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RESEARCH ARTICLE

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Adipose-derived stem cells decrease cardiomyocyte damage induced by porphyromonas gingivalis endotoxin through suppressing hypertrophy, apoptosis, fibrosis, and MAPK markers

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Abstract

Heart failure is one of the complications related to periodontal disease. In addition to drugs or herbal medicines, stem cell therapy shows potential in the treatment of cardiomyopathy. This study investigates if stem cells exhibit beneficial effects on cardiomyocyte damage induced by porphyromonas gingivalis endotoxin (Pg-LPS). From the experimental results we find that Pg-LPS reduce cardiomyocyte viability via the activation of apoptosis, hypertrophy, fibrosis and MAPK signaling. Pg-LPS damaged cardiomyocytes co-cultured with adipose-derived stem cells (ADSC) increases cardiomyocyte viability through suppressing the pathological markers described above. Further evidence implies that survival marker, IGF1, secreted from ADSC, may play an important role in the Pg-LPS induced protective effect on cardiomyocyte damage.

KEYWORDS

cardiomyopathy, endotoxin, IGF1, periodontal disease, stem cell

*These authors share equal contribution

1 | INTRODUCTION

Epidemiological data from the World Health Organization (WHO) indicate that nearly 100% of adults have dental cavities worldwide, including periodontal disease. Periodontal disease is caused mainly by bacterial infection of the gums. Porphyromonas gingivalis (Pg) is one of the bacteria related to periodontal disease.[1] Pg invasion into the blood results in sepsis and systematic inflammation. These pathological conditions further initiate the complications related to periodontal disease. Heart failure is one of the complications associated with periodontal disease.[2,3]

In addition to Pg invasion, circulating Pg endotoxin (Pg-LPS) is greatly associated with heart failure progression. Lee et al.[4] stated that Pg conditioned medium damages cardiomyocytes by activating hypertrophy and fibrosis pathways through MAPK signaling.[5] Further, Pg-LPS induces cardiomyocyte apoptosis mediated through TLR4. Although some herbal medicines and functional foods can rescue cardiomyocyte damage induced by Pg-LPS through suppressing inflammatory signaling, the protective effect of stem cells in the treatment of cardiomyocyte damage induced by Pg-LPS is rarely mentioned.

Stem cells are capable of performing tissue regeneration through trans differentiation of different lineages and the release of growth factors or cytokines via the paracrine route. Weil et al.[6] illustrated that stem cells show anti-inflammatory properties by expressing IL-10 in cardiomyopathy induced by LPS. Thus, these data suggest that stem cells may show potential in the treatment of systematic inflammation. The cardiac protective effect of stem cells in cardiomyocyte damage induced by Pg-LPS is discussed in this study.

2 | MATERIALS AND METHODS

2.1 | Chemicals, reagents, and antibodies

All chemicals and reagents used in this study were purchased from Sigma-Aldrich LLC. (St. Louis, MO, USA). Primary antibodies used for western blotting were purchased from Abcam Plc, Cambridge, MA, USA (including Bid, t-Bid, caspase 3, α-tubulin, NFAT3, ANP, BNP, MMP2, MMP9, GAPDH, and IGF1), Cell Signaling Technology, Danvers, MA, USA (including Erk, p-Erk, JNK, p-JNK, p38, p-p38) and Santa Cruz Biotechnology, Santa Cruz CA, USA (cyclin B, cyclin D). Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz CA, USA).

2.2 | Adipose-derived stem cell (ADSC) isolation and characterization

Briefly, epididymal fat tissue was isolated, minced and digested for 3 h at 37°C with collagenase type 2 (0.2% in PBS). The cells isolated from the fat tissue were cultured in DMEM medium (Invitrogen) with 10% FBS (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen) and 2 mM L-glutamin (Invitrogen) at 37°C. Before experimentation, surface protein markers were screened by flow cytometry (BD FACSAria, Becton, Dickinson and Company, 1 Becton Drive, Franklin Lakes, NJ 07417-1880) and differentiation analysis [SR811D250, AMS Biotechnology (Europe), 184 Park Drive, Milton Park, Abingdon OX14 4SR, UK] were performed to confirm that the cells isolated from adipose tissue were ADSC. This animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (protocol number: 2016-208).

