

# An *in vitro* study on the biocompatibility of WE magnesium alloys

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**Abstract:** Magnesium alloys are being actively studied for intravascular stent applications because of their good mechanical strength and biocompatibility. To rule out the high allergenicity of nickel and neurotoxicity of aluminum element, four kinds of WE magnesium alloys (where “W” represents the metallic element Y and “E” represents mixed rare earth (RE) elements; Y: 2.5, 5.0, 6.5, and 7.5 wt %; Nd: 1.0, 2.6, 2.5, and 4.2 wt %; Zr: 0.8 wt %) were chosen for *in vitro* investigation of their biocompatibility using cell culture. The results showed that, with the increase of rare earth elements in WE magnesium alloys, fibrinogen adsorption decreased and coagulation function was improved. It was also found that WE magnesium alloys promoted the adhesion of endothelial cells. With the increase of adhesion time,

adhered cell numbers increased gradually. With 25% extracts, all the WE alloys promoted cell migration, while 100% extracts were not conducive to cell migration. Based on the above results, WE magnesium alloys 5.0WE (5.0Y-2.6Nd-0.8Zr) and 6.5WE (6.5Y-2.5Nd-0.8Zr) have better biocompatibility as compared with that with 2.5WE (2.5Y-1.0Nd-0.8Zr) and 7.5WE (7.5Y-4.2Nd-0.8Zr), and could be as the promising candidate materials for medical stent applications. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2015.

**Key Words:** magnesium alloys, rare earth element, biocompatibility, coagulation function, cytocompatibility

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## INTRODUCTION

Materials used for intravascular stent applications can be mainly classified as non-degradable and degradable materials. The non-degradable stent materials include common stainless steel, cobalt, nickel titanium alloys. Although they have certain curative effect in clinic, non-degradable material stents are implanted in human body permanently and could cause a series of adverse reactions, such as persistent physical stimulation, local chronic inflammation, and endothelial dysfunction.<sup>1–3</sup> To overcome these problems, biodegradable stents materials have been actively investigated in recent years.<sup>4–7</sup> Common biodegradable stent materials mainly include biodegradable polymer materials, pure iron and magnesium alloys. Although polymer materials have many advantages over metallic stent materials,<sup>8,9</sup> the same radial force with blood vessels is not guaranteed in the stent implantation, which restricts its retraction rebound. For this type of stent implantation, in addition, the balloon cannot expand fully and heat is needed, which could bring

unpredictable damage to patients. Because the density of polymer materials is small and cannot be detected by X-ray imaging technique, the polymer stent implantation is difficult to position.<sup>10–12</sup> As for iron stent materials, they can overcome these problems encountered with polymer stents. However the degradation rate is too slow in the body. As a result, the vascular repair and the degradation of stent cannot achieve the synchronization.<sup>13,14</sup>

Therefore, the development of new biodegradable stent materials is very necessary. Because they are cost-effective and degradable, magnesium alloys with good mechanical compatibility and biocompatibility have become a research hotspot for stent applications.<sup>15–19</sup> The research results reported by Mario et al.<sup>20</sup> show that WE43 magnesium alloy stents inhibited smooth muscle cell proliferation, accelerated endothelialization speed. Waksman et al.<sup>21</sup> studied the safety of magnesium alloy stent and their results showed that the scaffolds had certain influence on the formation of porcine artery intima. When the magnesium alloy materials

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can be safely used in clinical medicine, their mechanical properties should be matched the requirements, along with excellent biological effectiveness and safety. Whether a material can be used as biomedical material, its biocompatibility is also the key property for its success.<sup>22</sup>

