

# $\beta$ -Nerve growth factor attenuates hepatocyte injury induced by D-galactosamine *in vitro* via TrkA<sup>NGFR</sup>

JUN-FENG LI<sup>1\*</sup>, JIAN-CHANG SHU<sup>2\*</sup>, SHAO-HUI TANG<sup>1</sup>, YAN-MEI DENG<sup>2</sup>, MEI-YA FU<sup>2</sup> and XIA LV<sup>2</sup>

<sup>1</sup>Department of Gastroenterology, The First Affiliated Hospital of Jinan University, Guangzhou, Guangdong 510630;

<sup>2</sup>Department of Gastroenterology, The Fourth Affiliated Hospital of Jinan University, Guangzhou, Guangdong 510175, P.R. China

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**Abstract.** Nerve growth factor (NGF) regulates the proliferation, differentiation and survival of cells and is also involved in the wound healing and tissue remodeling processes. The biological effects of NGF are dependent upon receptor signal-mediating functions, which differ between cells. This study attempted to investigate the hepatoprotective effect and possible mechanism of  $\beta$ -NGF on D-galactosamine (D-GalN)-injured human liver L-02 cell lines. We demonstrated that L-02 cells expressed the neurotrophin receptors tyrosine kinase-A nerve growth factor receptor (TrkA<sup>NGFR</sup>) and p75 pan-neurotrophin receptor (p75<sup>NTR</sup>). Recombinant human  $\beta$ -NGF markedly reduced cell injury and promoted the proliferation of L-02 cells damaged by D-GalN. However, this proliferation effect was blocked by the anti-TrkA<sup>NGFR</sup> antibody. Lactate dehydrogenase (LDH) and malondialdehyde (MDA) were released at reduced levels in the L-02 cell culture supernatant pretreated with  $\beta$ -NGF. Furthermore, the albumin (ALB) content in the cell medium and intracellular glutathione (GSH) levels were markedly augmented, and the permeability of the mitochondrial membrane of the L-02 cells was improved by  $\beta$ -NGF. Our results suggested that exogenous  $\beta$ -NGF protects L-02 cells from D-GalN-induced injury through the NGF/TrkA<sup>NGFR</sup> signaling pathway.

## Introduction

The liver is predominantly comprised of polarized epithelial cells, which regulate the intermediary metabolism, detoxi-

fication, the manufacture of critical circulating proteins and the production of bile for digestion (1). However, the liver is susceptible to different types of injuries. Hepatocyte apoptosis is considered to be a pivotal pathological process in a variety of liver injuries. The damaged hepatocytes release apoptotic bodies that are engulfed by resident kupffer and hepatic stellate cells (HSCs), which release chemokines and cytokines that subsequently promote HSC activation and transdifferentiation into myofibroblasts, resulting in dysregulated hepatic fibrosis and cirrhosis (2). Cirrhosis is the worst consequence of continuous liver injury, as it may lead to portal hypertension, liver failure and mortality (3). The optimal therapeutic strategy for several liver diseases is to directly inhibit the mechanism that triggers the liver injury. Clinically, antiviral therapies have been demonstrated to be effective for viral hepatitis. However, there are no effective treatments for a variety of liver diseases, including liver fibrosis, and patients with hepatitis C (HCV) or hepatitis B viruses (HBV) who are unresponsive to antiviral therapies. For these patients, taking effective measures to protect the hepatocytes from injury and inhibit apoptosis are important therapeutic strategies (4).

Nerve growth factor (NGF) and its receptors are produced in nervous and non-nervous system cells, which regulate the differentiation, survival, development and apoptosis processes of cells by means of autocrine or paracrine signaling and are also involved in wound healing and tissue remodeling processes. The biological effects of NGF are mediated through two membrane receptors, the tropomyosin tyrosine kinase-A nerve growth factor receptor (TrkA<sup>NGFR</sup>) and the p75 pan-neurotrophin receptor (p75<sup>NTR</sup>) (5). Recent evidence has suggested that NGF has broader physiological effects, which may be involved in the liver injury-repair process, since its mRNA and protein expression levels become elevated whilst regenerating hepatocytes. Furthermore, activated HSCs induced by lead nitrate in rats (6), partial hepatectomy (7) or CCl<sub>4</sub> treatment in mice (8) and exogenous recombinant NGF may promote activated HSC apoptosis (8,9). Certain authors have demonstrated that manipulation of the NGF/p75<sup>NTR</sup> axis represents a novel means for regulating the progression and resolution of liver fibrosis (10). However, little is known with regards to the protective effect of NGF on damaged hepatocytes.

