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A study on the relationship between changes in serum hs-CRP levels and Chinese ischemic stroke subclassification

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Abstract

Objective: To explore the effects of combined application of culture supernatant of human umbilical cord mesenchymal stem cells (hUCMSCs) and ciprofloxacin on *Staphylococcus aureus* (SA) in vitro.
Methods: hUCMSCs were isolated from umbilical cord tissues of full-term healthy fetuses after cesarean section and then cultured. Cells in the third passage were chosen for the use of experiment after identification. SA strains isolated from wounds of burn patients in our burn wards were used in the following experiment. Cells were divided into 0, 10, 100 and 1,000 ng/ml lipopolysaccharide (LPS) groups by use of the random number table (similarly hereinafter). Cells were cultured with culture medium containing mesenchymal stem cells (MSCs) after being treated with medium containing corresponding mass concentrations of LPS for 12 h. At post culture hour (PCH) 6, 12 and 24, 6 wells of culture supernatant of cells in each group were obtained to measure the content of LL-37 with enzyme-linked immunosorbent assay (ELISA). Ninety blood agar culture plates were divided into ciprofloxacin control group (CC), ciprofloxacin + supernatant group (CS), and ciprofloxacin + supernatant + LL-37 antibody group (CSL), with 30 blood agar culture plates in each group. Blood agar culture plates in group CC were coated with 1.5×10^8 colony forming unit (CFU)/ml bacteria solution prepared with normal saline. Blood agar culture plates in group CS were coated with 1.5×10^8 CFU/ml bacteria solution prepared with normal saline and hUCMSC culture supernatant (cultured by MSC culture medium, the same below) in double volume of normal saline. Blood agar culture plates in group CSL were coated with 1.5×10^8 CFU/ml bacteria solution prepared with normal saline, hUCMSC culture supernatant in double volume of normal saline, and 2.6 μL of LL-37 antibodies at the concentration of 2 μg/ml. At PCH 12, 24 and 48, 10 blood agar culture plates were taken out from each group to observe the distribution of SA colonies on blood agar culture plates and to measure diameters of zones of inhibition of ciprofloxacin. The minimum inhibitory concentration (MIC) of ciprofloxacin against SA in each group was recorded. Fractional inhibitory concentration (FIC) indexes of ciprofloxacin in group CS and group CSL at PCH 12, 24 and 48 were calculated, with the synergistic effect evaluated. Data were processed with factorial design ANOVA, one way ANOVA, LSD-*t* test, Kruskal-Wallis test and Mann-Whitney *U* test.
Results: (1) At each PCH, the content of LL-37 in cell culture supernatant in 10 ng/ml LPS group, 100 ng/ml LPS group or 1,000 ng/ml LPS group was higher than that in 0 ng/ml LPS group (with *t* values ranging from 11.22 to 33.36, *p* values all below .01); the content of LL-37 in cell culture supernatant in either 100 ng/ml LPS group or 1,000 ng/ml LPS group was higher than that in 10 ng/ml LPS group (with *t* values ranging from 2.24 to 18.73, $p < .05$ or $p < .01$); the content of LL-37 in cell culture supernatant in 1,000 ng/ml LPS group was higher than that in 100 ng/ml LPS group (with *t* values ranging from 12.46 to 14.70, *p* values all below .01). (2) At PCH 12, 24 and 48, the bacterial colonies in groups CC, CS and CSL became integrated over time. In CC group, diameters of zones of inhibition of ciprofloxacin at PCH 12, 24 and 48 were 26 mm, 24 mm and 23 mm respectively, with no obvious changes. At PCH 12, 24 and 48, diameters of zones of inhibition of ciprofloxacin in groups CS and CSL were 82 mm, 71 mm, 68 mm and 74 mm, 59 mm, 56 mm respectively, which were significantly larger than those in group CC. (3) At each PCH, MIC of ciprofloxacin against SA in group CC was significantly higher than that in groups CS and CSL respectively (with *Z* values ranging from 6.22 to 6.71, *p* values all below .01); MIC of ciprofloxacin against SA in group

