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Reviewer A

The study by Wu and co-workers examined the effects of an Mg-containing incubation on a RAW macrophage cell line and the transfer of conditioned media to an SMC cell line. The results show convincing biological effects of Mg, but the possible mechanisms transducing this response were not examined.

Specifically, the following points should be addressed in a revision:

1. The effects of the Mg-based alloy were identical to equivalent concentrations of Mg. The title and conclusion should hence state that the results show Mg-induced responses.

Response 1: Thank you for your kind suggestions. We revised the title (Page 1, line 3-4) and the conclusion (Page 4, line 66, and Page 20, line 416) of the manuscript in line with your suggestions.

2. The authors should also explain how the measured ppm obtained for the stent extracts relates to the molar concentrations for the Mg^{2+} solutions.

Response 2: Thank you for your kind questions. In line with our previous studies (PMID: 31849975), we first detected the concentration of magnesium ions from JDBM by ICP-AES, and the concentration of magnesium ions from 15% JDBM extract was about 150ppm (ppm equals 1 mg of substance per liter, mg/L). As the molecular weight of magnesium is 24g/mol, we prepared $MgCl_2$ solution at 6mM of (150mg/L) final magnesium ion concentration similar to that of 15% extract. Then 2mM $MgCl_2$ solutions were prepared to simulate the magnesium ion concentration of 5% extract. Finally, the magnesium ion concentration in all the extracts and $MgCl_2$ solutions was determined by ICP-AES. We have modified our text as advised (see Page 8, lines 158-165).

3. The authors analyzed the elemental composition of the stent extracts, apparently by ICP-AES and a pH detector as mentioned in the legend in Figure 1. However, this method is not explained in the Material and Methods section.

Response 3: Thank you for your kind suggestions, and we have revised it in the material and methods part (see Page 8-9, line 158-171).

4. In their flow cytometry experiments, they used Annexin V-FITC and PI for stainings. Which target proteins were stained with these dyes, and how does this relate to

apoptosis and necrosis? How was the gating done that is presented in Fig. 1?

Response 4: Thank you for your kind questions. In the flow cytometry experiments, the target protein of Annexin V-FITC is Annexin V, and Propidium iodide (PI) is a fluorescent dye that binds to DNA. Annexin V (or Annexin A5) is a member of the annexin family of intracellular proteins that binds to phosphatidylserine (PS) in a calcium-dependent manner. PS is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, membrane asymmetry is lost, and PS translocates to the external leaflet. Fluorochrome-labeled Annexin V can then be used to target and identify apoptotic cells specifically. However, Annexin V binding alone cannot differentiate between apoptotic and necrotic cells. To help distinguish between the necrotic and apoptotic cells, PI staining was used in this study. Early apoptotic cells will exclude PI, while late-stage apoptotic cells and necrotic cells will stain positively due to the passage of these dyes into the nucleus, where they bind to DNA. Therefore, each quadrant represents a specific cell population: ① Annexin V-/PI-: living cells; ② Annexin V+/PI-: early apoptotic cells; ③Annexin V+/PI+: late apoptotic cells; ④ Annexin V-/PI+: Necrotic cells. For the gating strategies, forward versus side scatter (FSC vs. SSC) gating was used to identify cells of interest based on size and granularity (complexity), then a two-parameter density blot was used to distinguish specific cell populations by creating a plot on Annexin V vs. PI.

Again, thank you for your kind questions, and we have modified our text as advised (see Page 10, line 194-206).

5. In the gene expression analysis, the control group is set at 1 but lacks standard deviations of the mean. One-way ANOVA may not be appropriate if the control group does not have variance.

Response 5: Thank you very much for your kind suggestions, and we have reanalyzed the gene expression data with Mann-Whitney U test, and some change has been made in Fig 5e and Figure 7a. However, these changes do not affect our conclusion.

6. In the conditioned media transfers, the control is not adequate. For each cell media transfer, the same Mg-containing medium incubated in the absence of macrophages should be transferred.

Response 6: Thank you very much for your kind suggestions. We agree with you that it would be better if the same Mg-containing medium incubated in the absence of macrophages were used in the conditioned media transfers. However, in this study, we aimed to detect the effects of a biodegradable Mg-based alloy on the function of vascular smooth muscle cells (VSMCs) via immunoregulation of macrophage, but not the direct effect of the role of magnesium on VSMCs. Besides, the direct role of Mg on VSMCs has been studied before, as we mentioned in the introduction and discussion

part (see page 6, line 120-122, and page 16, line 332-334). Therefore, according to the principle of changing one single variable simultaneously, we did not set the Mg-containing medium incubated in the absence of macrophages as a control group in this study.