In this study, we provide evidence that human hepatocyte L-02 cells express NGF and its receptors, TrkA<sup>NGFR</sup> and

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*Correspondence to:* Dr Jun-Feng Li, Department of Gastroenterology, The First Affiliated Hospital of Jinan University, 613 West Huangpu Avenue, Guangzhou, Guangdong 510630, P.R. China  
E-mail: ljfmed@163.com

\*Contributed equally

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p75<sup>NTR</sup>. Furthermore, we report that exogenous human recombinant  $\beta$ -NGF attenuates injury and inhibits the apoptosis of L-02 cells induced by D-galactosamine (D-GalN), and that the protective mechanism of  $\beta$ -NGF is likely to be mediated through the TrkA<sup>NGFR</sup> signaling pathway.

## Materials and methods

**Chemicals.** DMEM, fetal bovine serum (FBS), penicillin, streptomycin, 0.25% EDTA-Trypsin, D-GalN and silymarin (Sily) were purchased from Life Technologies (Gibco, Carlsbad, CA, USA). Recombinant human  $\beta$ -NGF was obtained from R&D Systems (Minneapolis, MN, USA) and rabbit polyclonal anti-NGF, anti-TrkA<sup>NGFR</sup> and anti-p75<sup>NTR</sup> antibodies were derived from Boster Bioengineering Limited Company (Wuhan, China). Human albumin (ALB), lactate dehydrogenase (LDH) activity, malondialdehyde (MDA) and glutathione (GSH) assay kits were supplied by Jiancheng Bioengineering Institute (Nanjing, China). The BD<sup>TM</sup> MitoScreen (JC-1) kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The cell proliferation reagent XTT was purchased from Bio Basic Inc (BBI, Markham, ON, Canada). The MaxVision<sup>TM</sup> kit and 3,3'-diaminobenzidine (DAB) were purchased from the Maixin Bioengineering Institute (Fuzhou, China).

**Cell cultures.** The human liver L-02 cell line (Institute of Biochemistry and Cell Biology, SIBS, Shanghai, China) was cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, and maintained in a humidified 5% CO<sub>2</sub> incubator at 37°C. The culture medium was replaced every 2-3 days. The cells were sub-cultured and allowed to adhere for 24 h prior to performing subsequent experiments.

**Immunocytochemistry.** L-02 cells (1 $\times$ 10<sup>5</sup> cells/well) were seeded into 6-well plates with coverslips and fixed with 4% formaldehyde in PBS at room temperature (RT) for 15 min, then washed three times with PBS. L-02 cells were incubated overnight at 4°C with primary rabbit polyclonal antibodies against NGF or TrkA<sup>NGFR</sup>, or p75<sup>NTR</sup> diluted (1:100) in PBS containing 0.1% Triton X-100 detergent. Cells were washed three times with PBS and subsequently incubated for 30 min at RT with the MaxVision kit (HRP-Polymer anti-rabbit IgG) according to the manufacturer's instructions. The signal was detected using DAB as the chromogen. Counterstaining was performed with Mayer's hematoxylin (Invitrogen, Carlsbad, CA, USA). Negative controls were obtained by omitting the primary antibody. Stained cells were visualized using an inverted optical microscope (Olympus CK40, Tokyo, Japan).

**Light microscopy.** L-02 cells were pretreated with  $\beta$ -NGF for 30 min and subsequently incubated with D-GalN for 24 h. The alterations to cell morphology were viewed using an inverted Olympus CK40 microscope. Microphotographs were captured with a digital Canon EOS 600D camera (Canon, Tokyo, Japan).

**Cell proliferation assay.** The primary culture of L-02 cells were seeded into 96-well plates at a density of 5 $\times$ 10<sup>3</sup> cells/well and pretreated with  $\beta$ -NGF at various concentrations (0, 25, 50, 100, 200 and 400  $\mu$ g/l) for 30 min prior to

incubation with D-GalN (40 mmol/l). Sily (100 mg/l) was used as a positive control. In order to evaluate whether the neurotrophin receptors, TrkA<sup>NGFR</sup> and/or p75<sup>NTR</sup>, were involved in the response of the L-02 cells to  $\beta$ -NGF, the cells were cultured in the presence of  $\beta$ -NGF (100  $\mu$ g/l) and anti-TrkA<sup>NGFR</sup> (200  $\mu$ g/l) or anti-p75<sup>NTR</sup> (200  $\mu$ g/l) for 30 min, then D-GalN was added and incubated for a further 24 h. Following this period, the XTT reagent was added to the cultured medium and incubated in a humidified atmosphere for 4 h. The absorbance of the samples was measured using a microplate reader at the dual wavelength mode of 450 and 630 nm, respectively. The experiment was performed in tetrameric and repeated three times with consistent results.

