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## A Protective Mechanism of Glucagon-like Peptide-1 on AGEs-Induced H9C2 Cardiomyocytes Injury through Inhibiting ROS-Autophagy Pathway

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### Abstract

Diabetic cardiomyopathy is involved in multiple molecular mechanisms, including advanced glycosylation end products (AGEs), reactive oxygen species (ROS), and autophagy. Glucagon-like peptide-1 (GLP-1) is thought to prevent from the deleterious effects of AGEs through suppressing ROS in diabetes, but the role of GLP-1 in ROS-mediated cardiomyocytes injury is unknown. In this study, we tested the hypothesis that Glucagon-like peptide-1 (GLP-1) would attenuate AGEs-induced injury in H9C2 cardiomyocytes by inhibiting ROS-autophagy pathway. Cultured H9C2 cardiomyocytes were treated with one of the following: (1) 1mg/L BSA, (2) 100mg/L AGEs-BSA, (3) 100mg/L AGEs-BSA and 10nmol/L GLP-1, (4) 100mg/L AGEs-BSA and 5nmol/L N-acetyl-L-Cysteine (antioxidant), (5) 100mg/L AGEs-BSA, 10nmol/L GLP-1 and 5μmol/L Rapamycin (autophagy agonist) for 24-hours. We found that AGEs impaired cell viability, increased Intracellular ROS productions, induced apoptosis and triggered autophagy; however, which were reversed remarkably by GLP-1 or N-acetyl-L-Cysteine. Furthermore, the effects of GLP-1 of AGEs-induced cell injury were inhibited by Rapamycin except for ROS. These results suggest

that GLP-1 may prevent H9C2 cardiomyocytes from AGEs-induced injury partly through inhibiting ROS-autophagy pathway.

**Keywords:** GLP-1; Ages; ROS; Autophagy; H9C2 Cardiomyocytes; Protective Mechanism

## 1. Introduction

Diabetic cardiomyopathy (DCM) is increasingly recognized as an emerging risk of heart failure and mortality among diabetic patients independent of vascular pathology [1-2]. Advanced glycosylation end products (AGEs) play an indispensable role in the underlying mechanisms of DCM. The increased formation of AGEs secondary to hyperglycemia may alter structural proteins and lead to a series of pathological changes which involve oxidative stress, inflammation, apoptosis, fibrosis, and hypertrophy [3,4]. Recently, autophagy has been regarded as a novel mechanism in diabetic heart [5,6]. Some studies have demonstrated an inhibited autophagic flux in the hearts of type1 diabetic animals, which may be a cardioprotective response against hyperglycemia injury [7-9]. As a result of hyperglycemia, the formations of AGEs have been reported to result in the damages in some tissues by inducing autophagy [10-12].

Glucagon-like peptide-1 (GLP-1) is a kind of gut hormone secreted by the intestinal L cells, which participates in the maintenance of glucose homeostasis, insulin secretion and pancreatic beta-cell function [13,14]. In addition, GLP-1 also has a protective effect on cardiovascular disease [15,16]. Increasing evidences have suggested that the protective role of GLP-1 is concerned with prevention from the deleterious effects of AGEs in diabetes [17], but effects and mechanisms of GLP-1 on DCM are not fully understood. In this study, we investigated the effect of GLP-1 on autophagy in H9C2 cardiomyocytes incubated with AGEs, and evaluated the underlying role of autophagy regulated by GLP-1 in cardioprotection of DCM.

## 2. Materials and Methods

### 2.1 Cell Culture and Model of Ages-Induced Cell Injury

The rat cardiomyoblast cell lines H9c2 were offered by the Laboratory of Molecular Biology, the Second Affiliated Hospital of Nanchang University (China). The cell culture procedure was performed according to the previously reported technique [18]. H9C2 cardiomyocytes cultured for 3 days were treated with different doses of AGEs-BSA (Calbiochem, America) to establish a model of AGEs-induced cell injury.

### 2.2 Group of Experiments and Treatment Protocols

H9C2 cardiomyocytes were respectively treated with 1mg/L BSA (control); 100mg/L AGEs-BSA (AGEs); 100mg/L AGEs-BSA and 10nmol/L GLP-1 (AGEs+GLP-1); 100mg/L AGEs-BSA and 5nmol/L N-acetyl-L-Cysteine (AGEs+NAC); 100mg/L AGEs-BSA, 10nmol/L GLP-1 and 5μmol/L Rapamycin (AGEs+GLP-1+ Rapa). After incubation for 24 h, the effects of GLP-1 (Sigma, America) on AGEs-induced cell injury were appraised.

### 2.3 Cell Viability Measurements

Cell viability was determined by Cell Counting kit-8 (Beyotime Biotechnology, China). Briefly, following above treatments, the cells were plated onto 96-well plates, and incubated with 10µl CCK-8 solution for 2 hours at 37°C. Optical density (OD) was measured (450 nm wavelength) using Micoplate reader (Biotek, America).

### 2.4 Intracellular ROS Measurements

ROS measurement was carried out according to the procedure of ROS assay kit (Beyotime, China). After the treatments for 24 hours, 96-well plates with H9C2 cardiomyocytes were washed two times using PBS. 2, 7-dichlorodi-hydrofluorescein diacetate (DCFH-DA) was added into plates at a dose of 10µmol/L, and incubated in CO<sub>2</sub> incubator (Eppendorf, Germany) for 20 minutes. The cells were subsequently washed three times, and then ROS-sensitive dye of cells was assessed under an inverted fluorescence microscope (Olympus, Japan) with an excitation wavelength of 488nm and an emission wavelength of 525nm. Fluorescent readings were acquired through image analysis software (Image J 1.410).

### 2.5 Apoptosis Measurements

Apoptosis was measured by flow cytometry. In brief, cells from all groups were treated as follow: 0.25% trypsin (Solarbio, China) digestion, cells collection with centrifugation (300g, 4°C), and cell resuspension (1×Binding Buffer). Apoptosis was finally determined by using Annexin V/PI (Zoman, China) double-staining method. The cells were analyzed with a Calibur/Aria flow cytometer (BD, America).

Hoechst 33258 kit (Beyotime, China) was performed to assess the morphology of apoptotic cells. Following the treatment protocols, the culture medium was removed; the cells in wells were washed three times with cold PBS, and fixed with 4% Paraformaldehyde for 5 minutes. After the cells were washed and dried, Hoechst 33258 was added to each well, and the cells were incubated away from light for an additional 5 minutes. The nuclear fluorescence was visualized under a BX50-FLA microscope (Olympus, Japan).

### 2.6 Autophagosome Formations Measurements

The formations of autolysosomes were detected by Monodansylcadaverine (MDC) staining (Solarbio, China). Cells from all groups in 24-well plates were incubated with 100 µl MDC solution for 30 min at 37 °C, cells were washed two times with PBS. MDC was observed with a 400-435 nm filter set by fluorescence microscopy (Olympus, Japan).

