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Effects of magnesium on growth and proliferation of human embryonic stem cells

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Abstract— **The effects of magnesium on the growth and proliferation of human embryonic stem cells (hESCs) was explored to advance magnesium as an implant biomaterial. When magnesium ions from magnesium salt were added to the culture media at 10, 100, 250, 500, 750, and 1000 ppm (0.4, 4, 10, 20, 30, 40 mM) the rate of increase in viable cell coverage over time was higher for the larger doses of magnesium salt. Thus, the addition of magnesium ions exerted a positive effect on viable cell coverage. When hESCs were cultured with pure magnesium metal strips through transwell inserts, the cells underwent an initial increase in viable cell coverage, followed by rapid cell death within the first 24 hours. This initial increase in viable cell coverage corresponded to the colonies dispersing and losing their tightly packed morphologies. The cell death may be attributed to an increased alkalinity in the culture media incubated with the magnesium metal strips. In conclusion, since the degradation of magnesium results in both magnesium ions and OH- ions (an increase of pH), controlling the degradation of magnesium to obtain the perfect balance of ions is critical for advancing magnesium as an implant biomaterial.**

I. Introduction

Annually, over 200,000 peripheral nerve injuries (PNIs) occur due to accident trauma or surgical procedures in the United States [1]. Due to the limitations of current clinical practices for PNIs, there is ongoing research for the development of artificial nerve guidance conduits (NGCs) to guide axonal re-growth and to facilitate nerve regeneration. A wide range of different biomaterials have been explored for this purpose, in conjunction with the incorporation of various stem cell therapies for enhanced regeneration. This proceeding paper presents magnesium as a possible conduit material. Magnesium is promising for neural applications due to its biodegradable, mechanical, and conductive properties as well as its positive role in neuroprotective and repair mechanisms. In order to advance magnesium as an implant biomaterial, we explored the effects of magnesium (magnesium salt at different concentrations and magnesium metal strips) on the growth and proliferation of hESCs.

II. Materials and Methods

A. Magnesium Preparation and Characterization

Pure, polished magnesium metal sheets (P-Mg, 99.9% purity, Good Fellow) were cut into 5 x 5 mm substrates, characterized, individually weighed, and sterilized before cell studies. To disinfect, the P-Mg surface was wiped with ethyl alcohol (Koptec) and exposed to UV light in a class II biosafety cabinet for 12 hours on each side. A 100,000 ppm sterile stock solution of Mg^{2+} ions was prepared by dissolving MgCl•6H20 (Sigma-Aldrich) into DI water and filtering through a 0.20 micron filter (Sartorius Stedim).

The surfaces of the P-Mg were characterized using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS). SEM images and EDS data were obtained using the SEM FEI XL30 attached with an EDAX detector. SEM images were taken at an accelerating voltage of 15kV. EDS analysis was performed at a magnification of 2000X so that a substantial portion of the sample surface would be analyzed to show the average elemental distribution.

B. Stem Cell Culture and Characterization

The H9 hESCs (WiCell) were stably transfected with an octamer-binding transcription factor-green fluorescent protein (OCT4-GFP) reporter plasmid as previously described [2]. H9-OCT4 ESCs were maintained in a T-25 flask (Falcon) under feeder-free conditions using Geltrex™ matrix (Invitrogen) and 1 mL of mTeSR®1 media (STEMCELL Technologies). Upon observation under a light microscope and verification of about 80-90% confluency, H9-OCT4 hESCs were passaged at a split ratio of 1:5 using Accutase (Innovative Cell Technologies) and glass beads.

Morphology and GFP fluorescence of H9-OCT4 hESCs were observed using the microscope Nikon Eclipse Ti. Phase contrast images were used to determine morphology, while fluorescent images were used as a marker for undifferentiated stem cells. Phase contrast images and fluorescent images were merged using NIS-Elements Imaging Software.