**Biochemical assay.** Hepatocyte toxicity was determined by LDH activity and the concentration of MDA in the culture medium. The production of ALB, an important functional marker of hepatocytes, was evaluated using a colorimetric method. The antioxidative condition of hepatocytes was determined by measuring the levels of GSH in the cell lysate.

**Mitochondrial membrane potential (MMP).** The change in MMP was evaluated from uptake of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide), a cationic lipophilic fluorescent probe. In intact cells, the healthy mitochondrial membrane was polarized and JC-1 was rapidly taken up by mitochondria. This uptake increased the concentration gradient of JC-1, which led to the formation of JC-1 aggregates in the mitochondrial matrix. JC-1 aggregates exhibited a red spectral shift resulting in higher levels of red fluorescence (Em<sub>max</sub> 590 nm). In cells with an altered MMP, JC-1 did not aggregate in the mitochondria and thus remained in the cytoplasm as monomers, producing green fluorescence (Em<sub>max</sub> 527 nm). L-02 cells were incubated with JC-1 at 37°C for 15 min and centrifuged at 400  $\times$  g for 5 min at RT. Subsequently, the cells were carefully removed, the supernatant was discarded and the cells were resuspended in the JC-1 working solution. L-02 cells were analyzed by flow cytometry at the excitation and emission wavelengths of 527 and 590 nm, respectively.

**Statistical analysis.** All values are expressed as the mean  $\pm$  SD. Differences between the groups were compared using a one-way analysis of variance (ANOVA) test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Expression of NGF, TrkA<sup>NGFR</sup> and p75<sup>NTR</sup> in L-02 cells.** The expression of NGF, TrkA<sup>NGFR</sup> and p75<sup>NTR</sup> was examined in L-02 cells using immunocytochemistry. Immunostained NGF was detected prominently in the cytoplasm and on the nuclei, and TrkA<sup>NGFR</sup> and p75<sup>NTR</sup> immunoreactivities were observed on the cell membrane and in the cytoplasm of L-02 cells (Fig. 1).

**Morphological changes of L-02 cells in the culture medium.** The morphology of L-02 cells incubated with D-GalN demonstrated discontinuities of the cytoplasm membrane, its spherical shape and highly granular cytoplasm formed a notable contrast

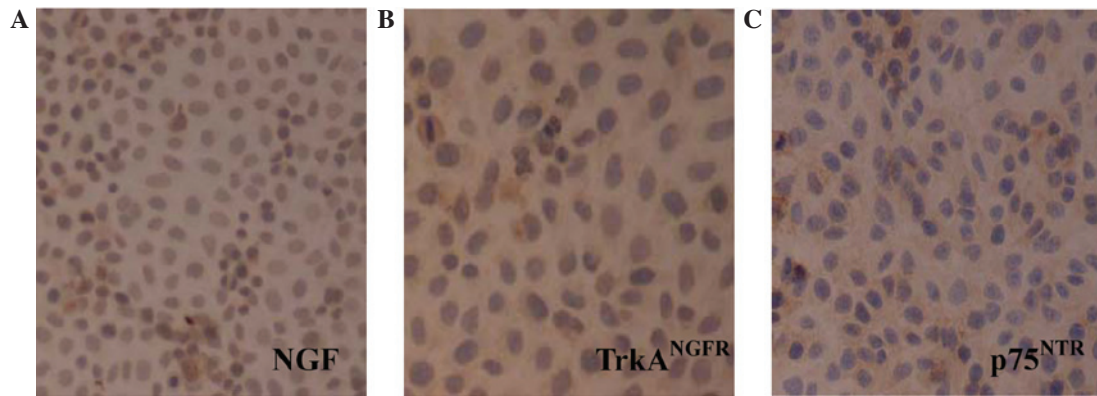


Figure 1. (A) NGF, (B) TrkA<sup>NGFR</sup> and (C) p75<sup>NTR</sup> protein expression in L-02 cells (immunocytochemistry; magnification, x200). NGF, nerve growth factor; TrkA<sup>NGFR</sup>, tyrosine kinase-A nerve growth factor receptor; p75<sup>NTR</sup>, p75 pan-neurotrophin receptor.

