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In vitro and *in vivo* cytocompatibility evaluation of biodegradable magnesium-based stents: a review

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ABSTRACT Biodegradable magnesium (Mg)-based vascular stents have been designed as temporary scaffolds to treat angiostenotic lesions for the maintenance of normal blood flow. Numerous studies have presented *in vitro* and *in vivo* tests for the evaluation of the safety and feasibility of Mg-based vascular stents and the related materials. Therein the cytocompatibility is a basic and important parameter in the evaluation system. In this review, we summarize the applications and limitations of *in vitro* evaluation methods including basic characterization methods and direct and indirect cytotoxicity tests. We discuss the influencing factors on cytotoxicity, such as surface roughness, preconditioning of sample surface, cell type for the biocompatibility evaluation in direct contact as well as conditions for the formation of extracts/degradation products for indirect assays. Besides, we highlight the recent *in vivo* animal tests and clinical trials about Mg-based stents along with some associated results. The aim of this review is to provide a meaningful reference in the further developments and related evaluation methods of Mg-based stents.

Keywords: magnesium, stent, cytocompatibility, *in vitro*, *in vivo*

INTRODUCTION

Cardiovascular diseases (CVD) present heart and vascular dysfunction, which are the main cause of death and morbidity in the world [1], posing a heavy burden on individual patients and the social systems. The implantation of vascular stents developed as one of the effective methods to treat the CVD [2]. For the research and development of cardiovascular stents, the choice of stent materials is a critical step which determines the feasibility of the stent implantation in human vascular

lesions. The basic requirements of the stent materials are non-toxicity or carcinogenicity and, as such, able to be tolerated by the body without causing further harm [3].

The conventional applications of permanent bare metal stents and drug-eluting stents lead to long-term endothelial dysfunction, endothelialization delay, late thrombosis, permanent physical and mechanical stress stimulation, toxic metal ion release, local chronic inflammation, and lifelong anticoagulant therapy [4–7]. In particular, these stents limit the possibility of late vascular remodeling. Biodegradable materials are new promising materials for implanted medical devices, and magnesium (Mg)-based stents are attracting more and more attention because of the intriguing combination performances of biodegradation, biocompatibility and appropriate mechanical property [8–16]. Mg-based stents are able to provide stability during the healing period, and then degrade and dissolve within the vessel after the target tissue is healed, and finally the stent would not exist anymore. No foreign material would be left behind, which could effectively prevent chronic inflammation, delayed allergic reactions and further invasive treatments at the same site [11,17]. The major limitation in the application of Mg-based stents is the rapid degradation associated with a decrease of mechanical performance [18,19]. At present, there are two methods to reduce the degradation rate and improve their mechanical properties. One is alloying with elements such as calcium, zinc, strontium, zirconium, rare earth elements, and aluminum [13,20,21], and another is surface modification [22–24]. Both *in vitro* and *in vivo* evaluations must be carried out to verify the feasibility of Mg-based stents before the application in human.

At an ideal vascular stent, the endothelial cells (ECs)

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adhere on the stent strut as quickly as possible to establish re-endothelialization without excessive proliferation of smooth muscle cells (SMCs) or restenosis. Besides this, there should be also no or only mild inflammatory reaction during the implantation period. In terms of biodegradable Mg-based stents, relatively high concentrations of Mg ions are released inevitably, thus the influence of the released Mg ions on these cells is necessary to be assessed in *in vitro* tests [25,26].

The *in vitro* evaluation of cytocompatibility is a rapid, standardized, sensitive, and inexpensive method to determine whether a material performs physiologically, without release of any harmful extractable substance(s). The evaluation of the biodegradable Mg-based materials is mainly conducted by two methods [27], direct and indirect assessments. The direct method may be limited for Mg based substrates, due to the fast change of the sample surface during the degradation process. Therefore, the indirect method generally is preferred to evaluate the cytocompatibility of the biodegradable Mg-based materials. Based on the ISO protocol of cytotoxicity evaluation, an extract that allows for more than 75% cell viability is considered as non-cytotoxic [28].

Biodegradable Mg-based vascular stents have been investigated in many different animal models and clinical trials. For *in vivo* animal studies, the different candidate Mg-based stents were implanted into the arteries without atherosclerotic lesion at various sites. Most of the results were positive about the cellular and tissue response surrounding the stents [13,26,29–40]. It means that the applications of Mg-based stents in the vessels are safe for the body. A representative clinical trial is the Absorbable Metal Stents (AMSS, Biotronik, Germany) using stents made by WE43 alloy with rare earth elements, implanted into 20 patients with critical limb ischemia [41]. This is a milestone—the first clinical trial of biodegradable Mg-based vascular stents. A series of follow-up clinical trials also obtained positive results, such as Magic Stent, DREAMS, DREAMS 2G (Magmaris) and so on [11,41–50]. It is worth mentioning that the Magmaris stent received CE mark approval in June 2016, and it shows very encouraging clinical data on endothelialization, radial resistance, recoil, thrombosis and degradation properties.

