracts with dye formation. As background controls extracts were pipetted into four parallel wells of the 96-well plates not containing cells and mixed with XTT reagent. Non-specific dye formation in the control wells (not resulting from metabolic activity of the cells) was subtracted from the respective samples.

In experiments comparing extraction under simulated clinical conditions with standard extraction conditions samples were extracted likewise, but half of the samples were extracted in pure FCS instead of DMEM. The samples were also agitated during the extraction period. L929 fibroblasts were preincubated likewise, but in two different batches, one of which had been previously adapted to DMEM containing 80% FCS instead of the usual 10% concentration, to permit higher concentrations of serum extract (FCS-E) in the assay.

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