### 2.7 Autophagy Associated-Protein Measurements

The expressions of autophagy associated-proteins, Beclin-1 and LC3II/I, were analyzed by western blot. Total protein was extracted by cell collection, cell lysis, and protein precipitation. The protein concentration was estimated using a bicinchoninic acid (BCA) assay kit. An equivalent of 30-50µg total protein was resolved on a 5-12% SDS-PAGE and transferred to the polyvinylidene fluoride (PVDF) membrane. Then, the membrane was blocked with 5%

non-fat milk in TBST for 2 hours, followed by incubation overnight at 4°C with primary antibodies according to the manufacturer's recommendations. Subsequently, the membranes were washed thrice with TBST for 5 minutes each, incubated with horseradish peroxidase-conjugated secondary antibody in 5% non-fat milk-TBST for 1 hour and washed with TBST four times for 7 minutes each. The intensity of the immunoreactive bands of Beclin-1 and LC3B were quantified by Image J1. 410.

## 2.8 Autophagic Flux Measurements

To further confirm the activity of autophagy, autophagic flux was monitored by Ad-mCherry-GFP-LC3 system (Beyotime, China). Briefly, the cells were incubated with mCherry-GFP-LC3 adenoviral vectors for 24 hours at 37°C, and then new medium was replaced in wells, followed by incubation with different reagents for 24 hours at 37°C. Autophagy was observed under a BX50-FLA fluorescence microscope (Olympus, Japan). Autophagic flux was determined by counting the number of GFP, RFP and merged points (dots/cells).

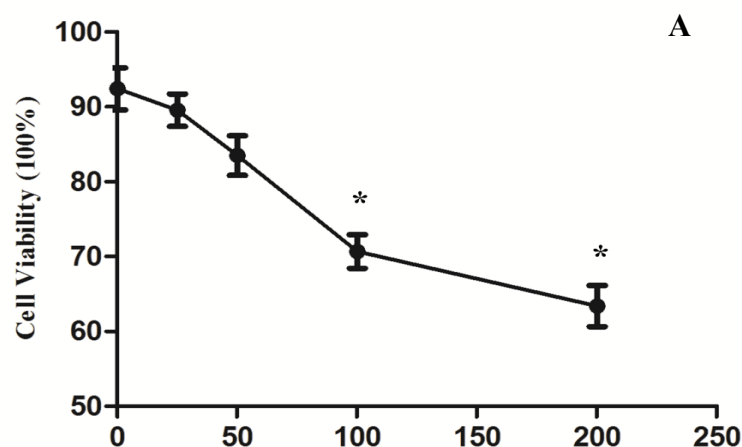
## 2.9 Statistical Analysis

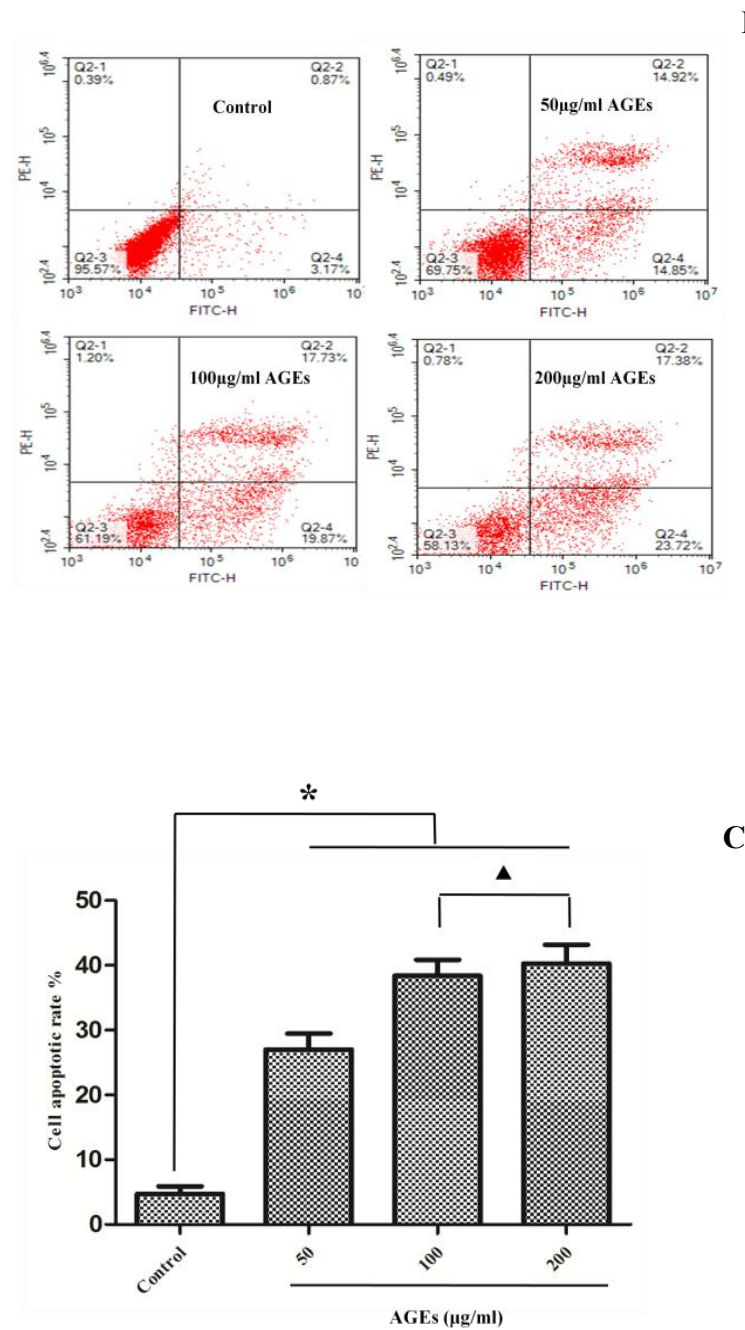
All experiments were performed at least three times. All data were expressed as the mean  $\pm$  SD. One-way ANOVA was applied to assess statistical significance between groups. A value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1 Ages Induce Cardiomyocytes Injury in A Dose-Dependent Manner

After administration of different doses of AGEs-BSA in H9C2 cardiomyocytes, AGEs resulted in H9C2 cardiomyocytes injury in a dose-dependent manner, which were demonstrated by measurements of cell viability (Figure 1A) and apoptosis rate (Figure 1B and 1C). 100mg/L AGEs was indicated as the dose of following experiment (Figure 1C).



