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LPS at low concentration promotes the fracture healing through regulating the autophagy of osteoblasts via NF-κB signal pathway

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Abstract. – OBJECTIVE: To investigate the effect of low-concentration lipopolysaccharide (LPS) on proliferation and apoptosis of osteoblasts and to discover the mechanism of low-concentration LPS in facilitating the proliferation of osteoblasts.

MATERIALS AND METHODS: MC3T3-E1 osteoblasts were treated with LPS, 3-methyladenine (3-MA, autophagy inhibitor), and BAY11-7082 (inhibitor of nuclear factor-kappa b, NF-κB), respectively. The cell cycles were detected using a flow cytometer. Cell proliferation and activity of MC3T3-E1 osteoblasts were explored by cell counting kit-8. Western blotting and immunofluorescence assay were performed to detect the protein level. RNA expression was measured through polymerase chain reaction (PCR) and immunofluorescence assay.

RESULTS: At the third day after cell culture, cell infusion reached 80%, and cells were taken as the subjects. At 6 h after treatment with low-concentration LPS, the proliferation and activity of cells were higher than those at 1 h and 12 h after treatment, and the apoptotic level was significantly lower than that in cells at 12 h after treatment. The proliferation and activity of cells in the low-concentration LPS group were significantly higher than those in the control group, 3-MA group and BAY11-7082 group, and the apoptotic level was lower than those in these groups. Compared with those of cells in control group and BAY11-7082 group, the messenger RNA (mRNA) and protein expressions and nuclear transfer of cells in low-concentration LPS group were significantly elevated, but there were no statistically significant differences in comparisons with the 3-MA group. In the experiment of cell autophagy, the autophagic level in cells in low-concentration LPS group was higher than those in the control group, 3-MA group and BAY11-7082 group.

CONCLUSIONS: Through the NF-κB signaling pathway in osteoblasts, low-concentration LPS can activate the autophagy and promote cell proliferation, thereby inhibiting cell apoptosis and accelerating the fracture healing.

Key Words:
Lipopolysaccharide, Fracture, Autophagy, Osteoblasts, NF-κB.

Introduction

Bone regeneration refers to a complicated restorative process involving osteoblasts as well as other cells and cytokines, where osteoclast-induced bone resorption and bone formation, as the core, forming a dynamically balanced process[1]. Fracture healing and bone metabolism all through one's life are dependent on the bone regeneration[2]. However, medical professionals are always confused by the osteoporosis caused by higher bone absorption rate than bone formation rate with an increase in age, and the delayed fracture healing, or even bone nonunion led by stagnated bone formation in the middle-aged patients[3]. In recent years, cytokines involved in the regulation of bone regeneration have become research hotspots[4,5], but the mechanism of these pro-inflammatory cytokines in bone regeneration that are critical to the initial stage in activating the fracture healing has not yet been fully elucidated. Lipopolysaccharide (LPS) and prostaglandin E2 (PGE2) are major pro-inflammatory cytokines in bone regeneration[6-8].

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LPS can induce the release of various inflammatory cytokines, and has been compared to a "double-edged sword" for its effects on cells and tissues[9]. Previous studies indicated that LPS can directly induce the apoptosis and necrosis of cells and tissues, while the latest study has revealed that low-concentration LPS can also promote the proliferation of cells or tissues[9,10]. There remain few studies reporting the effect of low-concentration LPS on promoting cell proliferation. NF-κB is a fast-response transcription factors, which widely exists in cytoplasm as p65-p50 dimer. It is usually activated by LPS-induced PGE2 secretion, and inhibiting the activation of NF-κB can hinder the bone loss caused by insufficiency of estrogen[11]. The autophagy mechanism is highly conserved in evolution and widely exists in eukaryotes. Recently, autophagy has attracted the attention of researchers, which found that the mechanism of autophagy has vital effects on disease courses, vascular and endocrine diseases[12,13]. During autophagy, the regulation is mediated by autophagy-related genes (ATGs), in which Beclin1 and light chain 3 (LC3) are key ATGs in autophagy[14]. In stress environment, cells can initiate the autophagic functions to sustain the activity of cells in reverse environment, like disease[15]. In this study, the enhanced effect of low-concentration LPS on proliferation of osteoblasts was associated with the NF-κB signaling pathway and cell autophagy; the roles of NF-κB signaling pathway and cell autophagy were explored using specific inhibitors of them.

Materials and Methods

Experiment Materials and Reagents

MC3T3-E1 osteoblasts (Institute of Biochemistry and Cell Biology, Shanghai, China); cell count kit-8 (CCK8), flow cytometry kit for cell cycle and alkaline phosphatase (ALP) kit (Beyotime Biotech Co., Ltd, Shanghai, China); 3-methyladenine (3-MA) and BAY11-7082 (Sigma-Aldrich, St. Louis, MO, USA); antibodies of p65, Beclin1, autophagy protein 5 (Atg5) and LC3 (Cell Signaling Technology, Danvers, MA, USA).

