Evaluation of magnesium alloys with alternative surface finishing for the proliferation and chondro-differentiation of human mesenchymal stem cells

J. Trinidad¹, G. Arruebarrena¹, E. Sáenz de Argandoña¹, G. Ruiz de Eguino², A. Infante², C. I. Rodríguez²

¹ Mondragon University, Loramendi 4, Mondragón, Spain
² Stem Cell Laboratory, Research Unit, Hospital de Cruces, Plaza Cruces s/n, Barakaldo, Spain

E-mail: jtrinidad@eps.mondragon.edu

Abstract. Articular cartilage has little capacity for self-repair. As a result, continuous mechanical stress can lead to the degradation of articular cartilage, culminating in progressive damage and joint degeneration. Tissue engineering has arisen as a promising therapeutic approach to cartilage repair. Magnesium alloys are one of the most important metallic biomaterials emerging in this area due to their biocompatibility, bio-absorbability and especially to their mechanical properties. These properties make magnesium alloys a promising biomaterial in the regeneration of cartilage tissue. Objective. This study was undertaken to analyze the influence of surface characteristics of magnesium alloys in the adhesion, proliferation and differentiation of human mesenchymal stem cells (MSCs). Methods. Two commercial magnesium alloys (AZ31B and ZM21) were subjected to different treatments in order to obtain four different surfaces in each alloy. Human MSCs were seeded into the magnesium alloys and analyzed for their proliferation and chondrogenesis differentiation ability. Results. Human MSCs showed a greater proliferation and chondro-differentiation when cultured in the ZM21 magnesium alloy with a surface finishing of fine sanding, polishing, and etching.

1. Introduction
Damage to articular cartilage is of great clinical consequence because it has a poor regenerative capacity due to the lack of blood supply and the inability of native chondrocytes to proliferate and participate in regeneration at the injury site [1, 2].

Tissue engineering combines the principles of engineering and biology to design and fabricate constructs to promote and expedite the repair and regeneration of damaged tissue. The underlying principle of tissue engineering involves the utilization of biocompatible and mechanically suitable scaffolds, combined with an appropriate source of cells. These components, when combined, form a tissue-engineered construct which can function as the tissue replacement material, and, in principle, facilitate a faster rate of tissue repair [1–3].
Current focal cartilage lesion treatments are based on surgical procedures like abrasion arthroplasty, microfracture, mosaicplasty, osteochondral allografts and autologous chondrocyte implantation (ACI, MACI) [4]. These techniques are based on the generation of fibrous cartilage that covers the defect. This fibrocartilage is histologically different from articular cartilage, containing a higher proportion of type I collagen fibers with less glycosaminoglycans. Hence, the fibrous cartilage has less than optimal physical and biochemical characteristics compared to articular cartilage. Furthermore, the integration rate of the new extracellular matrix with the injured adjacent cartilage is low due to the anti-adhesive properties of the new matrix. Moreover, with these techniques long term clinical results indicate that degenerative joint diseases such as osteoarthritis may appear [4, 5].

Mesenchymal stem cells (MSCs) are a promising cell source for the engineering of cartilage tissue, due to their intrinsic capacity to differentiate to chondrocytes [6]. To improve the results in the healing of articular cartilage, as seen in Ohba’s review [7], research groups have generated articular cartilage through the in vitro differentiation of mesenchymal stem cells (MSCs) on scaffolds.

Successful cell-based cartilage tissue engineering depends not only on the selection of an appropriate biocompatible scaffolding material, but also on materials with suitable mechanical properties that result in a desirable clinical outcome when combined with cells.

Magnesium (Mg) and its alloys are new biomaterials which have been studied recently for tissue engineering [8–15]. The advantage of Mg compared to other biomaterials traditionally used in tissue engineering, such as polymers, injectable gels and ceramics, are its mechanical properties. Magnesium has a higher mechanical strength than polymers and gels, and greater fracture toughness than ceramics. In addition, Mg is an essential component in human metabolism [9]. It is the fourth most abundant cation in the body and the second most important after potassium in the intracellular environment [16]. The Mg of the human body appears mainly in the skeleton, being an essential component for bone growth and maturation [17, 18]. A Mg deficit may cause disease in the cartilage [19], and moreover Mg ions can increase the rate of proliferation of chondrocytes in in vitro culture [20]. Finally, taking into account its degradation in the human body, the cations generated due to Mg corrosion are efficiently regulated by the organism [14]. These characteristics make Mg a promising material for use in musculoskeletal tissue regeneration. However, the main problem of Mg is its high rate of degradation in body fluids [8–11, 15]. The rapid degradation of Mg could generate subcutaneous hydrogen bubbles that can damage surrounding tissues.

In this study, human MSC are induced to proliferate and to differentiate into chondrocytes on two commercial Mg alloys. The influence of material and surface finishing is evaluated based on the cellular proliferation potential observed and the quality of generated tissue.

