Int J Clin Exp Pathol 2018;11(3):1446-1452
www.ijcep.com /ISSN:1936-2625/IJCEP0069726

Original Article

Human umbilical cord mesenchymal stem cells polarize RAW264.7 macrophages to an anti-inflammatory subpopulation

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Received November 23, 2017; Accepted December 17, 2017; Epub March 1, 2018; Published March 15, 2018

Abstract: Objective: To investigate the effects of human umbilical cord mesenchymal stem cells (hUCMSCs) on the polarization of lipopolysaccharide-stimulated RAW264.7 macrophages. Methods: Lipopolysaccharide-stimulated RAW264.7 macrophages were co-cultured with hUCMSCs in a Transwell system for 4 d, and then labelled with anti-F4/80, anti-CD86, and anti-CD206 antibodies for flow cytometry. The co-cultured supernatants were detected by enzyme-linked immunosorbent assay for prostaglandin E2. The co-cultured RAW264.7 macrophages were also lysed to measure the intracellular level of inducible nitric oxide synthase. Results: There were significantly more F4/80+CD86+CD206+ RAW264.7 macrophages in the hUCMSCs-treated groups than the control group ($P<0.001$). The secretion of prostaglandin E2 by lipopolysaccharide-stimulated RAW264.7 macrophages was significantly inhibited in a dose-dependent manner with the addition of hUCMSCs ($P<0.001$). The expression of iNOS, the intracellular marker of M1 cells, was also significantly inhibited by hUCMSCs ($P<0.05$). Conclusion: hUCMSCs significantly polarize the lipopolysaccharide-stimulated RAW264.7 macrophages from a pro-inflammatory M1 subpopulation to an intermediate subpopulation of anti-inflammatory M2 macrophages, which are associated with a gradual decrease of iNOS and PGE_2 levels.

Keywords: human umbilical cord mesenchymal stem cell, macrophage polarization, prostaglandin E2

Introduction

Mesenchymal stem cells (MSCs) have been recognized as an appropriate source for stem cell therapy because of their self-renewal capacity and the ease of their isolation [1]. Derived from the Wharton's jelly of human umbilical cord, human umbilical cord mesenchymal stem cells (hUCMSCs) are promising candidates for treatment of rheumatoid arthritis, stroke, and congenital corneal diseases [2-4]. Nevertheless, the underlying mechanisms of hUCMSCs therapy remain debatable, among which the immunomodulatory effects of hUCMSCs have been widely investigated [5]. hUCMSCs produce various cytokines such as interleukin (IL)-6 and interferon-α to interact with other cells such as leukocytes, monocytes and macrophages, playing a critical role in pathophysiological processes [5].

Macrophages differentiate to either the classically pro-inflammatory M1 or the alternatively anti-inflammatory M2 sub-population under different stimulating conditions [6]. The M1 subpopulation secretes IL-1, IL-6, tumour necrosis factor-α, and expresses intracellular markers such as inducible nitric oxide synthase (iNOS) and surface markers such as cluster differentiation (CD) 86 [7]. The M2 subpopulation, which can be further divided to M2a, M2b, and M2c, generally expresses surface markers such as mannose receptor (CD206) and IL-10. When stimulated by lipopolysaccharide (LPS), macrophages are polarized toward the M1 subpopulation and against the M2 subpopulation possibly through secretion of PGE_2 by MSC [8, 9].

Our study is to investigate the effects and the underlying mechanisms of different concentrations of hUCMSCs on the polarization of LPS-stimulated RAW264.7 macrophages.