In this review, we summarize the present *in vitro* evaluation methods of biodegradable Mg-based stents or candidate materials, as well as some limitations in the application of these methods. Besides, we highlight the recent *in vivo* animal tests and clinical trials about Mg-based stents along with some associated results. The aim of this review is to provide some helpful information for

future research.

IN VITRO CYTOTOXICITY EVALUATIONS OF Mg-BASED MATERIALS

Evaluation methods of cytotoxicity

Two types of assays are mainly applied to *in vitro* cytotoxicity tests, that is, direct and indirect culture assays. The direct culture assays refer to the cell viability of a cell culture directly on the surface of the alloys, while the indirect culture assays refer to the investigation of the effect of degradation products from the alloy by a culture medium on cell growth [51]. Both methods are affected by various experimental factors, such as the use of different alloys, cell lines, and medium types [12,16,51–61].

Direct culture assays

For a stent implanted into vessel, it is essential to evaluate the direct interaction between the substrate (Mg-based metals) and cells/tissue. The direct culture assays were introduced as part of a field-wide effort to improve and standardize the *in vitro* testing of Mg-based biomaterials. They are applied to simulate cellular processes at the implant site, to probe the cell-biomaterial interface directly [62] and to provide a more comprehensive *in vitro* method for the initial rapid screening of cytocompatibility and degradation of Mg-based materials than the ISO 10993-based methods [62–65].

Direct contact assays are necessary for the initial rapid screening of cytocompatibility and cytotoxicity of biomaterials [66]; however, they have some limitations, mainly appearing as inhibition of cellular activity on the surface of Mg-based sample because of an initial rapid degradation of the Mg alloy. This causes a marked increase in local pH [16,63,67–69] and locally high osmolality due to the release of ions into the medium [62,67]. The initially rapid evolution of hydrogen leads to gas cavitation and dramatically hinders cell attachment [69–73]; it also promotes the shedding of the surface degradation layer impeding the adhesion of cells, making it difficult to carry out cell culture experiments [27,67]. The change in surface chemistry and morphology affects cell spreading and long-term survival. In addition, there is a technical problem, as the redox pair of metallic Mg and the dissolved Mg ions in the medium interferes with standard vitality assays. It converts the tetrazolium salts of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) or sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-

tetrazolium inner salt (XTT) assays to formazan in the cytotoxicity evaluation, leading to a higher background and falsifying the results of cell viability [74]. The direct contact model does not reflect the dynamic fluid condition of the *in vivo* setting, which removes degradation products. Local effects, therefore, are overestimated in this approach.

In the following, the effects of various factors on cell toxicity in the direct cell culture assays for Mg-based metals will be discussed, such as surface roughness, preconditioning of sample surface, and applied cell type.

(i) Surface roughness

Literature data about the influence of surface roughness of Mg-based concerning cellular activity, adhesion and spreading in direct culture assays are not conclusive, because roughness induces processes with opposite effects. Roughness from 1 to 100 nm typically promotes cell adhesion or growth [71,75,76]. However, depending on the degree of roughness and the alloy composition, the degradation is enhanced by the roughness. This may lead to enhancing ion concentration and osmotic stress for the cells, impairing cell adhesion and growth [77,78]. Different cell types also show different sensitivity to these effects. Hence, although there is an influence of roughness on cell adhesion and proliferation, the actual effect is hard to be predicted and it is necessary to adjust the *in vitro* test conditions as close as possible to the *in vivo* condition in the screening of Mg-based alloys as vascular stents.

(ii) Sample preconditioning

Mg-based samples under the standard cell culture conditions undergo rapid degradation with toxic effects to the cells. However, protein adsorption on the sample surface retards the degradation *in vivo*. Pretreatment of Mg-based samples is a critical step for initial cell adhesion and survival on sample surfaces in the direct culture assays [26,51,69,71,79]. The pretreatment usually consists of a 24 h incubation at 37°C, 5% CO₂, in solutions like simulated body fluid (SBF) and Dulbecco's Modified eagle medium (DMEM). A large range of factors, including H₂ gas evolution, pH increase due to Mg dissolution in the cell culture medium, as well as the chemical and physical properties of the native or corroded Mg surface, would all contribute to a decrease in initial cell adhesion and growth on the Mg substrate in the *in vitro* assays [71]. High Mg ion concentrations up to 30 mmol L⁻¹ (750 mg L⁻¹) do not affect cell density, cell spreading, and actin stress fiber formation [69]. The difference to the *in*

vivo environment is that *in vivo* the H₂, pH, and part of products are buffered or removed by the body fluid, and meanwhile, various proteins adhere to the surface of Mg and retard the degradation, which supports the cell adhesion and growth. Pretreatment of the surface aims to simulate the *in vivo* condition by reducing the degradation rate of the Mg-based samples.