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CSL was significantly higher than that in group CS (with Z values all equal to 6.72, p values all below .01). (4) FIC indexes of ciprofloxacin in groups CS and CSL at PCH 12, 24 and 48 were 0.011, 0.032, 0.032 and 0.122, 0.350, 0.350, respectively. The results indicated that hUCMSC culture supernatant had a synergistically antibacterial effect when combined with ciprofloxacin.
Conclusions: hUCMSCs can secrete LL-37, and the secretion level is improved with increase of LPS concentration. The combination of hUCMSC culture supernatant with ciprofloxacin can decrease the dosage of ciprofloxacin in resisting SA effectively. Once LL-37 is neutralized, the synergistically antibacterial effect of hUCMSC culture supernatant is decreased thereby.

Key Words: Mesenchymal stem cells, *Staphylococcus aureus*, Dermcidins, Ciprofloxacin

Human umbilical cord mesenchymal stem cell (hUCMSC) is a type of primitive stem cells and has the advantages of strong proliferation ability, short amplification time, low immunogenicity, and no need for tissue typing when performing allogeneic transplantation.[1] To this end, it comes to a focus in the stem cell research. Studies have shown that hUCMSCs have a paracrine effect and can secrete various cytokines, nerve growth factors, glial-derived neurolrophic factors etc., playing an important role in regenerative medicine.[2] In addition, hUCMSCs are able to secrete a variety of peptides, which are present in cell culture supernatant. Biological antimicrobial peptides occupy the main position among many types of peptides, typically known as LL-37, human β-Defensin-2 etc.[3] Therefore, the effect of hUCMSC combined with ciprofloxacin on *Staphylococcus aureus* was observed in vitro in this research, which provided a scientific basis for the antibacterial application of hUCMSC culture supernatant combined with antibiotics in the future.

1 Materials and methods

1.1 Main materials

This study was approved by Ethics Committee in corresponding author's affiliation (approval number: 2015MER-13), and umbilical cord tissues were obtained from full-term healthy fetuses after cesarean section in Department of Obstetrics and Gynecology in our hospital. It is required to obtain informed consent from puerperae and their relatives before specimens were collected. hUCMSCs were isolated in our experimental center. After cultivation and identification, cells in the third passage were chosen for the use of experiment. SA strains isolated from wounds of burn patients in our burn wards were provided by Clinical Laboratory in our hospital.

DMEM/F12 medium, FBS, 10 U/ml penicillin-streptomycin double-antibody were purchased from Gibco Corporation (USA), LPS was purchased from Sigma Corporation (USA). LL-37 test kits were purchased from Shanghai Jijin Chemical Technology Co., Ltd., rabbit anti-human LL-37 antibodies were purchased from Bioss Antibodies (Beijing, China), and ciprofloxacin E-Test susceptibility test strips were purchased from BioMérieux (France). The CO_2 incubator was purchased from SANYO Corporation of Japan, the photoelectric turbidimeter was purchased from Shanghai Jinjia Scientific Instruments Co., Ltd., blood agar culture plates were purchased from Guangdong Huankai Microbial Technology Co., Ltd., and McFarLand was purchased from Wenzhou Kangtai Biotechnology Co., Ltd. The constant temperature incubator was purchased from Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd. The CKX41 inverted phase contrast microscope was purchased from Olympus Corporation in Japan.

1.2 Determination of LL-37 content in culture supernatant of hUCMSC

The cells were harvested and seeded in twelve 6-well culture plates at a density of 5×10^6 cells/well. Meanwhile, these cells were divided into 0, 10, 100 and 1,000 ng/ml LPS groups by use of the random number table (the same below), with 3 plates in each group. And then, 3 ml of mesenchymal stem cell (MSC) culture medium (DMEM/F12 medium + 10% FBS [V/V] + 10 U/ml penicillin-streptomycin double-antibody) were added into each well in the culture plate. In 0, 10, 100 and 1,000 ng/ml LPS groups, cells were cultured with medium only containing mesenchymal stem cells (MSCs) after being treated with MSC medium added by corresponding mass concentration of LPS for 12 h. At PCH 6, 12 and 24, the cell culture supernatant was collected from one plate in each group, and ELISA was used to determine the content of antimicrobial peptide LL-37.

