

## Original Article

# The inhibitory effect of mesenchymal stem cell conditioned medium on the secretion of inflammatory factor of human gingival fibroblast induced by lipopolysaccharide

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**Abstract:** A variety of cytokines and inflammatory mediators participate in the process of periodontitis. Human gingival fibroblasts (HGFs) provide nutrients and play a significant role in the repair of dentin. This study investigated the inhibitory effect of mesenchymal stem cell (MSC) conditioned medium on the secretion of inflammatory factors by HGFs that induced by lipopolysaccharide (LPS). HGFs were cultured *in vitro* and divided into three groups, including control group maintained with complete DMEM medium containing 10% FBS, LPS group treated by 60 µg/ml LPS, and induction group further treated by MSC conditioned medium based on LPS intervention. Cell proliferation was assessed by MTT assay. Cell apoptosis was determined by flow cytometry. Cell apoptosis activity was evaluated by the detection of caspase 3. Interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2) expressions were detected by real-time PCR and ELISA. Our result showed that, in LPS group, cell proliferation reduced, cell apoptosis increased, caspase 3 activity enhanced; IL-8, TNF-α, and PGE2 expressions were up-regulated, compared with that in control group ( $P < 0.05$ ). In contrast, the treatment of MSCs conditioned medium significantly elevated cell proliferation, declined cell apoptosis, attenuated caspase 3 activity, and decreased IL-8, TNF-α, and PGE2 levels compared with LPS group ( $P < 0.05$ ). In conclusion, MSCs conditioned medium protected HGF against the damage of LPS through inhibiting the secretion of inflammatory factors.

**Keywords:** Human gingival fibroblasts, mesenchymal stem cell, conditioned medium, IL-8, TNF-α, PGE2

## Introduction

Periodontitis is a kind of chronic infectious disease mainly caused by bacteria, leading to the chronic inflammation of periodontal supporting tissue, which represents one of the most common oral diseases [1, 2]. The pathogenic factors of periodontitis include bacterial endotoxin and bacterial enzymes, which can directly cause injury of periodontal epithelial tissue and connective tissue [3]. Untimely treatment may induce the inflammation in gingiva, even adversely affecting periodontium, alveolar bone, and cementum, resulting in periodontitis [4, 5]. Periodontitis is particularly found in young adults. Unobvious symptom at early stage may delay the diagnosis, aggravate the symptom, loosen tooth, and even affect other organs [6, 7].

Inflammation, orchestrated by host, plays an important role in the occurrence and progression of periodontitis. Numerous cytokines and inflammatory mediators are involved in the inflammatory response, even leading to the secondary damage towards periodontal tissue [8, 9], the main factors of which include IL-8, TNF-α, and PGE2, and related metabolites [10, 11]. These factors not only contribute to proinflammatory function, and also induce bone demineralization and facilitate bone absorption [12, 13]. Fibroblasts participated in the process of injury repair. Accumulative evidence demonstrated that fibroblasts can restrain scar dysplasia and improve wound healing via the regulation of cell function and biological behavior [14, 15]. Of note, human gingival fibroblasts (HGFs) are featured as self-healing and self-renewal that provide nutrition and promote the

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repair for dentin, which also exert maintaining the normal structure and function of myeloid tissue. The damage of HGFs leads to embrittlement and breaking of hard dental tissue, and even influences mouth chewing. Therefore, the protection of HGFs from injury or apoptosis was considered a potential way to assure normal structure and function of myeloid tissue [16, 17].

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types and play an important role in tissue repair and regeneration [18].

In this regard, this study aimed to explore the possible effect of MSC conditioned medium as well as the mechanism on HGF secreting inflammatory factors.

