MiR-145 inhibited lipopolysaccharide-induced apoptosis of gastric mucosal via up-regulating JNK-mediated cytoprotective autophagy

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Abstract: It has been reported that specific microRNA could inhibit apoptosis of gastric mucosa. Our study was designed to investigate the effect and mechanisms of miR-145 in gastric mucosa. Gastric mucosal cells (GES-1) were treated with null-vector or miR-145 over expression plasmid. Cell viability was determined by CCK-8 assay and detection of apoptosis by flow cytometry. Autophagic and apoptosis protein expression and c-Jun NH2-terminal kinase (JNK) phosphorylation were determined by Western blotting. Autophagy response and JNK activities were inhibited by specific inhibitor, 3MA or SP600125, respectively. LDH release assay was used to detect cytotoxicity. We confirmed that miR-145 triggered an autophagic response in GES-1 cells and depended on JNK activation. Blocking autophagy or JNK activation with specific inhibitor, 3MA or SP600125, potentiated cell death and caspase-3 activation. Furthermore, we confirmed that miR-145 enhanced the viability of GES-1 cells, phosphorylation of JNK and inhibited apoptosis of gastric mucosal miR-145 inhibited apoptosis of gastric mucosal via up-regulating JNK-mediated cytoprotective autophag.

Keywords: miR-145, autophagy, apoptosis, gastric mucosal.

INTRODUCTION

Apoptosis is the main cause for gastric mucosa injury. Compared with direct damage on gastric mucosa cells by pathological factors, apoptosis accounts for more in chronic gastric mucosa lesion (Torre *et al*., 2015). In the field of gastric mucosal apoptosis, lipopolysaccharide (LPS) was reported with enhancing effect of apoptosis, and proved with clinical significance in many gastric mucosa diseases (Pan *et al*., 1997; Laussmann *et al*., 2012). On the other hand, complex pathways were involved in apoptosis progress, and latest research showed that even autophagy was associated with apoptosis level (Camidge *et al*., 2014; Soria *et al*., 2011).

Autophagy constitutes a stress adaptation response and/or increased metabolic demands that avoids and suppresses cell death (O'Donovan *et al*., 2011). However, autophagy also leads to cell death in certain circumstances (Aft *et al*., 2002, Kaushik *et al*., 2015). Consistent with its contradictory roles in cell death control, the effects of autophagy in cells' response to microRNA are also complex: either promoting or protecting against therapy-induced death (Zhao *et al*., 2013, Liu *et al*., 2007). Recent studies suggested that cytoprotective autophagy may relate to the activation of JNK (O'Donovan *et al*., 2011). The JNK, also known as stress -activated kinase, has been shown to regulate autophagy in mammalian cells in response to various stress stimuli and indicates that JNK activation may be a critical determinant of the cellular response. However, some studies showed opposing effects of JNK-mediated autophagy in cancer death (Mizushima *et al*., 2011). Thus, the role of JNK-mediated autophagy is contradictory in cell death control and needs further study. MicroRNA is a kind of small non-coding RNA fragments with a structure of 22 nucleotides. Complete complementary between microRNA and mRNA always results in degradation of target gene (Di *et al*., 2013). With proven technique developed, more and more microRNA became predictor for diseases at early stage, and some even were transformed into potential therapeutic target (Mora *et al*., 2009, Wei *et al*., 2008). miR-145 was reported to inhibit apoptosis in many cell lines, however, it is still unclear whether miR-145 could influence apoptosis of gastric mucosal and the detailed mechanism need further exploration.

The purpose of the present study is to observe whether autophagy is activated by miR-145 in gastric mucosal cells and to explore whether such up-regulating effect on autophagy is responsible for apoptosis inhibition.

MATERIALS AND METHODS

Main reagents

RPMI 1640 medium and fetal bovine serum (FBS) were from Gibco Company (Grand Island, U.S.). Cell counting kit and Annexin V FITC/PI apoptosis detection kit were bought from Dojin Laboratories (Kumamoto, Japan) and Beckman Coulter company (U.S.), respectively. PierceTM LDH cytotoxicity assay kit was obtained from Thermo-Scientific™ (Pittsburgh, PA). 3-methyladenine

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(3MA) and chloroquine (CQ) were bought from Sigma-Aldrich (U.S.). SP600125 was from Calbiochem Company (Germany) for JNK inhibition. Pro-tease and phosphatase inhibitor cocktail were from Roche (Switzerland). Polyvinylidene difluoride membranes were bought from Millipore (U.S.).