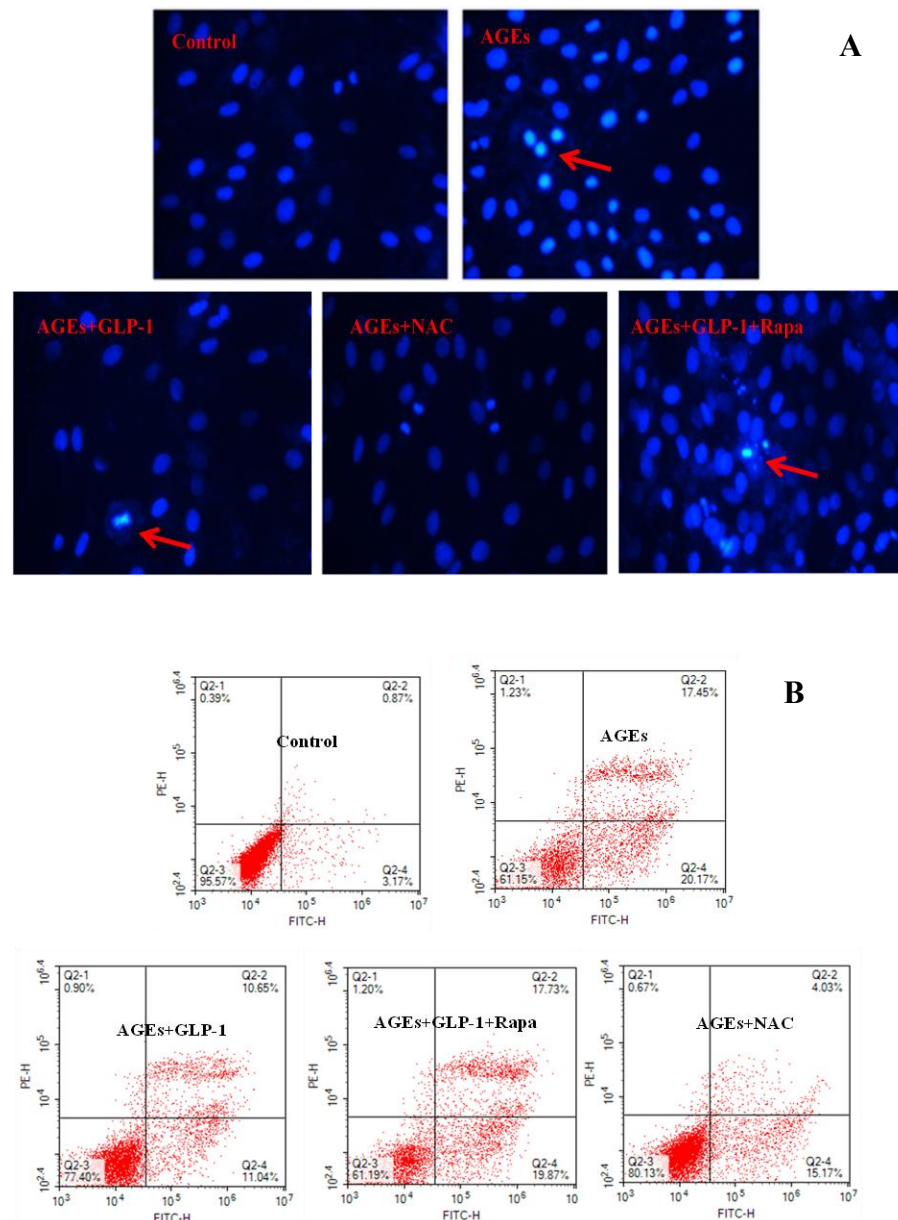


**Figure 1: AGEs result in H9C2 cardiomyocytes injury.** (A). AGEs significantly suppressed cell viability in a dose-dependent manner compared with control group (0 ug/ml AGEs) (\* $P < 0.05$ ) (B). Apoptosis were assessed by flow cytometry in H9C2 cardiomyocytes at different doses of AGEs. (C). AGEs significantly induced

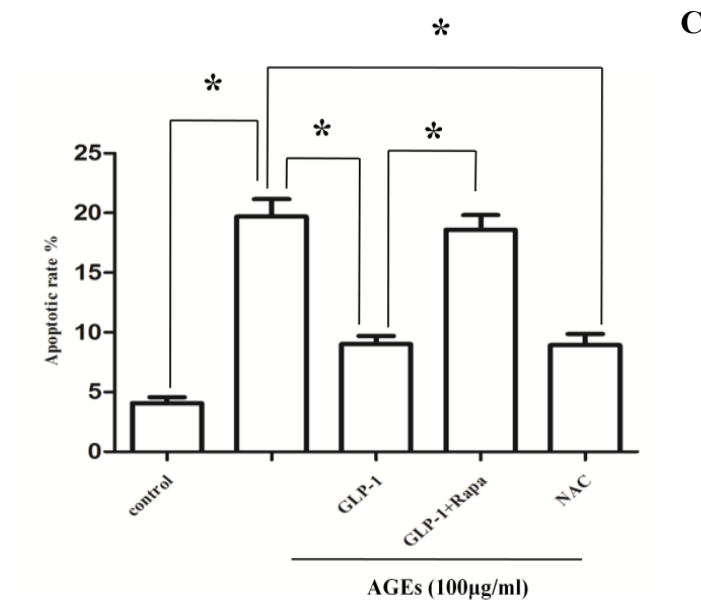
cardiomyocytes apoptosis at the doses of 50 ug/ml, 100 ug/ml and 200 ug/ml (\* $P < 0.05$ ), there were not significantly different between 100 ug/ml and 200 ug/ml in apoptotic rate ( $\Delta P > 0.05$ ).

### 3.2 Ages Induce Cardiomyocytes Injury Through the Increased Formation of ROS

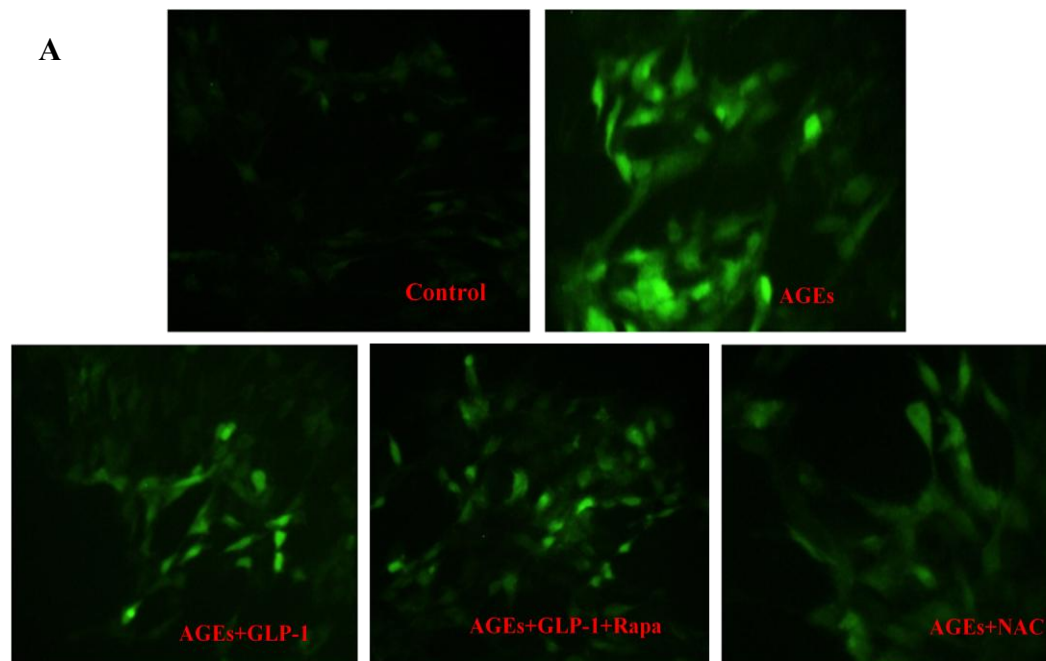
To further determine whether AGEs-induced cell injury is associated with increased ROS, antioxidant (N-acetyl-L-Cysteine, NAC) was applied to investigate the damage mechanism of AGEs. The results showed that NAC reduced Intracellular ROS levels (Figure 3) and apoptotic rate (Figure 2) significantly compared with AGEs. It suggests that AGEs might result in injury to H9C2 cardiomyocytes through the increased formation of ROS.