The pretreatment methods affect initial cell response. Mg-alloy pretreated in different solutions induced different cell responses in the systematic studies of Sanna-kaisa Virtanen et al. [69,71]. For example, only few individual cells with poor cell spreading or even a cell-free surface was observed on Mg without pretreatment. A significantly higher number of cells were observed on the surface of samples immersed in simulated body fluid (M-SBF) for 5 days at 37°C or for 24 h in 1 mmol L⁻¹ NaOH. More cells adhered on samples soaked in M-SBF than in 1 mmol L⁻¹ NaOH, but better cell spreading, more actin cytoskeleton, as well as filopodia formation, were seen on the NaOH-passivated surface. Mg surfaces pretreated by DMEM with or without serum or pretreated in pure serum (incubation at 5% CO₂, 37°C, 95% relative humidity, 24 h) induced greatly increased cell density compared with SBF-treated samples. The coating formed in the cell culture medium was more protective than that in SBF. Cell culture medium effectively tailored the degradation rate of pure Mg and furthermore strongly enhanced cell adhesion and spreading.

(iii) Cell type

In the evaluation of the cytocompatibility of Mg alloys, the different sensitivity of different cell types also plays an important role. ECs, SMCs, and inflammatory cells, among others, interact with Mg-based stents in injured blood vessels.

For cell viability, ECs benefit from elevated levels of magnesium: No toxicity was seen up to ca. 25 mmol L⁻¹, even enhanced proliferation and migration occurred around 10 mmol L⁻¹ Mg²⁺. In contrast, there was no stimulating effect of the Mg²⁺ ions for SMCs [25,51,80,81]. This has been traced down to cell cycle regulation and proliferation-associated genes, which showed only minor changes in ECs but were down-regulated in SMCs, indicating that Mg inhibits SMC proliferation without a major influence on EC proliferation [25,82].

In general, the inflammatory response to biomaterials is initiated by the reaction of vascularized connective tissue to the injury caused by the implantation [83,84]. There is more and more evidence that magnesium deficiency promotes inflammation, exacerbates immune stress re-

Table 1 Standard surface areas and extract liquid volumes [90]

Thickness (mm)	Extraction ratio (surface area or mass/volume)±10%	Forms of material
<0.5	6 cm ² /mL	Film, sheet, tubing wall
0.5 to 1.0	3 cm ² /mL	Tubing wall, slab, small molded items
>1.0	1.25 cm ² /mL	Larger molded item(s)
Irregularly shaped solid devices	0.2 g/mL	Power, pellets, foam, non-absorbent, moulded items
Irregularly shaped porous devices (low-density materials)	0.1 g/mL	Membranes

sponse and decreases the specific immune response [85–87]. Magnesium in a range of 0.21 to 21 mmol L⁻¹ Mg²⁺ (5 to 500 µg mL⁻¹) has no major effects on macrophage viability, the release of the proinflammatory cytokine tumor necrosis factor α, or on the proliferation of allogenic lymphocytes in a mixed leukocyte incubation [86,87]. It can be concluded that the Mg ions produced from Mg-based implants are biocompatible and show a low inflammatory potential.

Indirect culture assays

Current cytocompatibility and cytotoxicity tests of Mg alloys are mostly based on the extract culture method (ISO 10993-5 and 10993-12), which is also called indirect or media extract method, because the cells are only exposed to certain concentrations of media extracts from degraded samples and are not in the same culture as the samples. These ISO standards were originally developed for testing the cytocompatibility of non-degradable materials [63]; however, they were widely adopted to study Mg or Mg alloys mainly due to the simplicity of the method [27,63]. A benefit of the indirect test approach is that the effects of eluted compounds from the material into a liquid phase (blood, body fluid, or cell culture medium) on cells can be easily predicted.

There are also some factors affecting the utilization of indirect culture assays, such as the surface area to extraction medium ratio, the serum concentration in the medium, and the ion accumulation in the extract.

(i) Surface area to extraction medium ratio

The surface area of Mg-based sample to extraction medium ratio is a critical factor influencing the results of cytocompatibility during the extract preparation for the indirect assessment [27]. A surface area to extraction medium ratio of 1.25 cm² mL is used in many studies [12,53,68,88,89], based on recommendations of ISO 10993-12 for the standard surface area and extract liquid

Figure 1 Comparison of extraction in either pure serum or DMEM containing 10% serum (standard extraction method), modified from reference [52], Copyright 2013, Elsevier.

volumes (Table 1) [90]. According to the ISO standard, the extraction medium should be able to extract the maximum amount of extractable substance(s) within physiologically relevant surface: volume ratios for biological testing [90]. Table 1 shows the homologous surface area or mass/volume of different sample types. Obviously, the ratio depends on the thickness of the sample. It is important to select the appropriate surface area/medium volume ratio for specific demands [64].

(ii) Serum concentration

Varying concentrations of serum in the extraction of Mg alloy significantly affects the cytotoxicity in the indirect culture assays. When Mg-based samples are extracted in serum or in a high concentration of serum instead of pure cell culture medium, the metabolic activity of the cells was significantly less inhibited for some Mg-based alloys as shown in Fig. 1 [52]. This phenomenon can be attributed to a “protective” effect of the serum proteins on Mg degradation, thus reducing the cytotoxicity. Such protective effects are confirmed by the literature, indicating a pronounced influence of blood components,

especially of proteins such as albumin, on the biodegradation of Mg alloys [74,91–93]. From this point of view, the serum-based *in vitro* test system has the potential to closely predict the *in vivo* degradation behavior and biocompatibility of Mg-based materials.