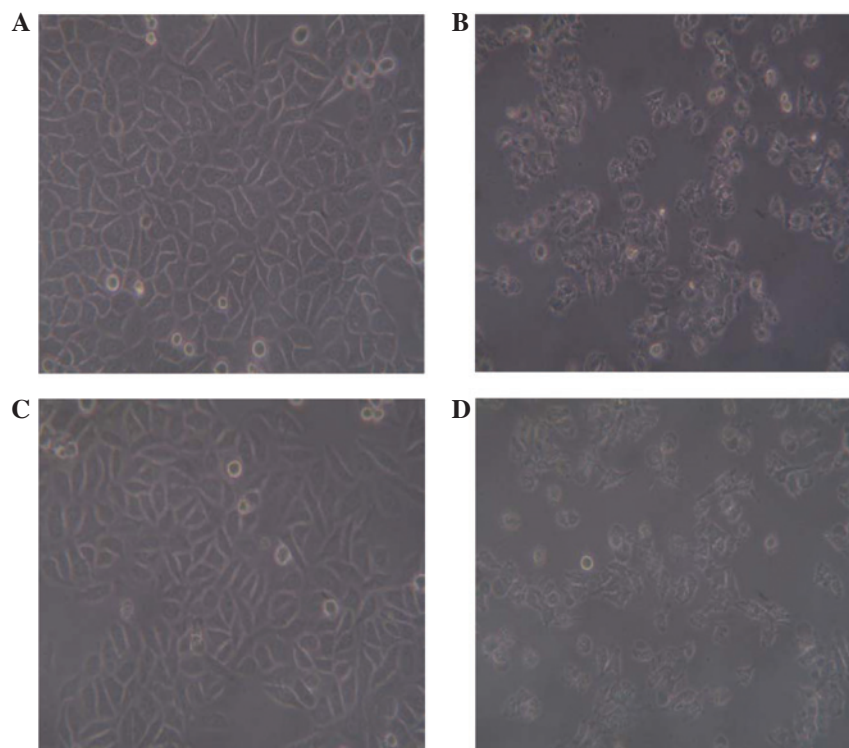


Figure 2. Morphological changes of L-02 (micromorphology; magnification, x200).