7. The direct effects of Mg on the SMC-like cell line should be established with the same concentrations as used in the macrophage cell line cultures, and the concentrations after transferred conditioned media should be established.

Response 7: Thank you for your kind suggestions. Because the direct effect of Mg on the SMC-like cell lines has been reported in the previous studies (PMID: 30270501 and PMID: 14566962) as we mentioned in the manuscript (see page 6, line 120-122, and page 16, line 332-334), we did not replicate it in this study, which we think are beyond the current scope of this manuscript.

For the conditioned media, we collected the supernatants from LPS-activated RAW264.7 cells treating with or without extracts or $MgCl_2$ solutions, centrifuged for 5 min at 1500 rpm, then mixed them with fresh DMEM complete medium at a ratio of 1:1. The magnesium concentrations in the different conditioned media were about as follows: CTR: 10mM, 5% extract: 32mM, 15% extract: 76mM, $MgCl_2$: 26mM, 6mM $MgCl_2$: 70mM.

8. Using only cell lines and not confirming in human primary cells is a limitation.

Response 8: Thank you for your kind suggestions. We agree with you that using only cell lines and not confirming in human primary cells is a limitation in this study because it is hard for us to obtain the human primary cells, and we have added it as a discussion in the main text of the manuscript (page 19-20, line 404-405).

9. Minor: I do not recommend AS as an abbreviation for atherosclerosis.

Response 9: Thank you for your kind suggestions, and we have revised the abbreviation in the text as advised (page 5-6, lines 84, 85, 89, and 107).

10. Suggestion: The role of M2 macrophages in the resolution of inflammation should be mentioned. Suggested reference: Nat Rev Cardiol. 2019 Jul;16(7):389-406.

Response 10: Thank you for your kind suggestions, and we have mentioned the role of the M2 macrophage in the resolution of inflammation in the introduction part as advised (page 7, line 132-133).

Reviewer B

The revised manuscript elucidated the mechanisms underlying the seemingly contradictory results from in vivo and in vitro studies of biodegradable Mg-based

alloys as cardiovascular stent material. The authors describe that JDBM extracts and $MgCl_2$ solutions inhibit the phenotypic switching of VSMCs to a proinflammatory phenotype via macrophage-mediated immunoregulation. Macrophage-mediated immunoregulation in the vascular wall has already been linked. Although they suggested that the decrease of some macrophage-derived genes promoted VSMC phenotypic switching, proliferation, and migration, more experiments are needed to elucidate this point.

The authors need to perform additional experiments to address the following:

1. The authors show the cytotoxic effects of JDBM extracts and $MgCl_2$ solutions on macrophages. It would be interesting to know the cytotoxic effects of JDBM extracts and $MgCl_2$ solutions on VSMCs.

Response 1: Thank you for your kind suggestions. We are sorry that we did not detect the direct cytotoxic effect of JDBM extracts and $MgCl_2$ solutions on VSMCs because the study aims to detect the effects of a biodegradable Mg-based alloy on the function of vascular smooth muscle cells (VSMCs) via immunoregulation of macrophage but not the direct effect of magnesium on VSMCs. Besides, the direct role of Mg on VSMCs has been studied before, as we mentioned in the introduction and discussion part (see page 6, line 120-122, and page 16, line 332-334). Therefore, we detected the cytotoxic effect of the conditioned media obtained from RAW264.7 cells, which were shown in Figure 4.

2. The authors used a small number of genes to determine the macrophage profile and conclude an M2-polarization of the macrophages. It would be interesting to consider the expression of other M1 genes (Il1b, Cxcl10, Ptgs2…) and other M2 genes (Arg1, Hmox1…). The mouse genes must be written correctly, with the initial letter in capital letters and everything in italics.

Response 2: Thank you for your kind suggestions. We agree with you that it would be better if more genetic markers for M1/M2 polarization were detected to determine the macrophage profile. We did not test more markers because previous studies have demonstrated that magnesium could promote the polarization of the macrophages towards an M2 type (PMID: 32817917, PMID: 27709132, and PMID: 33061375), and we tried to verify the conclusion from previous studies. iNOS, Cd86, Cd163, and Cd206 the most widely used markers to determine the M1/M2 polarization, so we chose these markers to determine the macrophage profile.

Thank you for pointing out the mistakes we have made, and we have revised the gene names as your kind suggestions.

Minor corrections

1. One minor but no less important correction is the improvement in the

quality/resolution of the figures, especially the flow cytometry dot plots and their axes and the figures of the cell cycle and their legends. Also, indicate the statistics and the number of experiments at the bottom of the figures.