(iii) Ion accumulation in the extract

The preparation of extracts from Mg-based samples, which is currently standardized in ISO 10993 about *in vitro* cytotoxicity tests, is the critical step of the indirect culture assays, influencing outcomes of cytotoxicity tests [94]. However, these available standards do not consider the physiological clearance of the ions released from the implanted Mg-based biomaterials by circulation *in vivo* [64]. The standards not only neglect the impact of the different accumulated ions in the extracts on the cytotoxicity results [62,81,82,95,96], the accumulation also contributes to the difference and even contradictory results of *in vitro* and *in vivo* studies [12,86,87,97]. There are two feasible approaches to reduce the accumulation of Mg and alloy ions in the extracts. The degradation rates may be reduced *in vitro* by modifying the extracted environment such as more supplement of serum into cell culture medium, as mentioned above. Alternatively, it has been recommended to dilute the extracts to different concentrations [53,63,64], which helps for matching the *in vivo* findings more closely.

Limitations of direct and indirect culture assays

The direct culture assays were established to investigate cytocompatibility at the dynamic surface of biodegradable Mg alloys, such as cardiovascular stents [62–65], that is, cells in direct contact with the samples. Nevertheless, it is difficult to identify the factors causing a reduction of cell viability and adhesion. These factors include the local increase in alkalinity, the change of surface topography, and/or the surface composition [12,81,98–100]. Herein, it has to be emphasized that the “dynamic environment” in direct culture assay is formed in the process of the degradation of Mg alloys accompanied by the hydrogen generation, product diffusion, as well as pH changes. These dynamic factors affect cell behavior, which is in contrast to the indirect culture system [62]. It is totally different from the natural dynamic bioenvironment *in vivo* [101], in which the stent is exposed to various organic [102] and inorganic [103] constituents, followed by a complex response of proteins, blood [104], cells [64] and tissue [46] and meanwhile the dynamic circulation system removes the degradation products and prevents a drift of local pH [101].

The indirect culture assay presents the effects of the compounds extracted from the Mg-base samples in culture medium on cells. Many studies indicated that extracts from Mg alloys have little or no effect on cell viability by utilizing the indirect culture assay [79,105–107]. Similarly, it is obvious that the test system is static compared to *in vivo* condition as mentioned above [46,62,64,101–104], that is, it cannot adapt to the changes in the environment of the degradable Mg-based stent as the human body does.

Summing up, these *in vitro* culture assay methods in cytotoxicity of biodegradable Mg-based materials can be used as an economic and simple tool in the initial material screening studies before *in vivo* and clinical evaluation [66]. The constant pool condition of classical cell culture with accumulation of degradation and cellular products also allows for more sensitive evaluation of several parameters than a dynamic animal system. Furthermore, *in vitro* tests are more favorable than animal studies from an ethical point of view [70]. Of course, these *in vitro* methods are limited in some challenges, as they cannot mimic the *in vivo* conditions perfectly [70]. The influencing effects of the degradation of Mg-based samples on cell behavior are only partly effective [108], and thus the reliability and predictability of *in vitro* methods is reduced [109]. Finally, the evaluation is restricted to the cellular level and does not indicate the interaction between degradation kinetics and vascular tissue. Therefore an *in vitro* dynamic flow system such as bioreactors would be best simulating the situation in the human body [63,110–112] to reduce or even eliminate the gap between *in vitro* and *in vivo* studies, which should be further improved [70,109].

Characterization methods of cytotoxicity

The ISO 10993-5:1999 [94] requires that the cytotoxicity is measured *via* cell death, inhibition of cell growth, cell proliferation or colony forming ability. Tetrazolium salt-based assays are widely used to measure cytotoxicity or cell proliferation [113]. Tetrazolium dyes include MTT and XTT, and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (CCK-8) [114]. Besides, 5-bromo-2'-deoxyuridine (BrdU) test, lactate dehydrogenase (LDH) test, DNA measurement, and Cell Titer-Glo Luminescent Cell Viability Assay (CTG) are some novel characterization methods that are able to eliminate the limitations of tetrazolium salt-based assays.

MTT is a water-soluble tetrazolium salt reduced into insoluble blue formazan by NAD(P)H-dependent oxi-

Figure 2 Analysis of the formazan production (MTT and XTT) without and with cells for the direct assay. The ODs of the specimens incubated without or with cells are compared to the ODs of the control (pure culture medium and cells incubated without specimen, respectively). The controls are set to 100%, modified from reference [74], Copyright 2010, Elsevier.

doreductases within the metabolically active cells [114,115], and solubilization solution is always used to dissolve the MTT formazan. XTT as the second-generation tetrazolium salt is reduced by dehydrogenase enzymes and produces soluble orange formazan end products [115]. MTT and XTT assays are frequently used to test the effects of samples on cell viability because of their convenience and reliability [12,75,116,117], and the amount of formazan formation can reflect the level of cell viability. These tests are easy, fast, non-radioactive and interact specifically with living cells [75]. However, there are some limitations in the utilization of these tetrazolium salt-based kits to determine the cytotoxicity of biodegradable Mg-based samples, because Mg degradation strongly affects the test systems, including Mg^{2+} ions and pH.