Cell Culture and Grouping

Dulbecco's modified Eagle medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin were used for regular culture of MC3T3 E1 osteoblasts in a CO_2 incubator, and medium was replaced every other day. When cell infusion reached 80%, cells were digested with 0.25% trypsin containing 0.02% ethylene diamine tetraacetic acid (EDTA) for subculture. In control group, cells were cultured in general DMEM/F12 medium, while those in LPS group were treated with the culture medium containing 500 ng/mL LPS for 1 h, 6 h and 12 h; cells in 3-MA group were treated with 3-MA for 6 h, during which cells were additionally treated with LPS (500 ng/mL) for 1 h, 6 h and 12 h; in the BAY11-7082 group, 5 μmol/L BAY11-7082 was used to treat cells for 6 h, in which cells were additionally treated with lipopolysaccharide (LPS, 500 ng/mL) for 1 h, 6 h and 12 h.

CCK8

Cells in the logarithmic phase were used for CCK8 experiment (KeyGenBioTech, Jiangsu, China). In brief, cells were firstly digested using 0.25% trypsin-EDTA followed by centrifugation with the supernatant being discarded. Cell suspension was prepared using DMEM medium supplemented with 10% fetal bovine serum (FBS); for cell count, cells were diluted to a density of 1.0×10^5/mL. Cells were seeded onto a 96-well plate for detecting cell vitality in following procedures: cell suspension was inoculated on a 96-well plate (100 μL/well); thereafter, cells were cultured in an incubator for 4 h (37°C and 5% CO_2) till cells adhered to the wall. Following the requirement of experiment, cells were intervened, in which 4 wells were set for each group; CCK8 reagent was added into each well in a 96-well plate followed by incubation in an incubator (37°C and 5% CO_2) for 4 h. Then, the optical density at a wavelength of 450 nm was detected using a microplate reader.

Cell Cycle Detection via Cell Cytometry

Cells were digested using trypsin and collected for washing with 0.01 mol/L phosphate-buffered saline (PBS) once. Thereafter, cells were centrifuged at 1000 rpm for 5 min for collection again to prepare cell suspension at density of 1×10^6/mL. 1 mL cell suspension was extracted and slowly added into 10 mL pre-cooled 70% ethanol on ice for fixation at 4°C for at least 24 h. The fixed single cell suspension was washed using phosphate-buffered saline (PBS) twice followed by centrifugation at 1000 rpm for 5 min with the supernatant being discarded. In the sediment, 300 μL propidium iodide (PI) was added with 30 μL RNase (10 mg/mL) in water bath at 37°C for 30 min to eliminate the interference of

RNAs. Thereafter, samples were loaded onto the machine for detection of cell cycle.

Cell Apoptosis Detection Via Flow Cytometry

Cells in the logarithmic phase were used for experiment intervention. Those cells were digested and washed for preparation of single-cell suspension and adjustment of cell density to (1-5) $\times 10^5$/mL, where 500 μL binding buffer was added to prepare cell suspension and slightly mixed; after 5 μL Annexin-V fluorescein isothiocyanate (FITC) was added and well mixed, 5 μl propidium iodide (PI) was again added for incubation for 15 min in a dark place followed by detection on a flow cytometer within 1 h. The experiment results were analyzed using the BD CellQuest (BD Biosciences, Franklin Lakes, NJ, USA).

ALP Activity Assay

After treatment of cells in each group, they were collected through digestion and centrifugation followed by washing with phosphate-bufferes saline (PBS) twice and adjustment of cell density to 1×10^5/mL. Afterwards, 0.5% Triton X-100 was added, cells were placed at 4°C for 12 h. At low temperature, ultrasound was used for cell lysis, and 10 μL cell lysate were used to detect the concentration of proteins using Bradford method. Next, 50 μL cell lysate were extracted, where buffer and substrate were sequentially added in accordance with the instruction of kit and well mixed, followed by water bath at 37°C for 15 min. Thereafter, color developing agent was added and immediately mixed for detection of optical densities of all groups at 570 nm.

Autophagy Detection Via Cyto-ID Kit

Cyto-ID kit was used to detect the intracellular autophagy-related LC3 proteins. In cells growing on the slides, when cell density reached 50% to 70%, intervention was conducted as described in experiment design with negative control. After the supernatant was removed, cells were washed using 1×Assay buffer twice, and the single-layer cells were covered with 100 μL microscopic detection reagent. In a dark place, cells were incubated at 37°C for 30 min followed by washing cells with 100 pL 1×Assay buffer, next the extra buffer was removed; the sections were mounted using the anti-quenching agent containing 4',6-diamidino-2-phenylindole (DAPI), and the slides were placed under a microscope for observation (selecting FITC and DAPI and observing at 60× vision).

Nuclear Transfer Through Immunofluorescence Assay of NF-κB p65

Inoculated on a slide, pre-treated MC3T3-E1 cells were washed three times with 0.01 mol/L PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. The sections were incubated with 1% bull serum albumin (BSA) containing 0.2% Triton X-100 overnight, followed by further incubation using the primary antibodies, secondary antibodies of NF-κB p65 and DAPI for observation under a fluorescent microscope and image collection.

Real-time Polymerase Chain Reaction (RT-PCR)

After treatment, cells in each group were collected for extraction of total RNA with TRIzol reagent and measurement of concentration in samples. According to the sample concentration, the total RNA was added into the reverse transcription system for reverse transcription. The first 40 cycles were designed for complementary DNA (cDNA) synthesis, in which the conditions of reverse transcription were set for PCR amplification. After each cycle, the fluorescent signal was collected immediately with the amplification and melting curves being recorded.