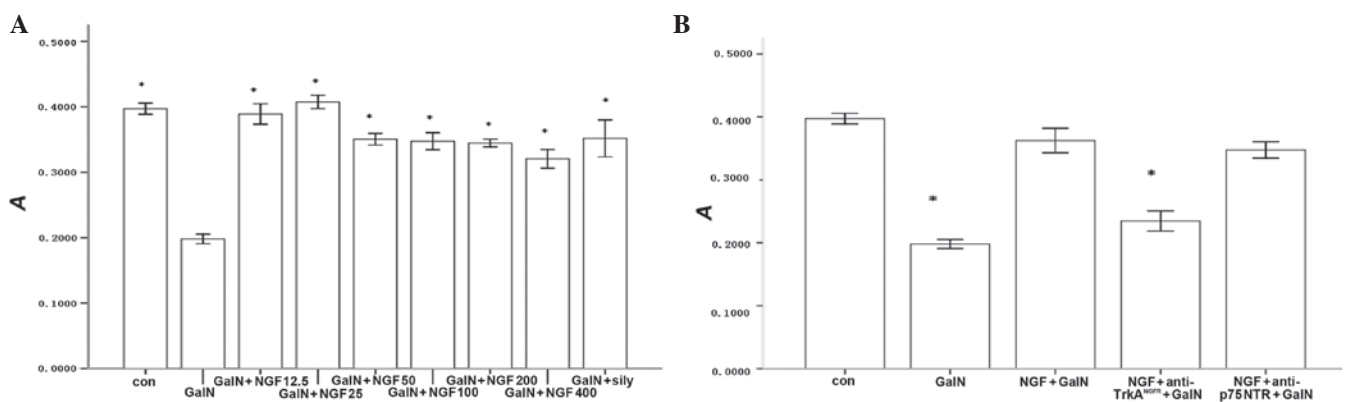


Figure 3.  $\beta$ -NGF promotes the proliferation of L-02 cells. (A) The effect of  $\beta$ -NGF on the proliferation of L-02 cells injured by 40 mmol/l of D-GalN after 24 h (\* $P$ <0.05 vs. D-GalN). (B) The effect of  $\beta$ -NGF promoting the proliferation of L-02 cells was eradicated by an anti-TrkA<sup>NGFR</sup> blocking antibody (\* $P$ <0.01 vs.  $\beta$ -NGF + GalN). Con, control;  $\beta$ -NGF,  $\beta$ -nerve growth factor; D-GalN, D-galactosamine; anti-TrkA<sup>NGFR</sup>, anti-tyrosine kinase A nerve growth factor receptor; p75<sup>NTR</sup>, p75 pan-neurotrophin receptor; sily, silymarin. Values are presented as the mean  $\pm$  SD (n=4).

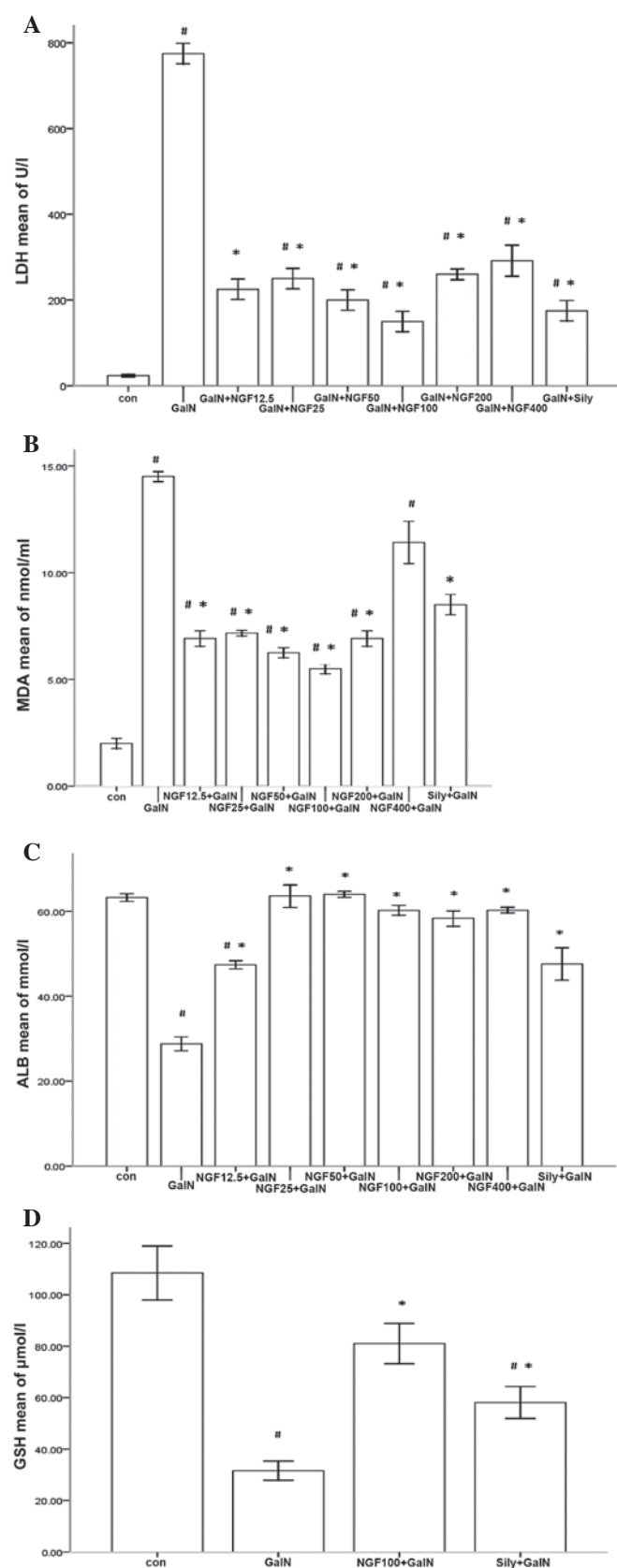


Figure 4. The effects of  $\beta$ -NGF on the biochemical changes of L-02 cells injured by 40 mmol/l GalN after 24 h. (A) Effect of  $\beta$ -NGF on the secretion of LDH from L-02 cells. (B) Effect of  $\beta$ -NGF on the secretion of MDA from L-02 cells. (C) Effect of  $\beta$ -NGF on the secretion of ALB from L-02 cells. (D) Effect of  $\beta$ -NGF on the secretion of GSH from L-02 cells. Con, control; GSH, glutathione; GalN, galactosamine; NGF, nerve growth factor; Sily, silymarin; ALB, albumin; MDA, malondialdehyde. Values are presented as the mean  $\pm$  SD (n=4). <sup>\*</sup>P<0.05 vs. con; <sup>#</sup>P<0.05 vs. GalN.

