Botulinum toxin type A induces protective autophagy in human dermal microvascular endothelial cells exposed to an *in vitro* model of ischemia/reperfusion injury

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Received February 14, 2018; Accepted June 29, 2018

DOI: 10.3892/etm.2018.6741

Abstract. Botulinum toxin type A (BTXA) has been reported to increase the survival of ischemic skin flaps; however, the exact mechanism underlying this effect remains unclear and needs to be further established. The present study aimed to elucidate whether autophagy caused by BTXA functions as a protection mechanism and to identify the mechanisms of its regulation by BTXA in human dermal microvascular endothelial cells (HDMECs) subjected to hypoxia/reoxygenation (H/R)-induced injury. HDMECs were harvested from the upper eyelid tissues of female blepharoplasty patients. HDMECs were exposed to BTXA treatment for 12 h and then subjected to hypoxia for 8 h, followed by reoxygenation for 24 h. Chloroquine diphosphate salt (CQ) was used as an autophagy inhibitor. H/R led to extreme injury to the HDMECs as indicated by the rise in the apoptosis rate, which was significantly attenuated by BTXA pretreatment. The outcomes demonstrated that H/R caused autophagy, as evidenced by a higher type II/type I ratio of light chain 3 (LC3), increased expression of Beclin-1 and increased autophagosome formation. BTXA enhanced autophagy and attenuated apoptosis in a dose-dependent manner, whereas CQ attenuated the BTXA antiapoptotic effects and inhibited the formation of autophagolysosomes, which caused clustering of the LC3-II in cells. In conclusion, autophagy promoted by BTXA serves as a potential protective effect on ischemia/reperfusion injury.

Introduction

Free flap grafting has been widely applied in the restructuring of soft tissue or skin defects caused by trauma or burns. However, flap surgery results in a high rate of morbidity correlated with regional flap necrosis, which is observed in 7-20% of free flaps (1). Ischemia/reperfusion injury (IRI) is caused by the reperfusion of blood to previously ischemic tissue, which causes extreme cellular injury (2,3). Reperfusion of blood into ischemic tissue can cause a cascade of inflammatory processes, leading to damage to vascular and endothelial cells, capillary narrowing and tissue necrosis (4,5). In addition to serious necrotic injury, ischemia also activates the caspase system, which rapidly leads to apoptosis and cell death (6).

Botulinum toxin type A (BTXA) has been demonstrated to increase flap survival through chemical denervation (7) and chemical delay (8). In fact, alterations in the vascular endothelial cells following IRI are also critical, as IRI can also affect microvessels. Endothelial cells, which are the basic component of the vasculature, serve a significant role in maintaining vessel function, for instance, in cytokine secretion and regulation of vascular tone. For a free flap, endothelial cells are essential to provide a blood supply, oxygen transportation and adjustment of skin microcirculation (9,10). IRI of a skin flap causes a change in the function of the endothelial cells, such as increased production of reactive oxygen species, induction of inflammatory response and even apoptosis (11). Endothelial cell injury resulting from ischemia/reperfusion causes disruption of the vascular endothelium and dysregulation of vascular tension, which ultimately leads to worsening of the damage. Thus, protection of the endothelial cells is an important way of alleviating skin flap necrosis.

BTXA, a polypeptide, is produced by the bacterium *Clostridium botulinum*. BTXA has been widely used in clinical practice since its authorization by the Food and Drug Administration (12). It has been demonstrated to exert protective effects on skin flaps in animals by reducing inflammation, ameliorating blood flow and attenuating necrosis (13). Despite significant advances in the use of BTXA in IRI (7,8,13), the exact mechanism of BTXA needs to be further established.