2. Experimental procedure
2.1. Choice of the magnesium alloys
The magnesium alloys evaluated in this study are AZ31B (nominal 3%wt. Al and 1%wt. Zn) and ZM21 (nominal 2%wt. Zn and 1%wt. Mn). Aluminium is an alloying element that increases the corrosion resistance of magnesium. In fact, magnesium alloys combining with aluminium have been commonly used, especially aluminium-zinc series, in several studies [10–13, 15], despite Al being a toxic element [20]. AZ31B was chosen because it contains low percentage of Al (~ 3%), decreasing the toxicity caused by Al. The second alloy, ZM21, has been chosen due to the corrosion resistance that magnesium alloys with similar composition have shown in body
Table 1. Surface finishing treatment.

| Surface | Treatment |
|---------|-----------|
| Surf. 1 | 1000 grit SiC paper SiC |
| Surf. 2 | 1000 grit SiC paper + 4000 grit SiC paper |
| Surf. 3 | 1000 grit SiC paper + 4000 grit SiC paper + polishing (Al$_2$O$_3$ 0.05 µm) |
| Surf. 4 | 1000 grit SiC paper + 4000 grit SiC paper + polishing (Al$_2$O$_3$ 0.05 µm) + etching (HNO$_3$ 69.5 %) |

2.2. Development of magnesium samples

20x20x2 mm samples were prepared. They were processed to be divisible into 4 pieces of 10x10 mm for various analyses (cellular adhesion and proliferation, chondrogenesis and material degradation). In order to determine the influence of surface finishing on cellular adhesion and proliferation, four different surfaces were evaluated. These surfaces are described in Table 1.

After modifying the surfaces of the samples, they were subjected to a sterilization process with ethylene oxide to avoid possible alterations due to thermosensitivity of the material [22].

2.3. Mesenchymal stem cells

The cells chosen for culture on magnesium alloys are primary cultures of human mesenchymal stem cells (MSCs) obtained from bone marrow. The MSCs are a type of adult stem cell with a high rate of proliferation in vitro during the first passages. Furthermore, they are able to differentiate under appropriate culture conditions into mesodermal cell lineages (adipocytes, osteocytes and chondrocytes) [6]. In the present work the proliferation and chondrogenesis potential of the MSCs on magnesium alloys has been studied.

Human bone marrow-derived MSCs (BM-MSCs) were obtained from Inbiobank, a node of the National Cell Line Bank. These cells were harvested from cadaveric donors upon the informed consent of close relatives under the supervision of the Spanish National Transplant Organization (ONT). After expansion, MSCs displayed a typical CD29+, CD73+ (SH3 and SH4), CD105+ (SH2), CD166+, CD34-, CD45- and CD31- phenotype. In the presence of specific differentiation factors, and they were able to differentiate into adipocytes, chondrocytes and osteocytes (not shown).

2.4. MSCs proliferation

MSCs were spread at high density on Mg alloys and were maintained in culture for two weeks in a controlled atmosphere with 5% CO$_2$ at 37 °C. The culture medium for cell proliferation was enriched with proteins, amino acids and antibiotics to avoid possible contamination. The components are detailed in Table 2.

For the cell proliferation analysis on Mg alloys, the cells were stained with Hoechst. This is a fluorescent stain that labels double-stranded DNA concentrated in the cell nucleus, allowing
Table 2. Components of the proliferation medium and differentiation medium.

| Medium type      | Medium components                          |
|------------------|--------------------------------------------|
| Proliferation    | DMEM                                       |
|                  | L-Glutamine                                |
|                  | Penicillin/Streptomycin                    |
| Chondrogenesis   | Basal Medium                               |
|                  | Dexamethasone                              |
|                  | Sodium piruvate                            |
|                  | L-Glutamine                                |
|                  | Proline                                    |
|                  | Gentamicin/Amphotericin                    |
|                  | Ascorbic acid                              |
|                  | TGF-β                                      |

the identification of cells on metallic alloys using fluorescence microscopy. The presence of this stain does not affect cell viability.

2.5. Differentiation into chondrocytes: chondrogenesis

Once the cells proliferated on the alloys, chondrogenesis was induced. Cells were cultured in differentiation medium supplemented with recombinant growth factor TGF-β, described in Table 2. The cells were maintained for 6 weeks under these conditions, replacing the culture medium every 2 or 3 days.

3. Results

During the cell culture, the generation of gas bubbles was observed in Mg alloys (Figure 1). These bubbles were generated due to Mg corrosion in the culture medium. The Mg corrosion is described by the equation (1) [8]:

\[ Mg(s) + 2H_2O \rightarrow Mg(OH)_2 + H_2(g) \] (1)

According to this equation, the gas generated in the culture medium is hydrogen.

The generation of hydrogen was variable depending on the materials and surface finishing. The ZM21 alloy, generated less hydrogen, and had a greater corrosion resistance in physiological medium than the AZ31B alloy. Regarding the surface finishing, Surf. 1 presented less corrosion resistance in both materials. The corrosion resistance of Surf. 2, Surf. 3 and Surf. 4 did not differ significantly. In the same way, a pH increment of the culture medium was produced due to the generation of hydroxides (equation (1)).