1.3 Antibacterial activity of hUCMSC culture supernatant combined with ciprofloxacin in vitro

Staphylococcus aureus strains were evenly inoculated in blood agar culture plates, which were laid upside down in the constant temperature incubator set at 37°C. After 24 h, it was needed to observe the growth of colonies, with the well-grown picked out and used for the following experiment after amplification. Cells were seeded in three 6-well

plates at a density of 5 × 10^6 cells/well, and 3 ml of MSC culture medium was added to each well. After 24-hour cultivation, the cell culture supernatant was collected for the following experiment.

1.3.1 Distribution of SA colonies, diameters of zones of inhibition of ciprofloxacin and MIC of ciprofloxacin against SA

Ninety blood agar culture plates were divided into ciprofloxacin control group (CC), ciprofloxacin + supernatant group (CS), and ciprofloxacin + supernatant + LL-37 antibody group (CSL), with 30 blood agar culture plates in each group. Blood agar culture plates in group CC were coated with 1.5 × 10^8 CFU/ml SA bacteria solution prepared with normal saline. Blood agar culture plates in group CS were coated with 1.5 × 10^8 CFU/ml SA bacteria solution prepared with normal saline and hUCMSC culture supernatant (cultured by MSC culture medium) in double volume of normal saline. Blood agar culture plates in group CSL were coated with 1.5 × 10^8 CFU/ml bacteria solution prepared with normal saline, hUCMSC culture supernatant in double volume of normal saline, and 2.6 μL of rabbit anti-human LL-37 antibodies at the concentration of 2 μg/ml. After 15 minutes, ciprofloxacin E-Test susceptibility test strips were flattened on the centers of the blood agar culture plates and placed in the constant temperature incubator. At PCH 12, 24 and 48, 10 blood agar culture plates were taken out from each group to observe the distribution of SA colonies on each blood agar culture plate and to measure diameters of zones of inhibition of ciprofloxacin. The minimum inhibitory concentration (MIC) of ciprofloxacin against SA in each group was recorded.

1.3.2 Fractional inhibitory concentration (FIC) index of ciprofloxacin

FIC index of a certain drug can be calculated by dividing MIC of the drug (used in combination) by MIC of the drug used alone. Fractional inhibitory concentration (FIC) indexes of ciprofloxacin in group CS and group CSL at PCH 12, 24 and 48 were calculated as a consequence. The evaluation criteria are as follows: that FIC is no more than 0.5 indicates synergistic effect, that FIC is more than 0.5 and no more than 1.0 indicates additive effect, that FIC is more than 1.0 and no more than 4.0 indicates indifferent effect, FIC more than 4.0 indicates antagonistic effect. Synergistic and additive effects are considered to be valid, while indifferent and antagonistic effects are invalid.

Table 1: The comparison of LL-37 content in hUCMSC culture supernatant in each group at PCH (ng/ml, $\bar{x}$ ± s)

Group	n	PCH 6	PCH 12	PCH 24
0 ng/ml LPS Group	18	4.2 ±0.4	4.7 ±0.4	5.2 ±0.4
10 ng/ml LPS Group	18	6.9 ±0.5	7.1 ±0.5	7.4 ±0.6
100 ng/ml LPS Group	18	7.7 ±0.6	7.8 ±0.5	7.9 ±0.6
1,000 ng/ml LPS Group	18	9.9 ±0.6	10.3 ±0.6	10.6 ±0.7
F value		367.89	376.43	253.38
p value		< .001	< .001	< .001
t_1 value		15.85	14.63	11.22
p_1 value		< .01	< .01	< .01
t_2 value		20.37	18.66	13.46
p_2 value		< .01	< .01	< .01
t_3 value		32.83	33.36	27.41
p_3 value		< .01	< .01	< .01
t_4 value		4.52	4.03	2.24
p_4 value		< .01	< .01	< .01
t_5 value		16.97	18.73	16.19
p_5 value		< .01	< .01	< .01
t_6 value		12.46	14.70	13.95
p_6 value		< .01	< .01	< .01