Autophagy is a lysosomal-dependent catabolic pathway that recycles proteins and organelles in cells (14), and is considered to serve a significant role in maintaining cellular homeostasis (15). It can be activated by various stressors, such as ischemia, hypoxia or cell starvation, and autophagy activity usually contributes to cell adaptation or survival (16-18). By contrast, inordinate and dysregulated autophagy may contribute

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Key words: autophagy, botulinum toxin type A, human dermal microvascular endothelial cells, ischemia/reperfusion injury

to cellular dysfunction or apoptosis (19-21). Protective autophagy can be induced in various tissues, including cardiomyocytes and cerebral neurons (22,23). However, the effect of autophagy in reperfusion damage of human dermal microvascular endothelial cells (HDMECs) remains unclear. Previous research revealed that BTXA increases survival and attenuates apoptosis in skin flaps (24). Therefore, it can be hypothesized that the protective effects of BTXA against IRI in HDMECs may be a result of the induction of autophagy.

A previous experiment confirmed that BTXA attenuates endothelial apoptosis during IRI (13); however, the underlying mechanism remains unclear. Thus, the present study used an *in vitro* model of hypoxia/reoxygenation (H/R) in HDMECs to determine the role of BTXA and to confirm the effects of autophagy.

Materials and methods

Cell extraction and culture. HDMECs were harvested from the upper eyelid tissues of 23 female patients with blepharoplasty (age range, 18-25 years) from October 2016 to November 2017. The present study was approved by the Ethics Committee of Anzhen Hospital (Beijing, China). Subsequent to washing with cold phosphate-buffered saline (PBS), the skin flaps were cut into sections of 0.5x0.5x0.1 cm. As previously described (25), the skin flaps were treated with the neutral protease, dispase II (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), to separate and remove the epidermal layer from the dermis. Next, the dermis was digested using collagenase I (Sigma-Aldrich; Merck KGaA) to obtain a cell suspension containing HDMECs, which were cultivated for 1 week. Following treatment with trypsin to create a single cell suspension, HDMECs were purified using a CD31 microbead kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cells were directly transferred into culture or subjected to a second purification. The present study was authorized by the Ethics Committee of Anzhen Hospital (Beijing, China; approval no. 2015021X), and all patients provided signed informed consent.

The cells were cultured and maintained in endothelial cell medium (ECM; cat. no. 1001; ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) containing 5% fetal bovine serum, 1% endothelial cell growth supplement and 1% penicillin/streptomycin at 37˚C in a humidified incubator (5% CO_2). The experimental cells were treated with BTXA (0.1, 0.2, 0.4, 0.8, 1.6 or 3.2 U/ml) for 12 h before induction of hypoxia. Control cells were treated with PBS for the same period of time.

H/R treatment. The culture medium was replaced with fresh serum-free ECM and then the cell cultures were placed in a hypoxic incubator, containing a gas mixture comprising 90% N_2, 5% O_2 and 5% CO_2, for 8 h. To imitate ischemia/reperfusion *in vitro*, cells were then incubated for a further 24 h under normal conditions.

BTXA preparation. BTXA is available as a freeze-dried power, which must be kept in cold storage. The powder was dissolved in ECM and then stored at 4˚C and used within 4 h. The solution was adjusted to a final concentration of 10 U/ml by the addition of ECM.

Chloroquine diphosphate salt (CQ). As an autophagy inhibitor, CQ (cat. no. C6628, Sigma-Aldrich; Merck KGaA) primarily inhibits autophagy by suppressing the formation of autolysosomes. The powder was dissolved in DMSO and then stored at -20˚C. Cells were treated with 25 μM CQ for 2 h prior to BTXA treatment.

Determination of apoptosis using flow cytometry. The apoptosis rate was assessed by flow cytometry using a fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection kit I (cat. no. 556547; BD Biosciences, Franklin Lakes, NJ, USA). According to the manufacturer's protocol, the cells were harvested and resuspended in 1X binding buffer at a concentration of $1x10^6$ cells/ml. Next, 100 μl of the solution ($1x10^5$ cells) was transferred to a 5 ml culture tube, and 5 μl FITC Annexin V and 5 μl propidium iodide (PI) were added. The mixture was incubated for 15 min at room temperature in the dark. Prior to analysis by flow cytometry, 300 μl of 1X binding buffer was added to each tube. The following controls were used in the flow cytometry experiment: Unstained cells, cells stained with FITC Annexin V and cells stained with PI.