Western Blotting

Cells in each group were collected for extraction of proteins using the corresponding kit, and measurement of protein concentration through bicinchoninic acid (BCA) method. After protein quantification, 5 μL 5× loading buffer was added followed by boiling at 99°C for 5 min and separation via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in which the concentration of stacking gel was 5%, and that of separation gel was 10%. Proteins were separated at a constant voltage of 90 V for 30 min in upper gel, and 150 V for 50 min in separation gel till the bromophenol blue reached to the bottom of gel. Gel, filter paper and polyvinylidene difluoride (PVDF) membrane were placed sequentially, and membrane transfer was performed in an electrophoresis tank. Thereafter, PVDF membrane was taken out, marked and placed in 5% skimmed milk for blocking for 1 h, followed by incubation at 37°C on a shaker. Afterwards, the membrane was washed using PBS three times, and after removal of blocking agent, primary antibody was added on the membrane (1:850) for incubation at 4°C overnight. On the second day, primary antibody was collected, fol-

lowed by washing with phosphate-buffered saline and Tween 20 (PBST) three times (5 min/time). Thereafter, the secondary antibody (horseradish peroxidase (HRP) labeled goat anti-rabbit immunoglobulin G, 1:5000) was added for incubation on a shaker at 37°C for 2 h. Secondary antibody was removed, and membrane was washed three times using PBST (15 min/time). Color development was performed for membrane in a visualizer and the images were scanned using ImageJ to obtain the gray value.

Statistical Analysis

Statistical Product and Service Solutions 13.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Data were presented as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). $p<0.05$ suggested that the difference had statistical significance.

Results

Morphological Observation of Cells in Culture

Under the microscope, the morphology of cells in culture were observed, and the results showed that in DMEM/F12 medium supplemented with 10% FBS, MC3T3-E1 cells were in well adhering growth in fusiform (Figure 1A); 2 to 3 days later, cells were reticulated, and the cell infusion reached about 80% (Figure 1A). Statistics indicated that at 3 days after cell culture, the comparison with that at 1 day after cell culture showed a statistically significant difference (Figure 1B).

Low-concentration LPS Promoted Cell Proliferation and Vitality

Results of CCK8 detection for cell vitality manifested that at 6 h and 12 h after treatment with low-concentration LPS, cell vitality was significantly higher than those in the control group,

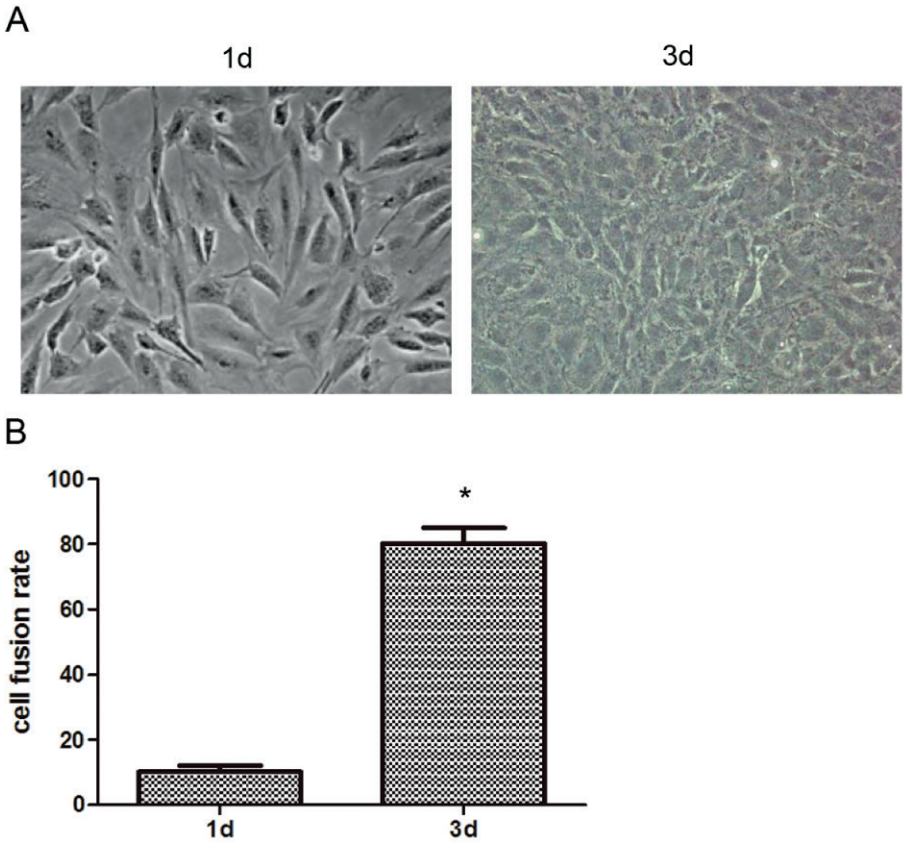

Figure 1. Detection of cell infusion under microscope. ***(A)***: Cell morphology at 1 day and 3 days after culture under a microscope. ***(B)***: Statistics on cell infusion. *Compared with the level at 1 day after culture, $p<0.05$.