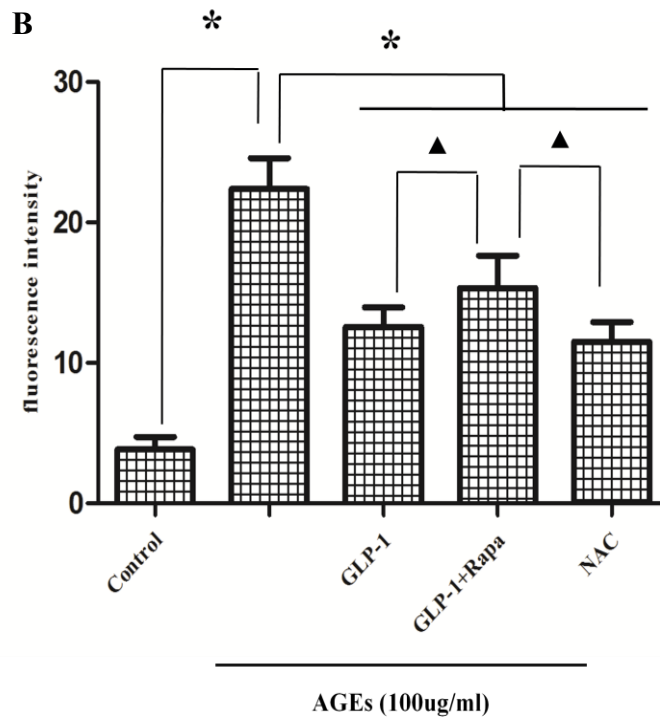






**Figure 2: GLP-1 or NAC suppresses AGEs-induced apoptosis in H9C2 cardiomyocytes.** (A). The morphologies of apoptotic cells were assessed by Hoechst 33258 kit in all groups, red arrow indicated apoptotic cells. (B). Apoptosis were assessed by flow cytometry in all groups. (C). Apoptotic rates were analyzed in all groups, GLP-1 or NAC significantly suppressed AGEs-induced apoptosis, which were inhibited by Rapamycin (\* $P < 0.05$ ).





**Figure 3: GLP-1 or NAC attenuates the formation of AGEs-induced ROS in H9C2 cardiomyocytes. (A).** ROS-sensitive dye of cell was assessed by DCFH-DA in all groups, green fluorescence represented intracellular ROS production. **(B).** Fluorescence intensity of ROS was analyzed in all groups, AGEs significantly increased formations of ROS (\* $P < 0.05$ ), which were remarkably inhibited by GLP-1 or NAC (\* $P < 0.05$ ). Rapamycin could not significantly impaired the effect of GLP-1 on ROS ( $\Delta P > 0.05$ ).

### 3.3 GLP-1 Attenuates Cardiomyocytes Injury By Suppressing the Formation of Ages-Induced ROS

To investigate the effect of GLP-1 on AGEs-induced injury in H9C2 cardiomyocytes, the model of AGEs-induced cells injury was treated with a dose of 10nmol/L GLP-1. The results showed that GLP-1 remarkably impaired the adverse effects of AGEs on apoptosis (Figure 2) and ROS levels (Figure 3), which had a similar effect to NAC (Figure 2 and Figure 3). It indicates that GLP-1 protected cardiomyocytes against AGEs-induced injury through interfering with the formation of ROS.

### 3.4 GLP-1 Inhibits Ages-Induced Autophagy in H9C2 Cardiomyocytes

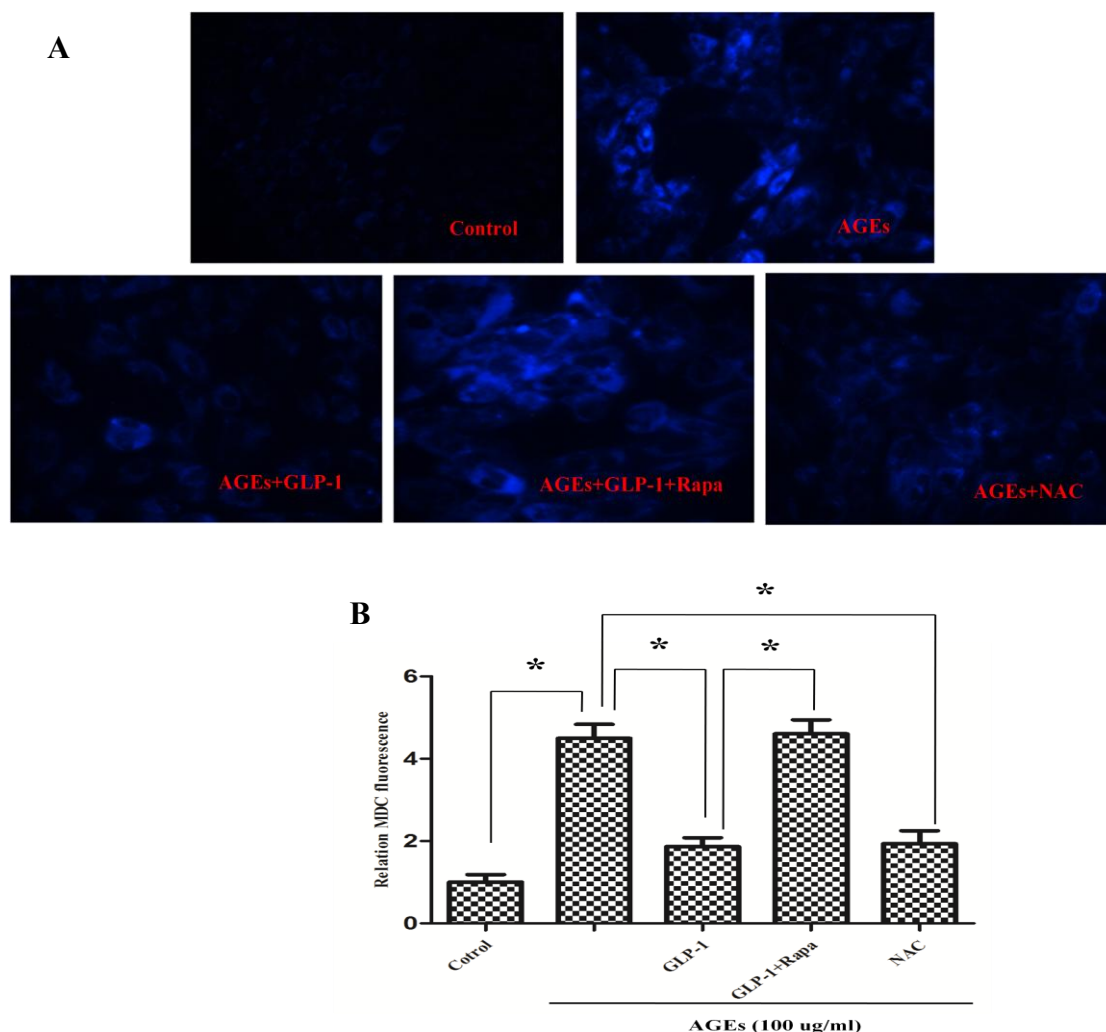
Since there is a tight interaction between ROS and autophagy in related pathological conditions [19,20], we tried to investigate whether GLP-1 had an effect on autophagy through AGEs-induced ROS pathway. The results showed that AGEs induced autophagy significantly at a dose of 100mg/L (Figure 5). After administration of GLP-1, there were lower expressions in the proteins of LC3II/I and Beclin-1 than that in AGEs group, which had no statistical difference compared with administration of NAC (Figure 6). Ad-mCherry-GFP-LC3 also suggested that autophagy was induced at a late stage in cardiomyocytes after incubation with AGEs for 24 hours. As shown in the pictures