Influence of Mg^{2+} on the results of tetrazolium dyes has been reported. Degradation products from Mg degradation impact the result of tetrazolium-based cytotoxicity assays [74], resulting in a higher background and the false results of cell viability (Fig. 2). Because the released Mg^{2+} ion will reduce the XTT and MTT tetrazolium salt to formazan and thereby increase the readout of the MTT and XTT assays (Fig. 3). In addition, pH variation from Mg-based degradation has a significant influence on the results of XTT test and even more of the MTT test [74,118]. As shown in Fig. 4, the optical density (OD) value is gradually higher for the culture medium with

higher pH in the MTT and XTT tests. To derive reasonable results, the pH influence needs to be studied further and has to be included in the array test development. In general, the MTT is more sensitive to pH changes to basic values than the XTT test and pH has an influence on MTT due to a shift in absorption maxima at low pH or high cell density [118].

In summary, the tetrazolium-based tests are not an ideal tool in cytotoxicity measure of Mg-based alloys as the existence of such interference factors in MTT and XTT tests. In order to eliminate the false results, appropriate controls are needed to subtract the mean OD of the controls for each alloy from the sample values in the presence of cells, and thus the non-specific and inherent interference in these tests could be compensated [52]. Besides, some test methods are also reasonable, which are not influenced by the Mg degradation. For instance, the BrdU test reflects the cell proliferation based on DNA replication activity [52,65,74,82]; LDH test detects the release of lactate dehydrogenase from cells when the cytomembrane is damaged [98,119]; CTG test determines the number of living cells by quantitative measurement of ATP [51]; as well as DNA measurement uses a DNA intercalating fluorochrome (bisbenzimid), and the emission wavelength of bisbenzimid change can be detected by a fluorometer, thereinto the amount of measured DNA is correlated to the number of living cells [74,120].

IN VIVO PERFORMANCES OF BIODEGRADABLE Mg-BASED MATERIALS AND STENTS

It is necessary to carry out a series of *in vivo* implantations in animal models and clinical trials to evaluate the biocompatibility and applicability of Mg-based stents. Several years of animal and pre-clinical studies with these stents provided encouraging results (summarized in Table 2).

Animal studies: implantation models, characterization methods and summarized results

Stents with the aluminium and rare earth containing magnesium alloy AE21 were firstly successfully implanted into the carotid arteries of Beagle dogs in 2003 [13], and many follow-up *in vivo* evaluations of different Mg-based stents through animal implantations have been carried out in the recent decade. Animal studies comprise the implantation process and the post-implantation characterization. Different animal models including dog, rabbit and pig models are used for *in vivo* tests of the Mg-based stents. The selected implantation sites mainly in-

Figure 3 Assumed formation of formazan (a: MTT; b: XTT) by Mg materials, modified from reference [74], Copyright 2010, Elsevier.

clude right coronary artery (RCA), left anterior descending (LAD) and left anterior circumflex (LCX) in the consideration of mimicking the most frequent clinical cardiovascular occlusions, as well as the other sites such as abdominal aorta [30], femoral artery [13,26,31] iliac artery [32], renal artery [32] and so on.

Most existing studies applied coronary angiography (CA), optical coherence tomography (OCT), intravascular ultrasound (IVUS), and histopathology including various histological staining and immunohistochemistry (IHC) for evaluation of the degradation process and biocompatibility of Mg-based stents. In particular, OCT is an effective tool for a

quantitative assessment of stent degradation, as it allows the measurements of stent strut profile due to its high-resolution capability [33,121–123]. CA is utilized to characterize the peripheral embolisation or in-stent thrombosis; and IVUS is used to measure the data of the perfused lumen, plaque, and stent areas, to investigate the neointima formation and vascular remodeling. Histopathology of stented arteries segments shows the situation of re-endothelialization, restenosis, and inflammation, and the main process includes tissue fixation, embedding, cutting slices and staining. It is noteworthy that some researchers reported a novel tissue fixation method, in which the stented arteries were *in situ* fixed with 10%

Figure 4 Influence of pH on the MTT and XTT test. Absorption values were measured and calculated as percent of control (pH 7.35). The absorption values of the unchanged medium were set to 100%. Significant differences ($p < 0.05$) for both tests are indicated by the asterisks, modified from reference [74]. Copyright 2010, Elsevier.

buffered formaldehyde solution perfused at 100 mm Hg for 15–30 min after an overdose of anesthesia and then removed for further conducts [26,31,35,36,39,124].

Re-endothelialization is a critical index in the characterization of biosafety of vascular stents after implantation. Supporting the *in vitro* results, the Mg-based stents *in vivo* also promote the proliferation of endothelium because complete endothelialization occurs in approximately 30 days for bare Mg-based stents [32,36,39] while in 40–60 days for the drug-eluting Mg-based stents [38,39]. The biodegradable Mg-based vascular stent barely induces in-stent thrombosis [30,31,35,36,39], restenosis or occlusion [13,26,30,33–37,39,45] after implantation into animal arteries. A reasonable explanation for the lack of thrombotic complications is that Mg-based alloys are electronegative and provide hypothyrombogenic surfaces [36,125].