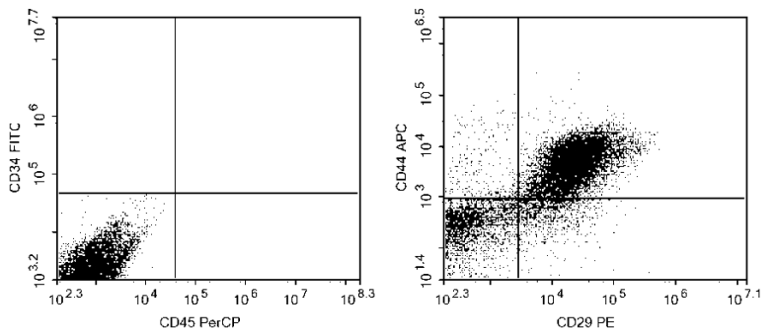

Figure 1. Flow cytometric analysis of surface markers of hUCMSCs. hUCMSCs express CD29 and CD44 but do not express CD34 and CD45.

Materials and methods

Isolation and identification of hUCMSCs

Under the approval of our Institutional Review Board and after obtaining a written informed consent from the patients, we isolated hUCMSCs from human umbilical cords according to the previously published protocols [10]. Briefly, a 15-cm umbilical cord was harvested and rinsed in phosphate-buffered saline (PBS) and then cut into small pieces of 2-3 cm. The blood vessels and perivascular tissues were carefully removed, and the cord was added with 0.2% type II collagenase (298 U/mg; Sigma Corporation, NJ, USA) and incubated under 37°C for 2 h on a shaker. The solution was then diluted with phosphate buffered saline (PBS) at a ratio of 1:4 and centrifuged at 2500 rpm for 5 min. The supernatants were discarded, and the cells were cultured in DMEM/F12 complete media supplemented with 10% fetal bovine serum (Sigma-Aldrich Corporation, MO, USA) and 1% penicillin/streptomycin (Solarbio Life Science, Beijing, China) under 37°C. The isolated hUCMSCs were expanded to passage 4 (P4) for the experiments.

The P2 hUCMSCs were labelled with FITC-labelled anti-CD34, PerCP-labelled anti-45, APC-labelled anti-CD44, and PE-labelled anti-CD29 human antibodies, and characterized for surface markers using flow cytometry on a fluorescence-activated cell sorter (FACS) (Beckman Coulter, CA, USA). Five thousand events were recorded for each sample, and cell markers were defined as positive by comparing with the fluorescence of isotype controls.

Transwell co-culture of hUCMSCs and LPS-stimulated RAW264.7 macrophages

RAW264.7 macrophages were cultured in DMEM/F12 media (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, MA, USA) under 37°C and 5% carbon dioxide. They were pre-treated with 100 μg/ml LPS for 3 d. A 6-well transwell coculture system (Corning Inc., NY, USA) was employed for non-contactable co-culture of hUCMSCs and LPS-stimulated RAW264.7 macrophages. 1×10^4 LPS-stimulated RAW-264.7 macrophages and 10×10^5, 8×10^5, 6×10^5, 4×10^5, 2×10^5, 1×10^5 hUCMSCs were seeded into the upper and lower chambers of 6-well plates, respectively. The control group was added only with 1×10^4 LPS-stimulated RAW264.7 macrophages to the upper chamber and cells were incubated under 37°C in a 5% CO_2 incubator for 4 d. Each experiment was repeated for three times.

Flow cytometry

Following a 4-day co-culture, the LPS-stimulated RAW264.7 macrophages were dissociated with trypsin-EDTA into single cell suspension. They were washed with PBS three times and incubated with PE-labelled anti-F4/80, Alexa Fluor 488-labelled anti-CD206 and PE/Cy7-labelled anti-CD86 mouse monoclonal antibodies (Abcam PLC, MA, USA) on ice in a dark room for 30 min. The cells were then washed three times with PBS and examined with flow cytometry (Beckman Coulter, CA, USA). The expression of cell markers was compared with an isotype control. The acquired flow cytometric images were analysed with FlowJo version 10.0.7 (FlowJo LLC, OR, USA).