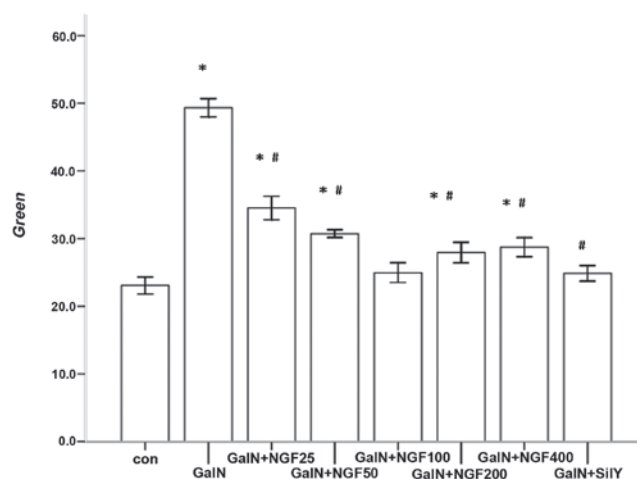


Figure 5. Accumulation of JC-1 in the cytoplasm decreased following NGF treatment of L-02 cells injured by D-GalN. <sup>\*</sup>P<0.05 vs. control; <sup>#</sup>P<0.05 vs. GalN. Con, control; D-GalN, D-galactosamine; NGF, nerve growth factor; sily, silymarin; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide.

to the intact cells.  $\beta$ -NGF and Sily were capable of markedly improving the D-GalN-induced morphological changes to L-02 cells (Fig. 2).

*$\beta$ -NGF promotes the proliferation of L-02 cells.* Exogenous human recombinant  $\beta$ -NGF was capable of promoting the proliferation of D-GalN-injured L-02 cells in a dose-dependent manner. We observed a statistically significant increase at concentrations between 25–400  $\mu$ g/l.  $\beta$ -NGF and Sily significantly promoted the proliferation of L-02 cells compared with D-GalN alone. However, there were no differences between the  $\beta$ -NGF and Sily groups (Fig. 3A). To demonstrate which signaling pathway contributed to  $\beta$ -NGF irritating the proliferation events of L-02 cells, we inhibited the biological activity of TrkA<sup>NGFR</sup> with anti-TrkA<sup>NGFR</sup> and observed that the promoted proliferation effect of  $\beta$ -NGF on L-02 cells was blocked. However, pretreatment of L-02 cells with the anti-p75<sup>NTR</sup> antibody, which neutralized the binding of  $\beta$ -NGF to p75<sup>NTR</sup>, did not affect the proliferation of L-02 cells responding to  $\beta$ -NGF (Fig. 3B). These data suggested that TrkA<sup>NGFR</sup> participates in the response of the L-02 cells to  $\beta$ -NGF.

*Biochemical changes of  $\beta$ -NGF on D-GalN-induced L-02 cells.* D-GalN severely injured L-02 cells in the primary culture following 24 h incubation. D-GalN induced a marked increase in LDH and MDA activity in the supernatant (Fig. 4A and B). Furthermore, ALB production and the GSH content of the cells decreased (Fig. 4C and D).  $\beta$ -NGF reduced the levels of LDH and MDA released from D-GalN-induced L-02 cells, while ALB synthesis was markedly enhanced and the cell content of GSH was increased.

*Effect of  $\beta$ -NGF on the MMP of D-GalN-induced L-02 cells.* The MMP was measured using the fluorescent probe JC-1. There was low accumulation of JC-1 in the mitochondrial matrix of L-02 cells incubated with D-GalN. However,  $\beta$ -NGF significantly decreased the permeability of the inner mitochondrial membrane and JC-1 accumulated in the mito-

chondrial matrix, recovering the MMP damaged by D-GalN. There was no statistical difference in JC-1 accumulation in the mitochondrial matrix between the  $\beta$ -NGF and Sily treatment groups (Fig. 5).

