Long-Lasting Exendin-4-Loaded PLGA Nanoparticles Ameliorate Cerebral Ischemia/Reperfusion Evoked Brain Injury and Voiding Dysfunction in Diabetic Rats

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Abstract

Exendin-4 (Ex-4) is an incretin mimetic agent approved for diabetes treatment and neuronal protection. However, the required frequent injections restrict its clinical application. We prepared Ex-4-loaded poly(d,l-lactide-co-glycolide) nanoparticles (PEx-4) and investigated the effect on cerebral ischemia/reperfusion (IR) injury associated with cystopathy in diabetic rats. Using ten minutes of bilateral carotid artery occlusion combined with hemorrhage-induced hypotension of IR model in streptozotocin-induced type 1 diabetic (T1DM) Wistar rats, we compared Ex-4 and PEx-4 effect on prefrontal cortex edema, voiding function and oxidative stress including cerebral spinal fluid (CSF) reference \( \text{H}_2\text{O}_2 \) (RH\(_2\text{O}_2\)) and HOCl (RHOCl) levels, endoplasmic reticulum (ER) stress, apoptosis, autophagy and pyroptosis signaling in brain and bladder by western blot and immunohistochemistry. Single injection of PEx-4 displayed higher CSF antioxidant activity and long-lasting hypoglycemic effect than Ex-4 in rats. T1DM enhanced CSF RH\(_2\text{O}_2\), and pIRE-1/cleaved caspase-12/pJNK/ATF4/ATF6/CHOP-mediated ER stress, caspase 3/PARP mediated apoptosis, Beclin-1/LC3-II mediated autophagy and caspase 1/IL-1\( \beta \) mediated pyroptosis signaling in the prefrontal cortex and bladders. IR led to prefrontal cortex edema, impairment in micturition center and further increased CSF RH\(_2\text{O}_2\) and HOCl level, ER stress, apoptosis, autophagy and pyroptosis signaling in the T1DM brains and bladders. PEx-4 were more efficient than Ex-4 in attenuating IR-evoked prefrontal cortex edema and oxidative stress in brains and improving voiding dysfunction in bladders of T1DM rats. In summary, PEx-4 with stronger antioxidant activity and long-lasting bioavailability confer efficiently therapeutic efficacy to ameliorate IR-evoked brain and bladder injury through inhibiting oxidative stress, ER stress, apoptosis, autophagy and pyroptosis signaling in diabetic rats.

Introduction

Diabetes mellitus (DM) resulting from defects in insulin secretion, insulin action, or both is a metabolic disorder with multiple serious complications [1] possibly by hyperglycemia induced oxidative stress and inflammation. The most common complications of DM are lower urinary tract symptoms (LUTS) including diabetic bladder dysfunction [2]. The risk for stroke is doubled in DM patients in comparison with the general population, and these patients are at increased risk of death due to cerebrovascular diseases. Urinary incontinence is a common sequela of acute hemispheric stroke [3]. Among analyzed variables of urodynamic study from patients, total bladder capacity, post-void residual urine volume and bladder compliance were significantly affected in the ischemic and hemorrhagic stroke patients with detrusor overactivity and detrusor underactivity [4]. It is suggested that micturition center located in the brain could be impaired in the ischemic and hemorrhagic stroke patients leading to voiding dysfunction.

At the first stage of treatment in DM evoked global brain ischemia associated with cystopathy, non-invasive strategies like anti-diabetic and neuroprotective drugs should be considered in diabetic brain injury abd cystopathy [5]. GLP-1 (glucagon-like peptide-1) is a gut hormone and binds to a seven transmembraneous domain-G-protein-coupled receptor glucagon-like peptide 1 receptor (GLP-1R) to activate downstream cyclic AMP signaling [6]. GLP-1 reduced circulating glucose ore and has been
suggested as a therapeutic alternative in DM [7]. Exendin-4 (Ex-4 with amino acid sequence of HEGTFTSDLSKQMEEEAVRLFIEWLK NGGPSSGAPPSS-NH2, 4186.7 Da), a peptide isolated from the salivary secretions of the lizard *Heloderma suspectum* (Gila monster) shares a 53% amino acid identity with GLP-1 [8, 9]. Ex-4 has been shown to have several beneficial antidiabetic actions including glucose-dependent enhancement of insulin secretion, slowing of gastric emptying, reduction of food intake and an increase in β-cell mass (animal studies) as well as markers of β-cell function [10, 11, 12].

Although hypoglycemic, cardiovasoprotective, and neuroprotective effects of Ex-4 have been reported [13, 14], this drug is also limited by a short half-life, high burst release, and lower bioactivity. Recently, we have developed long-lasting Ex-4-loaded poly(D,L-lactide-co-glycolide) (PLGA) microspheres and found that the Ex-4 microsphere was more effective than traditional Ex-4 to improve bilateral carotid artery occlusion combined with hemorrhage-induced hypotension-induced oxidative injury and cognitive deficits through the activated p-Akt/p-eNOS and suppressed NF-κB/ICAM-1 signaling, ER stress, and apoptosis pathways [15]. PLGA is one of the most successfully used biodegradable polymers for a longer degradation time from several months to several years and approved by the US FDA and European Medicine Agency in various drug delivery systems in humans. Its hydrolysis to metabolite monomers, lactic acid and glycolic acid, which are endogenous and easily metabolized by the body via the Krebs cycle, a minimal systemic toxicity is associated with the use of PLGA for drug delivery or biomaterial applications [16]. In this study, we prepared PLGA-loaded Ex-4 nanoparticles (PEx-4) to determine whether PEx-4 is stronger than Ex-4 in bioavailability and long-lasting effect, which provide systemic protection against hypoxic/ischemic and hemorrhagic stroke evoked brain and bladder injury. We also explored the PEx-4 and Ex-4 effects on hypoxic/ischemic and hemorrhagic stroke induced oxidative stress for the first time including cerebral spinal fluid H$_2$O$_2$ and HOCl amount and ER stress, apoptosis, autophagy and pyroptosis signaling in the impaired brain and bladder.