Western blot analysis. HDMECs were washed twice with cold PBS and exposed to lysis buffer containing a protease inhibitor for 20 min, followed by centrifugation at 15,000 x g for 15 min at 4˚C. The protein concentration was determined using a bicinchoninic acid assay kit (cat. no. 23227, Thermo Fisher Scientific, Inc.), and protein content was adjusted to achieve equal concentration and volumes. Next, protein samples were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Samples were then incubated with monoclonal rabbit primary antibodies at 4˚C for 12 h, including anti-light chain 3 (LC3; 1:1,000; cat. no. 12741), anti-Beclin-1 (1:2,000; cat. no. 3495) and anti-GAPDH (1:2,000; cat. no. 5174; all from Cell Signaling Technology, Inc., Danvers, MA, USA). Secondary antibody incubation was then performed using alkaline phosphatase goat anti-rabbit immunoglobulin G (cat. no. 7074; Cell Signaling Technology, Inc.). Protein bands were visualized using Enhanced Chemiluminescence (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, Protein expression was quantified using an Odyssey Infrared Imaging system (Gene Company, Ltd., Beijing, China).

Immunofluorescence staining. Immunofluorescence staining was performed to determine the expression of LC3. Briefly, the cells were washed with PBS then fixed with 4% neutral-buffered formaldehyde for 15 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in Tris-buffered saline for 15 min and then incubated with 5% bovine serum albumin in PBS for 30 min at room temperature. Next, the cells were incubated with anti-LC3 antibody (cat. no. 12741; Cell Signaling Technology, Inc.) in 3% bovine serum albumin-PBS at a dilution of 1:100 overnight at 4˚C in a humidified chamber. Subsequent to washing with PBS, the cells were incubated with a FITC-conjugated goat anti-rabbit antibody (cat. no. 4413; Cell Signaling Technology, Inc.) at a dilution of 1:200 in PBS at 37˚C for 1 h in the dark. Finally, nuclei were counterstained with DAPI. Slides were observed