LPS+3-MA group and LPS+BAY11-7082 group; cell vitality in the 3-MA group was higher than those in control group and BAY11-7082 group, but lower than that in the LPS group. The differences had statistical significance (Figure 2A). At 12 h after treatment with low-concentration LPS, a slight decrease was identified in cell vitality when compared with the level at 6 h after treatment. The results of flow cytometry for detecting the proportion of cell proliferation showed that at 6 h and 12 h after treatment with low-concentration LPS, cell proliferation rate was significantly higher than those in the control group, LPS+3-MA group and LPS+BAY11-7082 group. The cell proliferation rate in the 3-MA group was higher than those in control group and BAY11-7082 group, but lower than that in the LPS group, and the differences had statistical significance (Figure 2B and C). At 12 h after treatment with low-concentration LPS, a slight decrease was observed in cell proliferation rate when compared with the level at 6 h after treatment.

ALP activity detection for cell vitality was also carried out, and the results showed that at 6 h and 12 h after treatment with low-concentration LPS, the cellular ALP activity was significantly higher than those in the control group, LPS+3-MA group and LPS+BAY11-7082 group, the cellular ALP activity in the 3-MA group was higher than those in control group and BAY11-7082 group, but lower than that in the LPS group. The differences had statistical significance (Figure 2D).

Low-concentration LPS Inhibited Cell Apoptosis

Flow cytometry and Western blotting assay were performed to determine the proportion of cell apoptosis. The results revealed that, compared with the proportions of cell apoptosis at 12 h after treatment in the LPS group, LPS+3-MA group and LPS+BAY11-7082 group, those in the control group and at different time points were significantly elevated; the comparisons among other groups showed no statistically significant differences (Figure 3A and B). Western blotting assay was performed to detect the expressions of apoptosis-related proteins, B-cell lymphoma-2 (Bcl-2) and Bax; the results indicated that there was a slight decrease in the protein expression of Bcl-2 in the LPS group, 3-MA group and BAY11-7082 group at 12 h after treatment, while the expression of Bax was significantly elevated in those groups at 12 h after treatment (Figure 3C and D). However, Bax/Bcl-2 ratio indicated that, compared with that in the LPS group, the proportions of cell apoptosis in 3-MA group and BAY11-7082 group were significantly elevated. The comparisons among different groups at 12 h after treatment showed statistically significant differences (Figure 3E). At 6 h after treatment with low-concentration LPS, cell vitality was favorable. Thus, 6 h of treatment was selected as the experiment condition.

Low-concentration LPS Induced the Activity of NF-κB Signal Pathway in Cells

The activity of NF-κB signal pathway in cells was detected through PCR, Western blotting and immunofluorescence assay. PCR results indicated that the messenger RNA (mRNA) expression of NF-κB in the low-concentration LPS group was significantly elevated in comparison with that in the control group, and BAY11-7082 could inhibit the regulatory effect of LPS on mRNA expression of NF-κB. Compared with that in the LPS group, the mRNA expression of NF-κB in LPS+-BAY11-7082 group was significantly decreased, but there was no statistically significant difference in comparison between the LPS+3-MA group and LPS group (Figure IVA). The results of Western blotting for the protein expression of p65 were similar to those of PCR: low-concentration LPS significantly augmented the protein level of p65, which might be blocked by BAY11-7082 (Figure 4B and C). Immunofluorescence assay was performed to test the nuclear transfer, and the results suggested that in comparison with the control group, nuclear transfer of NF-κB was remarkably elevated in the low-concentration LPS group, while BAY11-7082 could suppress the regulation of LPS to NF-κB signaling pathway; there was no statistically significant difference in comparison between 3-MA group and LPS group.

Low-concentration LPS Induced Cell Autophagy

Immunofluorescence assay, PCR and Western blotting assay were conducted to detect the changes in cell autophagy, which revealed that compared with the control group, low-concentration LPS significantly increased the expression of LC3 in cells, and BAY11-7082 and 3-MA could restrain the inducing effect of LPS. The proportions of LC3-positive cells in the LPS+-BAY11-7082 group and the LPS+3-MA group were significantly less than that in the LPS group (Figure 5A). PCR was performed to measure the mRNA expression of Atg5 in cells, and the results