**Materials And Methods**

**Ethical approval**

All surgical and experimental procedures were approved by Institutional Animal Care and Use Committee of Far-East Memory Hospital and are in accordance with the guidelines of the National Science Council of Republic of China (NSC, 1997) and all experiments were performed in accordance with ARRIVE guidelines. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

**Chemicals**

PLGA (cat. no. P2191, lactide/glycolide=50/50, Mw=30000 to 60000 Da), poly(vinyl alcohol) (87 to 89% hydrolyzed), polyvinyl alcohol (PVA), Ex-4, and dichloromethane were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA).
Experimental design and preparation of Ex-4-loaded PLGA nanoparticles

PLGA nanoparticles containing exendin-4 were prepared using a water-oil-water (w/o/w) emulsion solvent evaporation method as described previously [17]. Briefly, 1 mg of Ex-4 was dissolved in 200 μL distilled water. The aqueous solution was emulsified with 1 mL dichloromethane containing PLGA, using a MICCRA D-1 homogenizer (ART Prozess- & Labortechnik GmBH & Co KG, Müllheim, Germany) at 20,000 rpm for 180 seconds. The primary emulsion was then added to 5 mL of 3% polyvinyl alcohol (PVA) and sonicated for 60 seconds to form a double emulsion, using a Qsonica Sonicator (Q700 Ultrasonic Processor, Qsonica, LLC, Newtown, CT, USA). The resulting emulsion was combined with 50 mL of 0.5% PVA and stirred for 3 hours at room temperature, allowing the dichloromethane to evaporate. The resulting PLGA nanoparticles were washed three times in distilled water, by centrifugation at 10,000 × g (Centrifuge 5810R; Eppendorf AG, Hamburg, Germany).

Nanoparticles characterization

We used a scanning electron microscope (JEOL-6500F, JEOL, Japan) to characterize the morphology and size of the PLGA nanoparticles. The images were analyzed using the ImageJ software (National Institutes of Health, NIH, Bethesda, MD, USA).

In vivo drug release study

The in vivo drug release method was described as previously [15]. The Ex-4 and PEx-4 in saline were subcutaneously once administered to some male Wistar rats (50 μg/kg). Blood was collected by retro-orbital bleeding into tubes containing EDTA at different time points for 7 days, transferred into 1.5-mL centrifuge tubes, and centrifuged at 15 000 × g for 5 minutes. To measure Ex-4 entry into the brain parenchyma, we measured the Ex-4 concentration in the cerebrospinal fluid (CSF). Under anesthesia, 50 to 100 μl CSF was collected from the medullary cisterna magna of rats. The Ex-4 concentration in CSF and plasma was assayed with an Enzyme Immunoassay Kit (EK-070-94, Phoenix Pharmaceuticals, Burlingame, CA, USA).

Animals and grouping

The groups being compared, including control groups. Total number of eighty female adult Wistar rats (200-250 g) with age > 8 weeks were purchased from BioLASCO Taiwan Co. Ltd. (I-Lan, Taiwan) and housed at the Experimental Animal Center of Far-East Memory Hospital at a constant temperature (24±1°C) and with a consistent light cycle (light from 07:00 to 18:00 o'clock). All rats were maintained two per cage throughout the experiment. During the experimental periods rats had free access to tap water and chow. An adaptation period of one week was allowed before the initiation of the experimental protocols. Food and water were provided ad libitum. Body weight was measured every week. The last day of the experimental period the animals were placed on metabolic cages to collect urine. All
efforts were made to minimize both animal suffering and the number of animals used throughout the experiment. We used female rats for the ease of recording of bladder function.

One previous study suggested that the STZ-induced diabetic rats are the best model for the DM and induce diabetes similar to the human [18]. Rats were made diabetes (DM) by intraperitoneal injection of streptozotocin (STZ) (60 mg/kg, Sigma-Aldrich). The rats with similar age and body weight were randomly assigned to one of the following 8 groups (n = 10 in each group): 1) control group (Con), 2) DM group, 3) control + IR group (ConIR), 4) DM + IR group (DMIR), 5) control + IR + 50 μg/kg/week Ex-4 group (ConIRE), 6) control + 50 μg/kg/week Ex-4-loaded PLGA nanoparticles treated group (ConIRPE), 7) DM + IR + 50 μg/kg/week Ex-4-treatment group (DMIRE), and 8) DM + IR + 50 μg/kg/week PEx-4 treated group (DMIRPE). The onset of DM occurred rapidly and was associated with polydipsia, polyuria, and a tail vein blood glucose concentration >250 mg/dL (One Touch II; LIFESCAN, Milpitas, CA). After two weeks of STZ, Ex-4 or PEx-4 was administered once per week via subcutaneous injection (50 μg/kg/week) for another two weeks. In this study, we excluded the rats died during diabetes induction or global cerebral ischemia (IR) injury. Animal care and experimental protocols were conducted in accordance with the guidelines prescribed by the National Science Council of the Republic of China (NSC1997).