Cell culture

Human gastric mucosa cells, GES-1, which have been shown to be resistant to LPS (Park *et al*., 2009), were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and grown in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100U/mL penicillin and 100μg/mL streptomycin at 37°C under an atmosphere of 95% air and 5% CO2.

Cell viability assay

Cell viability was assessed via a cell counting kit according to the manufacturer's instructions. Briefly, cells were plated at a density of 3×103 cells/well in 96-well plates and were allowed to adhere for 24h before treatment. After treating with 2-DG alone or co-treated with soluble recombinant human TRAIL/Apo2 ligand, cells were cultured for 24, 48 or 72 h and then treated with 10 μL of solution containing WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) for an additional 2 h. The absorbance at 450 nm was measured on a microplate reader.

Detection of apoptosis by flow cytometry

Annexin V FITC/PI apoptosis detection kit was applied to detect the cellular apoptosis. In short, gastric cancer cells treated with 2-DG (10mM) and TRAIL (100ng/mL) for 48 h and then were collected and resuspended at a concentration of about 1\×\10 6cells/mL. After mixing with 5 μL of Annexin V FITC and 5μL propidiumiodide (PI) solutions, the mixture was incubated for 15 min in the dark. Analysis was carried out using a Becton Dickinson (MountainView, CA, U.S.) FACScan flow cytometer.

Cytotoxicity assay

A cytotoxicity assay based on the release of lactate dehydrogenase (LDH) was assessed by a PierceTM LDH cytotoxicity assay kit. Briefly, cells were seeded in 48-well plates at 50–60% confluence and were pretreated with 3MA or SP600125 for 2 or 3 h, respectively, and then treated with TRAIL (100ng/mL) for 48 h. The supernatant of the treated cells was relocated into 96- well plates. Then, 100μL of the LDH reaction solution was added for 30 min. Finally, LDH activity was measured at 490 nm by a microplate reader.

Western blotting analysis

Total cell lysates were prepared by the addition of RIPA buffer and fresh protease and/or phosphatase inhibitor cock-tail. The proteins were fractionated with 10 or 12% SDS-PAGE and electro blotted onto polyvinylidene difluoride membranes. The blots were blocked for 2 h at room temperature with 5% non -fat milk. The membranes were incubated overnight at 4°C with primary antibodies (1:500-1000 dilutions) against cleaved-caspase-3 (Abcam, Cambridge, UK), cleaved-PARP (Abcam, Cambridge, UK), phospho-JNK (Cell signaling technology, U.S.), JNK(Cell signaling technology, U.S.), glucose transporter (GLUT1) (Santa Cruz Biotechnology, CA, U.S.), LC3 (Santa Cruz Biotechnology, CA, U.S.), p62 (Santa Cruz Biotechnology, CA, U.S.), DR4, DR5 (Bioworld Technology, U.S.) or β-actin (Santa Cruz Biotechnology, CA, U.S.) and then incubated with relative secondary antibody (Santa Cruz Biotechnology, CA, U.S.) for 1 h at room temperature. β-Actin was used as a loading control.

Autophagy assays

MGC803 and SGC7901were infected with a retrovirus expressing GFP-LC3 according to standard infection protocols. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, and then the cells were analyzed by fluorescence microscope for the presence of LC3 dots. More than five LC3 spots in cells were considered autophagy positive, and 200 cells were counted in each group.

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS 18.0 software. The representative of experiments was repeated at least three times and the results were presented as mean ± SEM. Differences between multiple groups were analyzed by one-way ANOVA. $P<0.05$ was considered as a significant difference.