Note. hUCMSC: human umbilical cord mesenchymal stem cell; main effect of treatment factor, F = 381.37, p < .01; main effect of time factor, F = 101.31, p < .01; mutual effect of treatment and time factors, F = 10.75, p < .01; F value and p value are resulted from the overall comparison among groups at each PCH; t_1 and p_1 values, t_2 and p_2 values, t_3 and p_3 values are resulted from the comparison between 0 ng/ml LPS Group and 10 ng/ml LPS Group, between 0 ng/ml LPS Group and 100 ng/ml LPS Group, between 0 ng/ml LPS Group and 1,000 ng/ml LPS Group respectively; t_4 value and p_4 value, t_5 value and p_5 value, t_6 value and p_6 value are resulted from the comparison between 10 ng/ml LPS Group and 100 ng/ml LPS Group, between 10 ng/ml LPS Group and 1,000 ng/ml LPS Group, between 100 ng/ml LPS Group and 1,000 ng/ml LPS Group respectively

1.4 Statistical treatment

The data were represented by $\bar{x}$ ± s. SPSS 17.0 statistical software was applied to factorial design ANOVA of all data. In addition, one way ANOVA was performed to the data fitted normal distribution when in the comparison among groups, while LSD-t test was performed in the comparison between two groups; as to the data not fitted normal distribution, Kruskal-Wallis test was performed in the comparison among groups, and Mann-Whitney U test was performed in the comparison between two groups. The difference ($p < .05$) was of statistical significance.

2 Results

2.1 LL-37 content in hUCMSC culture supernatant

At each PCH, the content of LL-37 in cell culture supernatant in 10 ng/ml LPS group, 100 ng/ml LPS group or 1,000 ng/ml LPS group was higher than that in 0 ng/ml LPS group ($p < .01$); the content of LL-37 in cell culture supernatant in either 100 ng/ml LPS group or 1,000 ng/mL LPS group was higher than that in 10 ng/ml LPS group ($p < .05$ or $p < .01$); the content of LL-37 in cell culture supernatant in 1,000 ng/ml LPS group was higher than that in 100 ng/ml LPS group ($p < .01$, see Table 1).

2.2 Distribution of SA colonies and diameters of zones of inhibition of ciprofloxacin

At PCH 12, 24 and 48, the bacterial colonies in groups CC, CS and CSL became integrated over time. In group CC, diameters of zones of inhibition of ciprofloxacin at PCH 12, 24 and 48 were 26 mm, 24 mm and 23 mm respectively, with no obvious changes. At PCH 12, 24 and 48, diameters of zones of inhibition of ciprofloxacin in groups CS and CSL were 82 mm, 71 mm, 68 mm and 74 mm, 59 mm, 56 mm respectively. In these two groups, diameters of zones of inhibition of ciprofloxacin at PCH 12 were larger than those at PCH 24, and diameters of zones of inhibition of ciprofloxacin at PCH 24 were in proximity to those at PCH 48. At each PCH, diameters of zones of inhibition of ciprofloxacin in groups CS and CSL were significantly larger than those in group CC (see Figure 1).