Global cerebral ischemia

In groups 3–8, temporary global cerebral ischemia (IR) was induced under avertn (2,2,2-tribromoethanol) anesthesia by ten-min bilateral common carotid arteries with hemorrhage-induced hypotension according to our previous report [15]. In brief, animals were anesthetized by avertn (0.02 ml/g) during surgery to minimize discomfort and were fixed on an operating table. The left and right femoral arteries were catheterized with a PE-50 catheter respectively to continuously record arterial blood pressure and blood sampling. After heparinization, blood was quickly withdrawn via the femoral artery. When the mean arterial blood pressure reached 30 mmHg, the bilateral common carotid arteries were clamped with surgical clips for 10 min, after which the clips were removed and blood was reinfused via the femoral vein. In the sham-operated animals, the vessels were exposed, but neither blood withdrawal nor clamping of the carotid arteries was performed. The rectal temperature was maintained at 37±0.5°C in all animals during surgery with a homeothermic blanket. After experiment, the animals were sacrificed by intravenous KCl.

Cerebral edema measurement by T2-weighted magnetic resonance imaging (MRI)

MRI was carried out in the animals using a Bruker Biospec 7-T MRI system as described previously [15]. Anesthesia was induced with 5% halothane and maintained with 1.5% halothane (both concentrations prepared in O₂: N₂O, 70:30 by volume). Rats were intubated and mechanically ventilated at a rate of 60 breaths/min. Heart rates and respiratory rates were monitored throughout the procedure, and body temperature was maintained at 37 °C. A rapid-acquisition relaxation enhancement T2-weighted sequence was used to determine the precise lesion location, with a rapid-acquisition relaxation enhancement factor (RARE) of 16, a repetition time of 5086 ms, and an echo time of 70.1 ms. The in-plane resolution was 250 × 250 × 250 μm and 15 slices were acquired. A second T2-weighted image set of 25 contiguous slices
was acquired at the lesion site (RARE factor = 16, repetition time = 5086 ms, echo time = 70.1 ms) with an in-plane resolution of $117 \times 117 \times 500 \, \mu m$. Infarct areas were manually delineated on the MRI images using Paravision software (Bruker Corp., Billerica, MA) and multiplied by the interslice distance to calculate the infarct volume [15]. *Image J* was used to analyze the area of brain edema as described in Figure 1B.

**Measurement of specific CSF ROS activity**

Hydrogen peroxide ($H_2O_2$) and hypochlorite (HOCl) are two major ROS generated from activated neutrophils via the myeloperoxidase (MPO) [19]. In this part of study, 100–200 μL of cerebrospinal fluid (CSF) was withdrawn from the cisterna magna in the urethane-anesthetized (1.2 g/kg, Sigma, Missouri, KC, USA, intraperitoneally) rats. We measured CSF $H_2O_2$ and HOCl amount by an amplified chemiluminescence (CL) technique as described previously [19]. In brief, CL signals emitted from the “test mixture” of cerebral spinal fluid (CSF) [or phosphate-buffered saline (PBS) (50 mmol/L, pH 7.4) as a background control], $H_2O_2$ (or HOCl), and CL-emitting substance [i.e., luminol (5-amino-2,3-dihydro-1,4-phthalalazinedione); Sigma, Chemical Co., St. Louis, MO, USA] was measured with a multi-wavelength CL spectrum analyzer (CLA-SP2, Tohoku Electronic Ind., Co., Sendai, Japan). In this study, we used 25 μL of CSF sample or 25 μL of PBS throughout. We first mixed 25 μL of CSF and 1.0 mL of 25 μmol/L luminol in a 4.0 mL quartz cell (1 × 1 × 4 cm) for 100 seconds. Next, 1.0 mL of 0.03% $H_2O_2$ or 0.012% NaOCl was immediately added into the quartz cells. Luminol stock solution (250 μmol/L) was prepared as 1 mg of luminol dissolved in 22.7 mL of PBS. The CL emitted from the above reaction mixture was recorded and measured as “reference $H_2O_2$ counts” (RH$_2$O$_2$) or “reference HOCl counts” (RHOCl). PBS was added to the test system, and the RH$_2$O$_2$ and RHOCl yielded were recorded as the background counts. A RH$_2$O$_2$ or RHOCl level indicated the ROS value.

**Cystometric parameters**

Under anesthesia, PE-50 catheters were placed in the left femoral artery for measurement of ABP and in the left femoral vein for administration of drugs. ABP was recorded in an ADI system (Power-Lab/16S, ADI Instruments, Pty., Ltd., Castle Hill, Australia) with a transducer (Gould-Statham, Quincy, USA). Body temperature was kept at 36.5-37°C by an infrared light and was monitored with a rectal thermometer.

We introduced a transcystometric model to evaluate the micturition alteration in the bladder in response to IR injury. The method has been well-established as described previously [15]. Briefly, these rats were anesthetized by subcutaneous injection of urethane (1.2 g/kg body weight). After the bladder was exposed through a midline incision of the abdomen, a PE-50 catheter (bladder catheter) was inserted through the apex of the bladder dome and was connected via a T-tube to a P23 ID infusion pump and pressure transducer (Gould-Statham, Quincy, USA). The intravesical pressure was recorded continuously in an ADI system (Power-Lab/16S, ADI Instruments, Pty., Ltd., Castle Hill, Australia). The following parameters of bladder responsiveness were measured: intercontraction interval (ICI, the time lag between
two micturition cycles identified with active contractions (>10 mmHg), baseline bladder pressure (BP), micturition duration (MD), and contractile amplitude (Am = maximal bladder pressure-BP) for a micturition. The cystometric parameters were evaluated during each 8-min interval.