C. Culturing with Magnesium

A 12-well plate (BD Falcon) for magnesium pre-degradation was prepared by covering 3 wells with cold Geltrex™ matrix (Invitrogen) in DMEM media (Invitrogen) (1:50) for 24 hours. The excess Geltrex™ solution was removed and H9-OCT4 cells (passage 15) were seeded onto the wells with 1 mL of mTeSR®1 media and maintained for 24 hours under standard cell culture conditions (37°C, 5% CO_2). After the initial 24 hours of incubation, the mTeSR®1 media was removed and replenished, and 3 P-Mg samples were introduced through transwell inserts (Corning), which were positioned within the wells where H9-OCT4 hESCs were cultured. After 24 hours of culture, the magnesium

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specimens were collected, weighed, and set aside as pre-degraded magnesium (D-Mg).

To test growth and proliferation rates of H9-OCT4 cells with the addition of Mg^{2+} ions at 1000, 750, 500, 250, 100, and 10 ppm, another two 12-well plates were similarly prepared and seeded (passage 9+5, the "+" indicates freezing and thawing of the culture) for 24 hours. The Nikon Biostation CT was used to monitor growth when using mTeSR®1 media with additional Mg^{2+} ions (from 100,000 ppm sterile stock solution). The culture media was collected and replenished every 24 hours (with MTeSR®1+Mg^{2+} ions), and stored at 4°C.

To test the cytocompatibility of the P-Mg and the D-Mg samples, another 12-well plate was similarly prepared and seeded (passage 16) for 24 hours. P-Mg and D-Mg samples were then introduced through transwell inserts and moved to the Nikon Biostation CT. The Nikon BioStation CT incubated the cells under standard cell culture conditions, while taking phase contrast and fluorescent time lapse images every six hours for 72 hours at two random points. The culture media was collected every 24 hours for pH measurements and replenished with fresh media.

D. Proliferation Analysis

Phase contrast and fluorescent images collected from the Biostation CT were used to quantify the percentage of area covered by viable H9-OCT4 colonies. Since the Biostation CT captured images at two different points on each well, and each magnesium condition was tested in triplicates, a total of 6 points were analyzed and averaged for each condition. The area of viable cells expressing the undifferentiated hESC marker OCT4 were outlined manually using ImageJ software. The percentage of area covered by viable cells was quantified using the outlined area divided by the total image area. The standard error of the mean was calculated and used to generate error bars. The numerical data sets were analyzed using standard analysis of variance (ANOVA) followed by standard post hoc tests with the Holm-Bonferroni correction.

III. Results and Discussion

A. P-Mg Surface Characterization

Surface conditions have been shown to play an important role in Mg degradation. Previous studies have shown significant differences in morphology, topology, and composition when comparing polished and unpolished pure magnesium before and after mesenchymal stem cell (MSC) culture [3]. In another study describing surface interactions of polished and oxidized magnesium alloys with MSCs, the polished surfaces had superior cell adhesion due to its slower degradation (slower rate of mass loss and lower overall pH increase) [4]. AFM results showed that the oxidized alloy had a significantly rougher surface than the polished alloy, with a mean roughness of 196 nm compared to 65 nm [4]. Using polished magnesium samples ensures the removal of the oxidized surfaces of as manufactured magnesium. Fig. 1A is a SEM image of P-Mg showing a smooth surface, and Fig. 1B is the corresponding EDS analysis showing 94.2 wt% Mg, and 5.8 wt% of oxygen present on the surface.