2.3 | Isolation of neonatal rat cardiomyocytes (NRCMs)

Cardiomyocytes were obtained from neonatal rat hearts in accordance with the manufacturer's protocol (neonatal cardiomyocyte isolation system kit Cellutron Life technology, Highland Park, NJ). Briefly, heart tissues from neonatal rats were minced and then digested with collagenase type 2. After digestion, the digestion solution was centrifuged (2000 rpm for 10 min) and the cell pellets were transferred into culture dishes containing culture medium (DMEM with 10% fetal bovine serum, 100 μg/ml, penicillin, 100 μg/ml streptomycin, and 2 mM glutamine) for further experiments. This animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (protocol number: 2016-208).

2.4 | Cell culture

H9c2 cardiomyoblasts from the American Type Culture Collection (ATCC, CRL-1446) (Rockville, MD), NRCMs from neonatal rat hearts or ADSC from rat adipose tissue were incubated in 10 cm culture dishes containing Dulbecco's modified Eagle's medium (DMEM) with penicillin (100 μg/mL), streptomycin (100 μg/mL), HEPS buffer (1 mM), glutamine (2 mM) and 10% FBS at 37°C under 5% CO_2. H9c2 cardiomyoblasts (passage 32 to passage 40 were used in this study) or ADSC were placed in serum-free essential medium overnight before treatment.

2.5 | Western blot

Briefly, the protein concentration for the experimental samples was measured using Lowry's protein assay. Forty μg of protein for each sample was then separated using 12% SDS-PAGE with a constant voltage of 75 V. The gel was then transferred onto a Hybon-C membrane with constant voltage 50 V for 3 h. After transfer the PVDF membranes were placed in TBS buffer with 3% bovine serum albumin (BSA). Primary and secondary antibodies were added to all PVDF membranes and the images for all target proteins were taken using Fujifilm LAS-3000 (GE Healthcare). ImageJ was applied to quantify the intensity of bands on western blot.

2.6 | Coculture of adipose-derived stem cells with H9c2 cardiomyoblasts or neonatal rat cardiomyocytes

Briefly, H9c2 cells or NRCMs were cultured in 6-well culture dishes. Adipose-derived stem cells (ADSC) were cultured in hanging insert (Millipore). The hanging insert with ADSC was placed in 6-well dishes and co-cultured with H9c2 cells without contact.