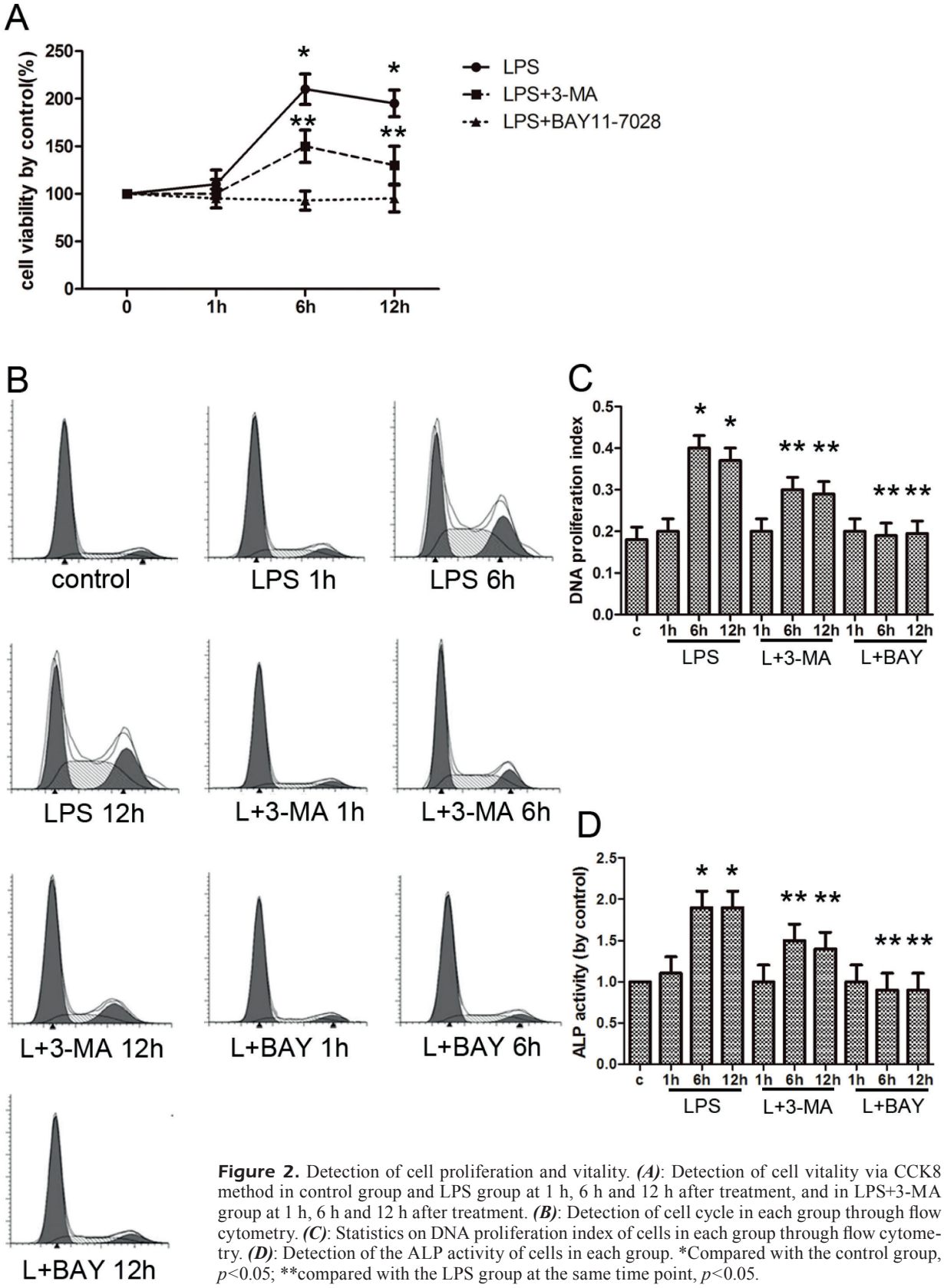

Figure 2. Detection of cell proliferation and vitality. ***(A)***: Detection of cell vitality via CCK8 method in control group and LPS group at 1 h, 6 h and 12 h after treatment, and in LPS+3-MA group at 1 h, 6 h and 12 h after treatment. ***(B)***: Detection of cell cycle in each group through flow cytometry. ***(C)***: Statistics on DNA proliferation index of cells in each group through flow cytometry. ***(D)***: Detection of the ALP activity of cells in each group. *Compared with the control group, $p<0.05$; **compared with the LPS group at the same time point, $p<0.05$.