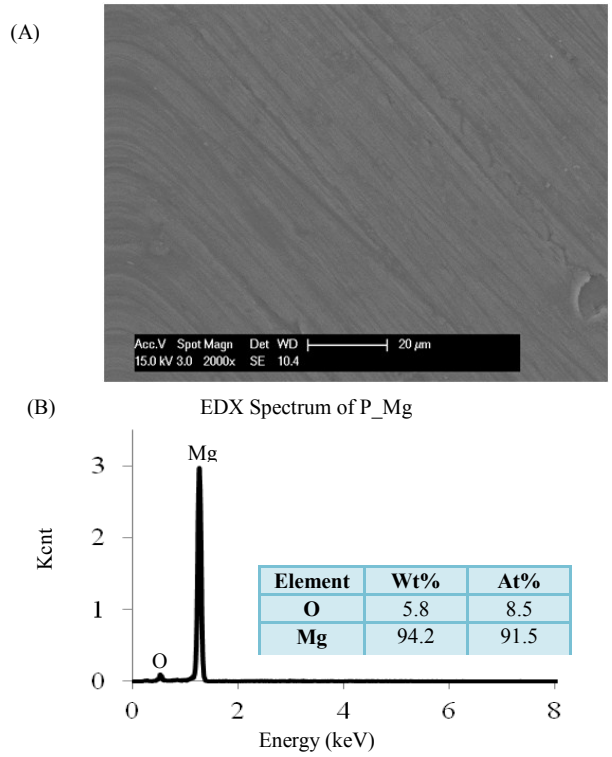

Fig 1: (A) SEM image of the polished Mg at 15 kV and 2000x magnification (20µm scale bar). (B) EDS data analysis at 2000x magnification.

B. *Initial Stem Cell Culture Characterization*

Undifferentiated H9-OCT4 hESCs have prominent nucleoli and high nucleus to cytoplasm ratio; they also grow in tightly packed colonies with defined external boundaries [5]. Initial observation of H9-OCT4 cells through a brightfield microscope revealed these characteristics, indicating continuous undifferentiated proliferation (Fig. 2). The GFP driven by the OCT4 promoter serves as a fluorescent marker for undifferentiated cells since OCT4 is highly expressed in undifferentiated pluripotent cells (capable of differentiating into different cell types) and its expression is down-regulated upon differentiation [6]. Thus, fluorescent images show that starting H9-OCT4 hESC cultures were undifferentiated and remained pluripotent.

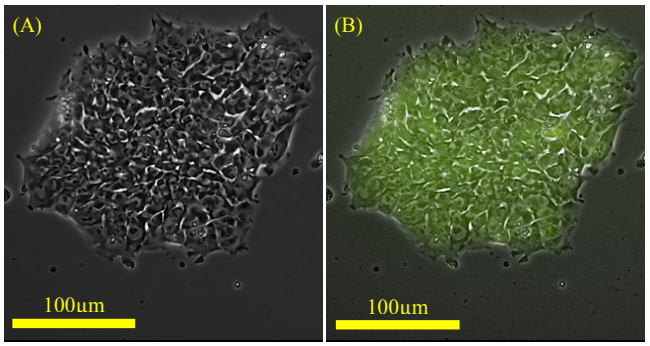

Fig. 2: Optical images of H9-OCT4 ESCs cultured with magnesium specimens over 72 hrs.

C. Culturing with Magnesium

A montage of the images captured by the Biostation CT reveals the progression of cell death following the direct exposure of the degradation products of the Mg specimen (Figure 3). At hour 6, the morphology of Mg-treated H9-OCT4 hESCs were comparable to the control with numerous round, healthy and tightly packed colonies. However, by hour 12, those colonies began to disperse and lost their typical tightly-packed morphology. Subsequently, the cells became elongated and we observed the presence of a few round single cells by hour 18. By hour 24, a few elongated cells remained and the cultures were composed of mostly round single cells. The endpoint of the experiment (hour 72) shows the presence of predominantly dead cells, as revealed by the loss of colonies and the presence of cell debris.

Biostation CT images of the stem cells cultured with additional Mg^{2+} ions showed dispersed morphologies by hour 6 (data not shown). The stem cells seemed to maintain their dispersed colonies until confluency. Further investigation will be done in future studies to explore this observation.