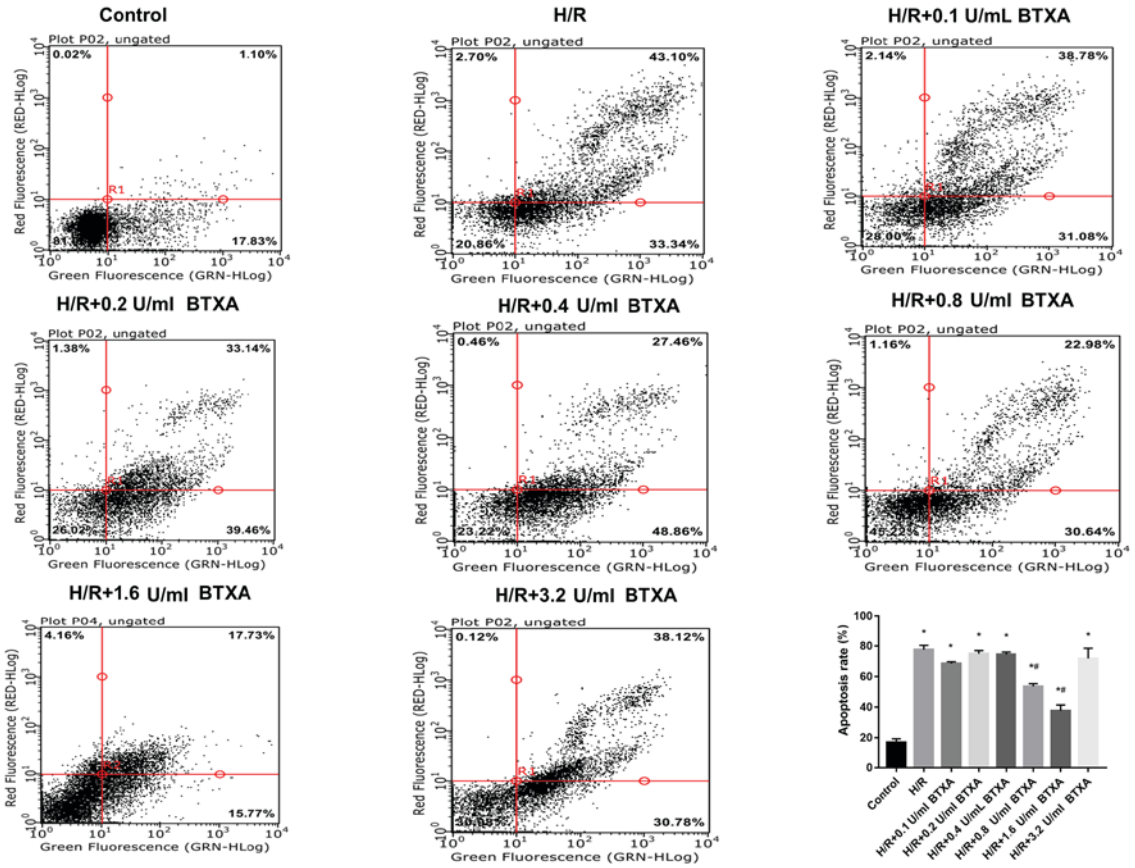

Figure 1. BTXA reduced apoptosis of human dermal microvascular endothelial cells in a dose-dependent manner during H/R. Apoptotic rates were determined by flow cytometry. Values are presented as the mean ± standard deviation. *P<0.01 vs. the control group; #P<0.01 vs. the H/R group. BTXA, botulinum toxin type A; H/R, hypoxia/reoxygenation.

and images were captured under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Transmission electron microscopy. Following treatment, cells were washed twice with cold PBS, harvested with trypsin and then centrifuged at 1,200 x g for 5 min at room temperature. The supernatant was discarded, and cells were fixed with 2.5% glutaraldehyde at 4˚C for 3 h. Subsequently, the samples were dehydrated by an acetone gradient and embedded in Epon 812 resin (cat. no. 14120; Electron Microscopy Sciences, Hatfield, PA, USA), followed by semi-thin section optical positioning and ultra-thin sectioning. The sections were then double-stained with uranyl acetate and lead citrate. A transmission electron microscope was used to record images.

Statistical analysis. Statistical analysis was conducted using IBM SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). Differences among the groups were analyzed by one-way analysis of variance, while pairwise comparisons within groups were conducted using the Student-Newman-Keuls q test. P<0.05 was considered to denote a statistically significant difference. All the data are presented as the mean ± standard deviation.

Results

BTXA attenuated ischemia/reperfusion-induced apoptosis of HDMECs. HDMECs were hypersensitive to H/R. To identify whether BTXA protects HDMECs from apoptosis, HDMECs were treated with different concentrations of BTXA (0.1, 0.2, 0.4, 0.8, 1.6 or 3.2 U/ml) for 12 h prior to exposure to hypoxia. Flow cytometric analysis was initially conducted to assess the apoptosis rate of cells in all the treatment groups. The results demonstrated that, compared with the control group, the rate of apoptosis was significantly increased following H/R exposure. Flow cytometric analysis indicated that BTXA treatment significantly decreased the rate of apoptosis following H/R induction in a dose-dependent manner (Fig. 1). While BTXA did not have a protective role at low concentrations (0.1, 0.2 and 0.4 U/ml), the protective effect gradually increased with increasing concentrations of BTXA (0.8 and 1.6 U/ml) and was strongest at a concentration of 1.6 U/ml. However, the protection disappeared at a concentration of 3.2 U/ml BTXA. Thus, BTXA decreased the rate of apoptosis following H/R induction in a dose-dependent manner, and treatment with 1.6 U/ml BTXA produced the peak beneficial effect (Fig. 1). These results suggested that an appropriate concentration of BTXA may attenuate H/R-induced damage.