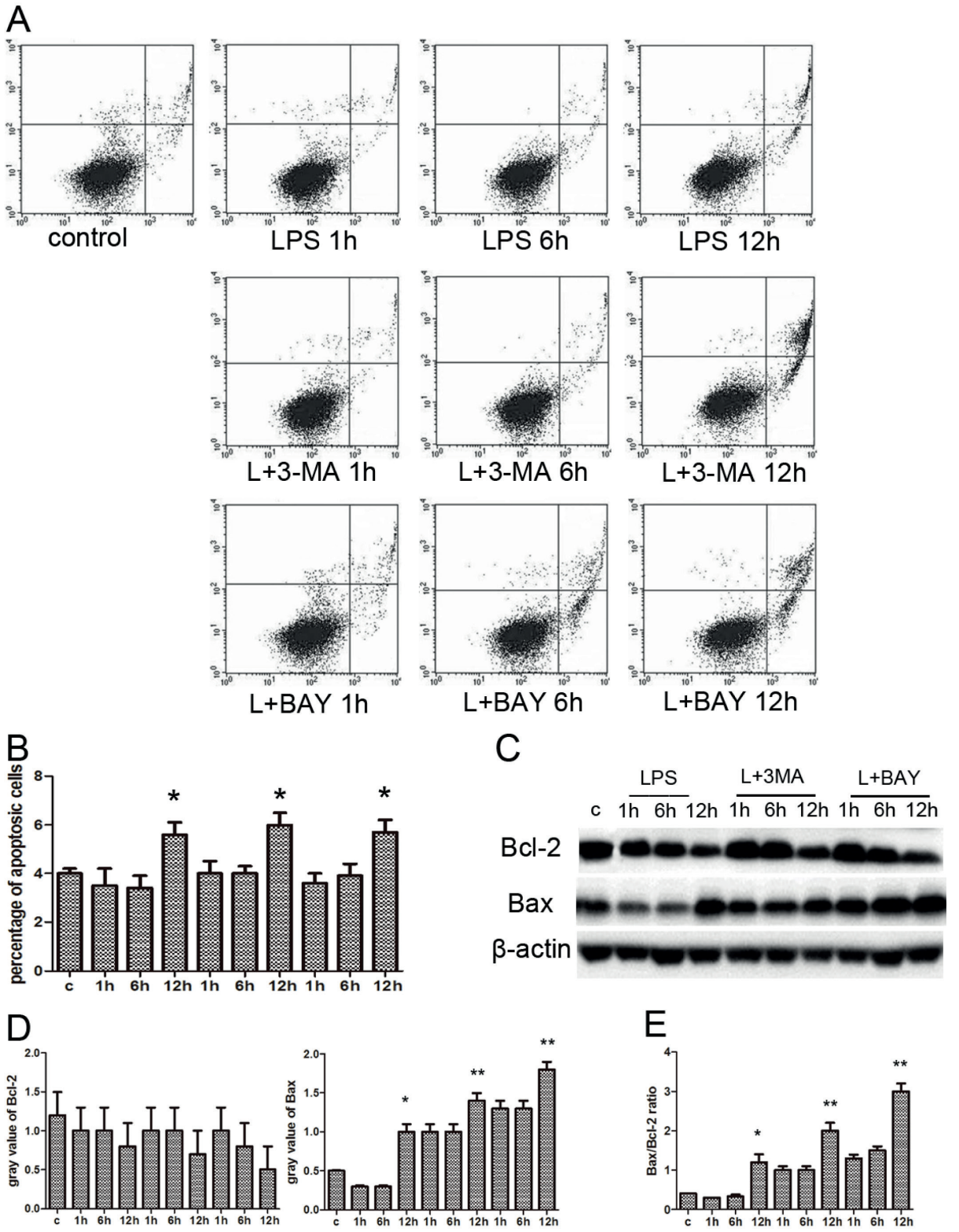

Figure 3. Detection of cell apoptotic level. ***(A)***: Detection of cell apoptotic level via flow cytometry in control group and LPS group at 1 h, 6 h and 12 h after treatment, and in LPS+3-MA group at 1 h, 6 h and 12 h after treatment. ***(B)***: Statistics on cell apoptosis proportions through flow cytometry. ***(C)***: Detection of protein expressions of Bcl-2 and Bax via Western blotting. ***(D)***: Gray value scan for Bcl-2 and Bax proteins in cells of each group. ***(E)***: Statistics on Bax/Bcl-2 ratios of cells in each group. *Compared with the control group, $p<0.05$; **compared with the LPS group at the same time point, $p<0.05$.