RESULTS

LPS induced cell death of GES-1 cells via decreased autophagy of GES-1 cells

We first investigated if LPS was able to influence autophagy. Next, to determine the role of autophagy in LPS-induced cell cytotoxicity, 3MA, the classical pharmacological inhibitor of autophagy, was used for blocking autophagy to detect the cell death. Compared with no LPS treatment cells, LPS decreased autophagy of GES-1 cells, verified by changes of autophagy proteins (fig. 1A). Moreover, LPS increased CASPASE3 activation and PARP expression (fig. 1A), suggesting cells death was triggered. Further kit experiment showed that LPS indeed enhanced GES-1 cells death and blocking autophagy exacerbated such pro-apoptosis effect (fig. 1B&C).

miR-145 induced autophagy of cells via JNK activation

We further showed that miR-145 transfected GES-1 cells had more significant increased levels of autophagy, and

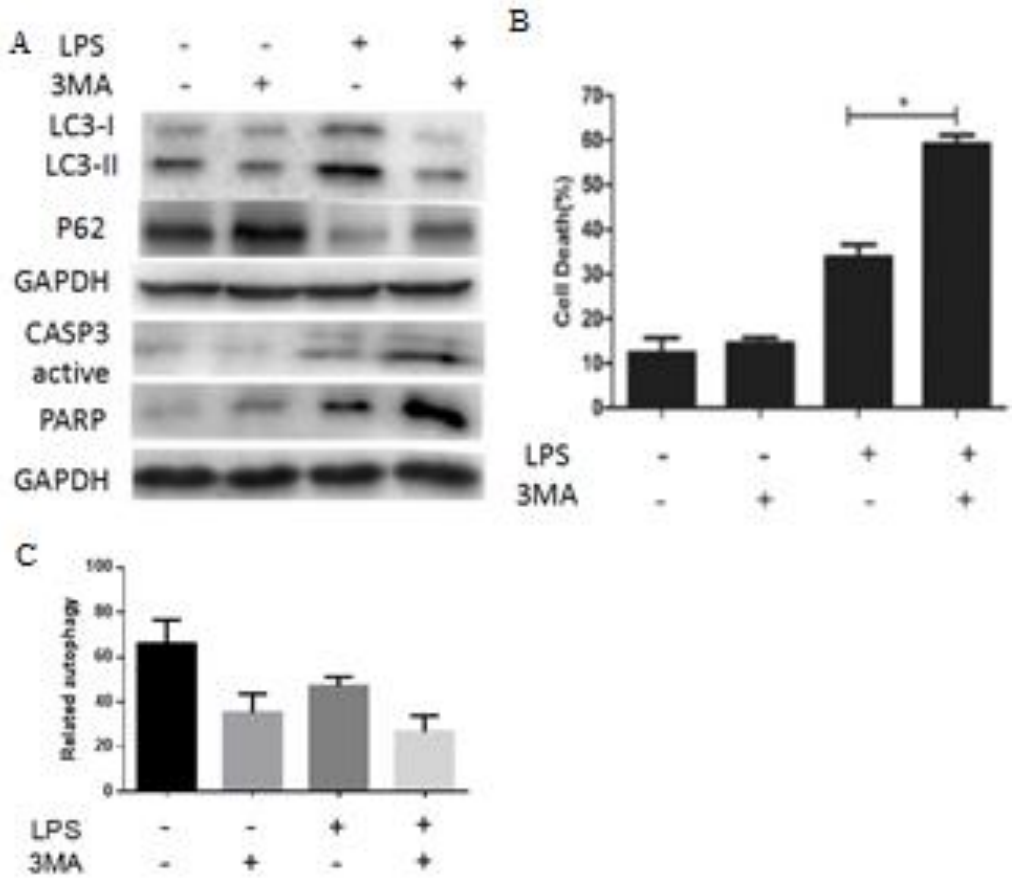

Fig. 1: LPS induced cell death of GES-1 cells via decreased autophagy of GES-1 cells. (A) Western blot for different treatment groups. (B) Cell death rate in different treatment groups. (C) Related autophagy level of different treatment groups. *P<0.05.

such effect could alleviate LPS-decreased autophagy (fig. 2A) in LPS treated GES-1 cells. In addition, we found that JNK activities were essential for miR-145 induced autophagy, while blocking JNK decreased levels of autophagy in GES-1 cells treated by miR-145 transfection (fig. 2B).