There were no or just slight signs of inflammation in most stented arteries [13,30,33,36,39,124], as a few inflammatory cells accumulated around the stent and subsided in the late-stage implantation. The minor inflammatory reaction in arteries can be reasonably attributed to the re-endothelialization and neointimal coverage on the stent struts [30]. Neointimal area was determined by subtracting the area of the lumen from the area within the stent struts. Neointima formation can be explained by two different processes: the local tissue “overload” with degradation products resulting from accelerated degradation; and the dominant relation between struts related injury caused by oversizing of the balloons

and induced regional inflammation [11,13,92]. In animal studies, there is no significant increase or even some decrease of the neointimal area with the prolonged implantation time [13,30,33,34,36], which can be attributed to the inhibition of the SMC proliferation. Besides, vascular remodeling is defined as the change in external elastic membrane area from index intervention to follow-up after implantation. Positive remodeling has been reported for Mg-based stented arteries [13,33,36]; however, Maeng *et al.* [124] reported the opposite result: negative vascular remodeling caused by rapid degradation may decrease the lumen area of arteries.

A simple evaluating model to prescreen the candidate Mg-based stent materials has been described, in which Mg-based wires as substitute for the stents were implanted into murine artery to evaluate the degradation and biocompatibility [111,126,127]. Wire implantation into artery lumen and wall in parallel allows to analyze the effects of fluid flow and diffusion. Although the geometry is different between the wire and stent, the simple model can investigate the interaction between the candidate Mg-based materials and the blood fluid or wall matrix. Furthermore, the wire implantation approach is faster and low-cost compared with the large animal stent implantation.

Most animal studies were conducted in normal models without atherosclerosis, which is a critical limitation in the *in vivo* animal evaluation of biodegradable Mg-based stents. The degradation kinetics of stents are different between normal arteries and atherosclerotic arteries. Therefore, it is reasonable to build a model of an atherosclerotic lesion or stenosis of arteries and then investigate the feasibility of stent implantation at the site of the lesion [37]. In addition, interspecies differences may also induce different tissue response to implanted stents [33,124].

Clinical trials

Up to now, the yttrium, rare earth and zirconium containing Mg alloy WE43 might be the only Mg-based alloy used for vascular stent in clinical trials, such as the Absorbable Metal Stent (AMS) [11,41,42,44–46,49,50] and Magic stent [47,48], which are two most frequently investigated stents in clinical trials. Besides coronary arteries [42,43,45,46] also peripheral arteries [41,44], pulmonary arteries [11,47,49] and other lesions [44,50] have been stented. CA and IVUS are the main follow-up tools to investigate the stented arteries. In general, the existing Mg-based stents including AMS, DREAMS and Magic stents are safe for the human body and induce only