Enzyme-linked immunosorbent assays (ELISA)

Following a 4-day co-culture, the culture media were harvested to detect the prostaglandin E2 level using an ELISA detection kit (Expand-Bio Company, Beijing, China) according to the manufacturer's instructions. LPS-stimulated RAW-264.7 macrophages were lysed by a freeze-thaw method for at least 5 times, and the mix-

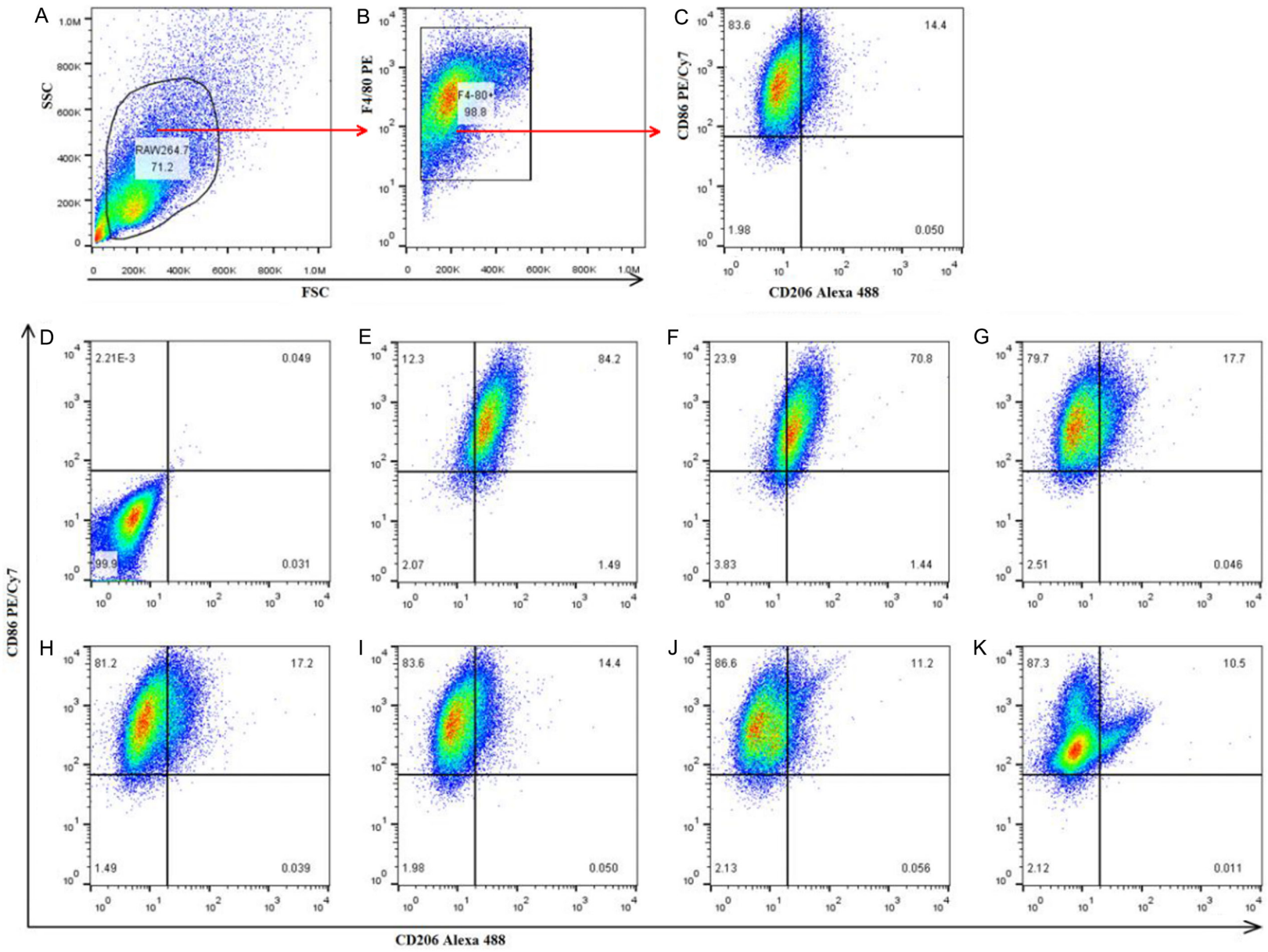
A
SSC
FSC
RAW264.7
71.2
B
F4/80 PE
F4-80+
98.8
C
CD86 PE/Cy7
CD206 Alexa 488
83.6
14.4
1.98
0.050
D
2.21E-3
0.049
99.9
0.031
E
12.3
84.2
2.07
1.49
F
23.9
70.8
3.83
1.44
G
79.7
17.7
2.51
0.046
H
81.2
17.2
1.49
0.039
I
83.6
14.4
1.98
0.050
J
86.6
11.2
2.13
0.056
K
87.3
10.5
2.12
0.011