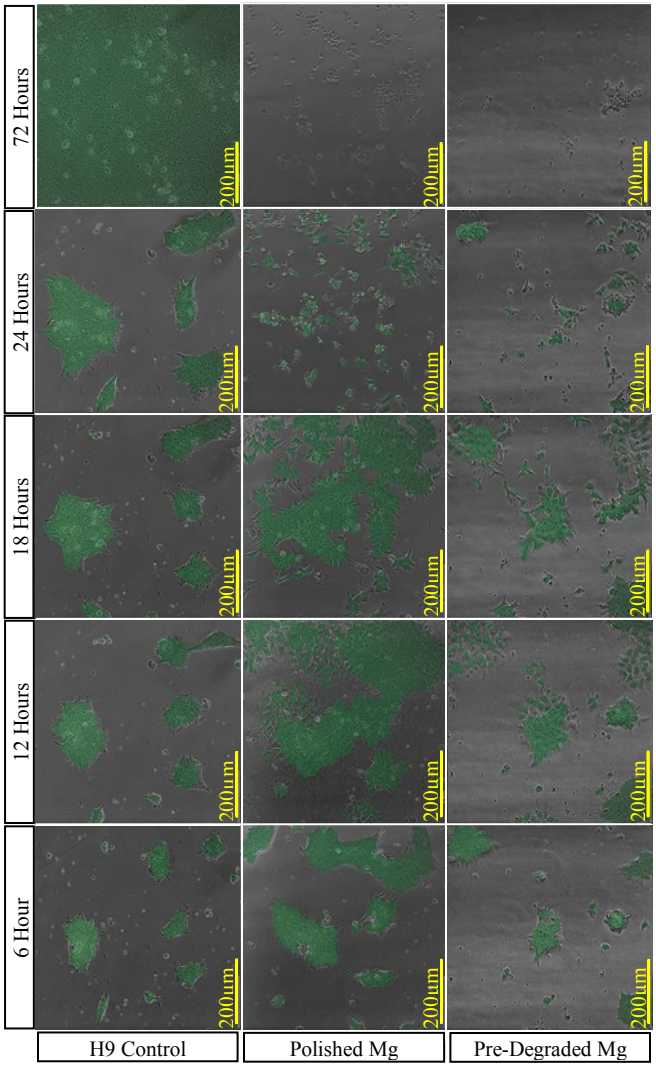

Fig. 3: Time-lapse images of H9-OCT4 ESCs cultured with magnesium specimens over 72 hrs. Control cells are H9-OCT4 hESCs cultured under standard culture condition without the presence of Mg specimen or additives.

D. Post Culture Media Analysis

When 10-1000 ppm Mg^{2+} was added to the culture media, the post culture collections maintained a pH comparable to the control. This acidity is a result of the regular metabolic processes of the cells. When the media was co-cultured with the Mg specimens, the pH of both P-Mg and D-Mg increased. This increase in alkalinity was expected from the degradation of the Mg specimen when considering the reaction cascade [3, 4]. The average pH measurements indicated that the P-Mg had a slightly higher pH than the D-Mg (Fig. 4). Since the P-Mg surface has been shown to be less oxidized, a greater surface of the Mg can react with water to begin the reaction cascade towards increased OH- ions (increased alkalinity).

E. Proliferation and Cell Viability Analysis

The percentage of viable cell coverage was used to analyze proliferation and cell viability over time. In Fig. 5a, the control showed a gradual increase in percentage of viable cell coverage over time, correlating to normal cell proliferation. Cells that were cultured with Mg specimen showed an initial increase in percentage of viable cell coverage (hour 12) followed by a decrease towards death or non-viability (hour 30). These results correlate with the Biostation CT images. Since the stem cells lost their tightly packed morphology at approximately hour 12, an increase in the percentage of viable cell coverage was observed. From that point on, the declining percentage of viable cell coverage is an indicator of cell death. Since it is impossible to have the same initial growth conditions, the percentage of viable cell coverage was normalized according to its first recorded value at hour 6 (Fig. 5B). In this figure, it is more apparent that the P-Mg and the D-Mg has similar growth within the range of deviation. For both the P-Mg and D-Mg cultures, the percentage of area coverage doubled from hour 6 to hour 12, followed by a gradual decline towards 0% coverage. Hence, we conclude that proliferation is not affected by pre-degraded nature of magnesium samples.