BTXA protects against H/R-induced injury in HDMECs through the activation of autophagy. Using CQ as an autophagy inhibitor, the present study aimed to confirm the effect of BTXA in activating autophagy in HDMECs exposed to H/R. CQ effectively inhibited autophagy by inactivating the lysosomal enzymes and blocking the formation of the

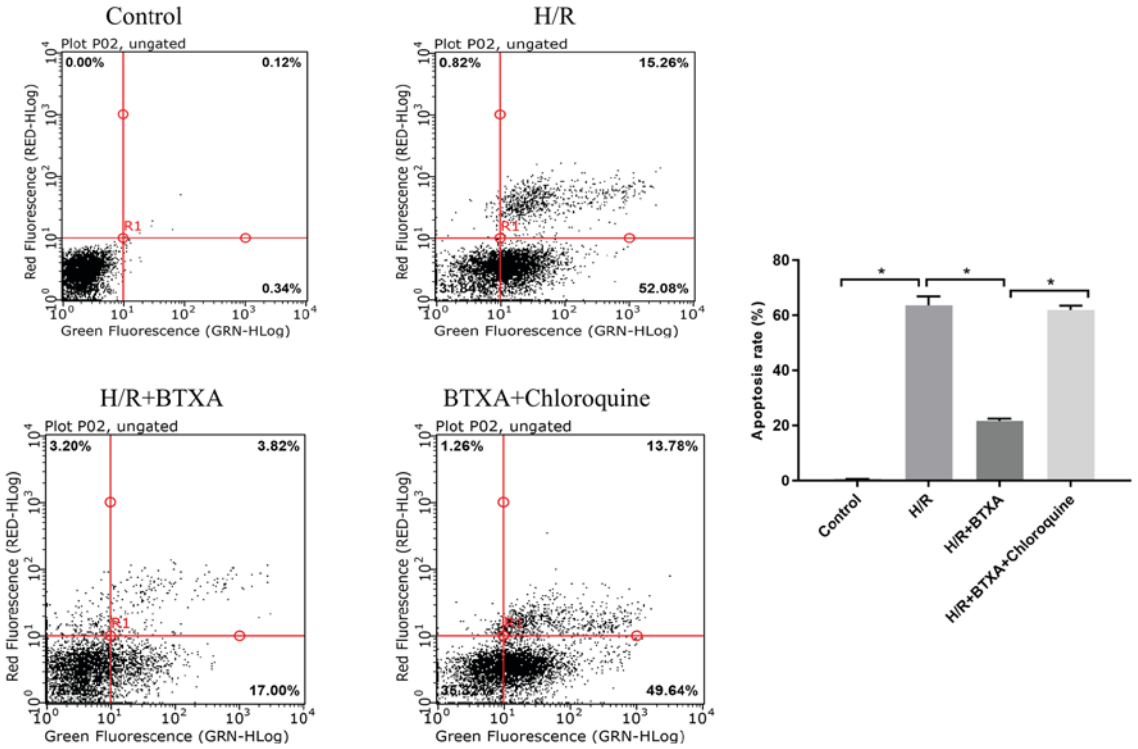

Figure 2. CQ, an autophagy inhibitor, attenuated the BTXA-induced antiapoptotic effects. CQ blocked the anti-apoptotic effect of BTXA following induction by H/R, as demonstrated by flow cytometry. Values are presented as the mean ± standard deviation. *P<0.01. CQ, chloroquine diphosphate salt; BTXA, botulinum toxin type A; H/R, hypoxia/reoxygenation.

autolysosomes. Compared with the H/R group, BTXA significantly attenuated the apoptotic rate, while the protective effect of BTXA was abolished by CQ (Fig. 2).

Next, the levels of LC3 and Beclin-1 in each group were measured by western blot analysis. Compared with the control group, H/R exposure caused significant conversion of LC3-I to LC3-II and increased the expression of Beclin-1 (Fig. 3A). The results also demonstrated that, when the cells were treated with 1.6 U/ml BTXA, the ratio of LC3-II/LC3-I and Beclin-1 expression were significantly increased compared with the H/R group (Fig. 3A). The addition of CQ suppressed the formation of the autolysosomes, which was characterized by a further increase of the LC3-II/LC3-I ratio. However, the expression the other autophagic marker, Beclin-1, was not markedly influenced by CQ. These findings were supported by the results of the immunofluorescence study, with immunofluorescence staining with the LC3 antibody revealing that BTXA and CQ treatment increased LC3-II punctate dots (Fig. 3B). Taken together, the results demonstrated that induction of autophagy explained the protective mechanism of BTXA against H/R-caused injury. At the same time, the study results confirmed that autophagy is a lysosome-dependent protein degradation pathway.