The moderate intake of rare earth elements help to improve the immunity of the organism because the low toxicity of rare earth elements is similar with iron and lower than some organic synthetic drugs or transition metal compounds.<sup>23</sup> In addition, the rare earth elements can prevent arteriosclerosis and also be used for anti coagulation, inflammatory.<sup>24</sup> When the rare earth elements are added to magnesium alloys, they will produce the strengthening effect, reduce weld cracking and improve the compactness of castings.<sup>25</sup> The mixed rare earth elements can also improve corrosion resistance of magnesium alloys in chloride environment, which is mainly due to the formation of mixed rare earth oxide film on the surface of magnesium alloys.<sup>26,27</sup> Thus in this study, the researchers have ruled out the high allergenicity of nickel and neurotoxicity of aluminum element, and chosen Y, Nd, and Zr as alloying elements for WE magnesium alloys with a variety of biological functions. The biocompatibilities of these WE magnesium alloys were investigated using *in vitro* cell culture. Such experimental research will provide a preliminary evaluation of WE magnesium alloys as stent materials, and provide the basis and reference for their future clinical applications.

## MATERIALS AND METHODS

### Materials

Activated partial thromboplastin time (APTT) and Prothrombin time (PT) assay kits were purchased from Jiancheng Bioengineering, Nanjing, China. Fibrinogen antibody, horseradish peroxidase (HRP) labeled monoclonal goat anti rabbit immunoglobulin, 3,3',5,5'-tetra methyl benzidine (TMB) were purchased from Beijing Biosynthesis Biotechnology, Beijing, China. Monoclonal Rabbit anti human P-selection and dimethyl sulfoxide (DMSO) were purchased from BOSTER, Wuhan, China. Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were obtained from Hyclone Company, China. Magnesium alloys used in this study were 2.5WE (Mg-2.5Y-1.0Nd-0.8Zr); 5.0WE (Mg-5.0Y-2.6Nd-0.8Zr); 6.5WE (Mg-6.5Y-2.5Nd-0.8Zr); 7.5WE (Mg-7.5Y-4.2Nd-0.8Zr).

### Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in RPMI1640 medium supplemented with 10% FBS in an incubator containing 5% CO<sub>2</sub> at 37 °C.

### Preparation of materials

The samples with a dimension of 8 mm in diameter and 2 mm in thickness were cut from extruded bars for the hemocompatibility test. Each sample was polished by 2000# grit sandpaper, ultrasonically cleaned in absolute ethanol for 10 min, and dried at room temperature. According to the methods in literature,<sup>14</sup> sample extracts were prepared using RPMI 1640 serum medium as the extraction

medium, with the quality of extraction medium ratio being 1.25 cm<sup>2</sup> mL<sup>-1</sup> in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C for 72 h. The extraction medium was diluted to 25 and 50% solution with RPMI1640 medium supplemented with 10% FBS and refrigerated at 4 °C.

### *In vitro* blood compatibility evaluation

Blood was obtained from normal volunteers in accordance with the protocol approved by the ethical committee of the College of Bioengineering of Chongqing University. The blood was treated with 3.8% solution of sodium citrate at a ratio of 9:1, which was used as an anticoagulant. To get the fresh human platelet-rich plasma (PRP) and platelet-poor plasma (PPP), the blood was centrifuged at 1200 rpm for 10 min and at 3000 rpm for 15 min using the Eppendorf 5417R refrigerated micro centrifuge (Eppendorf, Shanghai, China).

The samples were incubated with 500 µL PPP in culture plate for 2 h at 37 °C, and cleaned with a solution of lamb serum PBS closed liquid for three times. The samples were then transferred to new culture plates. The samples surface was covered by sheep serum PBS with goat anti human fibrinogen antibody. Then they were incubated for 1 h at 37 °C, washed with PBS for three times. The surface of the samples was first drained and then the samples were put into the new culture plates, adding chromogenic reagent TMB, reacted for 5–10 min. The color reaction was terminated with 1 mol L<sup>-1</sup> sulfuric acid. The absorbance was examined and recorded by an enzyme micro-plate reader (Bio-Tek ELX800, USA) at a wavelength of 405 nm.