Figure 2. Gating strategies and expression profiles in different groups. (A-C) Show the gating strategies in one representative group. Briefly, the macrophages are first grouped using the FSC and SSC, after which a group of macrophages with high expression of F4/80 are selected and analysed for expression of CD86 and CD206. (D-K) Show expression of CD86 and CD206 in different groups. The (D) shows expression of CD86 and CD206 in the isotype control, and the (E) to (J) show expression of CD86 and CD206 in the groups with added 1×10^4 LPS-stimulated RAW264.7 macrophages and 10×10^5, 8×10^5, 6×10^5, 4×10^5, 2×10^5, 1×10^5 hUCMSCs, respectively. (K) Shows the expression profile of the control group added with only LPS-stimulated RAW264.7 macrophages.

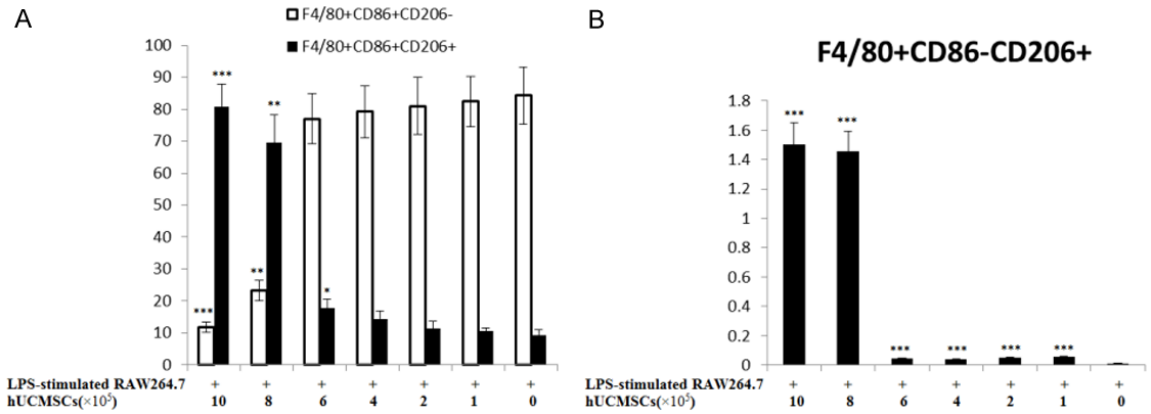

Figure 3. Percentages of macrophages expressing different combinations of surface markers. A: Shows the percentages of macrophages expressing F4/80+CD86+CD206+ (mixed subpopulation) and F4/80+CD86+CD206- (M1) phenotypes. The former phenotype gradually decreases while the latter phenotype increases as the numbers of hUCMSCs decrease. B: Shows the percentages of macrophages expressing F4/80+CD86-CD206+ (M2) phenotypes in different groups, with a significant decrease observed as the numbers of hUCMSCs decrease. All statistical analyses were performed with reference to the control group which was only added with LPS-stimulated RAW264.7 macrophages. **P*<0.05, ***P*<0.01, ****P*<0.001.

tures were centrifuged to get the supernatant for ELISA as mentioned above.