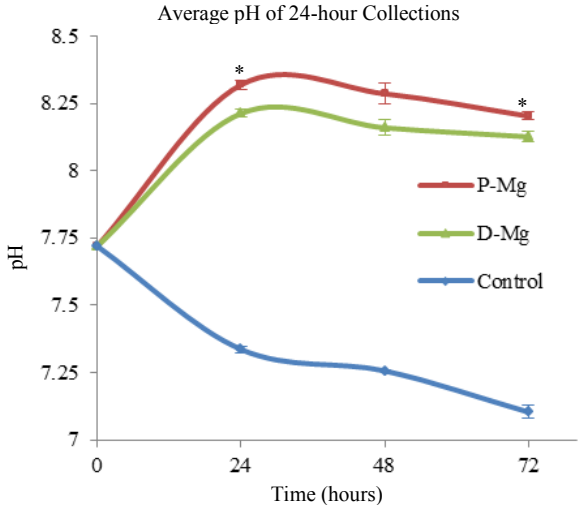

Fig. 4: Average pH measurements of 3 sets of 24 hour culture media with P-Mg and D-Mg. (* $p<0.05$ compared to D-Mg). Values are mean ± standard error of the mean; n = 3.

In Fig. 5C, the control, 10 ppm and 100 ppm (0.4 mM and 4 mM) magnesium doses appear to have similar normalized viable cell coverage, with no statistically significant difference between the groups (p>0.05).The cultures with more than 250 ppm (10 mM) magnesium dosages were higher than the control, with a statistically significant difference between the groups (p<0.05). In conclusion, additional magnesium ions (above 250 ppm or 10 mM) had a positive effect on viable cell coverage.

IV. Conclusion

Magnesium is essential for enzymatic reactions, membrane integrity, ATPase function, and cellular processes. Here we demonstrate that the addition of Mg^{2+} ions had a positive effect on the cell coverage of H9-OCT4 ESCs. To advance magnesium as an implant biomaterial, P-Mg and D-Mg was co-cultured with H9-OCT4 ESCs. Results showed that proliferation was not affected by pre-degraded nature of the magnesium. Both polished and pre-degraded magnesium caused similar initial increases in percentage of viable cell coverage (corresponding to cell dispersion), followed by rapid cell death. The degradation of P-Mg and D-Mg resulted in both Mg^{2+} ions and OH^- ions. This increased the alkalinity, which may have caused the cell death observed within the first 24 hours of culture. Consequently, controlling the degradation of magnesium to obtain the perfect balance of ions is critical for advancing magnesium as an implant biomaterial. Other factors that need to be explored in advancing magnesium include flow conditions and/or change of tissue fluid at the implantation site.

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References

[1] S. Kehoe, X.F. Zhang, D. Boyd, "FDA approved guidance conduits and wraps for peripheral nerve injury: A review of materials and efficacy," *Injury,* submitted for publication.

[2] P. Chatterjee, Y. Cheung, C. Liew, "Transfecting and nucleofecting human induced pluripotent stem cells," *J Vis Exp*, vol. 56, 2011.

[3] H. Liu, "The effects of surface and biomolecules on magnesium degradation and mesenchymal stem cell adhesion," *J Biomed Mat Res A,* vol 99A, pp. 249-260, Aug 2011.

[4] I. Johnson, D. Perchy, H. Liu, "In vitro evaluation of the surface effects on magnesium-yttrium alloy degradation and mesenchymal stem cell adhesion," *J Biomed Mat Res A,* vol 100A, pp. 477-485, Nov 2011.