When the thromboplastin of calcium ion tissue was put into plasma, the clotting mechanism started, making the prothrombin changed into thrombin, fibrinogen changed into fibrin. Then plasma solidification occurred for a certain period of time. If the exogenous coagulation process showed factor deficiency, the clotting time extended. The degree of the extended clotting time was proportional to single factor or all the lack degree of relevant factors. The 50 µL of testing PPP was incubated at 37 °C for 3 min. Then 100 µL of preheating prothrombin reagent was added, and mixed immediately. The plasma clotting time was recorded by a coagulometer (CA-50, Sysmex, Japan).

The APTT reagent was added into the plasma, blended in water bath with a constant temperature of 37 °C. With the effect of Ca<sup>2+</sup>, the intrinsic coagulation system was activated; fibrinogen was changed into insoluble fibrin. The solidification time can be detected. Such time was called APTT. The samples were covered by 200 µL PPP in a 24-well plate for 15 min at 37 °C. Then 50 µL PPP and 50 µL APTT kits were added and incubated for 3 min at 37 °C. The 50 µL warm-up solution of CaCl<sub>2</sub> was added, and mixed immediately. The plasma coagulation time was recorded by a coagulometer (CA-50, Sysmex, Japan).<sup>28</sup>

### Fluorescence staining

Human umbilical vein endothelial cells HUVECs were placed into six-well plates (1 × 10<sup>5</sup> cells/well) for 24 h. Then the

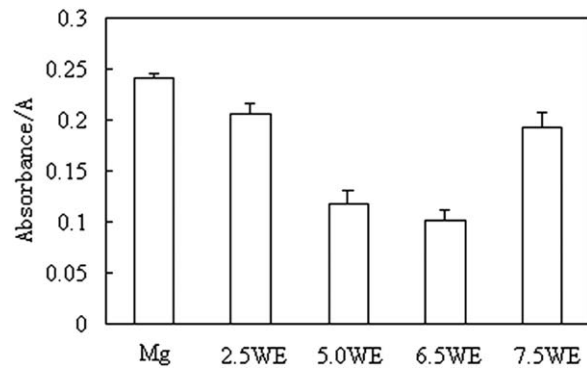


**FIGURE 1.** The fibrinogen adsorption on the surfaces of the magnesium alloy samples.

original medium was removed. About 1 mL of different concentrations of extracts were co-cultured for 24 h. The nuclear morphology was studied by fluorescence staining. After three times PBS washing, the samples were fixed with 4% paraformaldehyde at 4 °C, and then washed with PBS for three times. After 15 min of blocking of non-specific binding with PBS containing 1% bovine serum albumin, the samples were washed with PBS solution one time for 5 min. Then the co-cultured cells were treated with 0.5 mL of Hoechst 33258 at room temperature for 5 min. Controls were obtained by omitting the nuclear counterstaining. The samples were washed with PBS three times, each time for 3 min, to clear the redundant fluorescent dye. All samples were evaluated by a fluorescence microscope (Leica DMI 4000B, Germany).<sup>29</sup>

#### Cell adhesion

HUVECs were placed into 24-well plates ( $1 \times 10^6$  cells/well) for 24 h. Then the original medium was removed. 0.5 mL different concentrations of extracts was added into the 24-well culture plate and incubated for 24 h. The normal cultured cells and co-cultured cells with extracts were collected. The cell concentration was adjusted to  $1 \times 10^5$  cells/well in a 24-well plate. Then cleaning with PBS for three times after 30 min, 1 h, 2 h, and 3 h, non adherent cells were thoroughly washed. The cells were examined with inverted microscope in five randomly selected areas and counted.