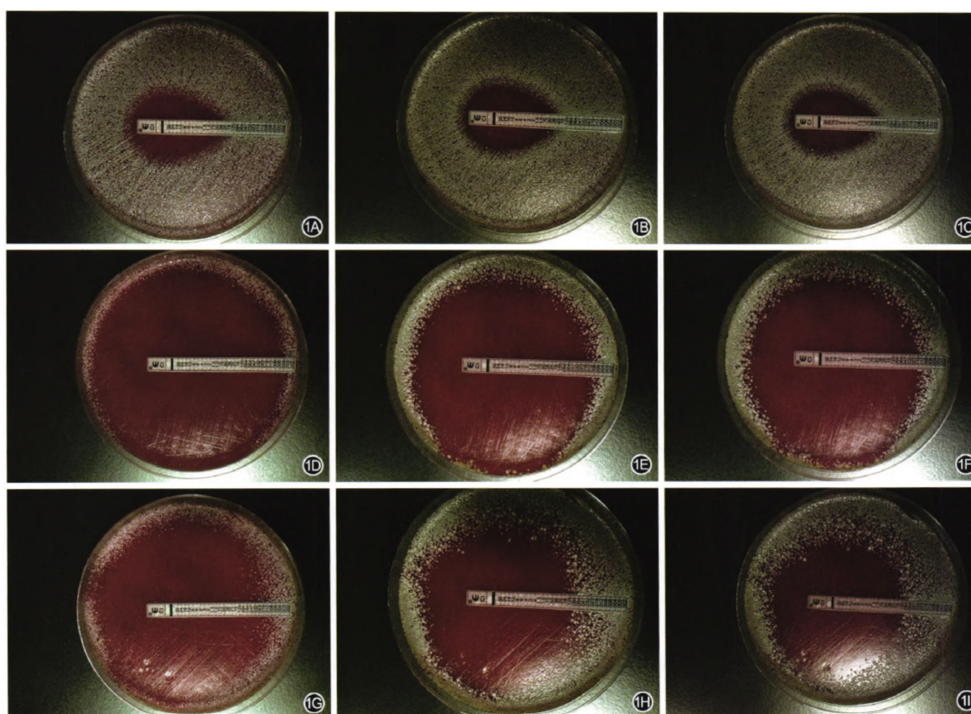

Figure 1: Figure 1 Distribution of SA colonies and diameters of zones of inhibition of ciprofloxacin in the three groups at each PCH

Figure 1A, 1B and 1C stand for CC group at PCH 12, 24 and 48 respectively, the bacterial colonies in this group become integrated over time, and the diameters of zones of inhibition are small with no obvious changes; Figure 1D, 1E and 1F stand for CS group at PCH 12, 24 and 48 respectively, the bacterial colonies in this group become integrated over time, and the diameters of zones of inhibition become smaller, but still larger than those in CC group; Figure 1G, 1H and 1I stand for CSL group at PCH 12, 24 and 48 respectively, the bacterial colonies in this group become integrated over time, and the diameters of zones of inhibition become smaller, but still larger than those in CS group and smaller than those in CC group; Ciprofloxacin E-Test susceptibility test strip was flattened on the center of the blood agar culture plate

Table 2: The comparison in MIC of ciprofloxacin against SA in each group at each PCH (μg/ml, $\bar{x} \pm$ s)

	n	PCH 12	PCH 24	PCH 48
CC Group	30	0.4600 ±0.1543	0.4601 ±0.1544	0.4601 ±0.1545
CS Group	30	0.0050 ±0.0010	0.0141 ±0.0013	0.0141 ±0.0013
CSL Group	30	0.0564 ±0.0497	0.1611 ±0.1648	0.1614 ±0.1665
χ^2 value		79.62	77.12	77.04
p value		< .001	< .001	< .001
Z_1 value		6.71	6.71	6.71
p_1 value		< .01	< .01	< .01
Z_2 value		6.67	6.24	6.22
p_2 value		< .01	< .01	< .01
Z_3 value		6.72	6.72	6.72
p_3 value		< .01	< .01	< .01

Note. Main effect of treatment factor, $F = 150.90$, $p < .001$; main effect of time factor, $F = 8.23$, $p = .001$; mutual effect of treatment and time factors, $F = 6.40$, $p < .001$; χ^2 and p values are resulted from the overall comparison among groups at each PCH; Z_1 and p_1 values, Z_2 and p_2 values, Z_3 and p_3 values are resulted from the comparison between CC Group and CS Group, between CC Group and CSL Group, between CS Group and CSL Group respectively

2.3 MIC of ciprofloxacin against SA

At each PCH, MIC of ciprofloxacin against SA in group CC was significantly higher than that in either group CS or group CSL respectively ($p < .01$); MIC of ciprofloxacin against SA in group CSL was significantly higher than that in group CS ($p < .01$, see Table 2).

2.4 FIC index of ciprofloxacin

FIC indexes of ciprofloxacin in groups CSL and CS at PCH 12, 24 and 48 were 0.122, 0.350, 0.350 and 0.011, 0.032, 0.032, respectively. FIC indexes of ciprofloxacin in these two groups mentioned above were both less than 0.5, i.e., hUCMSC culture supernatant had a synergistically antibacterial effect when combined with ciprofloxacin.