### Materials and methods

#### *Main reagents and instruments*

P.g-LPS was purchased from Invivogen (USA). MSC conditioned medium was bought from Yocon (China). DMEM medium, FBS, penicillin-streptomycin, and Dispasell digestive juice were purchased from Hyclone (USA). DMSO and MTT were obtained from Gibco (USA). RNA extraction kit and reverse transcription kit were derived from Axygen (USA). IL-8, TNF- $\alpha$ , and PGE2 ELISA kits were provided by R&D. Caspase 3 activity detection kit was purchased from Pall Life Sciences. The primers were designed by PrimerPrimer 6.0 and synthesized by Invitrogen (Shanghai, China). Labsystem Version 1.3.1 microplate reader was provided by Bio-rad (USA). ABI 7700 Fast real-time PCR amplifier was derived from ABI (USA). Benchtop was provided by Sutai purification equipment engineering co., Ltd (Suzhou, China). FACS Calibur flow cytometry was produced by BD (USA). MCO-5M CO<sub>2</sub> incubator was got from SANYO (Japan). Microsurgery instruments were bought from Shanghai Instrument factory. DNA amplifier was obtained from PE Gene Amp PCR System 2400. Other reagents were purchased from Sangon (China).

### Methods

#### *HGFs isolation and grouping*

The impacted mandibular tooth and accessory gingival tissue collected from the healthy adult were washed by PBS containing 2% penicillin-streptomycin and digested by  $2.4 \times 10^3$  U/ml Dispasell at 4°C overnight. Next, the tissue was

cut into 1 cm<sup>3</sup> and seeded in the 25 cm<sup>2</sup> cell culture flask at 37°C and 5% CO<sub>2</sub>. After 2 h, the flask was turned over and tissue was removed when the cells around the tissue reached 1/3. Then the cells were digested by 0.25% EDTA-trypsin and passaged when the cell fusion reached 80-90%. The cells in 2<sup>nd</sup>-3<sup>rd</sup> generation at logarithmic phase were used for experiments. HGFs were cultured *in vitro* and divided into three groups, including control group maintained with complete DMEM medium containing 10% FBS, LPS group treated by 60  $\mu$ g/ml LPS, and induction group treated by MSC conditioned medium based on LPS intervention.

#### *MTT assay*

HGFs at logarithmic phase were added with 20  $\mu$ l MTT for 4 h. Then, the plate was added with 150  $\mu$ l DMSO for 10 min and tested at 570 nm to obtain the absorbance value. Each experiment was repeated for three times.

#### *Caspase 3 activity detection*

Caspase 3 activity was tested according to the manual. The cells were digested by trypsin and centrifuged at 600 g, 4°C for 5 min. Next, the cells were lysed on ice for 15 min and centrifuged at 20000 g, 4°C for 5 min. At last, the cells were added with 2 mM Ac-DEVD-pNA and tested at 405 nm to calculate Caspase 3 activity.

#### *Flow cytometry*

HGFs were seeded in 50 mL flask at  $5 \times 10^5$ /mL and grouped. After treatment, the cells were collected and washed by PBS. Then the cells were fixed using precooled 75% ethanol at 4°C overnight. Next, the cells were washed by PBS and centrifuged at 350 g for 5 min. HGFs were resuspended in 800  $\mu$ l PBS containing 1% BSA and stained in 100  $\mu$ g/mL PI solution (3.8% Sodium Citrate, pH 7.0). At last, the cells were treated by 100 RnaseA (10 mg/ml) at 37°C for 30 min and tested by flow cytometry. The data were analyzed by FCSEXPRESS 3.0 software.

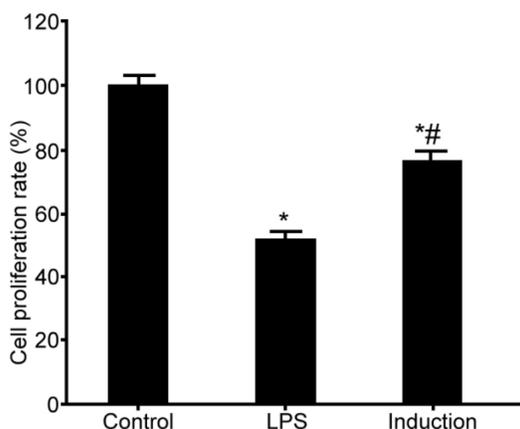
#### *Real-time PCR*

Total RNA was extracted from the HGFs and reverse transcribed to cDNA. The primers were designed using PrimerPremier 6.0 software and synthesized by Invitrogen (Shanghai, China) (**Table 1**). Real-time PCR was performed at 56°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as internal reference. The