**Immunohistochemistry**

The method for immunohistochemistry was described previously [15]. Tissue sections were deparaffinized in xylene and rehydrated in an ethanol series. The tissue sections were submitted to antigen retrieval step. The buffer solution used for heat-induced epitope retrieval was sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0). After 15 minutes of antigen retrieval step, the sections were blocked for non-specific binding with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at room temperature and incubated with the primary antibodies for 18 hours at 4°C. The tissue sections were washed with PBS three times and then were incubated with secondary antibodies Alexa Fluor488 (1:200; Abcam, Cambridge, United Kingdom) and nuclear staining dye Hoechst33342 (1:1000; Sigma-Aldrich) for 1 hour at room temperature. After washing with PBS, the tissue sections were mounted in mounting medium (Leica, Wetzlar, Germany). The slides were scanned by Leica TCS SP3 laser confocal microscope (Leica) to obtain the confocal images. Primary antibodies included mouse anti CHOP (1:100; Cell Signaling Technology, Denver, MA, USA), rabbit anti Caspase-3 (1:100; Abcam), rabbit anti Caspase-1 (1:100; Abcam) and rabbit anti LC3β (1:100; Abcam). Bladder sections were also stained with hematoxylin & eosin or Masson's trichrome for pathophysiologic evaluation.

**Western blotting**

The tissues were ground to powder with a prechilled mortar and pestle. The tissue powder was lysed in Lysis Buffer (Cell Signaling Technology) supplemented with protease inhibitor for 10 minutes at 4°C. The tissue homogenate was centrifuged at 14,000 rpm for 30 minutes. After centrifugation, the supernatant was collected into a new eppendorf. The concentrations of proteins were measured by Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA). Then the 40 μg protein samples were mixed with 1×sample buffer and were boiled for 5 minutes. The protein samples were resolved in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Millipore, Billerica, MA, USA). Then the blot was blocked with Hyblock (Hycell, Taipei, Taiwan) for 1 minute, and incubated with primary antibody overnight at 4°C. After washing three times with TBS, the blot was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour.

Detection of signals was performed by Immobilon Western Chemiluminescent HRP Substrate (Millipore). Primary antibodies included pIRE-1, IRE-1, Caspase-12, pJNK, JNK, CHOP, IL1β, Caspase-1, LC3β, Caspase-3, PARP, and β-actin. Secondary antibodies included HRP-conjugated rabbit anti-mouse IgG, HRP-conjugated donkey anti-goat IgG, and HRP-conjugated goat anti-rabbit IgG (all for 1:10000; all from Sigma Aldrich).

**Histologic staining**
A portion of the brain or bladder tissue was cut and fixed in 10% neutral buffered formalin solution, dehydrated in graded ethanol, and embedded in paraffin. Sections (4 μm) of the bladder or brain were stained with hematoxylin and eosin to evaluate the extent of morphological changes. Four-μm sections of formalin-fixed bladders were stained with Masson's trichrome for fibrosis staining (blue collagen accumulation).

**Statistical analysis**

All values and data were expressed as mean ± standard error mean (SEM). Data recording was not blinded because of technical requirements, but data analyses were blinded for biochemical analyses. The differences within groups were evaluated by paired t-test. The data were statistically analyzed by one-way analysis of variance, followed by Tukey's multiple comparison tests. Differences were regarded as significant if $P < 0.05$ was adapted. Statistical analyses were performed using the IBM® SPSS® Modeler 18.0 software (IBM, Armonk, NY, USA; IBM SPSS Modeler Knowledge Center) and curve fitting was carried out using GraphPad Prism (v. 6.0).

**Results**

**PEx-4 nanoparticles**

Our prepared PEx-4 were approximately 68.0±3.2 nm with a similar morphology (Figure 1A). After subcutaneous injection in vivo, PEx-4 and Ex-4 treated rats showed a peak plasma level at 2 hours (Figure 1B). The peak plasma Ex-4 concentration after 2 hours of Ex-4 treatment was significantly higher ($P < 0.05$) than that of PEx-4 treatment, implicating a large burst release by Ex-4. From 6 hours to 7 days, the plasma Ex-4 concentration of the PEx-4 group was maintained at a stable level and was higher ($P < 0.05$) than that of the Ex-4 group, indicating sustained release of PEx-4 (Figure 1C). Our data showed that the CSF Ex-4 level was consistent with the plasma Ex-4 value (Figure 1D). Two hours after Ex-4 treatment, the peak CSF Ex-4 value was higher ($P < 0.05$) than that of PEx-4 treatment also implicating a great surge of Ex-4 release. The CSF Ex-4 value in PEx-4 group was well maintained and higher ($P < 0.05$) than that of the Ex-4 group 6 to 24 hours after treatment, indicating sustained release of Ex-4 into the plasma and brain from PEx-4. Subcutaneous administration of Ex-4 or PEx-4 significantly decreased blood glucose 6 hours after treatment, but PLGA vehicle nanoparticles did not produce hypoglycemic effect (Figure 1E). PEx-4 significantly decreased blood glucose levels from 12 hours to 7 days after treatment in DM rats, but Ex-4 had no hypoglycemic effect on DM-induced hyperglycemia from 12 hours to 7 days after treatment. This data demonstrated that PEx-4 exerts a more long-lasting hypoglycemic effect than Ex-4.

**Effect of Ex-4 and PEx-4 on RH2O2 and RHOCl activity in CSF**
In Figure 2, typical emission of RH$_2$O$_2$ and RHOCl response from eight groups of rats 24 hours after IR injury was displayed. We noted that DM elevated higher value of RH$_2$O$_2$, not RHOCl in the basal state. CSF RH$_2$O$_2$ and RHOCl in ConIR and DMIR were significantly increased vs. Con and DM groups. However, the enhanced response of RH$_2$O$_2$ and RHOCl was partly decreased by Ex-4 or PEx-4 treatment. PEx-4 was more efficient than Ex-4 in reducing DM and IR induced oxidative stress.