Transmission electron microscopy observations. Transmission electron microscopy analyses revealed that HDMECs in the control group exhibited typical endothelial features, and were visible as oval cells with a large central nucleus and intact mitochondria and endoplasmic reticulum (ER; Fig. 4). HDMECs in the H/R group exhibited clear ultrastructural lesions, including cell swelling, appearance of numerous vesicles, ER dilation, mitochondrial swelling and vacuolization. Compared with the H/R group, pretreatment with BTXA evidently alleviated ultrastructural lesions and increased autophagosome formation. However, CQ aggravated ultrastructural lesions and reduced autolysosome formation, indicating that autophagy alleviated H/R-induced injury (Fig. 4). The results indicated that BTXA alleviated H/R-induced injury by inducing protective autophagy.

Discussion

Over the past few decades, BTXA has been widely used in clinical practice, particularly in plastic surgery, and satisfactory effects on facial rejuvenation have been obtained (26-29). As the research continues, there has been a broad spectrum of indications for the use of BTXA in neuromuscular disorders, such as spasmodic torticollis, spasms of the extremities and anal fissures (30-34). Furthermore, researchers have observed that BTXA has beneficial effects on ischemic skin flaps and Raynaud's disease (35-37). Results have demonstrated that BTXA is able to regulate the vascular tone and improve blood flow, while a number of studies have demonstrated that BTXA improved the survival of critical ischemic skin flaps in animal models (38-40). BTXA has beneficial effects on skin flap IRI, accounted for by its anti-inflammatory effect and chemical delay.

IRI is common in clinical practice and can lead to severe complications (41). Plastic surgeons have already recognized the problem and have searched for a better way to fight IRI. Our previous study demonstrated that ischemia/reperfusion caused serious necrosis of skin flaps, while BTXA pretreatment