Table 2 *In vivo* evaluation of Mg-based stents

Samples	Size of stent (mm)	Animal models/ Human	Implantation site	Time (Days)	Characterization methods	Brief summarized results		Ref.
						Biocompatibility	Degradation	
Animal implants								
Magmaris (Biotrinik)	D: 3, L: 20, WT: 0.15	Porcines, rabbits	Coronary artery	3, 28, 84, 175, 365, 730	SEM, OCT, CA	Increased endothelialisation and decreased thrombosis at 3 and 28 days; Absence of excessive lumen loss up to two years; Low acute thrombogenicity.	365–548 days	[40] 2017
Mg-2.2Nd-0.1Zn- 0.4Zr stents (JDBM-2)	D: 2, L: 14 (unexpanded)	2.5–3.5 kg New Zealand white rabbits	Abdominal aorta	30, 61, 122, 183	CA, IVUS, H&E staining, SEM	No acute and late thrombogenesis and in-stent restenosis; Slight inflammatory response and mild in-stent intimal hyperplasia (increased with time).	~183 days	[30] 2017
Mg-0.3Sr-0.3Ca stents, WE43 as control	D: 2.4, L: 10, WT: 0.25 (unexpanded)		Femoral artery	35	H&E staining, CA, SEM&EDX	No sign of occlusion; Protective Sr-HA layer is formed <i>in vivo</i> .	–	[28] 2016
Mg-0.5Sr tube WE43 as control	D: 2.4, L: 10, WT: 0.27 (unexpanded)	8.7 kg dogs	Femoral artery	21	SEM	No thrombosis.	245–266 days	[31] 2013
Bare Mg-alloy stents, Pt-Cr stents as controls	D: 3, L: 12	Female crossbred swines	LAD, LCX, RCA, ITA, renal and iliac arteries	30, 120	micro-CT, H&E, elastic trichrome, Von Kossa staining	30 days: Neointimal thickness and stenosis were higher with Mg stent; Arterial calcifications; Completely endothelialized; 120 days: Remaining stent fragments were covered by neointima, and sur- rounded by cells matrix with some calcifications	~120 days	[32] 2011
Mg-based AMSs (Biotronik)	ST: 0.24±0.032 (expanded)	25–38 kg Swines	LAD, LCX	28, 52, 91	CA, OCT, IVUS, H&E staining	No significant increase in neointimal area; Positive remodeling of the lumen and vessel; Trivial degree of inflammation.	~93 days	[33] 2008
AMS (Biotronik), BMS (Cordis) and Cypher (Cordis) as control	D: 3.41, L: 15; D: 3.70, L: 13; D: 3.51, L: 13 (expanded)	60 kg pigs	LAD, LCX, RCA	90	IVUS, CVIS Clear View Ultra, H&E staining	Safe and no inflammation; Negative vascular remodeling, causing luminal narrowing; Neointima consisted of SMCs without unresolved fibrin; Cannot scaffold long enough to avoid negative remodeling.	–	[124] 2008
Mg-Y-Re AMSs, 316L stainless steel stent as control	D: 3, L: 10, ST: 0.12 (unexpanded)	20–30 kg minipigs	RCA, LAD, LCX	28, 56.	CA, micro-CT, Van Gieson staining, IHS.	Neointimal area of AMS stented arteries was less than 316L stents; IS reduced with the time; The number of early macrophages did not differ at both time points.	–	[34] 2007
AMS adjunct vascular brachytherapy (VBT)		36–40 kg pigs	Coronary arteries	28	IVUS, H&E staining, pentachrome staining	No embolization or thrombosis; No acute recoil.	–	[35] 2007
Magic stent (Mg-Zr-Y-Re), stainless steel stent (Lektion Motion) as control	D: 3, L: 15, ST: 0.15–0.2 (unexpanded)	25–30 kg domestic pigs	LAD, LCX, RCA	3, 28	CA, IVUS, H&E staining, pentachrome staining	3 days: Start reendothelialization; 28 days: No intimal thickening, No intraluminal thrombus; Completely endothelialized;	~93 days	[36] 2006
		17–21 kg minipigs		90		90 days: No inflammation; Smaller vessel lumen; Positive remodeling		
AE21 stents	D: 2, L: 10, WT: 0.15–0.2 (unexpanded)	20–25 kg Pigs	LAD, LCX, RCA	10, 35, 56	CA, IVUS, histological analysis	Significant neointimal proliferation until day 35 (caused by local arterial injury), while not until day 56; Negligible inflammatory; Observed positive remodeling	<89 days	[13] 2003
Covered Mg-alloys stents, Willis cov- ered stents as con- trol (Microport)	–	2.5–3.5 kg New Zealand white rabbits	Carotid artery (lateral aneurysm model)	91, 183, 365	CA	Successfully formed right lateral aneurysm model; 183, 365 days: Disappearance of the aneurysms and patency of the carotid artery; No occlusion of the right carotid artery.	~365 days	[37] 2015
Paclitaxel-eluted WE43 stents	D: 2.5, L: 18, ST: 0.13 (unexpanded)	2.5–3.0 kg New Zealand white rabbits	Iliac artery	21, 42	SEM, EDS	A thin layer of tissue on some struts at 21 days; Endothelial coverage on struts and between struts until 42 days.	–	[38] 2014

(To be continued on the next page)

(Continued)

Samples	Size of stent (mm)	Animal models/ Human	Implantation site	Time (Days)	Characterization methods	Brief summarized results		Ref.
						Biocompatibility	Degradation	
AZ31B stents (BMAS)						No obstruction or thrombosis; Neointima formation; No difference of vessel injury, inflammation scores (mild), and internal elastic lamina between two groups; Larger lumen area (SEBMAS); Lower endothelialization in the SEBMAS at 30 days, while became similar in both groups at 60 through 120 days.		
Sirolimus-eluting AZ31B stents (SEBMAS)	D: 3, L: 15, ST: 0.155	2.0±0.2 kg New Zealand white rabbits	Abdominal aorta	30, 60, 90, 120	CA, H&E staining		<120 days	[39] 2011
Clinical implants								
DREAMS 2G (Biotronik)	D: 2.5/3.0/3.5, L: 15/ 20/25, WT: 0.15	184 patients with 189 lesions	Different lesions	30, 183, 365, 730, 1095		Safe and no thrombosis; Low inflammation peaked at 90 days and decreased thereafter; Low TLF rates up to two years.	~183 days	[29] 2017
AMS (Biotronik)	D: 3–3.5, L: 15		Coronary artery	122	CA	Other 5 patients: Demonstrable vasodilatation; Increasing cross-sectional area.	–	[41] 2014
DREAMS (Biotronik)	D: 3.25 or 3.5, L: 16	46 patients	LAD, LCX, RCA	30, 183, 365, 730, 1095	CA, IVUS, OCT	No cardiac death or scaffold thrombosis; No death and no thrombosis; Reduction of mean lumen area at 6 and 12 months; 7% rate of target lesion failure, 4.7% revascularization rate.	–	[42] 2013
AMS (INSIGHT)	D: 3–3.5, L: < 15	57 patients	Different lesions	30, 61	CA	61 days: 31.8% Angiographic patency rate for lesions.	–	[43] 2009
AMS (71)	D: 3–3.5, L: 10–15	63 patients (mean age: 61)	Coronary artery	122, 183, 365, 850	CA, IVUS	No myocardial infarction, subacute or late thrombosis, or death, and calcifications; Neointimal growth and negative remodeling in 4 months and decreased after over 12 months.	~120 days	[44] 2008 [45] 2007
Magic/Explorer stent (Biotronik)	D: 3, L: 15	2-month-old girl (hypoplastic pulmonary arteries)	Stenotic aorto-pulmonary collateral	–	CA	Initial significant increase in vessel diameter; Significant restenosis occurred 4 months after stent implant (1.4 mm)	~120 days	[46] 2007
Magic Stent (Lecton)	D: 3.5, L: 15	65-year-old man (stenosis in the LAD)	LAD	–	CA, IVUS, OCT	Vessel lumen: with no signs of narrowing or edge effect; 450 days: All struts were covered by a thin neointimal layer (thickness: 80–140 μm).	–	[47] 2007
AMS (Biotronik)	D: 3, L: 10 (unexpanded)	Female preterm baby	Left pulmonary artery (LPA)	–	HIS, H&E, Van Gieson, Von Kossa, Alizarin red staining	Died from multiple organ failure about 5 months later; Completely endothelialized; Reduced elasticity of the LPA; No calcification, necrosis or structural damage to the vessel wall; Neointima (up to 100 μm); No inflammatory cells.	–	[50] 2007
AMS (Biotronik)	D: 3.5, 4, L: 15	3-week-old male newborn (aortic coarctation)	Descending aortic arch	–	CA	Luminal reduction induced by degradation made the operated vessel to backslide into its previous course, and required implanting a second stent; Pathological magnesium levels in serum of the patient were not detected.	–	[49] 2006
AMS (Biotronik)	D: 3, L: 15	(critical limb ischemia)	Lower limb vascular	30, 90	CA, IVUS	89.5% patients had normal blood flow at 30 days and 68.4% at 90 days; Low immediate elastic recoil; no blood/vessel toxicity.	–	[50] 2005
AMS (Biotronik)	D: 3, L: 10	1.7 kg preterm baby	Left pulmonary artery	–	CA	Degradation process was clinically well tolerated; 7 days: reperfusion of the left lung, thrombus distal to the stent had dissolved; 42 days: the patient was eventually extubated;	~152 days	[11] 2005