**PEx-4 and Ex-4 on DM or IR induced brain edema by MRI analysis**

Previous studies had displayed the neuroprotective effect of Ex-4 on reducing brain injury in cerebral ischemic rats [14,15] and peripheral neuropathy in STZ-induced diabetes [20]. We compared the neuroprotective effect of PEx-4 and Ex-4 in DM and IR injury. The brain edema formation after IR was examined as demonstrated in Figure 3A, reflected by T2-weighted images and analyzed by thresholding tool of *image J* freeware in Figure 3B. On T2-weighted images, the bright zone in the brain cortex was regarded as edema region. Through the MRI data, the edema region in the CT1 (Figure 3C), CT2 (Figure 3D) and CT3 (Figure 3E) sections was significantly increased in ConIR and DMIR rats. The increased degree of brain edema by IR was significantly depressed with Ex-4 or PEx-4 in IRE and IRPE rats (Figures 3C to 3E).

**PEx-4 and Ex-4 on DM or IR induced histological outcomes in brains**

The appearance of neuronal shrinkage and vacuolization in rat brains after IR had been reported previously [21]. In the prefrontal cortex of Con and DM brains, most neurons appeared to be normal with well-defined nuclei and clearly visible cytoplasm (Figure 4A). After IR, the morphological abnormalities including shrinkage of neurons, vacuolization and angular with dark-stained nuclei could be observed in ConIR and DMIR brain (Figure 4A). The quantitative data were acquired by counting the number of dark-stained nuclei in three regular zones which were chosen randomly on the slides. The dark-stained nuclei were significantly ($P < 0.05$) decreased with the Ex-4 or PEx-4 treatment in IRE and IRPE brains (Figure 4B). PEx-4 was more efficient than Ex-4 in decreasing dark-stained nuclei number.

It had been proved that ER stress plays an important role in mediating ischemic neuronal cell death [22]. ER stress associated protein CHOP was found in Con and DM brains (Figure 4C). IR significantly ($P < 0.05$) increased the CHOP-positive stained cells in ConIR and DMIR groups as compared to respective Con and DM. As compared to IR groups, Ex-4 and PEx-4 treatment significantly ($P < 0.05$) decreased the enhanced CHOP-positive cells in the IRE and IRPE rats (Figure 4D).

**PEx-4 and Ex-4 on stress associated proteins in rat brain subjected to DM or IR injury**

The protein profile of the ER stress, apoptosis, pyroptosis and autophagy associated proteins in rat brain was analyzed by Western blotting (Figure 5A). ER stress associated proteins including pIRE-1/IRE-1 (Figure 5B), cCaspase-12/uCaspase-12 (Figure 5C), pJNK/JNK (Figure 5D), CHOP (Figure 5E), ATF4 (Figure 5F), ATF6 (Figure 5G), pyroptosis related IL1-β (Figure 5H), Caspase-1 (Figure 5I), autophagy related LC3B (Figure 5J), and apoptosis related cCaspase-3/uCaspase-3 (Figure 5K) and PARP (Figure 5L)
were displayed in DM as compared to Con. IR significantly enhanced all these stress markers in the ConIR vs. Con brains, whereas IR only significantly and further enhanced the pIRE-1/IRE-1, ATF6, Caspase-1, LC3B and cCaspase-3/uCaspase-3 in the DMIR group vs. DM group. As compared to ConIR, Ex-4 treatment efficiently and significantly \((P < 0.05)\) depressed ER stress related pIRE-1/IRE-1, cCaspase-12/uCaspase-12, pJNK/JNK, CHOP, ATF4, ATF6, pyroptosis related IL1-β and Caspase-1, autophagy related LC3B, and apoptosis related cCaspase-3/uCaspase-3 and PARP in IRE groups. As compared to DMIR group, Ex-4 treatment significantly \((P < 0.05)\) decreased ER stress related CHOP, ATF4, ATF6, pyroptosis related IL1-β and Caspase-1, autophagy related LC3B, and apoptosis related cCaspase-3/uCaspase-3 in DMIRE groups. These data informed that DM could amplify the signaling pathways involved ER stress, pyroptosis, autophagy and apoptosis in the brain. IR could further enhance these stress signaling pathways leading to brain injury. Our data found that PEx-4 in IRPE was more efficient than Ex-4 treatment of IRE in inhibiting ER stress, pyroptosis, autophagy and pyroptosis signaling pathways.

**PEx-4 and Ex-4 on pyroptosis, autophagy and apoptosis in DM or IR brains**

With the immunofluorescent staining technique, we explored the effect of DM, IR and Ex-4 treatment on the brain expression of pyroptosis related Caspase-1, autophagy related LC3B and apoptosis related Caspase-3 expression in these eight groups of rat brains. The green fluorescent density of these three markers was demonstrated in Figure 6A. The baseline level of Caspase-1, LC3B and Caspase-3 expression was less expressed in the Con brains. DM significantly \((P < 0.05)\) enhanced brain Caspase-1 (Figure 6B), LC3B (Figure 6C) and Caspase-3 (Figure 6D) stains vs. Con group, whereas IR further \((P < 0.05)\) enhanced Caspase-1, LC3B and Caspase-3 stain in the ConIR and DMIR brains vs. respective Con and DM brains. Ex-4 and PEx-4 significantly \((P < 0.05)\) reduced ConIR and DMIR-enhanced Caspase-1, LC3B and Caspase-3 fluorescence in the brains. PEx-4 was more efficient than Ex-4 in reducing IR-induced apoptosis, autophagy and pyroptosis in the ConIR brains and IR-induced pyroptosis through its neuroprotective effect.