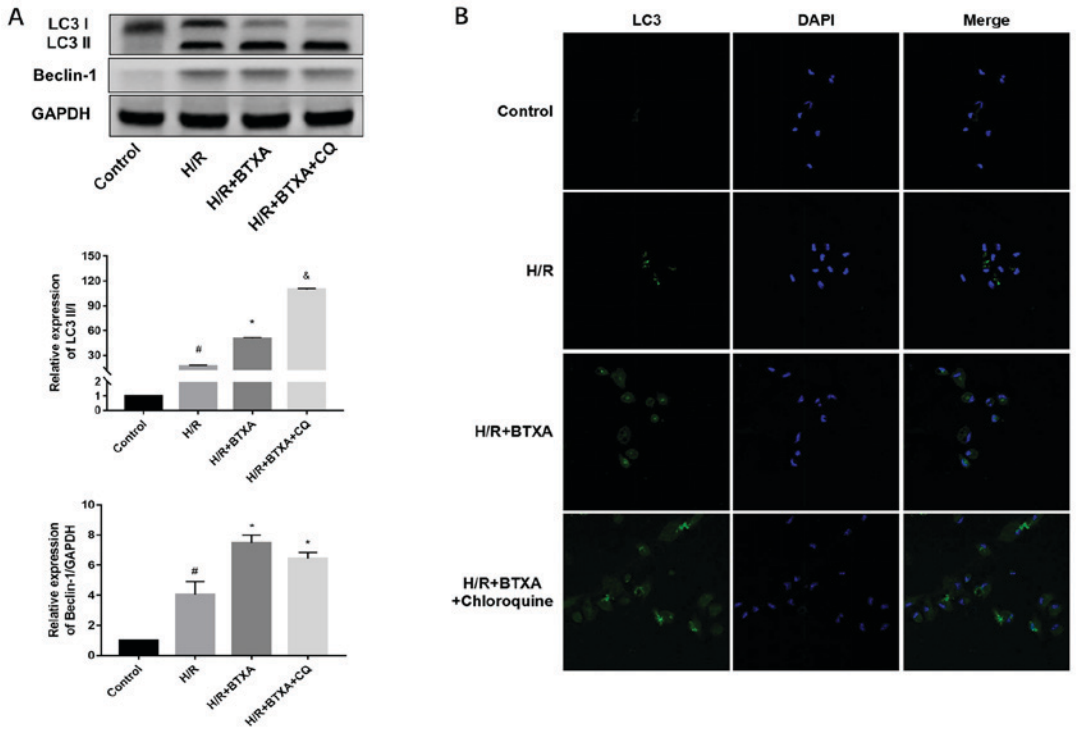

Figure 3. BTXA promoted autophagy in human dermal microvascular endothelial cells, whereas CQ inhibited autophagy. (A) Expression levels of LC3-II/LC3-I and Beclin-1 determined by western blot analysis. (B) Immunofluorescence staining of LC3-II (depicted as punctate dots). Values are presented as the mean ± standard deviation. Magnification, x400. $^{\#}$P<0.01 vs. control group; *P<0.01 vs. H/R group; $^{\&}$P<0.01 vs. BTXA group. BTXA, botulinum toxin type A; CQ, chloroquine diphosphate salt; H/R, hypoxia/reoxygenation; LC3, light chain 3.

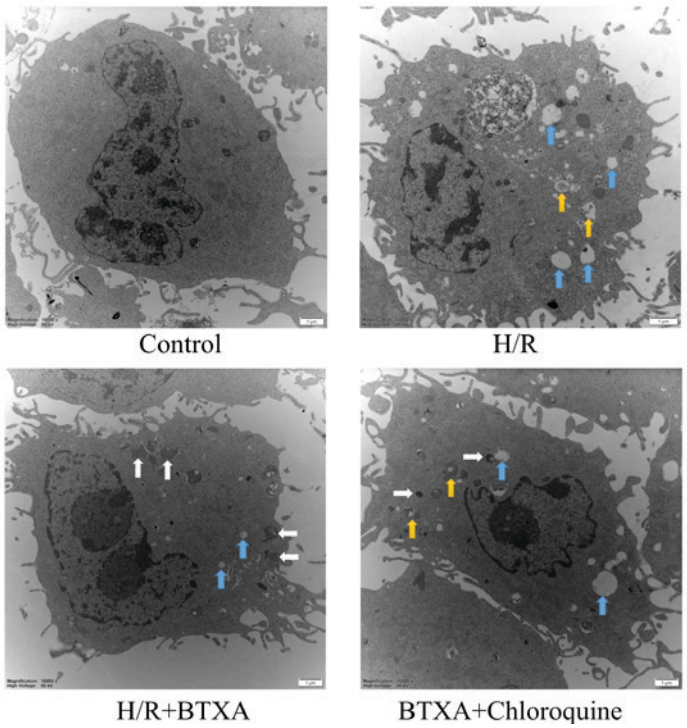

Figure 4. Effect of BTXA on autophagosome formation and ultrastructure in H/R-treated human dermal microvascular endothelial cells by transmission electron microscopy. BTXA promoted the formation of autophagosomes to maintain intracellular homeostasis, while CQ reduced this effect. Black arrows indicate the autophagosomes, yellow arrows indicate the swollen mitochondria, and blue arrows indicate the dilated endoplasmic reticulum. Magnification, x10. BTXA, botulinum toxin type A; H/R, hypoxia/reoxygenation; CQ, chloroquine diphosphate salt.

increased the survival of flaps in an animal experiment (42). The H/R HDMEC *in vitro* model was also established, and the results illustrated that the apoptosis rate of HDMECs following H/R was markedly increased.