Statistical analysis

Statistical analyses were performed using the SPSS 22.0 software (SPSS Inc., IL, USA). Data were expressed as mean±standard deviation of three independent experiments. The Student's t test was employed to compare the means between 2 groups, and one-way analysis of variance to compare among multiple groups. A P<0.05 was considered significantly different.

Results

Isolation and identification of hUCMSCs

Figure 1 shows that hUCMSCs express specific markers (CD29, CD44), without hematopoietic cell surface markers (CD34, CD45).

Surface markers F4/80, CD86 and CD206 on LPS-stimulated RAW264.7 macrophages

Figure 2 shows the gating strategies and the expression profiles of surface markers F4/80, CD86 and CD206 in different groups of LPS-stimulated RAW264.7 macrophages. F4/80, a lineage marker for mouse, was expressed on almost all macrophages. **Figure 3** shows the percentages of different populations of macrophages. F4/80+CD86+CD206- macrophages, a classically activated M1 subpopulation, were significantly reduced in the groups co-cultured with more hUCMSCs, as compared with the control group containing only LPS-stimulated RAW264.7 macrophages (P<0.001). F4/80+CD86+CD206+ macrophages, a recently defined mixed type of M1 and M2, significantly increased with the addition of more hUCMSCs (P<0.001) [11]. F4/80+CD86-CD206+ macrophages, an alternatively activated M2 subpopulation, were significantly higher in all groups treated with hUCMSCs, as compared with the control group added only with LPS-stimulated RAW264.7 macrophages (P<0.001).

Secretion of PGE_2 and the expression of iNOS in LPS-stimulated RAW264.7 macrophages

Figure 4 shows the expression levels of PGE_2 and iNOS in LPS-stimulated RAW264.7 macro-

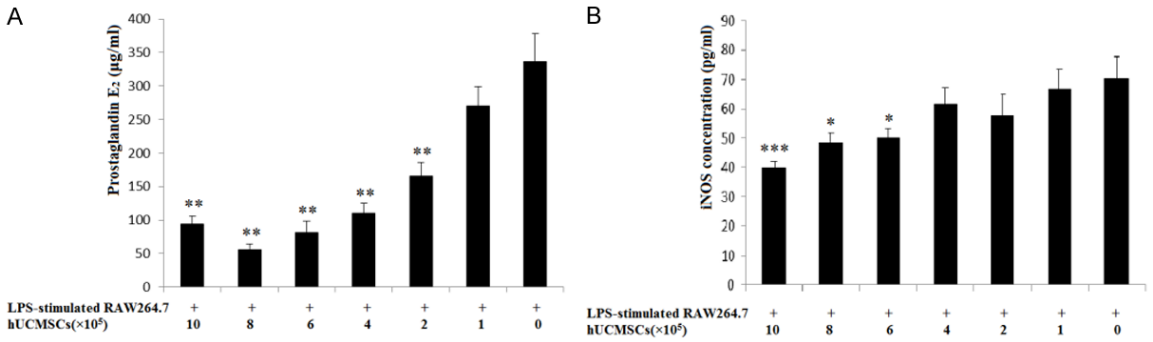

Figure 4. Concentrations of extracellular PGE_2 and intracellular iNOS. Both PGE_2 and iNOS concentrations increase with the decreasing number of hUCMSCs. All statistical analyses were performed with reference to the control group which was only added with LPS-stimulated RAW264.7 macrophages. **P*<0.05, ***P*<0.01, ****P*<0.001.

phages. The secretion of PGE_2 was significantly supressed by the addition of more hUCMSCs (P<0.001). The expression of intracellular iNOS was significantly lower in the groups added with hUCMSCs as compared with the control group (P<0.05).