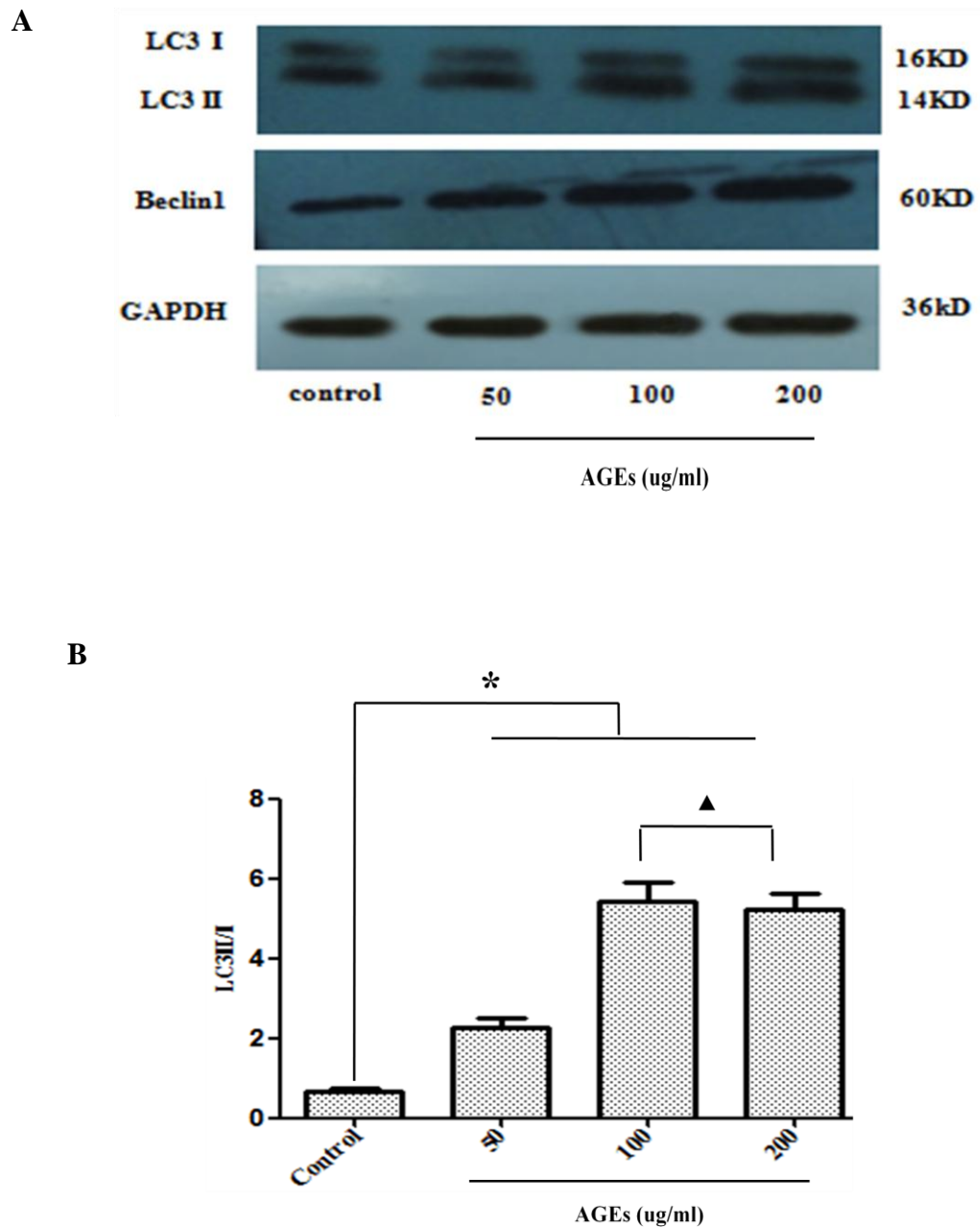
(Figure 8), there were more red points (representing autolysosomes) and fewer yellow points (representing autophagosomes) in AGEs group. However, after administrations of GLP-1 or NAC, there were fewer fluorescence points whether red or yellow than that in AGEs group (Figure 8). Taken together, our data suggest that GLP-1 might suppress autophagy through ROS-autophagy pathway during AGEs-induced cells injury.

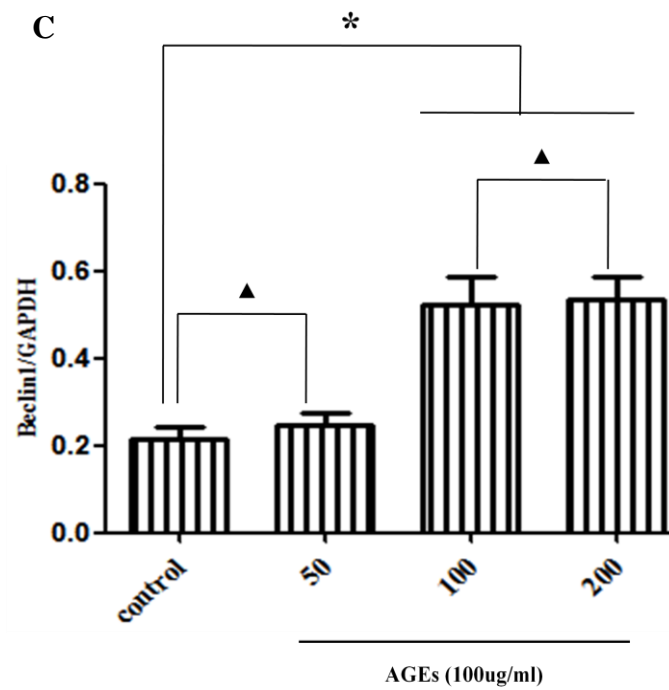
### 3.5 GLP-1 Prevents Cardiomyocytes from Ages-Induced Injury Partly Through Inhibiting ROS-Autophagy Pathway

To further demonstrate whether GLP-1 participates in the process of protection against AGEs-induced cells injury through inhibiting ROS-autophagy pathway, an autophagy agonist, rapamycin was administrated to the model of AGEs-induced cells injury. The results showed that rapamycin significantly impaired the effects of GLP-1 on autophagy and apoptosis except for ROS (Figure 4, Figure 7, and Figure 8). Combining the results of NAC-inhibiting autophagy, it indicates that GLP-1 might protect cardiomyocytes from AGEs-induced injury partly through inhibiting ROS-autophagy pathway.