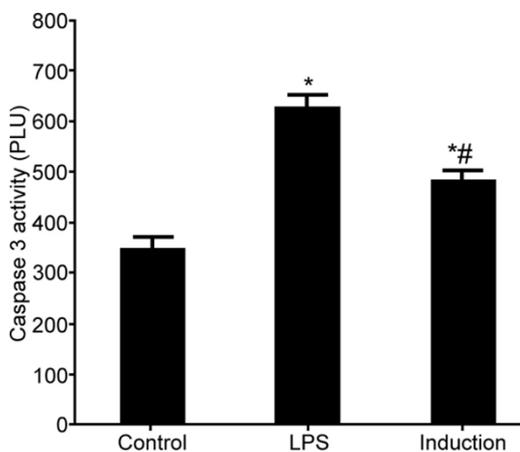
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**Table 1.** Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
IL-8	GTCACCTTCCTCAGATCCAT	AGTGTAGTTACCTCAATTT
TNF- $\alpha$	ATTGCTCGCCTCCAACC	GTGAGGTTGACAACCT
PGE2	ACCAGTCTGTCTCACCATT	TAGACCTTAATCGGAC



**Figure 1.** The impact of MSC conditioned medium on proliferation of HGFs. \*P < 0.05, compared with control. #P < 0.05, compared with LPS group.



**Figure 2.** The influence of MSC conditioned medium on Caspase 3 activity in HGFs. \*P < 0.05, compared with control. #P < 0.05, compared with LPS group.

relative expression of mRNA was calculated by  $2^{-\Delta Ct}$  method.

### ELISA

ELISA was used to test IL-8, TNF- $\alpha$ , and PGE2 contents in the serum. A total of 50  $\mu$ l diluted standard substance was added to each well to establish standard curve. Next, the plate was added with 50  $\mu$ l sample and washed for five

times. Then the plate was incubated in 50  $\mu$ l conjugate reagent at 37°C for 30 min. After being washed for five times, the plate was treated by 50  $\mu$ l color agent A and B at 37°C avoid of light for 30 min. At last, the plate was added with 50  $\mu$ l stop buffer to stop the reaction and tested at 450 nm to obtain the OD value. The OD value of standard sub-

stance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

### Statistical analysis

All data analyses were performed on SPSS19.0 software. All data were presented as mean  $\pm$  standard deviation and compared by one-way ANOVA. P < 0.05 was depicted as statistical significance.

### Results

#### *The impact of MSC conditioned medium on HGFs proliferation induced by LPS*

MTT assay was adopted to test the impact of MSC conditioned medium on HGFs proliferation induced by LPS. LPS significantly restrained HGFs proliferation compared with control (P < 0.05). Of note, in induction group, MSC conditioned medium obviously restored HGFs proliferation compared with HGFs solely treated by LPS (P < 0.05) (**Figure 1**).

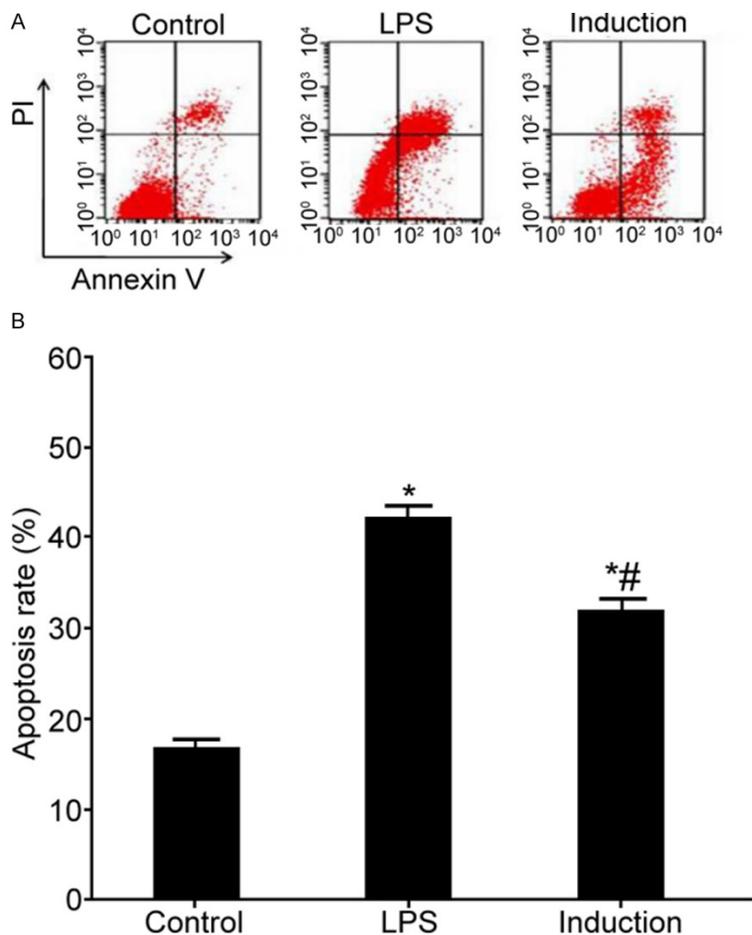
#### *The influence of MSC conditioned medium on Caspase 3 activity of HGFs induced by LPS*