Discussion

MSCs have been recognized as a potential cell source for regenerative medicine. Both clinical and experimental studies have shown promising beneficial effects of MSCs on improving the study objects' performance [12, 13]. However, the underlying mechanisms of MSCs on improving the patient's outcome remain disputable. The immunomodulatory properties of MSCs are possibly the most studied and recent studies have demonstrated that MSCs exert anti-inflammatory effects partially through the secretion of PGE_2 which is likely to polarize the macrophages from M1 to M2 subpopulation [14, 15]. Macrophages co-cultured with MSCs showed high level expression of CD206, a marker of alternatively activated subpopulation and low level expression of CD86, a marker of classically activated subpopulation [16, 17]. A third mixed subpopulation, namely CD86+ CD206+ macrophages, has also been proposed [18, 19]. Dong P et al. [20] showed that the CD86+CD206+ tumour-associated macrophages significantly correlate with the prognosis of α-fetoprotein-negative patients. Recently, Smith T et al. [11] observed that the LPS-stimulated macrophages may acquire a mixed activation state, with some cells expressing both M1 marker CD86 and M2 marker CD206.

Our results show that the LPS-stimulated RAW264.7 macrophages are divided into three groups based on their surface markers CD86 and CD206: classically activated subpopulation (M1), alternatively activated subpopulation (M2), and the mixed subpopulation (named M1.5). When the M1 subpopulation is pro-inflammatory and the M2 subpopulation is anti-inflammatory, the M1.5 subpopulation represents a large group (10~80%, varying by different treatments) of intermediate macrophages. It appears to be a transient state from M1 subpopulation to M2 subpopulation when treated with hUCMSCs, or vice versa. Cho D et al. [21] observed that MSCs can reciprocally regulate the M1/M2 balance of mouse bone marrow-derived macrophages, consistent with our finding. Our finding also indicates that macrophage differentiation should be understood as a dynamic continuum since the surrounding conditions of macrophages are complex [19, 22]. A pro-inflammatory state may coexist with an anti-inflammatory state to allow a more efficient and effective response to potentially harmful damages [23].

iNOS, an intracellular marker of M1 subpopulation, has been found supressed by MSC [24]. iNOS converts l-arginine to l-citrulline and nitric oxide that interacts with reactive oxygen species to exert pro-inflammatory effects [25]. Our results show that hUCMSCs supress generation of iNOS and prevent inflammation. Luan B et al. [26] showed that PGE_2 stimulates M2 polarization via the cyclic AMP-responsive element binding (CREB) pathway. However, our study showed that PGE_2 does not polarize macrophages to M2 sub-population but instead results in an M1 sub-population. Since we only measured the PGE_2 concentration on co-cultured supernatant, it's difficult to find out which

cell type (hUCMSCs or LPS-stimulated RAW264.7 macrophages) secreted it, but we observed a dose-dependent relationship between PGE_2 concentration and LPS-stimulated RAW264.7 macrophages. Previous studies reported that PGE_2 inhibits the production of pro-inflammatory cytokines in LPS-treated human and murine macrophages, polarizing macrophages to a M2 subpopulation [27]. Nevertheless, our study suggests that suppression of PGE_2 by hUCMSCs is accompanied by polarization of macrophages to a M2 phenotype, indicating that PGE_2 is pro-inflammatory rather than anti-inflammatory.

In conclusion, our study finds that hUCMSCs polarize the LPS-stimulated RAW264.7 macrophages to a M2 subpopulation, associated with the significant reduction of PGE_2 and iNOS. We have also observed a mixed phenotype (CD86+CD206+) of macrophages which plays an important role in the transition between M1 and M2 sub-populations.

Acknowledgements

This work was supported by the Henan Provincial Department of Science and Technology under grant number 1521024-10081.0. We would like to thank Prof. Kai Sun, MD, PhD, director of the Henan Provincial People's Hospital Central Laboratory for his kind assistance in our experiments.

Disclosure of conflict of interest

None.

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