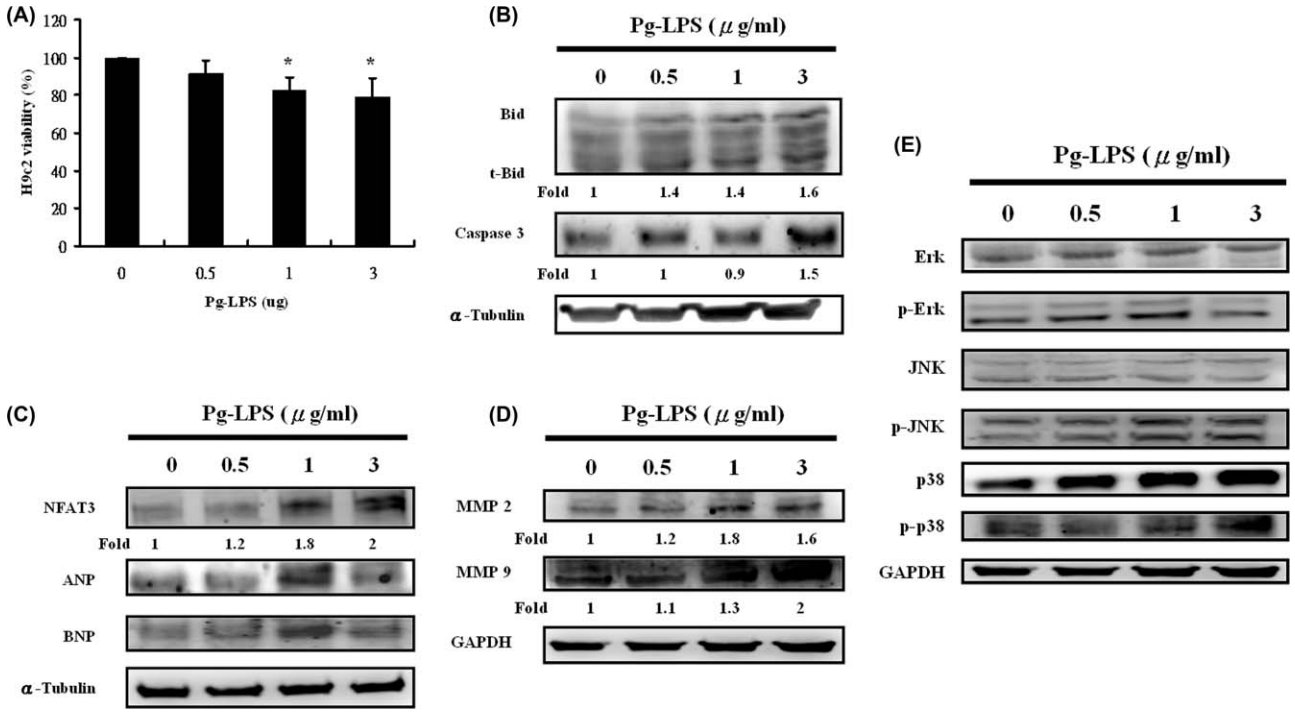

FIGURE 1 H9c2 cardiomyoblasts investigation under Pg-LPS treatment, (a) cell viability, (b) apoptotic marker expression, (c) hypertrophic marker expression, (d) fibrotic marker expression, (e) MAPKs expression

2.7 | Cell viability assay

This study applied the MTT assay to measure cell viability. Briefly, cells (1×10^5 cells per well) were cultured in 24-well plates for 24 h. The culture medium was then discarded and washed with PBS. After washing, 200 μL of MTT solution (0.5 mg/mL) was added to each well and incubated at 37°C for 4 h. After incubation, the MTT solution was removed and 150 μL of isopropyl alcohol (IPA) or dimethyl sulfoxide (DMSO) was added. The absorbance for IPA or DMSO solution was read at 550 nm wavelength using an automated micro plate reader.

2.8 | Statistical analysis

All experiments were repeated three times and the experimental results are expressed as mean ± SD (standard deviation). Statistical significance for the experimental results was calculated using ANOVA. Significant difference was considered at $P < 0.05$.

3 | RESULTS

3.1 | Investigation of H9c2 cell characterization in the presence of Pg-LPS

Figure 1A shows that H9c2 cell viabilities in the treatment of 0, 0.5, 1, and 3 μg/ml of Pg-LPS follow the order of 100, 91 ± 7, 82 ± 7, and 79 ± 10%, respectively. The H9c2 cell viabilities for 1 and 3 μg/ml of Pg-LPS are significantly higher than 0 μg/ml of Pg-LPS ($P < 0.05$). In addition to cell viability, protein marker expression was investigated for H9c2 stressed with Pg-LPS. From Figure 1B–E, we see that several protein markers were expressed in a dose-dependent manner in H9c2 cells stressed with Pg-LPS, including apoptotic markers (t-Bid and caspase 3), hypertrophy markers (NFAT3, ANP, and BNP), fibrosis markers (MMP2 and MMP9) and MAPKs.

3.2 | Characterization investigation for pg-LPS damaged NRCMs cocultured with ADSC

Before coculture, stem cell characterization should be screened, including membrane markers and differentiation ability. Flow cytometry was applied to screen surface markers on stem cell membrane, including positive markers, including CD29 and CD90, as well as negative markers including CD31 and CD45. From Figure 2A, the percentages for the control, CD29, CD90, CD45, and CD31 are 0, >99%, >79.6%, <1%, and <1%, respectively. Figure 2B illustrates that ADSC used in this study shows adipogenesis capability (oil red O stained). Figure 3 states the ADSC protective effect on Pg-LPS damaged H9c2 cells or NRCMs. Figure 3A shows that H9c2 cell viabilities in the presence of 0, 0.5, 1, and 3 μg/ml of Pg-LPS follow the order of 100, 94 ± 5, 85 ± 6, and 68 ± 5%, respectively. When H9c2 cells were cocultured with ADSC, H9c2 cell viabilities in the presence of 0, 0.5, 1, and 3 μg/ml of Pg-LPS are 100, 100 ± 8, 98 ± 7, and 91 ± 7%, respectively. Cell viability for H9c2 cells cocultured with ADSC is significantly higher than that with H9c2 cells alone under 3 μg/ml of Pg-LPS challenge. In addition to cell viability, protein expression for NRCMs cocultured with ADSC was investigated in the presence of Pg-LPS. Compared to the control, all protein markers are expressed in the presence of Pg-LPS, including MAPKs (p-JNK, p-Erk, and p-p38), hypertrophy marker (ANP) and fibrosis marker (MMP9). On the other hand, all protein markers are suppressed when NRCMs were cocultured with ADSC (Figure 3B).