In order to detect the apoptosis of HGFs, Caspase 3 activity detection kit was applied to evaluate the influence of MSC conditioned medium on Caspase 3 activity of HGFs. LPS markedly enhanced Caspase 3 activity compared with control (P < 0.05). However, MSC conditioned medium apparently suppressed Caspase 3 activity in HGFs compared to the cells with single LPS intervention (P < 0.05) (**Figure 2**).

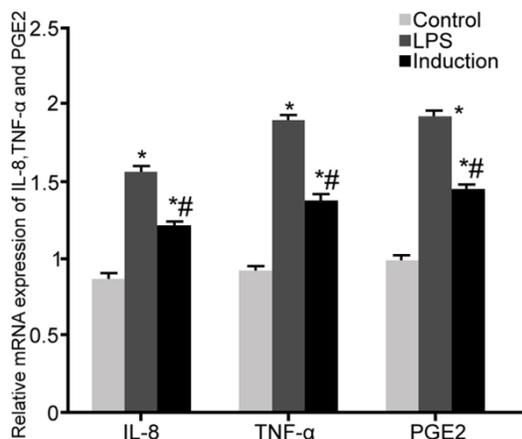
#### *The effect of MSC conditioned medium on HGFs apoptosis induced by LPS*

We further selected flow cytometry to assess the effect of MSC conditioned medium on HGFs apoptosis induced by LPS. Data showed that LPS significantly facilitated HGFs apoptosis compared with control (P < 0.05). Nevertheless, after the treatment of MSC conditioned medium, HGFs proliferation was obviously inhi-

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**Figure 3.** The effect of MSC conditioned medium on HGFs apoptosis. A. Flow cytometry detection of the effect of MSC conditioned medium on HGFs apoptosis induced by LPS; B. Effect analysis of MSC conditioned medium on HGFs apoptosis induced by LPS. \*P < 0.05, compared with control. #P < 0.05, compared with LPS group.



**Figure 4.** The impact of MSC conditioned medium on IL-8, TNF-α, and PGE2 mRNA expressions in HGFs. \*P < 0.05, compared with control. #P < 0.05, compared with LPS group.

bited compared with LPS group (P < 0.05) (Figure 3), suggesting that MSC conditioned medium suppressed HGFs apoptosis caused by LPS.

*The impact of MSC conditioned medium on IL-8, TNF-α, and PGE2 expressions of HGFs induced by LPS*

Real-time PCR was used to evaluate the impact of MSC conditioned medium on mRNA expressions of inflammatory factors including IL-8, TNF-α, and PGE2. The expressions of IL-8, TNF-α, and PGE2 mRNA were markedly increased compared with that of control (P < 0.05). In the contrast, by adding MSC conditioned medium, the levels of IL-8, TNF-α, and PGE2 mRNA in HGFs were apparently down-regulated compared with that in the group of single LPS intervention (P < 0.05) (Figure 4).