[5] T. Vazin, W.J. Freed, "Human embryonic stem cells: Derivation, culture, and differentiation: A review," *Restor Neurol Neurosci*, vol. 28, pp. 589-603, 2010.

[6] G. Shi, Y. Jin, "Role of OCT4 in maintaining and regaining stem cell pluripotency*," Stem Cell Research & Therapy,* vol. 1, 2010.

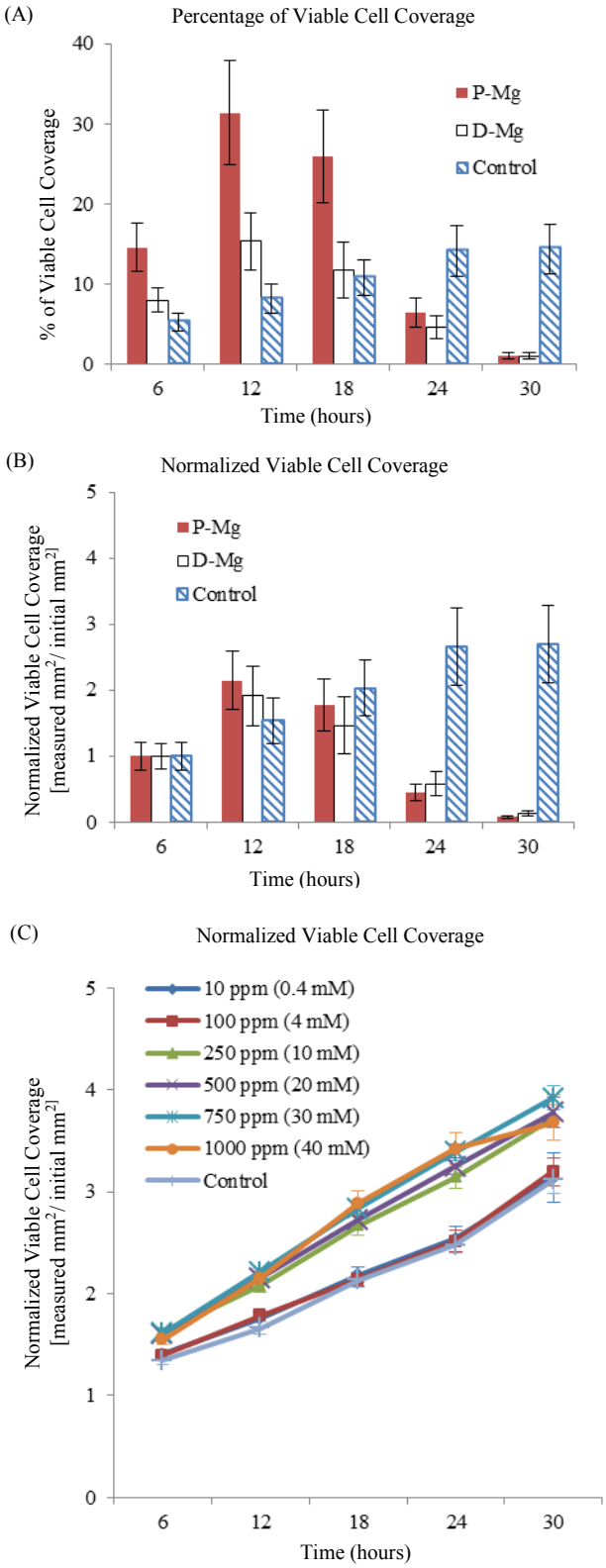

Fig. 5: Percentage of area coverage by viable H9-OCT4 hESCs over time (A) in P-Mg and D-Mg (B) in P-Mg and D-Mg normalized over the first time point, hour 6 (C) in cultures with additional Mg^{2+} ions. Values are mean ± standard error of the mean: n = 6.