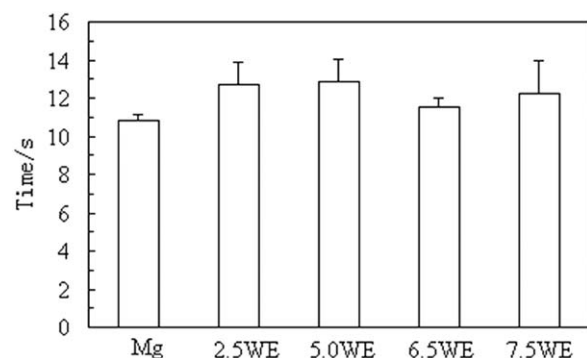


**FIGURE 2.** The PT of the magnesium alloys *in vitro*.

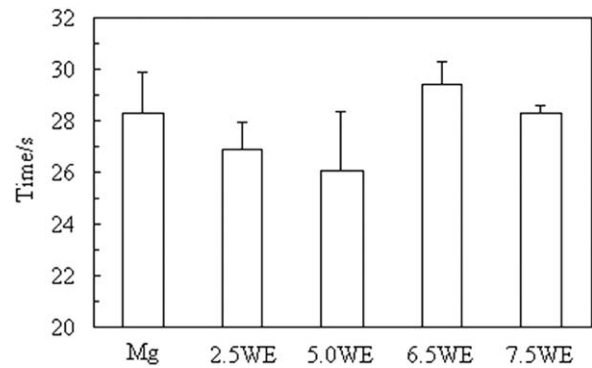


**FIGURE 3.** The APPT of the magnesium alloys *in vitro*.

#### Cell migration

HUVECs were placed into 24-well plates ( $1 \times 10^6$  cells/well) for 24 h. Then the original medium was removed. Nearly 0.5 mL different concentrations of extracts was added into the 24-well culture plate and incubated for 24 h. When the cell fusion degree reached 90%, the HUVECs would synchronize for 24 h with 1640 lacking serum. When the cells grew into monolayer, several vertical and parallel lines of about 0.7 mm were drawn on the sterile pipette tip in the same position, then washed two times using sterile PBS, and incubated normally by 1640 containing 10% fetal bovine serum. The cell migration was observed after 0 h, 12 h. The samples were compared by the migration area ratio. The migration area ratio of the samples was calculated using the following formula:

$$\text{Migration area ratio} = (1 - A_t/A_0) \times 100\% \quad (1)$$

Where  $A_0$  is the starting scratch area,  $A_t$  refers to the migration of the scratch area.

#### Statistical analysis

Statistical analysis was performed using Statistical Program for Social Sciences (SPSS, version 11.0). All data were expressed as mean  $\pm$  SD. Comparisons among group means were determined by one-way analysis of variance (ANOVA). Multiple comparisons between two means were conducted