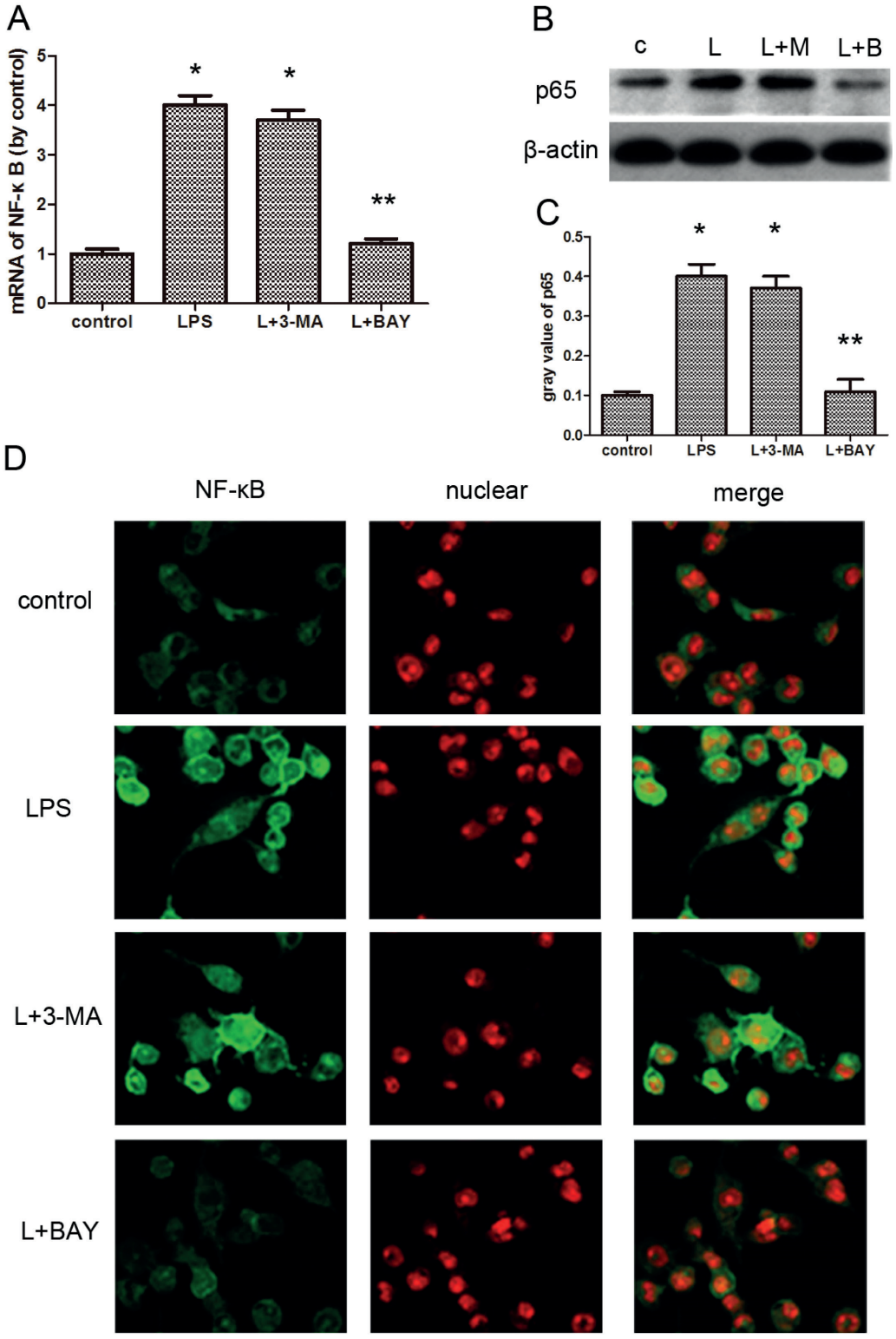

Figure 4. Detection of the activity of NF-κB in cells. ***(A)***: Detection of mRNA expression of NF-κB via PCR in control group, LPS group and LPS+3-MA group. ***(B):*** Detection of protein expression of p64 in the control group, LPS group, LPS+3-MA group and LPS+BAY11-7082 group. ***(C)***: Statistics on gray value scan of p64 protein of cells in each group. ***(D)***: Immunofluorescence assay for detecting the expression of NF-κB and nuclear transfer of cells in each group. *Compared with the control group, $p<0.05$; **compared with the LPS group at the same time point, $p<0.05$.