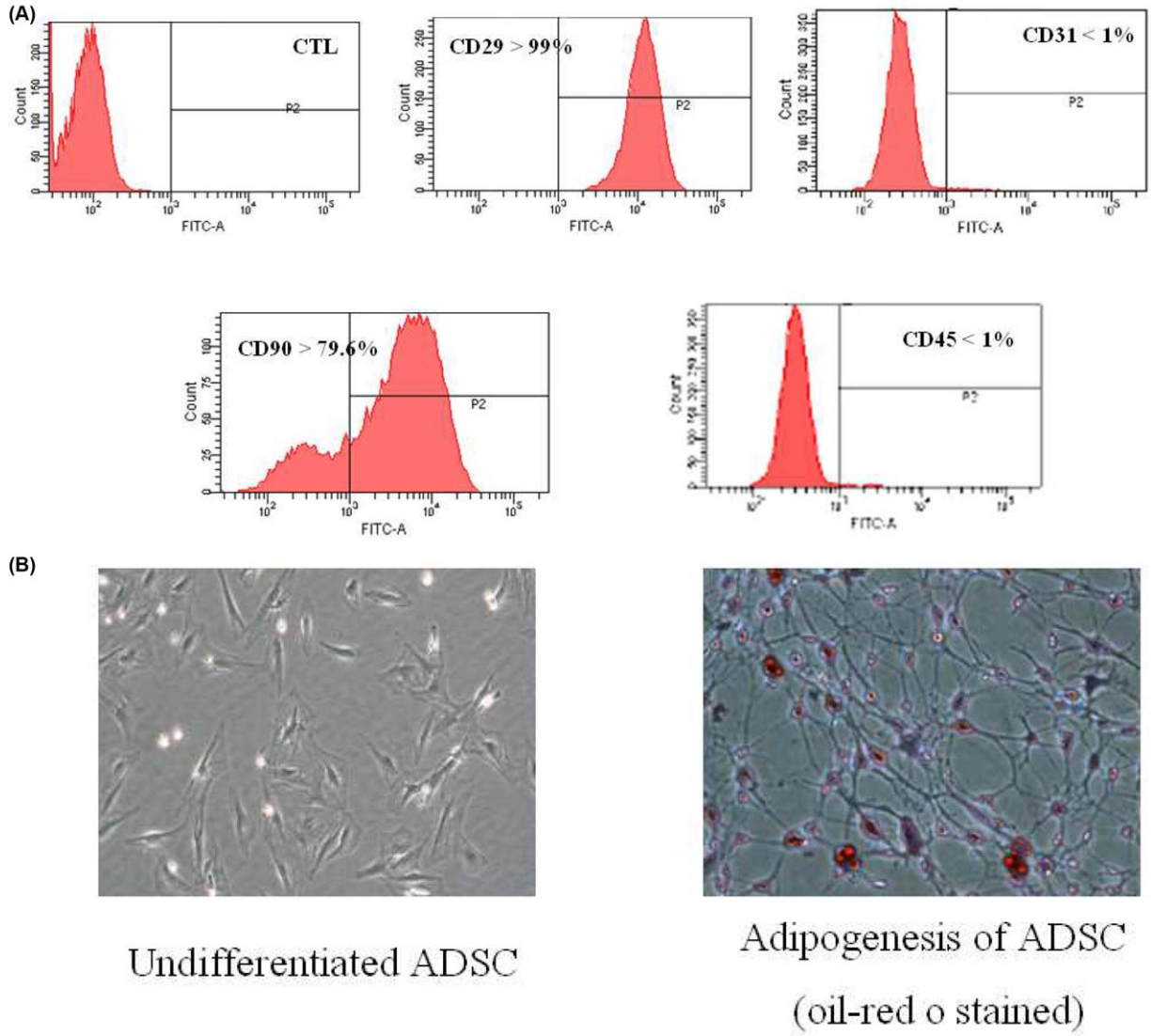

FIGURE 2 Adipose-derived stem cell (ADSC) characterization. (a) Surface markers analysis and (b) differentiation analysis [Color figure can be viewed at wileyonlinelibrary.com]

Figure 3C illustrates that IGF1, a kind of growth factor, secreted from ADSC conditioned medium via paracrine route. Figure 3D stated that ADSC conditioned medium can restore the viability of PG-LPS damaged H9c2 cells and neutralization of IGF1 by IGF1 antibody will partially block the therapeutic effect of ADSC conditioned medium on PG-LPS damaged H9c2 cells.