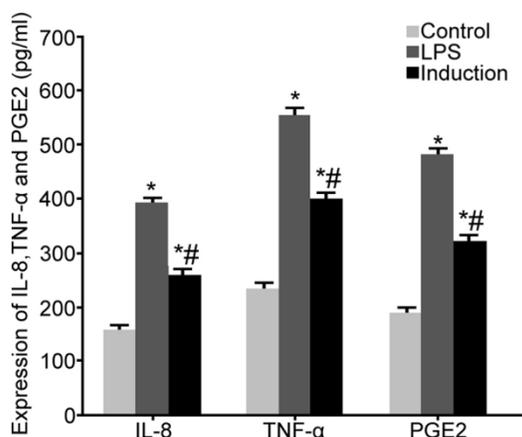
We further validate IL-8, TNF-α, and PGE2 secretion in HGFs at protein level by using ELISA. Our result indicated that the expressions of IL-8, TNF-α, and PGE2 proteins were markedly elevated by LPS compared with that of control

(P < 0.05). However, the promoting role of LPS was apparently inhibited by MSC conditioned medium along with reduction of IL-8, TNF-α, and PGE2 secretion in HGFs compared with that with single LPS intervention (P < 0.05) (Figure 5).

### Discussion

As the one of the main components in the cell walls of Gram negative bacteria (GNB), LPS is the major active constituent of endotoxin and is profiled with many biological activities. High concentration of LPS can cause the activation of inflammatory cascade and induce the immune response [19]. The periodontal disease represents a chronic inflammatory disease that destroys bone and gum tissues that

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**Figure 5.** The influence of MSC conditioned medium on IL-8, TNF- $\alpha$ , and PGE2 secretion in HGFs. \*P < 0.05, compared with control. #P < 0.05, compared with LPS group.

support the teeth. The exact pathogenesis of periodontal disease is complex and varies, among which, bacterial infection is considered as a major cause of periodontal disease, leading to periodontal pain and pulp necrosis [20, 21]. In the process of periodontal disease progression, the concentration of LPS accumulates in the blood circulation that promotes the release of a large amount of inflammatory factors from inflammation tissue, resulting in dysfunction of glycometabolism and energy metabolism, damage of mitochondria and lysosome, and direct or indirect toxic effects on dental pulp cells [22, 23]. In this study, we also confirmed that, after the stimulation of LPS, HGFs proliferation was significantly inhibited with the increase of apoptotic activity. Moreover, the expressions of inflammatory cytokines IL-8, TNF- $\alpha$ , and PGE2 were upregulated, indicating an aggravating state of inflammation. Therefore, the alleviation of inflammation becomes a research focus to protect the periodontal tissue from the infection of pathogenic bacteria.

Mesenchymal stem cell conditioned medium is the medium where the stem cells are cultured, among which, various stem cell-derived secreted factors and tissue regenerative agents including secretome, microvesicles, or exosome exist [24]. Treating tumor cells with conditioned medium from MSCs has been used as a useful model in many diseases such as neurodegenerative diseases, ovarian cancer [24, 25]. Accumulative evidence demonstrated that

MSC conditioned medium has been widely applied in the clinical therapy. For instance, the medium accelerates wound healing with fewer scars [26]. Besides, it also attenuated the degeneration of axons and myelin of spinal motor neurons [27]. Mesenchymal stem cells played anti-inflammatory roles during innate immune responses [28]. Recent studies also found that MSCs conditioned medium can promote human mesenchymal stem cell growth, proliferation, and differentiation potential [29]. However, there is still lack of reports about the role of MSCs conditioned medium on HGFs induced by LPS. This study showed that MSCs conditioned medium treatment significantly elevated cell proliferation, declined cell apoptosis, attenuated Caspase 3 activity, and decreased IL-8, TNF- $\alpha$ , and PGE2 levels, which was consistent with previous finding that the expressions of TNF- $\alpha$  and IL-6 in macrophages were suppressed by MSCs conditioned medium [28]. MSCs conditioned medium exerted therapeutic effect on liver injury by inhibiting inflammation [30]. In a similar fashion, our data unravel that, by inhibiting inflammatory cytokines secretion, MSCs conditioned medium can protect HGFs from LPS induction and induces cell apoptosis, although the specific mechanism remains to be further discussed. Inflammatory responses in the gingival tissues were demonstrated to be associated to patients with periodontitis [31]. This study illustrates that MSCs conditioned medium plays an inhibitory role during the development of periodontitis via the alleviation of inflammatory responses, which lays academic basis for further treatment of periodontitis.

### Conclusion

MSCs conditioned medium protected HGF from the damage of LPS through inhibiting inflammatory factors secretion, thus providing research direction and theoretical basis for the clinical treatment of periodontal disease.

### Disclosure of conflict of interest

None

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