**Ex-4 and PEx-4 on cystometry in eight groups of rats**

The representative cystometric graphs and the measurement of 4 urodynamic parameters for the eight groups of rats were shown in Figures 7A and 7B. The cystometry statistical data was indicated in Figures 7C-7F. The micturition interval ICI (Figure 7C), MD (Figure 7E) and BP (Figure 7F) was significantly \((P < 0.05)\) increased in DM group as compared to Con group. The level of Am in DM group was not affected by four-week DM induction as compared to Con group (Figure 7). IR injury depressed voiding function and caused urine retention in the ConIR and DMIR bladders associated with the decreased ICI and Am and increased MD and BP. PEx-4 (IRPE) was more efficient \((P < 0.05)\) than Ex-4 treatment (IRE) groups in recovering ICI, Am and MD toward the normal levels.

**Ex-4 and PEx-4 on bladder histopathology in eight groups of rats**
Our data found that the size and weight of DM bladders were larger than those of Con bladders. Hematoxylin and eosin staining revealed that the lamina propria layers in DM bladders were thicker than those in Con bladders (Figure 8A). The thickening region in DM bladder was determined by Masson's trichrome method. Blue-stained collagen fibers (fibrosis) were significantly increased in DM bladder (Figure 8B) possibly contributing to DM bladder hypertrophy. By use of ImageJ to analyze the degree of bladder fibrosis (Figure 8C), IR, Ex-4 or PEx-4 had no significant effect on blue collagen accumulation (fibrosis) (Figure 8D).

**Ex-4 and PEx-4 on stress associated proteins in rat bladders with DM or IR injury**

The protein profile of ER stress, apoptosis, pyroptosis and autophagy associated proteins in the eight groups of rat bladders was analyzed by Western blotting (Figure 9A). ER stress associated proteins including pIRE-1/IRE-1 (Figure 9B), cleaved Caspase-12 (Figure 9C), pJNK/JNK (Figure 9D), CHOP (Figure 9E), pyroptosis related IL1-β (Figure 9F), Caspase-1 (Figure 9G), LC3B (Figure 9H), Beclin-1 (Figure 9I), tATG/ATG (Figure 9J), and apoptosis related cCaspase-3/uCaspase-3 (Figure 9K) and PARP (Figure 9L) were significantly up-regulated in DM as compared to Con. IR significantly ($P < 0.05$) enhanced pIRE-1, cleaved Caspase-12, pJNK, CHOP, IL-1β, Caspase-1, LC3B, Beclin-1, Caspase-3 and PARP as compared to Con. DMIR significantly enhanced CHOP, Caspase-1, and Caspase-3 expression as compared to DM. As compared to ConIR or DMIR group, Ex-4 significantly ($P < 0.05$) down-regulated CHOP, IL-1β, Caspase-1 and PARP expression in the ConIR and DMIR groups. PEx-4 significantly ($P < 0.05$) down-regulated pJNK, CHOP, IL-1β, Caspase-1 and LC3B expression in the ConIRPE and DMIRPE groups vs. respective ConIR and DMIR groups. These data also informed that PEx-4 was more efficient than Ex-4 in reducing DM or IR enhanced signaling pathways involved ER stress, pyroptosis, autophagy and apoptosis in the bladders.

**Ex-4 and PEx-4 on pyroptosis, autophagy and apoptosis in DM or IR bladders**

We also used the immunofluorescent staining technique to determine the effect of DM, IR, Ex-4 and PEx-4 treatment on the bladder expression of pyroptosis related Caspase-1, autophagy related LC3B and apoptosis related Caspase-3 expression in these eight groups of rat bladders. The green fluorescent density of these three markers was demonstrated in Figure 10A. The baseline level of Caspase-1, LC3B and Caspase-3 expression was less expressed in the Con bladders. DM significantly ($P < 0.05$) enhanced bladder Caspase-1 (Figure 10B), LC3B (Figure 10C) and Caspase-3 (Figure 10D) stains vs. Con group, whereas IR further ($P < 0.05$) enhanced Caspase-1, LC3B and Caspase-3 stain in the ConIR and DMIR brains vs. respective Con and DM bladders. Ex-4 and PEx-4 treatment efficiently and significantly ($P < 0.05$) reduced ConIR- or DMIR-enhanced Caspase-1, LC3B and Caspase-3 fluorescence in the bladders. These data further implicated that PEx-4 is more efficient than Ex-4 in reducing IR-induced apoptosis, autophagy and pyroptosis in the bladders of ConIR and DMIR groups.

**Discussion**

Biodegradable nanoparticles like carbohydrate polymeric PLGA are frequently used to improve the therapeutic value of various water soluble/insoluble medicinal drugs and bioactive molecules by
improving bioavailability, solubility and retention time [15]. Our data evidenced that PEx-4 using PLGA as carrier conferred long-lasting effect in hypoglycemia, antioxidant activity and neuroprotection against DM and IR induced ER stress and three types of programmed cell death. The abnormalities in water balance had been regarded as an important role in the pathophysiology of traumatic brain injury or stroke [23]. Cerebral edema, defined as an abnormal increase in brain water content resulted in an increase in intracranial pressure, ischemia and death. Through the MRI data, the edema resulted from ischemia-reperfusion injury was restricted in the rat cortex region. And the severity of edema was increased gradually in a caudo-rostral pattern (Figure 1). It had been reported that Ex-4 was able to enhance NGF-induced neuronal differentiation and attenuate neural degeneration following NGF withdrawal [24]. The neuroprotection of Ex-4 was also regarded as a potential therapeutic target in neurodegenerative disease [25] and our data further confirmed PEx-4 was more efficient in reducing ischemic brain induced edema and injury, which may be related to aquaporin-4 mediated water uptake and compression of the adjacent capillary lumen [26]. This study using carbohydrate polymers PLGA nanoparticles delivered Ex-4 to the animal in vivo and discovered the therapeutic effect on IR induced brain injury and voiding dysfunction in DM rats. Pharmacokinetic evidence showed that PEx-4 was better than Ex-4 on long-lasting release of Ex-4 in plasma and CSF, reduction in DM evoked hyperglycemia, decrease of oxidative stress, ER stress, apoptosis, autophagy and pyroptosis in the IR brain and improvement of voiding dysfunction. In addition, our unpublished data indicated the dose of PEx-4 or Ex-4 did not impair renal tissue because the serum creatinine was not increased.