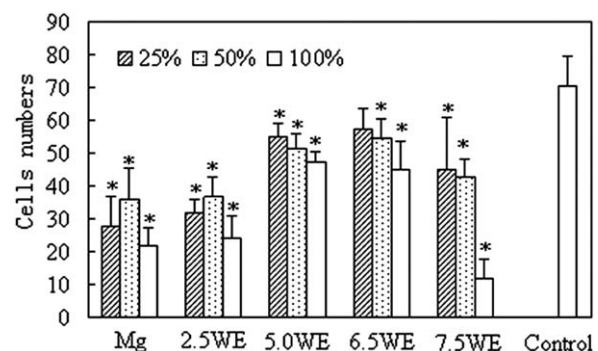


**FIGURE 4.** The HUVECs numbers after incubating magnesium alloys for 24 h, \*, significant difference ( $p < 0.05$ ).

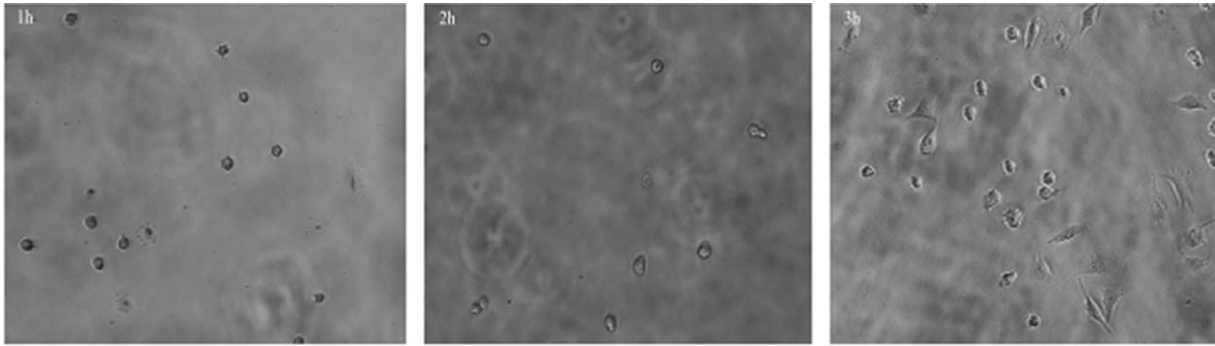


FIGURE 5. Typical optical microscopy images of adhesion of HUVECs at 1 h, 2 h, 3 h (×200).

by Duncan's multiple range method.  $p < 0.05$  was considered statistically significant.

## RESULTS

### *In vitro* blood compatibility evaluation

Fibrinogen is known as coagulation factor I. It is a protein which was synthesized by the liver. It is the most abundant plasma coagulation factors in blood and plays an important role in the blood coagulation process. The WE magnesium alloys used in this study were 2.5WE (Mg-2.5Y-1.0Nd-0.8Zr); 5.0WE (Mg-5.0Y-2.6Nd-0.8Zr); 6.5WE (Mg-6.5Y-2.5Nd-0.8Zr); and 7.5WE (Mg-7.5Y-4.2Nd-0.8Zr). Figure 1 showed the fibrinogen adsorption data on pure magnesium and WE magnesium alloys. It can be seen that, with the addition of rare earth elements, adsorption of fibrinogen decreased and coagulation function was improved.

The results of APTT and PT assays are shown in Figures 2 and 3. The results showed that addition of the rare earth elements in magnesium increased the APTT and PT with longer clotting time.

### Fluorescence staining

HUVECs fluorescence staining results showed that no blue fluorescence images of broken fragments were found after co-cultured for 24 h. For pure magnesium and all the WE alloys, the 100% concentration of extracts gave lower number of blue fluorescent points than the 25 and 50% concentrations of the extracts. To facilitate cell count, five random scopes were selected and counted under the microscope. The results were shown in Figure 4. With the addition of

rare earth elements, the number of adherent cells increased with addition increased and started decreasing for 7.5WE.

### Cell adhesion and migration

Because magnesium alloys degraded easily, the cell adhesion and growth on the surface were not easily observed directly. In this study, materials extracts were used to study the impact of the WE alloys on the adhesion of endothelial cells. As seen from the optical images in Figure 5, the HUVEC cells began adhesion at 1 h, and then showed spreading trend at 2 h, and some cells spreaded out completely at 3 h. Figure 6 showed the adhesion numbers of HUVECs on pure magnesium and WE magnesium alloys at different culture time. For pure magnesium, cell adhesion number remained unchanged at the three time points, while the magnesium alloy material promoted cell adhesion. With the increase of adhesion time, the number of cells adhesion increased. The 2.5WE, 5.0WE, and 6.5WE promoted cell adhesion more in comparison to 7.5WE. The cell migration results were shown in Figures 7 and 8. After 12 h, cell migration was obvious. The 25% concentrations of the extracts of all the materials had a tendency to promote cell migration. The 50% concentrations extracts of 2.5WE, 5.0WE, and 7.5WE had no significant migration effect. 100% concentrations extract of all materials were not conducive to cell migration.