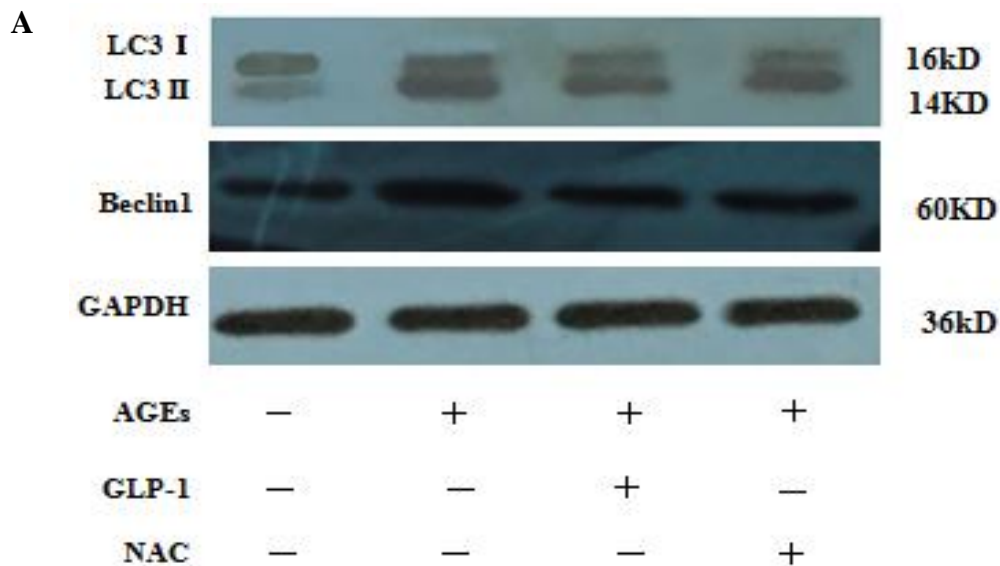


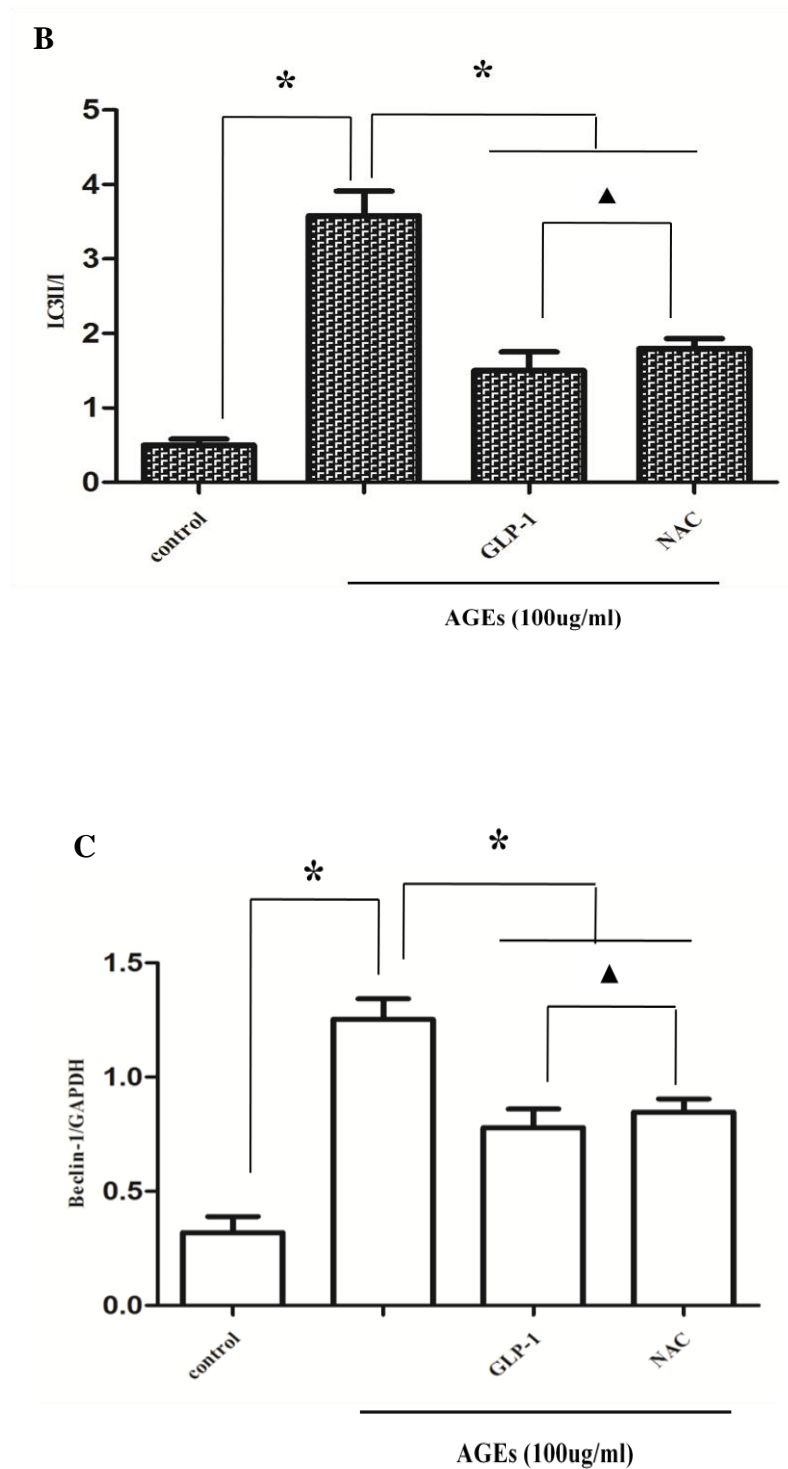
**Figure 4:** GLP-1 ameliorates the formation of autophagosome triggered by AGEs. **(A).** The formations of autolysosomes were detected by Monodansylcadaverine (MDC) staining in all groups, blue fluorescence represented cellular acid vesicles (including autolysosomes). **(B).** Fluorescence intensity of MDC was analyzed in all groups, AGEs significantly triggered the formation of autophagosome (\* $P < 0.05$ ), GLP-1 markedly ameliorated the formation of autophagosome triggered by AGEs (\* $P < 0.05$ ).