It has revealed that lesions of the medial prefrontal cortex caused nocturnal incontinence, urinary urgency and frequent micturition and only rarely caused urinary retention [27,28]. In our study, the ischemic brain with the edema of prefrontal cortex disturbed micturition and maintenance of urinary continence in rat. Besides, lesioning the medial prefrontal cortex would prolong the time interval between volume-evoked bladder contractions without changing the amplitude of contractions [29]. Our bladder dysfunction data was consistent with these previous studies. The two major ROS generated from activated neutrophils via the myeloperoxidase system are hydrogen peroxide (H₂O₂) and hypochlorite (HOCl) [19], which are important mediators in oxidative stress and inflammation. Our data found that DM and IR injury increased H₂O₂ and HOCl in the CSF implicating neuronal inflammation and oxidative stress. The novel finding indicated that increased ROS in CSF may impair brain function and micturition center leading to voiding dysfunction. A cross-talk role of exacerbated ROS production may be occurred between brain and bladder. Our prepared PEx-4 was more efficient than Ex-4 in reducing these oxidative stress and inflammation in IR and DM induced injury implicating the therapeutic potential of PEx-4 in future.

ER stress was the essential step in progression of ischemic stroke. Oxygen and glucose deprivation from IR injury contributed to ER stress [30]. After IR injury, the abnormalities of brain section including shrinkage of neurons, vacuolization and angular with dark-stained nuclei were observed in our study. In addition, CHOP, as a downstream molecule in ER-stress pathway [31], was higher expressed in IR and DM injury. Our data found that IR-enhanced ER stress was reduced by the treatment of Ex-4 and PEx-4. In one previous study, Ex-4 was proved to attenuate ER stress through silent mating type information
regulation 2 homolog1, which decreased by hyperglycemia [32,33]. Hyperglycemia also downregulated the expression of GLP-1R [34]. Since Ex-4 binds to GLP-1R and exerts biological functions, the downregulation of GLP-1R by hyperglycemia would attenuate the protection of Ex-4. A continuous release and long-lasting effect of Ex-4 by PEx-4 may trigger Ex-4/GLP-1R signaling to decrease ER stress. It is well known that Ex-4 protected the organs and cells from oxidative damage induced by DM [35-38]. These molecules are small enough to cross the blood–brain barrier [14,39], and GLP-1Rs are widely expressed throughout the brain. Our previous report delineated that Ex-4 treatment reduces stroke-induced frontal cortex edema, ER stress, apoptosis, and upregulation of aquaporin 4, glial fibrillary acid protein, and ICAM-1 in the damaged brain [15] implicating its neuroprotection. Our present data further evidenced the neuroprotective effect of PEx-4 on reduction of autophagy and pyroptosis in the DM brain.

The stroke patients with dysregulated bladders had underlying DM or were taking anti-cholinergic medications [40]. Three major mechanisms are responsible for post-stroke evoked urinary incontinence: 1) disruption of the neuromicturition pathways, resulting in bladder hyperreflexia and urgency incontinence; 2) incontinence due to stroke-related cognitive and language deficits, with normal bladder function; and 3) concurrent neuropathy or medication use, resulting in bladder hyporeflexia and overflow incontinence [40]. Our present data confirmed that DM or cerebral IR injury led to voiding dysfunction in these rats. In this study, we further investigated the molecular mechanism and pathophysiological functions in DM bladder after cerebral IR damage and evaluated the therapeutic potential of PEx-4. Ex-4 could rescue the bladder dysfunction resulted from DM and IR injury [41,42]. Our data further discovered that several stress markers (ER stress, autophagy, apoptosis and pyroptosis) were downregulated in DM bladder with the treatment of PEx-4. However, we had not found the significant effect to ameliorate morphological alteration in DM bladders with the treatment of PEx-4. We suspect that the long-term STZ induction might be the critical cause of the severe and irreversible damage in the DM bladder. The suppressed DM and IR induced oxidative stress in ER stress, apoptosis, autophagy and pyroptosis in the brain and bladder suggesting PEx-4’s global protection.

**Conclusion**

Type I Diabetic rats treated with PLGA carrying GLP-1 agonists (PEx-4) after bilateral carotid artery occlusion combined with hemorrhage-induced hypotension (IR) injury depressed the level of IR enhanced prefrontal cortex edema, oxidative stress, ER stress, apoptosis, autophagy and pyroptosis related proteins expression in the brain and bladder and ameliorated post cerebral IR induced voiding dysfunction. These results suggest that long-lasting release of GLP-1 agonists like Ex-4 may confer therapeutic potential to treat cerebral IR injury in the DM subjects in clinical trials.

**Declarations**

**Author contributions**
C.-H.C. and S.-D.C. performed experiments, analysed data and drafted the manuscript; W.-H.W. and P.-H.J performed nanoparticles by SEM; C.-P.Y. performed partial experiments and analysed data; C.-P.Y. and C.-T.C. provided animal model; S.-D.C., C.-P.Y. and C.-T.C. designed experiments and revised the manuscript.

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**Conflict of interest:** All the authors declare no conflicts of interest.