4 | DISCUSSION

Previous studies reveal that H9c2 cell damage induced by Pg conditioned medium through the expression of MAPK, hypertrophy and fibrosis pathways. We further confirm that Pg-endotoxin (Pg-LPS) induced H9c2 cells damage through similar signaling pathways (Figure 1). These findings are consistent with previous data.

As mentioned previously, heart tissue is difficult to regenerate after damage. In contrast, stem cells can repair damaged tissue through the paracrine effect and trans differentiation, including heart tissue. This study designed a coculture system (NRCMs/ADSC or H9c2/ADSC) to see if ADSC shows a protective effect on NRCMs/H9c2 cell damaged with Pg-LPS through the suppression of MAPK, hypertrophy and fibrosis signaling. We found that all pathological markers were suppressed in NRCMs when NRCMs were cocultured with ADSC in the presence of Pg-LPS. In contrast, increase in cell viability was observed when H9c2 cells were cocultured with ADSC (Figure 3). These results illustrate that ADSC increases NRCM/H9c2 cell viability through regulation of MAPK, hypertrophy and fibrosis signaling. We know that the paracrine effect is one of the important ADSC functions in performing tissue regeneration, including IFG1 secretion.[7] We therefore investigated IFG1 secretion in ADSC conditioned medium. We found that IGF1 expression is proportional to the ADSC cell number (Figure 3C). Furthermore, blockage of IGF1 by IGF1 antibody will decrease the therapeutic effect of ADSC conditioned medium on PG-LPS damaged H9c2 (Figures 3D and 4). This result confirms that ADSC secrets