3 Discussion

Recent studies have shown that hUCMSC can secrete various cytokines through the paracrine mode to further promote the effect on the repair of damaged cells, tissue repair and reconstruction. Therefore, hUCMSC comes to the focus in the research on regenerative medicine.[4–9] Its culture supernatant also has a similar treatment effect.[10,11] Studies have shown that MSC conditioned through hypoxia,[12] low nutrition and inflammatory simulators,[13,14] can significantly increase and add the variety to paracrine cytokines in these stem cells, and significantly enhance their tolerance ability to injury factors, so as to improve the therapeutic potential. This study showed that LL-37 could be detected in hUCMSC culture supernatant. After hUCMSCs were conditioned by LPS at an appropriate concentration, the volume of paracrine LL-37 would be improved with the increase of LPS concentration.

Studies have shown that hUCMSCs can delay the development of drug resistance in *P. aeruginosa*, and its mechanism is probably to inhibit the expression of outer membrane proteins through the secretion of antimicrobial peptide LL-37 and human β-Defensin-2.[15] This study showed that, diameters of zones of inhibition of ciprofloxacin in groups CS and CSL were significantly larger than those in group CC, indicating that hUCMSC culture supernatant had a synergistically antibacterial effect when combined with ciprofloxacin. However, in CS group and CSL group, diameters of zones of inhibition of ciprofloxacin at PCH 24 were in proximity to those at PCH 48, i.e., the synergistically antibacterial effect was weakened. The cause was probably that the bacterial reproduction came to a stable order-of-magnitude.

Currently, LL-37 is the only cathelcidin-family antimicrobial peptide which exists in the human body.[16] The recognized sterilization mode of LL-37 is a "carpet-like" pattern, namely, it can destroy the lipid bi-layer structure to rupture cell membranes. Studies have shown that antimicrobial peptides can enhance the effect of antibiotics on killing clostridium prazmowski.[17] Targeting at bacterial DNA, quinolones can impede DNA gyrases, so that bacterial cells will not divide any more. This study showed that, at each PCH, MIC of ciprofloxacin against SA in group CC was significantly higher than that in either group CS or group CSL, indicating that the combination of hUCMSC culture supernatant with ciprofloxacin has an obviously synergistic inhibition effect on the growth of SA in vitro environment to lower MIC of ciprofloxacin. At each PCH, MIC of ciprofloxacin against SA in group CSL was significantly higher than that in group CS, but it was still obviously lower than the normal value. It was indicated that the inhibition

ability of hUCMSC culture supernatant was significantly weakened due to the neutralization of LL-37, but there existed other biologically active substances (which had a synergistic inhibition effect) in the culture supernatant.

FIC index of a drug is an important parameter for evaluating the combined effect of antibiotics. In this study, FIC indexes of ciprofloxacin at PCH 12, 24 and 48 in CS group were significantly less than 0.5, indicating that hUCMSC culture supernatant had a synergistically antibacterial effect; After hUCMSC culture supernatant was added by LL-37 antibodies to silence LL-37, FIC indexes of ciprofloxacin in CSL group were still less than 0.5, indicating that in addition to LL-37 in hUCMSC culture supernatant, there may be other substances involved in the synergistically antibacterial activity, indicating that it was consistent with the results from relevant studies;[15] FIC indexes of ciprofloxacin at PCH 12, 24 and 48 in CSL group were about 10 times higher than those in CS group, indicating that the synergistically antibacterial effect of hUCMSC culture supernatant was weakened after LL-37 was silenced, i.e., LL-37 in hUCMSC culture supernatant had a strong synergistically antibacterial effect.

In summary, the combination of hUCMSC culture supernatant with ciprofloxacin in the antibacterial application can effectively reduce the dosage of ciprofloxacin. In the future, hUCMSC culture supernatant conditioned by an appropriate concentration of LPS can be used for antibacterial research, even combined with other types of antibiotics to further explore the antibacterial effect of the supernatant.

Conflicts of Interest Disclosure

The authors have no conflicts of interest related to this article.

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