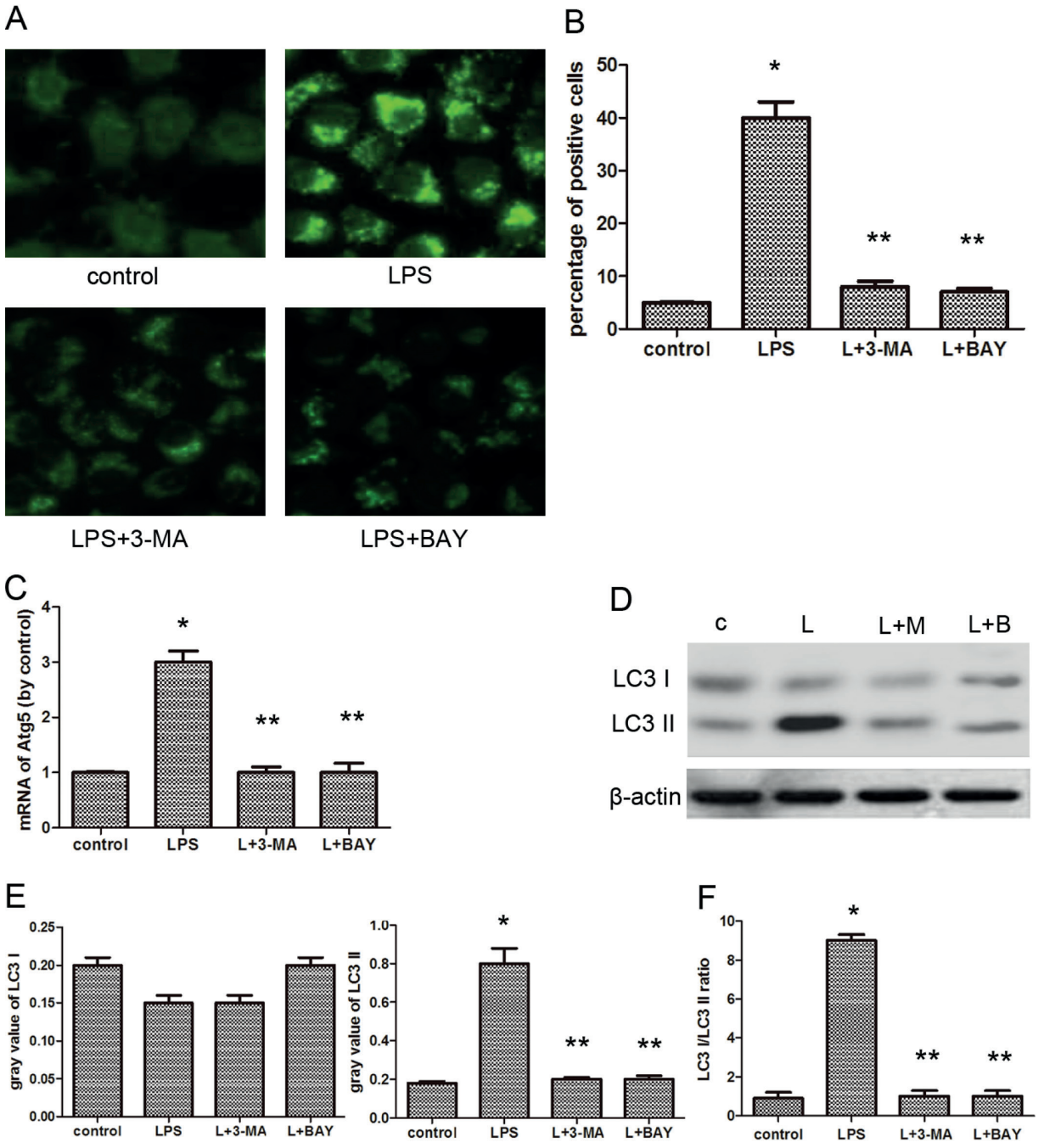

Figure 5. Detection of cell autophagy level. ***(A)***: Detection of LC3 expression in cells via immunofluorescent assay in control group, LPS group and LPS+3-MA group. ***(B)***: Statistics on the cell count of LC3-positive cells in each group. ***(C)***: Detection of mRNA expression of Atg5 in cells of each group via PCR. ***(D)***: Detection of LC3 I and LC3 II proteins of cells in each group. ***(E)***: Statistics on gray value scan of LC3 I and LC3 II proteins of cells in each group. ***(F)***: Statistics on the LC3 II/LC3 I ratios of cells in each group. *Compared with the control group, $p<0.05$; **compared with the LPS group at the same time point, $p<0.05$.

indicated that LPS induced an increase in mRNA level of Atg5, which could be blocked by BAY11-7082 and 3-MA. Besides, the mRNA expressions of Atg5 in cells in the BAY11-7082 group and the 3-MA group were significantly lower than that in the LPS group. The expression of autophagy-related proteins (LC3 I and LC3 II) was detected using Western blotting, whose results were similar to those above: in the LPS group, LC3 II and LC3 II/LC3 I ratio were significantly elevated,

but LC3 II and LC3 II/LC3 I ratio in the BAY11-7082 group and 3-MA group were significantly lower than those in the LPS group.

Discussion

Fracture healing is a complicated but well-organized regulatory process involving histological and biochemical variations[16]. Repair and regeneration of bone refer to a repair process in which osteoblasts, as well as other cells and cytokines are involved, and the core of this process is a dynamic balance between the osteoclast-mediated bone absorption and osteoblast-mediated bone formation. With the development of molecular biology, cytokines participating in bone repair have become research hotspots. Inflammatory period is a characteristic period in fracture healing involving many inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, LPS and tumor necrosis factor-α (TNF-α)[17]. During the inflammatory period, these inflammatory cytokines and some inflammatory mediators can mobilize the osteoblast precursors to the injured part, and guide the proliferation and differentiation of osteoblasts[5]. In addition, it has been identified that inflammatory cytokines are highly expressed during bone repair and physiological bone regeneration. The study results revealed that low-concentration LPS could promote proliferation and vitality of osteoblasts, and inhibit the apoptosis of osteoblasts, showing the enhancing effect of low-concentration inflammatory cytokines in fracture healing.

According to mass experiment data, regulating the activity of NF-κB signaling pathway is of great potential in treatment of some diseases, especially cancer, infection and inflammatory diseases[18]. Thus, a variety of researches have focused on the inhibitor of NF-κB due to its high specificity and effectiveness; these NF-κB inhibitors can disturb or inhibit the activity of NF-κB. BAY11-7082, a common inhibitor of NF-κB, can inhibit the phosphorylation of IκB α induced by some cytokines, thereby blocking the degradation of IκB α and following nuclear transfer of NF-κB, finally suppressing the NF-κB-dependent genetic transcription[19]. Moreover, results of this study also indicated that LPS can activate the NF-κB and promote nuclear transfer. BAY11-7082, the inhibitor of NF-κB, was applied to intervene in the induction of LPS, which showed that BAY11-7082 could well inhibit the activation of NF-κB induced by LPS, thus suppressing the osteoblast proliferation mediated by NF-κB signaling pathway. Cell autophagy is a process in which the macromolecular substances or damaged organelles in cells can be degraded by lysosomes, with which the new organelles can be assembled[20]. A latest work[21] reported that in stress environment like disease or trauma, cells could sustain their vitalities through activating and initiating the autophagic function. Furthermore, some studies[12,13,22] confirmed the prominent effect of cell autophagy in diseases like myocardial infarction, hypertension, diabetes mellitus and cranial nerve diseases. For bone cells at the fracture end, autophagy induced by the stress environment is critical to the survival of bone cells[23]. The results of this study showed that inhibiting the autophagy of osteoblasts could block the anti-apoptotic effect and promote the proliferation induced by low-concentration LPS. Therefore, in low-concentration LPS promoting the vitality of osteoblast, cell autophagy is essential. Additionally, BAY11-7082 can significantly decrease the activity of cell autophagy, suggesting that activation of NF-κB signaling pathway is the basis for upregulating cell autophagy through LPS.

Conclusions

We showed that low-concentration LPS can promote cell proliferation and vitality, and inhibit cell apoptosis, which may be caused by osteoblast autophagy activated by upregulation of NF-κB signaling pathway.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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