Blocking JNK activation enhanced LPS- induced cell death

We further tested effect of JNK activation on cell death. For LPS-treated GES-1 cells, blocking JNK activation significantly increased cell death accompanied with decreased autophay (fig. 3A). Moreover, compared with null treated cells, SP600124 treated GES-1 cells still had a significant increase of cell apoptosis markers under JNK activation blocking condition, while miR-145 abrogated GES-1 cells apoptosis (fig. 3B), suggesting miR-145 might influenced cell death.

miR-145 desensitizes GES-1 cells to LPS-induced apoptosis via up-regulating JNK mediated cytoprotective autophagy

The findings above demonstrated that miR-145 transfected GES-1 cells had enhanced JNK expression and autophagy levels. Further cellular viability experiment showed that, compared with null transfected cells, miR-145 transfected GES-1 cells had a significant increase of cellular viability, even under LPS-treatment (fig. 4A). Flow cytometry showed that, compared with null transfected cells, miR-145 decreased LPS-induced apoptosis in GES-1 cells (fig. 4B). Moreover, the immunofluorescence showed that LPS-induced apoptosis intensity has been decreased by miR-145 transfection (fig. 4C). All these demonstrated that miR-145 desensitizes GES-1 cells to LPS-induced apoptosis via up-regulating JNK mediated cytoprotective autophagy.

DISCUSSION

Our study showed that LPS-induced apoptosis of gastric mucosl was associated with decreased autophagy, while miR-145 inhibited LPS-induced apoptosis of gastric mucosa. Moreover, miR-145-induced JNK activation was responsible for such protective effect of autophagy. Taken together, we proved that miR-145 inhibited LPS-induced apoptosis of gastric mucosa via up-regulating JNK-mediated cytoprotective autophagy.

Previous studies offered scarce data on how microRNA influenced gastric mucosa (Park *et al*., 2009). Recent studies showed that miR-145 could enhance mucosa cell viability, while miR-145 was associated with increased cell proliferation (Xu *et al*., 2009, Giammarioli *et al*., 2012), suggesting miR-145 was involved in cytoprotection. However, there is little evidence on how miR-145 influenced gastric mucosa. Our study showed that the LPS treatment indeed increased cell death of GES-1 cells, and the autophagy was dramatically decreased by LPS. We further explored effect of miR-145 upon LPS injury, and found that miR-145 protected GES-1 cells against LPS injury. Intriguingly, miR-145 treatment up-regulated autophagy of GES-1 cells, while blocking JNK expression abrogated such autophagy enhancing effect of miR-145 in GES-1 cells, which might be responsible for the protection of miR-145.

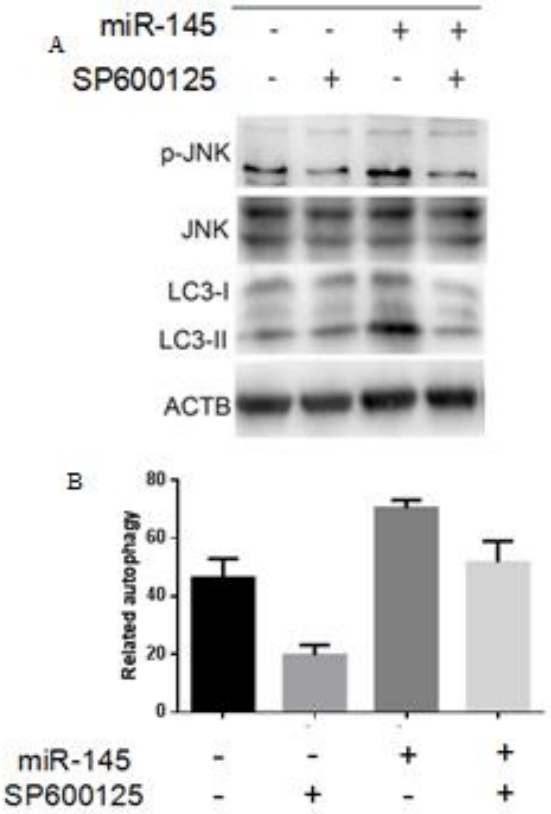

Fig. 2: miR-145 induced autophagy of cells via JNK activation. (A) Western blot of autophagy markers for different treatment in LPS treated GES-1 cells. (B) Related autophagy level of different treatment groups of LPS treated GES-1 cells.