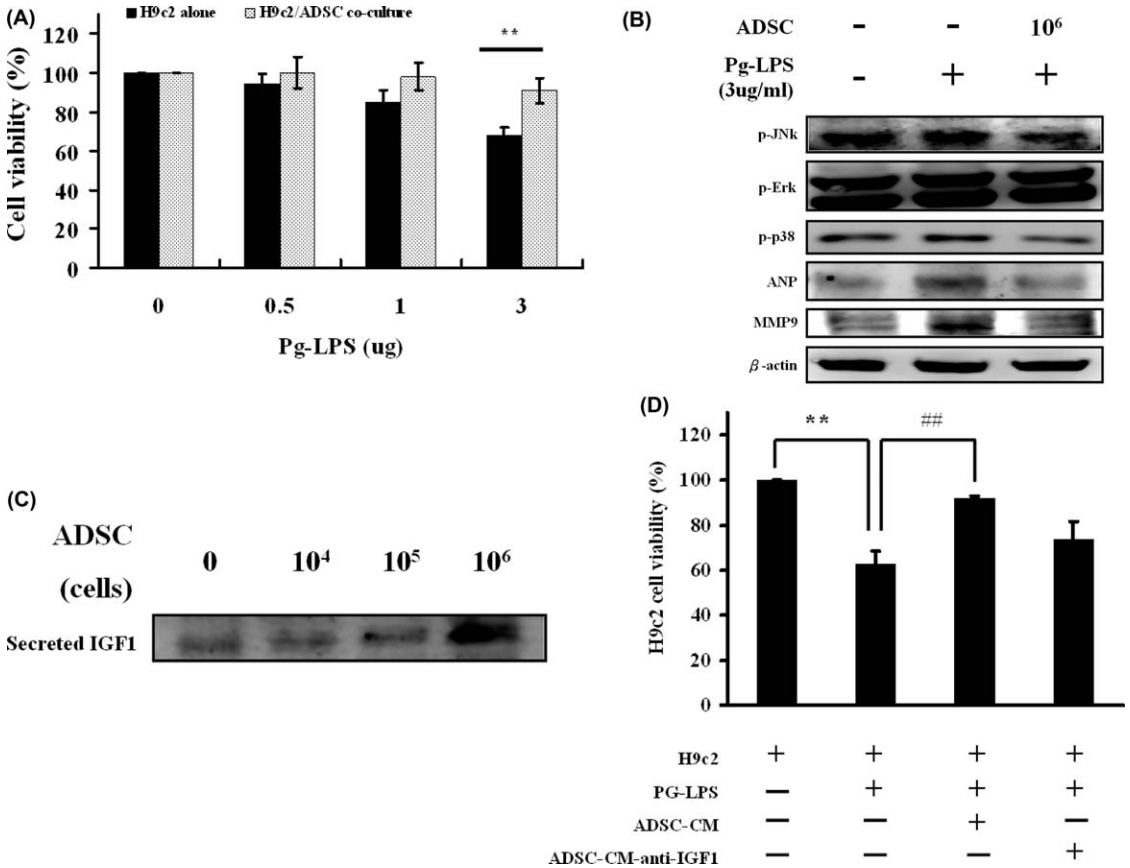

FIGURE 3 Cardiac protective effect on H9c2 cocultured with ADSC (1×10^6 cells). (a) H9c2 cell viability, (b) protein marker expression, (c) expression of IGF1 in ADSC conditioned medium, (d) H9c2 cell viability with different kinds of ADSC conditioned medium

growth factor into the culture medium. We know that IGF1 regulates cell proliferation through cell cycle activation. Therefore, we investigated the expression of proteins relating to the cell cycle in H9c2 cells cocultured with ADSC (Supporting Information Figure 2). When compared with H9c2 cells alone, we found that cell cycle related proteins (cyclin B and cyclin D) are expressed in the H9c2/ADSC coculture system. These data illustrate that ADSC rescues Pg-LPS damaged NRCMs through cell cycle activation.

We can conclude the experimental data as follows: (1) Pg-LPS damages H9c2 cells through the expression of MAPKs, hypertrophy, and fibrosis as well as cell viability suppression; (2) ADSC cocultured with NRCMs/H9c2 cells suppresses MAPKs, hypertrophy and fibrosis signaling as well as increases H9c2 cell viability; (3) IGF1 secretion from ADSC may activate the H9c2 cell cycle, leading to an increase in H9c2 cell viability and reduces pathological protein markers expression under Pg-LPS treatment (Figure 4).

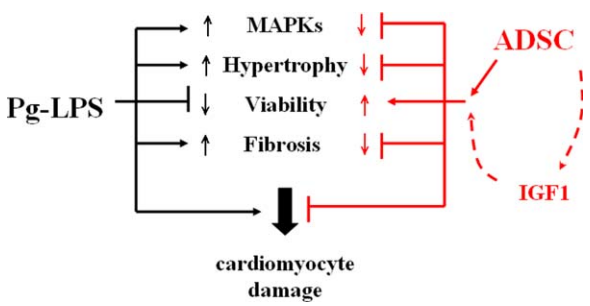

FIGURE 4 Graphic summary [Color figure can be viewed at wileyonlinelibrary.com]

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CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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REFERENCES

[1] Li X, Kolltveit KM, Tronstad L, Olsen I. Systemic diseases caused by oral infection. *Clin Microbiol Rev*. 2000;13(4):547–558.

[2] Chistiakov DA, Orekhov AN, Bobryshev YV. Links between atherosclerotic and periodontal disease. *Exp Mol Pathol*. 2016;100(1):220–235.

[3] Aarabi G, Eberhard J, Reissmann DR, Heydecke G, Seedorf U. Interaction between periodontal disease and atherosclerotic vascular disease—Fact or fiction? *Atherosclerosis*. 2015;241(2):555–560.

[4] Lee SD, Wu CC, Chang YC, et al. Porphyromonas gingivalis-induced cellular hypertrophy and MMP-9 activity via different signaling pathways in H9c2 cardiomyoblast cells. *J Periodontol*. 2006;77(4):684–691.

[5] Wu HC, Yeh YL, Kuo WW, et al. P38 mitogen-activated protein kinase pathways are involved in the hypertrophy and apoptosis of cardiomyocytes induced by Porphyromonas gingivalis conditioned medium. *Cell Biochem Funct*. 2008;26(2):246–255.

[6] Weil BR, Manukyan MC, Herrmann JL, et al. Mesenchymal stem cells attenuate myocardial functional depression and reduce systemic and myocardial inflammation during endotoxemia. *Surgery*. 2010;148(2):444–452.

[7] Cervelló I, Gil-Sanchis C, Santamaría X, et al. Human CD133(+) bone marrow-derived stem cells promote endometrial proliferation in a murine model of Asherman syndrome. *Fertil Steril*. 2015;104(6):1552–1560.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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