Response 1 : Thank you for your kind suggestions. We have improved the quality/resolution of the figures and indicated the statistic and the number of experiments at the bottom of the figures according to your kind suggestions.

2. The authors used RAW264.7 macrophage-like cells (murine cells) and A7r5 cells (rat cells). Why did you use cells from different species? This point can influence the results.

Response 2 : Thank you for your questions. We agree that it would be better if cell lines from the same genus origin were used in this study. Due to the limited experimental conditions, only RAW264.7 and A7r5 cells were available in our lab. Additionally, previous data showed they were used to study the influence of macrophages on vascular smooth muscle cells in the previous studies (PMID: 22305260 and PMID: 28035243).

3. RAW264.7 cells are activated with LPS (1 mg/ml). The standard concentration of LPS used is 100 ng/ml. This is a higher concentration. Why did you use this concentration? Did it affect/kill RAW264.7 cells?

Response 3: Thank you for your kind questions. A previous systemic study (PMID: 28346799) has investigated the stimulatory effects of LPS with different times, doses, and conditions on the activation of macrophages, and the results suggested that a high concentration of LPS (up to 1mg/ml) would be better for the activation of macrophage in a short time stimulation (within 6 hours). In this study, the RAW264.7 was activated by LPS for only 6h; then, the LPS was removed. Therefore, a high concentration of LPS (1mg/ml) was used in this study.

4. For cell viability, did you preserved cell media culture and used it to consider properly for cell viability determinations? What was the temperature of Annexin V incubation? What program did you use to process flow cytometry data?

Response 4: Thank you for your kind questions. For cell viability, we did not preserve cell media culture. The cell media culture was removed, then the cells were washed with PBS twice. Then, 100 µl of DMEM containing 10% CCK-8 solution was added to each well, followed by incubation for 3 h in a humidified 5% CO_2 incubator at 37°C.

For the Annexin V staining, the temperature of incubation is room temperature. For the gating strategies, forward versus side scatter (FSC vs. SSC) gating was used to identify cells of interest based on size and granularity (complexity), then a two-parameter density blot was used to distinguish specific cell populations by creating a

plot on Annexin V vs. PI. Each quadrant showed Figures 1, and 4 represents a specific cell population: ①Annexin V-/PI-: living cells; ②Annexin V+/PI-: early apoptotic cells; ③Annexin V+/PI+: late apoptotic cells; ④Annexin V-/PI+: Necrotic cells.

We have revised these methods in the text as your kind suggestions (page 9, line 188-189, and page 10, line 194-206).

5. Did you check the quality (Bioanalyzer) and quantity (NanoDrop) of RNA? RIN? Indicate if you made triplicates for each cDNA sample, the method used to analyze RT-qPCR (such as the 2-ΔCt method), and the control gene used.

Response 5: Thank you for your questions. NanoDrop in this study determined the quantity and quality of RNA. The expression levels of target genes were evaluated using the ΔΔCt method and were normalized to GAPDH. We have mentioned it in the material and method part as advised (Page 11-12, line 232-234).

6. What is the cause of the increase in pH with the 15% extract not observed with 6 mM $MgCl_2$? The changes in magnesium concentration (ppm) and the pH values do not show statistical significance.

Response 6: Thank you for your kind questions. We did observe a slight increase of the PH value in the 15% extract, which was in line with our previous study (PMID: 29538391). That might be because magnesium reacts with water from magnesium hydroxide, which could increase the pH value of the extract. However, the $MgCl_2$ solution is more stable than metal magnesium.

7. Discuss the unexpected increase in VEGF in macrophages after using JDBM and $MgCl_2$ solutions at low concentrations.

Response 7: Thank you for your kind suggestions. The increased *Vegf* expression in macrophages after using JDBM and MgCl2 solutions observed in this study is in line with the previous studies, which showed the interaction between M2 macrophage and VEGF. On the one hand, M2 macrophages could secret VEGF to stimulate angiogenesis; on the other hand, VEGF contributes to macrophage recruitment and M2 polarization. We have made a discussion about it as advised (Page 14, line 283-285).

8. Superscript the Mg^{2+} on line 345.

Response 8: We are sorry for the mistake we have made, and we have corrected it according to your kind suggestions (Page 18, line 371).

9. Subscript the $MgCl_2$ on line 619.

Response 9: Corrected.

10. Line 180-184: Please define PI at first use in the materials and methods.

Response 10： Thank you for your kind suggestions, and we have revised it as advised (page 10, line 194)