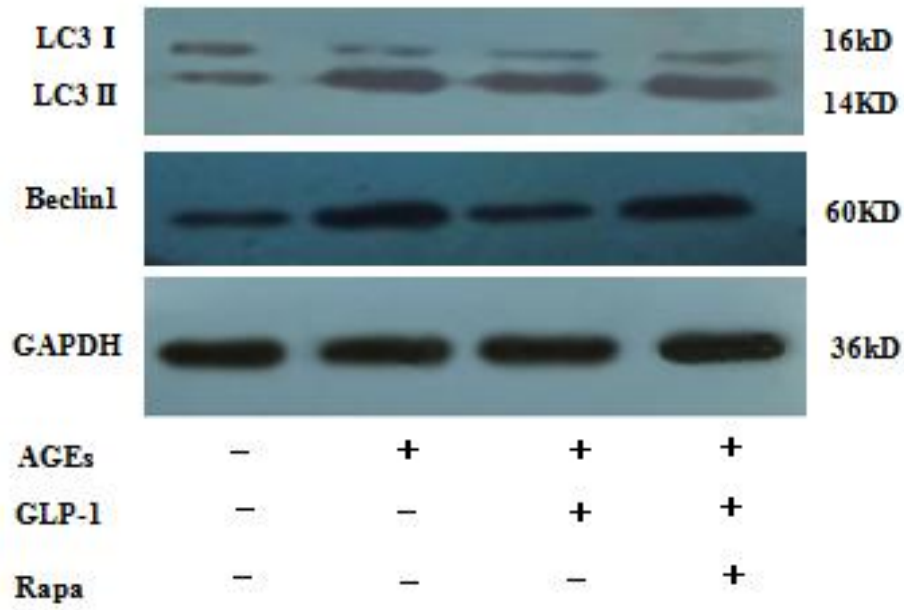
**Figure 5:** AGEs increase the expressions of LC3II/I and Beclin-1 proteins in H9C2 cardiomyocytes. (A). Expressions of LC3II/I and Beclin-1 were detected in the presence of different concentrations of AGEs by western blot. (B). Quantification of LC3II/I expression indicated that autophagy was significantly triggered at the AGEs doses of 50 ug/ml, 100 ug/ml and 200 ug/ml (\* $P < 0.05$ ), there were not significantly different between 100 ug/ml and 200 ug/ml ( $^{\Delta}P > 0.05$ ). (C). Quantification of Beclin-1 expression also suggested that 100 ug/ml AGEs was indicated as the dose of following experiment.



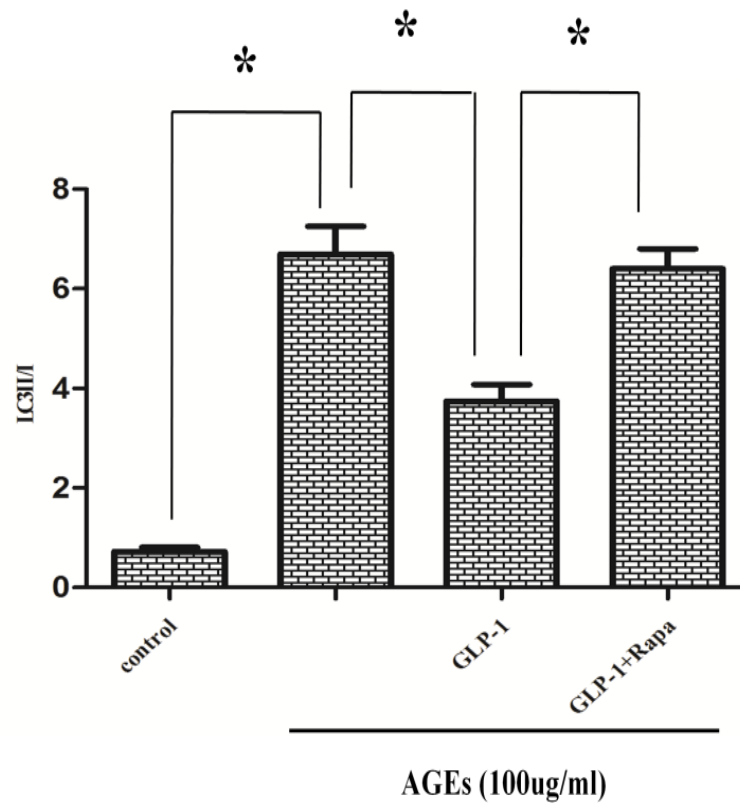


**Figure 6: GLP-1 treatment lowers the expressions of LC3II/I and Beclin-1 proteins in H9C2 cardiomyocytes.** (A). Expressions of LC3II/I and Beclin-1 proteins were measured in different groups by western blot. (B, C). Quantifications of LC3II/I and Beclin-1 expressions showed that GLP-1 might decrease AGEs-induced autophagy (\* $P < 0.05$ ), which had a similar effect to NAC (▲ $P > 0.05$ ).

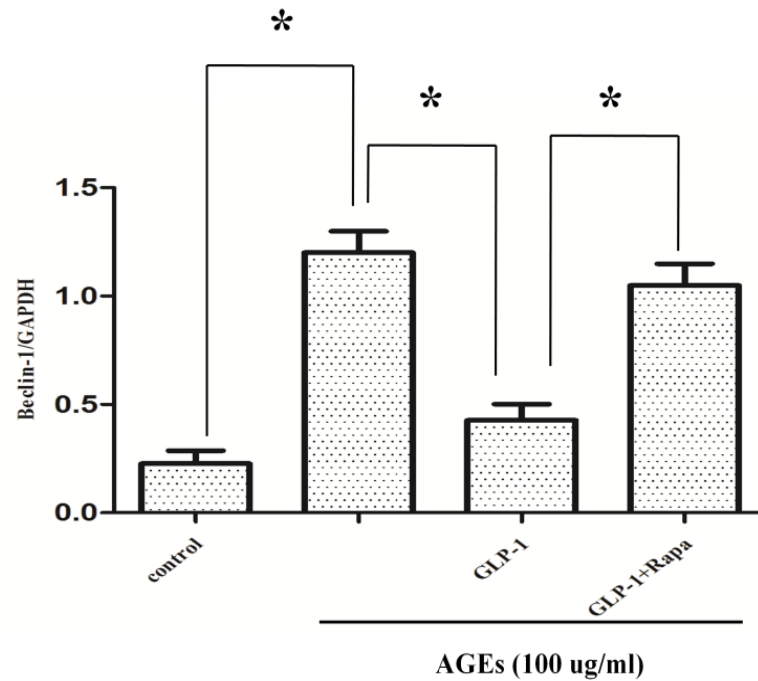
A



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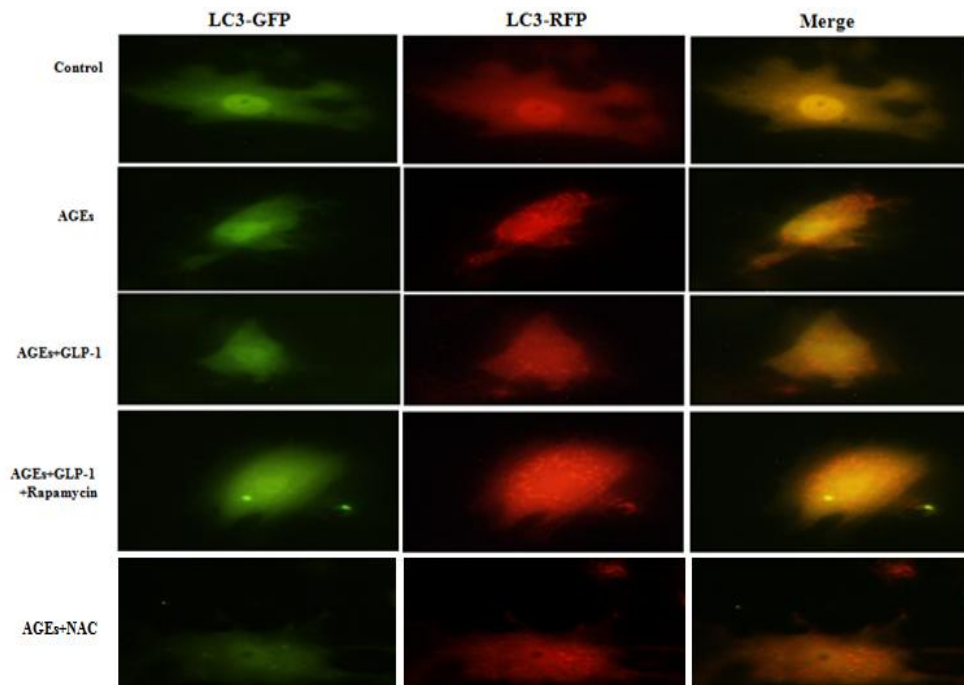