Note: D: diameter; L: length; WT: wall thickness; T: thickness; ST: strut thickness; CA: coronary angiography; OCT: optical coherence tomography; IVUS: intravascular ultrasound; CVIS: cardiovascular imaging system; RCA: right coronary artery; LAD: left anterior descending; LCX: left anterior circumflex; ITA: internal thoracic; AMS: absorbable metal stent made from WE43; BMS: bare-metal stents; Cypher: sirolimus-eluting stents; IHS: immunohistological staining; IS: injury score; CLI: chronic limb ischemia (or critical limb ischemia); TLF: target lesion failure.

little complications [11,41,43,44,46] in the early stage of the implantation, as well as hardly any scaffold thrombosis [11,43,45,46]. It is worth noting that the first clinically proven Mg-based stent DREAMS 2G (Magmaris) received CE mark approval in June 2016, and has shown very encouraging clinical data on endothelialization, radial resistance, recoil, and degradation properties. Nevertheless, luminal reduction [45,46], and in-stent restenosis [42,47] appeared in clinical trials. Pre-term degradation also caused a return of the original stenosis and required a repeated stent implantation [50]. Of course, discrepancies exist in these different clinical trials because of individual differences including the site and degree of the lesion, the age of patients, and the stent types.

SUMMARY

The biodegradable Mg-based stent is a promising successor to permanent metal implants because of its favorable biodegradation and cytocompatibility both *in vitro* and *in vivo*, observed in normal animal models and in clinical patients. Nevertheless, there still exist some limitations in the assessment of Mg-based stents or materials, such as the static cell culture *in vitro* and the non-atherosclerotic animal models *in vivo*. Therefore, it is necessary to optimize the existing methods of cytocompatibility evaluation, or develop more appropriate and feasible methods, such as an *in vitro* bioreactor that can closely mimic the *in vivo* microenvironment of the vessel and thereby solve the present problems of low correlation between *in vitro* and *in vivo* corrosion results, and the consideration of the animal ethics, which is also significant for the research and development of Mg-based stents, in addition to the optimization of materials design (materials optimization and surface modification).

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生物可降解镁基支架细胞相容性的体内外评价: 综述

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摘要 生物可降解镁基血管支架具有临时支撑血管的作用, 用来治疗血管狭窄病变, 维持正常血流。目前许多研究者已经报道了关于体内外评价镁基支架或其相关材料的生物安全性和应用可行性, 其中, 细胞相容性在这些评价系统中是一项非常重要的基本参数。本文总结了包括直接法和间接法在内的体外评价方法的应用和缺陷, 以及表征细胞毒性的方法, 如MTT和XTT等。我们也讨论了体外细胞毒性的一些影响因素, 在直接培养法中包括样品表面粗糙度、样品表面预处理、所采用的细胞类型等; 在间接法中有样品表面积和浸提溶液体积比值、培养基中的血清浓度、浸提液中的离子积累。此外, 我们也列出了目前关于镁基支架的体内动物实验和临床试验及其相关的结果。本综述的目的是希望能为将来镁基支架的研发和所涉及到的评价方法提供有意义的参考。