## Discussion

D-GalN is frequently used in liver damage models to replicate the effects of human viral hepatitis, which induces diffuse hepatic injury by depleting uridine nucleotides, resulting in the inhibition of the synthesis of messenger RNA and subsequent proteins (11). A hepatotoxic dose of D-GalN induces hepatic tissue lesions associated with a decrease in GSH synthesis or an increase in the breakdown of GSH (12,13). Mitochondrial permeability transition (MPT) leads to the dissipation of MMP and is considered to be a fundamental mechanism of cell apoptosis or necrosis (14). Liver regeneration driven by hepatocyte proliferation is required for hepatic tissue injury-repair and survival following acute/chronic liver injury (15,16).

This study demonstrated that pretreatment with exogenous human recombinant  $\beta$ -NGF promoted the proliferation and inhibited apoptosis of D-GalN-induced L-02 cells compared with D-GalN alone. LDH is an important marker for hepatocyte injury. We identified that D-GalN significantly increased the activity of LDH in the L-02 cell culture medium. However, pretreatment with  $\beta$ -NGF markedly decreased the LDH activity of D-GalN-induced L-02 cells. MDA is a marker for lipid peroxidation. Pretreatment with  $\beta$ -NGF reduced the concentration of MDA and increased intracellular GSH content compared with D-GalN alone. The production of ALB by D-GalN-incubated L-02 cells was markedly reduced, but  $\beta$ -NGF may improve the synthesis of ALB by D-GalN-induced L-02 cells. However, high MMP in L-02 cells was completely eradicated by D-GalN. The opening of high-conductance MPT pores increases the permeability of the inner-mitochondrial membrane, which results in the collapse of the MMP, a disruption in ionic homeostasis, cytochrome C release and subsequently, apoptosis (17). However, pretreatment with  $\beta$ -NGF may prevent MPT pores from opening by repairing the plasma membrane injured by GalN-induced L-02 cells, which may be achieved by enhancing GSH synthesis. The integrity of the cells was damaged by D-GalN, whose representations were associated with discontinuities in the plasma membrane, a spherical shape and a highly granular cytoplasm when compared with intact cells (18). Treatment with  $\beta$ -NGF markedly reduced D-GalN-induced morphological changes to the cells. These results indicated that  $\beta$ -NGF is a potential drug for protecting L-02 cells from D-GalN-induced injury.

To investigate the mechanism by which  $\beta$ -NGF promotes the proliferation of L-02 cells, we performed immunocytochemical staining for NGF, TrkA<sup>NGFR</sup> and p75<sup>NTR</sup> in L-02 cells. We discovered that L-02 cells expressed NGF, TrkA<sup>NGFR</sup> and the p75<sup>NTR</sup> protein. In order to evaluate which, or both, neurotrophin receptors were involved in the response of L-02 cells to  $\beta$ -NGF, L-02 cells were pretreated with  $\beta$ -NGF and anti-TrkA<sup>NGFR</sup> or anti-p75<sup>NTR</sup> for 30 min and subsequently damaged by D-GalN for 24 h. The promoted proliferation effect of  $\beta$ -NGF on the L-02 cells was inhibited by anti-TrkA<sup>NGFR</sup>. Notably, this selectively inhibited  $\beta$ -NGF binding to p75<sup>NTR</sup> with anti-p75<sup>NTR</sup>, yet it did not prevent  $\beta$ -NGF from promoting the proliferation

of L-02 cells. These data demonstrate that TrkA<sup>NGFR</sup> may be involved in the response of L-02 cells to  $\beta$ -NGF.

In conclusion, in this study we demonstrated that NGF and its receptors, TrkA<sup>NGFR</sup> and p75<sup>NTR</sup>, are expressed in L-02 cells. Exogenous human recombinant  $\beta$ -NGF exerts a hepatoprotective effect on D-GalN-induced L-02 cell injury. The beneficial effects of this agent may be partially due to the promotion of L-02 cell proliferation and the inhibition of apoptosis via the NGF/TrkA<sup>NGFR</sup> signaling pathway, which increases GSH synthesis, attenuates the disruption of oxidative stress and lipid peroxidation and reduces damage to the plasma membrane. Thus,  $\beta$ -NGF is a viable therapeutic option for various liver diseases.

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