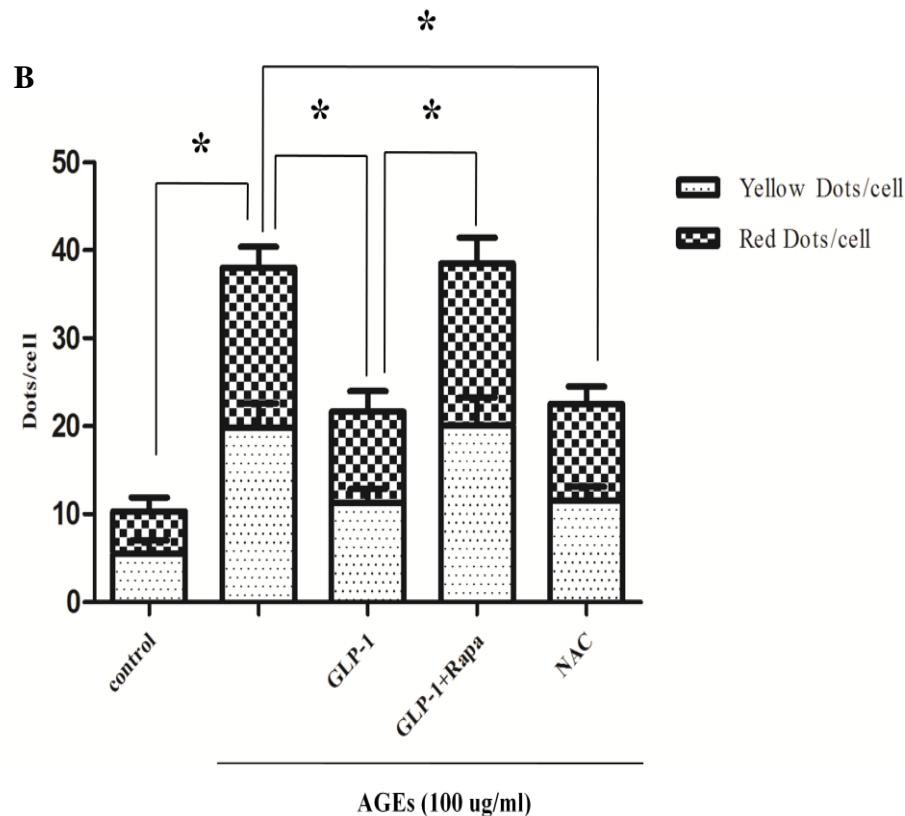
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**Figure 7: Rapamycin impairs the effects of GLP-1 on the expressions of LC3II/I and Beclin-1 proteins in H9C2 cardiomyocytes. (A).** Expressions of LC3II/I and Beclin-1 proteins were measured by western blot after treatment of Rapamycin. **(B, C).** Rapamycin significantly impaired the effects of GLP-1 on the expressions of LC3II/I and Beclin-1 proteins (\*P<0.05).

A





**Figure 8: GLP-1 treatment inhibits AGEs-induced autophagy in H9C2 cardiomyocytes. (A).** Ad-mCherry-GFP-LC3 system was performed to monitor autophagic flux in all groups. As shown in the pictures of merge, yellow points represented autophagosomes, and red points represented autolysosomes. There were fewer fluorescence points whether red or yellow in AGEs group compared with GLP-1 or NAC (\* $P < 0.05$ ). **(B).** Quantification of fluorescence points demonstrated that GLP-1 treatment significantly inhibited AGEs-induced autophagy in H9C2 cardiomyocytes (\* $P < 0.05$ ).

#### 4. Discussion

Autophagy is a catabolic process in which elderly or damaged organelles and proteins are engulfed by autophagosomes, delivered to and degraded in lysosomes under physiological or pathological conditions such as nutrient deprivation, energy exhaust and oxidative stress. Generally, autophagy is a survival pathway that provides substitutes for metabolism of cells or eliminates unwanted components of cells. However, autophagy is also represented as a mechanism of II programmed cell death while a persistent or intense autophagy is induced [21,22]. Our data demonstrate that autophagy was remarkably triggered in cardiomyocytes after administration of AGEs (100mg/L) for 24 hours; excessive ROS were simultaneously generated in cells, which were both impaired by N-acetyl-L-Cysteine or GLP-1. Our data also suggest that the damaged degree of cells was closely associated with the level of ROS. Recently the diabetic complications induced by AGEs have attracted much more attention, especially in diabetic heart [23]. AGEs-RAGE (receptor for AGEs) axis is regarded as a pivotal mechanism by which AGEs



induce the generation of ROS, thereby producing pro-inflammatory mediators, and promoting cardiac fibrosis and hypertrophy via activation of nuclear factor  $\kappa$ -B [4,24]. It indicates that ROS may be an important messenger for AGEs-RAGE axis in the development of DCM.

ROS serve as crucial signaling molecules in the pathogenesis of numerous diseases, including DCM [5,20,25]. Although moderate ROS increase may induce autophagy, thereby triggering a negative feedback mechanism that mitigates oxidative stress and promotes cell survival, excessive ROS could mediate autophagic cell death [26,27,28], which might be involved in impairment of mitochondrial function and concomitant ROS formation [29,30]. Our data suggest that there is a positive correlation between level of ROS and activation of autophagy. Furthermore NAC or GLP-1 administration could significantly lower activity of autophagy and ameliorate cells injury through interfering with ROS generation. It has been reported that GLP-1 counteracts the AGE-induced ROS generation, and prevents AGE-induced necrosis and apoptosis in many cell cultures [31,32,33]. In this study, we first report the effect of GLP-1 on autophagy of cardiomyocytes through AGEs-induced oxidative stress pathway.

To further demonstrate whether autophagy itself also contributes to the cells injury independent of ROS, we administrated rapamycin in H9C2 cardiomyocytes incubated with AGEs and GLP-1 together. The results show that enhanced autophagy with rapamycin markedly promoted apoptosis, and decreased cell viability; but could not significantly increase ROS level in cardiomyocytes. Our findings are consistent with some previous studies in which autophagy had a deleterious effect on cardiomyocytes under overactivated condition [10,34]. Meanwhile, our research also indicates that the effect of GLP-1 on autophagy is involved in ROS-autophagy pathway. Collectively, all above results reveal a potential mechanism that GLP-1 may have protective effect on AGEs-induced injury in H9C2 cardiomyocytes partly through inhibiting ROS-autophagy pathway, but the exact mechanisms of which are fully unknown, and remain to be further explored.

### Acknowledgements

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