## DISCUSSION

### Analysis of blood compatibility

Under normal circumstances, the coagulation process can be divided into three stages. The first stage is the activation of

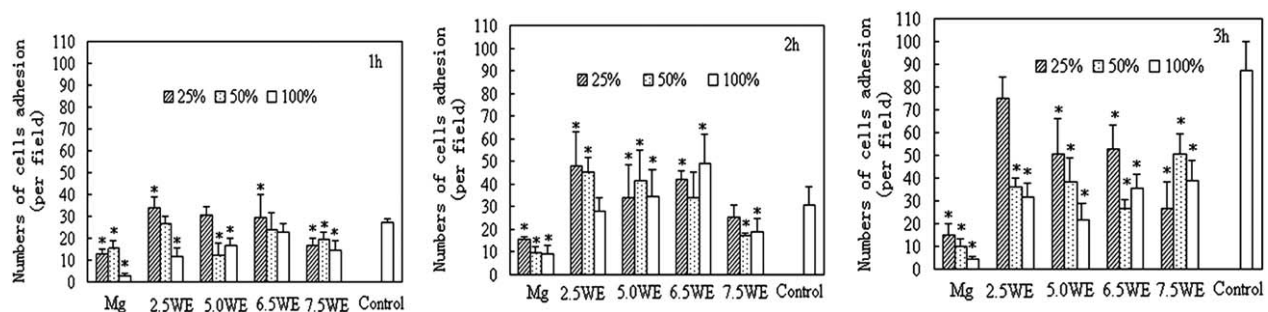


FIGURE 6. The adhesion numbers of HUVECs on pure magnesium and WE magnesium alloys at 1 h, 2 h, 3 h, \*, significant difference ( $p < 0.05$ ).