Autophagy has been reported to be protective against hypoxia or chemically-induced oxidative stress in several endothelial cell lines (43-46). LC3, as the main autophagy marker (47), is the main component of autophagosomes and exists in two molecular forms, including LC3-I (18 KDa) and LC3-II (16 KDa), and the LC-3II is eventually degraded through lysosomes. LC3-II is formed from cytosolic LC3-I (23) during autophagy activation and the ratio of LC3-II/I significantly increases. LC3-II is mainly degraded by the autolysosome pathway. As an autophagy inhibitor, CQ mainly inhibits autophagy by suppressing the formation of autolysosomes. As a result, LC3-II cannot be degraded by autolysosomes, and a large amount of LC3-II accumulates in cells. Beclin-1 is also an important autophagy marker and serves a critical role in autophagy.

Multiple studies (23,48) have already indicated that activation of autophagy exerts a protective effect in human umbilical vein endothelial cells. While apoptosis is considered to be a process of programmed cell death, autophagy is programmed cell survival. These processes share the same stimulating factors and regulatory proteins, however, they have different thresholds. Autophagy is a type of intracellular defense mechanism that degrades destroyed organelles and recycles proteins, allowing cells to store more nutrition and protect themselves from death (49). However, dysregulated autophagy may induce apoptosis and may also cause certain diseases. Several studies demonstrated that the upregulation of autophagy has beneficial effects in some diseases, including AIDS, autoimmune diseases and neurodegenerative diseases (50-54); however, its role in cancer is controversial (55-58). The present study revealed that BTXA promoted protective autophagy in HDMECs in an *in vitro* model of IRI. To the best of our knowledge, this is the first study to prove that BTXA protects H/R-treated HDMECs *in vitro* by inducing autophagy.

Based on the model of H/R injury, HDMECs were treated with BTXA for 12 h in an attempt to understand the effect of autophagy and its regulation by BTXA. The results demonstrated that autophagy had an antiapoptotic rather than a proapoptotic effect on HDMECs during the H/R period, and that BTXA protected HDMECs from H/R-induced injury in a dose-dependent manner. However, the BTXA treatment had a protective effect only at suitable concentrations, whereas other concentrations of BTXA may exacerbate apoptosis.

To further verify the role of autophagy in the protective effect of BTXA against H/R-induced injury, the autophagy inhibitor CQ was added into the culture medium and the protective effect of BTXA was blocked. CQ, a weakly-alkaline drug, inhibited the autophagic activity by disturbing the degradation of autophagosomes by autophagolysosomes. As a result, excessive LC3-II, an autophagy marker, was stored within cells. Furthermore, it was observed that simultaneous treatment with CQ also increased the cell apoptosis rate. Compared with the BTXA group, CQ significantly inhibited the formation of autolysosomes. These observations further suggested that protective autophagy caused by BTXA may be a potential mechanism underlying the beneficial effects of BTXA on IRI.

Although the present study demonstrated that activation of autophagy by BTXA may ameliorate IRI, certain limitations remain. Firstly, in the experimental design, only one autophagy inhibitor was used. Although this confirmed the results, the underlying reason is not clear. In subsequent experiments, the experimental design will be improved. In addition, the signaling pathways involved in autophagy remain unclear, and the study only presented the results of *in vitro* experiments. Consequently, further research is required to address these issues.

In conclusion, the present study revealed for the first time that autophagy serves as a protective mechanism of BTXA in H/R-treated endothelial cells *in vitro*. Thus, the study confirmed that autophagy activation may be a crucial strategy for ischemia/reperfusion-induced skin flap injury.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation Grants of China (grant nos. 81571922 and 81272130), and the Beijing Natural Science Foundation Grants of China (grant no. 7172065).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL conceived and designed experiments; YS and JC performed the experiments; YS and CC analyzed the data; and YS wrote the manuscript. All authors agreed and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was authorized by the Ethics Committee of Anzhen Hospital (Beijing, China; approval no. 2015021X), and all patients provided signed informed consent.

Patient consent for publication

All patients agree to the publication of this study.

Competing interests

The authors declare that they have no competing interests.

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