JNK related pathways played a pivotal role in autophagy, and the inhibition of JNK was reported with inhibitory effect of autophagy (Kurbanov *et al*., 2007). Our data further showed that the activation of autophagy was dependent on JNK signaling pathway in gastric mucosa, and the mechanism may be that JNK activation up-regulated Beclin-1 expression (Li *et al*., 2011) and induced Bcl-2 phosphorylation, which lead to disruption of the Bcl-2/Beclin1 complex and dissociation of these Bcl-2 family proteins from Beclin1 promotes autophagy (Quast *et al*., 2015). Meanwhile, previous study also showed the degradation of active caspase-8 may play a pivotal role in microRNA-induced autophagy (Thorburn *et al*., 2014). Although some evidence has proposed that JNK activation can also mediate apoptosis (He *et al*., 2012, Li *et al*., 2012), how to regulate the relationship between autophagy and apoptosis by JNK signaling has yet unknown and deserves further investigation.

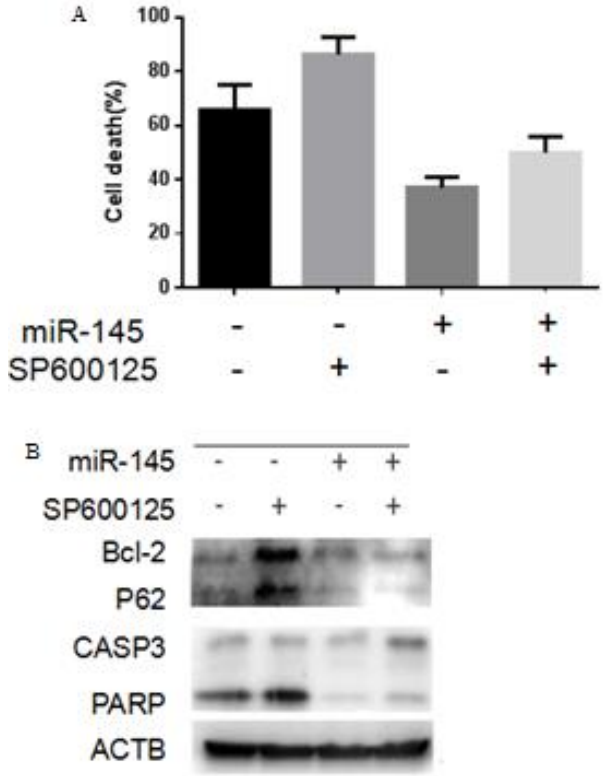

Fig. 3: Blocking JNK activation enhanced LPS- induced cell death. (A) Cell death rate in different treatment groups of LPS treated GES-1 cells. (B) Western blot of apoptosis markers for different treatment in LPS treated GES-1 cells

To detect details of cell death, we performed flow cytometry to verify that LPS mainly induced apoptosis of gastric mucosa, and miR-145 mitigated pro-apoptosis effect of LPS. Moreover, immunofluorescence experiment also demonstrated that apoptosis intensity was decreased by miR-145 transfection. Previous have demonstrated that apoptosis was associated with autophagy, and protective autophagy could help cells to undergo adverse condition instead of facing apoptosis fate (Cooper *et al*., 2018). We proved that miR-145 desensitized gastric mucosa to apoptosis via up-regulating JNK-related autophagy. Accordingly, appropriate increase of autophagy protects gastric mucosa against LPS injury, which is triggered by miR-145.