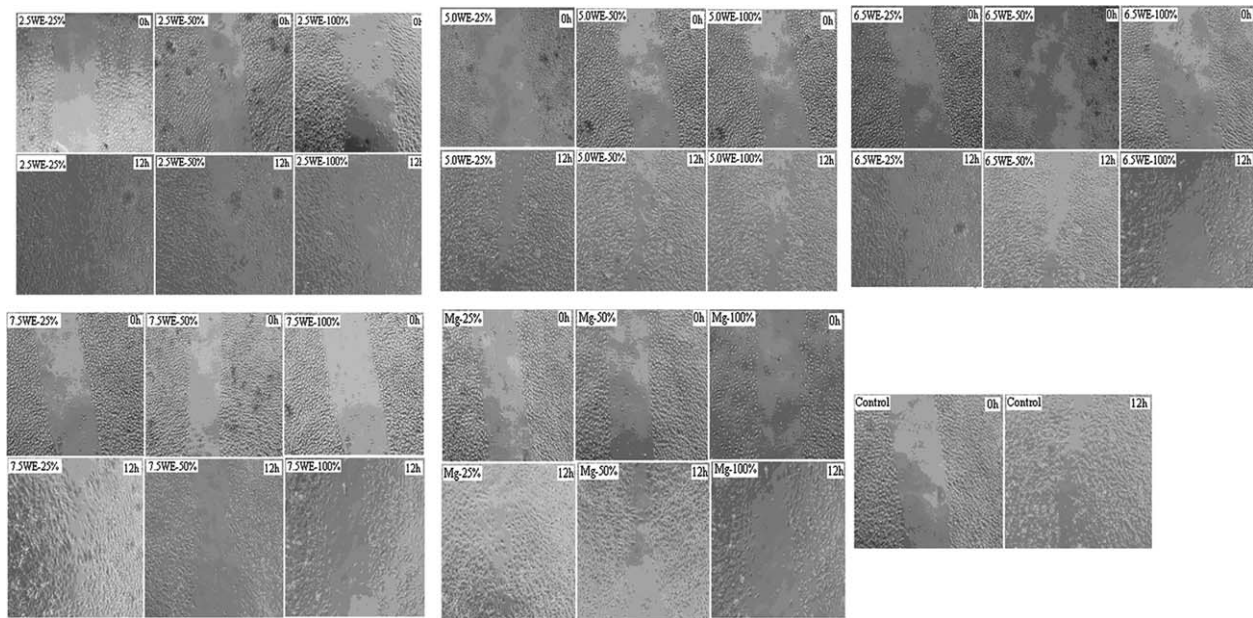


**FIGURE 7.** The micrographs of HUVECs transmigration at 0 h, 12 h ( $\times 100$ ).

the first coagulation factors to the formation of prothrombinase. The second stage is the formation of thrombin and the third stage is the fibrin formation. In addition, the PT and APTT test results can be screened for various clotting factor deficiencies and related inhibitors. Experimental results showed that with the increase of rare earth elements, PT increased compared with the control group. When the rare earth elements were added to a certain number, the APTT also showed an increasing tendency. Furthermore, fibrinogen is the most abundant plasma coagulation factors in blood and plays a very important role in the blood coagulation process. In the final stage of the coagulation process, as a precursor fibrinogen, soluble fibrinogen is further transformed into insoluble fibrin, resulting in blood coagulation.<sup>30,31</sup>

In this study, it was found that the amount of adsorbed fibrinogen decreased with the increase of the rare earth element, coagulation function also improved. The reasons may be that the magnesium alloy material had less binding for

fibrinogen, suppressed the common pathway of the blood coagulation process, and did not lead to the activation of the coagulation system. Therefore magnesium alloy material had anticoagulation effects.

#### Cytocompatibility analysis

Cytocompatibility of magnesium alloy materials has a crucial role to determine whether they can be used as vascular scaffold materials. Affinity between materials and cells mainly refers to the ability of cell adhesion and growth on the material surface. Generally, protein mediated adhesion mechanism and the affinity between materials and cells emerges. The cell adhesion difference will further affect cell proliferation, differentiation and other cell growth function.<sup>32,33</sup> This *in vitro* cell study demonstrated that WE magnesium alloys promoted endothelial cells adhesion. For 25% concentration extracts, all the WE magnesium alloys enhanced cell migration, while for 100% concentration extracts the WE magnesium alloys were not conducive to cell migration. The intrinsic mechanism of this phenomenon still needs to be studied and elucidated. Too much or too little alloying elements in the WE alloys was found to be not conducive to the growth of cells.<sup>34</sup> It was not a simple process to select one or several magnesium alloys with excellent overall performance, which required systematic and in-depth investigation to achieve mechanistic understandings.

#### CONCLUSION

The results of this study indicated that the WE magnesium alloy materials had no uniform rule between the added alloying elements and their *in vitro* biocompatibility. The adsorption content of fibrinogen decreased while the coagulation function gets improved with the increase of the rare earth elements in the WE magnesium alloys. In general,

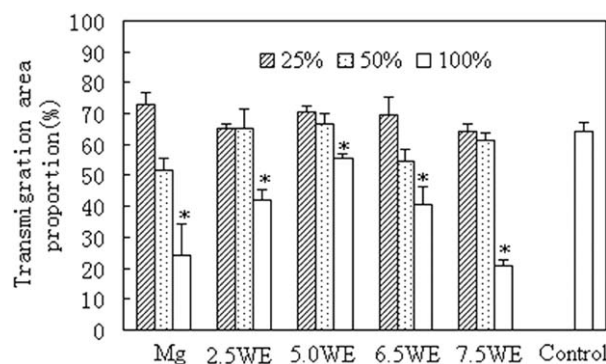


**FIGURE 8.** The transmigration area proportion of HUVECs. \*: significant difference ( $p < 0.05$ ).

addition of rare earth elements in magnesium alloys promoted endothelial cells adhesions and migration. The *in vitro* results obtained in this study evidently indicated that, as compared with 2.5WE and 7.5WE, 5.0WE and 6.5WE magnesium alloys have better biocompatibility and could be promising candidate materials for medical stent applications.

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