Analysis of the corrosion process on the magnesium alloys indicated that, corrosion products were observed in the culture medium after several days of proliferation (Figure 2a). Despite this observation, the cells were able to adhere to the samples, as shown in Figure 2b.
After two weeks in proliferation medium, cell confluence was great enough for the formation of a monolayer of undifferentiated cells. This cell layer covered the sample partially with significant extensions of tissue. Figure 3 shows one of the best examples of adhesion and cell proliferation (ZAM21 Surf. 4).

Comparing the two alloys, cell proliferation was lower, or nonexistent, in AZ31B alloy compared to the ZM21 alloy, as shown in Figure 4.

Taking into account the different surface morphologies of the ZM21 alloy, a greater cell proliferation rate was observed on surfaces 2, 3 and 4 (Figure 5). Surfaces 2 and 4 showed a more uniform cell distribution. The polishing of Surf. 3 apparently resulted in compromised cell adhesion and proliferation.

After the chondrogenesis process, the composition of the secreted matrix by differentiated cells was analyzed. For that purpose the sulfated glycosaminoglycans of the matrix were quantified, because these components are the principal constituents of the extracellular matrix of cartilage. In this analysis sulfated glycosaminoglycans were found. Preliminary results indicated a greater potential for the ZM21 alloy compared to the AZ31B alloy in regenerative medicine requiring
Figure 3. Detail of cell monolayer on ZM21 Surf. 4. Optimal cell proliferation region observed by SEM.

Figure 4. Hoechst staining after cell proliferation: a) AZ31B Surf. 2 b) ZM21 Surf. 2

Figure 5. Hoechst staining after cell proliferation: a) ZM21 Surf. 3 b) ZM21 Surf. 4

the generation of cartilage.

4. Conclusions
The preliminary results of this study indicate that the ZM21 alloy has a lower corrosion rate than AZ31B in simulated physiological fluids. The ZM21 alloy also favors cell adhesion and subsequent cell proliferation of mesenchymal stem cells on it; while the AZ31B alloy has a lower cell adhesion and proliferation rate.
Surface finishing, with fine sanding (Surf. 2), polishing (Surf. 3) and etching (Surf. 4) gave a homogeneous distribution of cells on the alloys. However, the polishing (Surf. 3) makes adhesion and proliferation of mesenchymal stem cells more difficult.

Subsequent identification of sulfated glycosaminoglycans in the samples confirmed the chondrogenesis process on magnesium alloys.

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References
[1] Tae S K, Lee S H, Park J S and Im G I 2006 Biomedical Materials 1 63–71
[2] Chen G, Liu D, Tadokoro M, Hirochika R, Ohgushi H, Tanaka J and Tateishi T 2004 Biochemical and Biophysical Research Communications 322 50–55
[3] Hardingham T, Tew S and Murdoch A 2002 Arthritis Research 4 Suppl 3 S63–S68
[4] Magnussen R A, Dunn W R, Carey J L and Spindler K P 2008 Clinical Orthopaedics and Related Research 466 952–962
[5] Glass G G 2006 Dis Mon 52 343–362
[6] Dominici M, Le-Blanc K, Mueller I, Slaper-Cortenbach I, Mariniani F, Krause D, Deans R, Keating A, Prockop D and Horwitz E 2006 Cytotherapy 8 315–317
[7] Ohba S, Yano F and Chung U 2009 IBMS BoneKey 6 405–419
[8] Zeng R, Dietzel W, Witte F, Hort N and Blawert C 2008 Advanced Engineering Materials 10 B3–B14
[9] Staiger M P, Pietak A M, Huadmai J and Dias G 2006 Biomaterials 27 1728–1734
[10] Witte F, Ulrich H, Rudert M and Willbold E 2007 Journal of Biomedical Materials Research - Part A 81 748–756
[11] Witte F, Ulrich H, Palm C and Willbold E 2007 Journal of Biomedical Materials Research - Part A 81 757–765
[12] Krause A, von der Hoh N, Bormann D, Krause C, Bach F W, Windhagen H and Meyer-Lindenberg A 2010 Journal of Materials Science 45 624–632
[13] Gu X, Zheng Y, Cheng Y, Zhong S and Xi T 2009 Biomaterials 30 484–498
[14] Li Z, Gu X, Lou S and Zheng Y 2008 Biomaterials 29 1329–1344
[15] Pietak A, Mahoney P, Dias G J and Staiger M P 2008 Journal of Materials Science: Materials in Medicine 19 407–415
[16] Gums J G 2004 American Journal of Health-System Pharmacy 61 1569–1576
[17] Aranda P, Planells E and Llopol S 2000 Ars Pharmaceutica 41 91–100
[18] Iannello S and Belfiore F 2001 Panminerva Med, 43 177–209
[19] Stahhmann R, Frster C, Shakibaei M, Vormann, Gutner T and Merker H J 1995 Antimicrobial Agents and Chemotherapy 39 2013–2018
[20] El-Rahman S S A 2003 Pharm Res 47 189–194
[21] Song G 2007 Corrosion Science 49 1696–1701
[22] Silvestre C, Fagoaga L, Garcia-Ma J, Lanzeta I, Mateo M C and Zapata M C 2000 ANALES Sis San Navarra 23 (Suppl. 2) 95–103