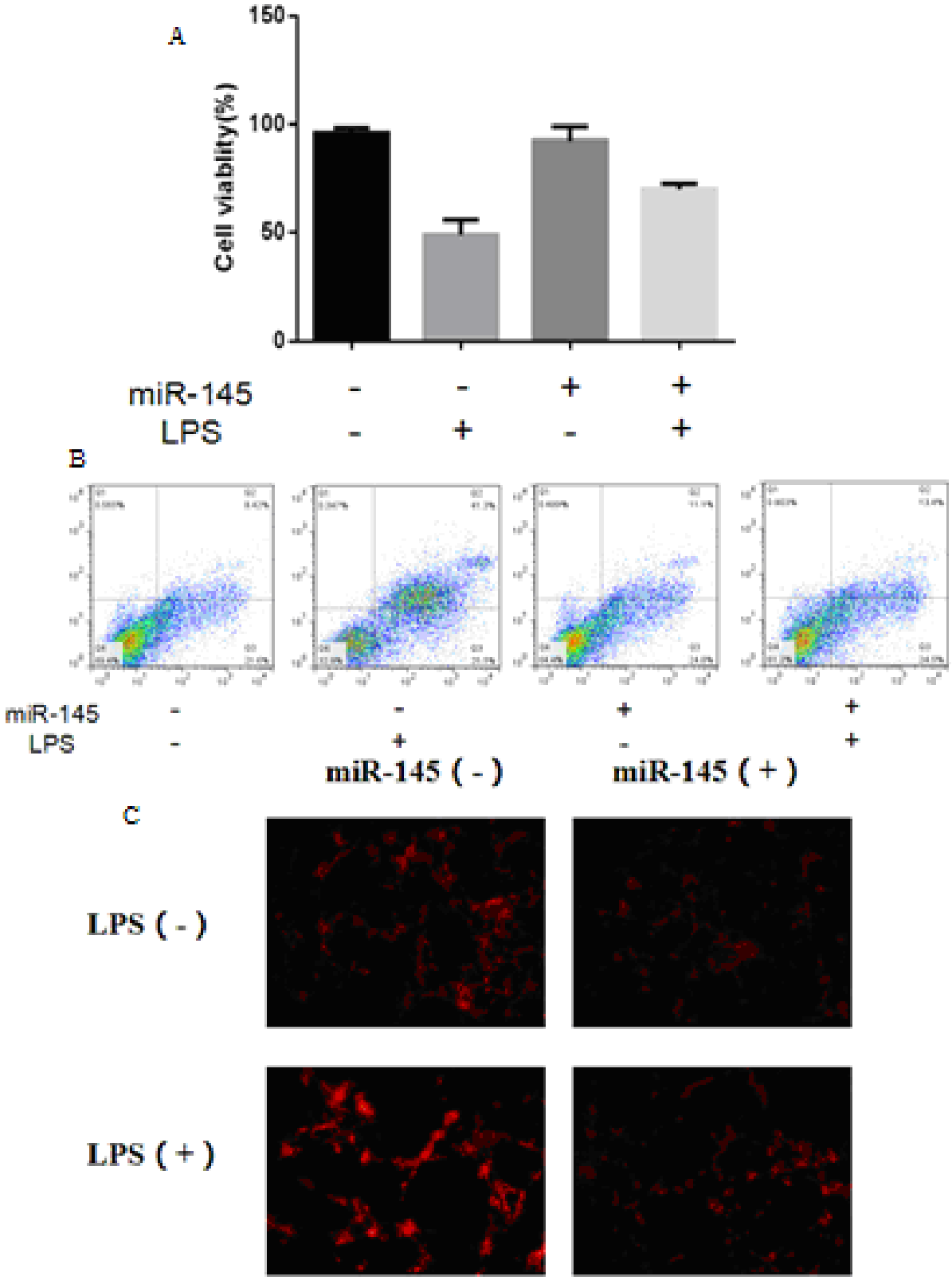

Fig. 4: miR-145, LPS-induced apoptosis and JNK mediated cytoprotective autophagy. (A) Cell viability analysis in different treatment groups of GES-1 cells. (B) Flow cytometry to detect apoptosis in different treatment groups of GES-1 cells. (C) Immunofluorescence for apoptosis intensity in different treatment groups of GES-1 cells.

CONCLUSIONS

Our study shows that miR-145 desensitizes LPS-induced apoptosis, at least in part, through suppressing JNK-mediated cytoprotective autophagy signaling. These findings suggest that miR-145 has tremendous adjuvant therapeutic potential for gastric mucosal protection and activation of JNK-mediated autophagy signaling could be useful for gastric mucosal treatment.

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