**Ethical approval:** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figures

(A) Morphology and size of exendin-4 (Ex-4)-loaded poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles (PEx-4) were examined under a scanning electron microscope. The PEx-4 microspheres were uniform in...
size (68±3.2 nm) and morphology. (B) After subcutaneous administration of Ex-4 or PEx-4, both groups of rats (n=5 each) showed highest plasma concentrations at 2 hours, but PLGA vehicle had no effect on Ex-4 concentration. The peak plasma Ex-4 level 2 hours following Ex-4 administration was significantly higher (P < 0.05) than that of PEx-4 treatment. (C) From 6 hours to 7 days, the plasma Ex-4 level of the PEx-4 group was well maintained and significantly (P < 0.05) higher than that of the Ex-4 group. (D) The maximal CSF Ex-4 level at 2 hours after Ex-4 treatment was significantly higher (P < 0.05) than that after PEx-4 treatment. However, the CSF Ex-4 concentration of the PEx-4 group was significantly higher (P < 0.05) than that of the Ex-4 group 6 to 24 hours after subcutaneous administration. (E) Hypoglycemic effects of subcutaneously administered Ex-4, PEx-4, or PLGA vehicle in control and diabetes mellitus (DM) rats (n=5 each). In normoglycemic control rats, subcutaneous Ex-4 or PEx-4 significantly reduced blood glucose levels after 6 hours of treatment, respectively, but PLGA vehicle nanoparticles did not. PEx-4 significantly (P < 0.05) decreased blood glucose levels from 12 hours to 7 days in DM rats, but Ex-4 had no effect on DM-evoked hyperglycemia during this period. Group differences were compared by one-way analysis of variance, followed by Tukey's multiple comparison tests. * P<0.05 compared with Ex-4 group.
Figure 2

Effect of Ex-4 or PEx-4 treatment on CSF RH2O2 and RHOCI response in the sham or IR rats of control (CON) and diabetes (DM) rats. The typical emission spectra of the chemiluminescence of a test mixture containing H2O2 and luminol for reference H2O2 (RH2O2), HOCl, and luminol for reference HOCl (RHOCI) in Sham, IR, Ex-4 treated IR (IRE) or PEx-4 treated IR rats (IRPE) of CON and DM rats are displayed in A and B. IR increased RH2O2 (C) and RHOCI activity (D), whereas DM further enhanced these responses. PEx-4 is more efficient in reducing RH2O2 and RHOCI activity in IRPE group as compared to Ex-4 in IRE.
Figure 3

The panel shows three caudo-rostral projections (CT1, CT2 and CT3) of coronal sections through a rat brain (A; modified from the rat brain picture of https://instruct.uwo.ca/anatomy/530/ratpix.pdf). The bright zones on T2-weighted images represent post-IR edema and are analyzed with ImageJ software (B). The percentage (%) of the brain edema zones are increased after IR and are significantly decreased with the pretreatment of Ex-4 (IRE) or PEx-4 (IRPE) in CT1 (C), CT2 (D) and CT3 (E) sections in both control and DM rat brain coronal sections. Data are expressed as mean ± SEM in each group (n = 6) using the single values. * P<0.05 compared with sham control, a P<0.05 compared with respective IR group and b P<0.05 compared with respective IRE group.

Figure 4
Effect of Ex-4 and PEx-4 on the level of morphological change of prefrontal cortex (A) with shrunken nuclei indicated by yellow arrows with hematoxylin-eosin stain and CHOP-positive stain (C, green fluorescence indicated by yellow arrows) in rat brain sections of control and DM rats. The number of shrinking nuclei is significantly increased after IR injury and decreased in the treatment of Ex-4 (IRE) or PEx-4 (IRPE) in brain sections (B). The CHOP-positive cells after IR are significantly increased and decreased in treatment of Ex-4 (IRE) or PEx-4 (IRPE) in brain sections (D). (scale bar = 50 μm). Data are expressed as mean ± SEM in each group (n=6) using the single values. * P<0.05 compared with sham control, a P<0.05 compared with respective IR group and b P<0.05 compared with respective IRE group.

Figure 5

The effect of Ex-4 (IRE) and PEx-4 (IRPE) treatment on IR evoked ER stress, apoptosis, pyroptosis and autophagy associated proteins in Con or DM rat brain (A). The levels of ER stress associated proteins including pIRE-1 (B), cleaved Caspase-12 (C), pJNK (D), CHOP (E), ATF4 (F), ATF6 (G), pyroptosis associated proteins including IL1-β (H), Caspase-1 (I), autophagy associated protein LC3B (J) and apoptosis associated proteins including cleaved Caspase-3/uncleaved Caspase-3 (K) and PARP (L) are compared in DM as compared to Con. IR in Con and DM groups further and significantly enhanced PIRE-
1, ATF6, Caspase-1, LC3B and cleaved Caspase-3 expression as compared to respective Con and DM groups. Ex-4 (IRE) and PEx-4 (IRPE) treatment with IR injury significantly down-regulated ER stress, pyroptosis, autophagy and apoptosis in the IRE and IRPE groups of Control and DM rats. Data are expressed as mean ± SEM in each group (n = 5) using the single values. * P<0.05 compared with sham control. # P<0.05 compared with sham DM. a P<0.05 compared with respective IR group. b P<0.05 compared with respective IRE group.

Figure 6

Effect of Ex-4 and PEx-4 treatment on the brain expression of pyroptosis, autophagy and apoptosis in control and DM rats subjected to global brain ischemia injury (IR). A: the level of green fluorescent intensity of Caspase-1 mediated pyroptosis, LC3B mediated autophagy and Caspase-3 mediated apoptosis is determined with an immunofluorescent stain in the rat brains. DM significantly enhanced brain Caspase-1 (B), LC3B (C) and Caspase-3 (D) stains indicated by green fluorescence, whereas IR further and significantly increased Caspase-1, LC3B and Caspase-3 stains in the Con and DM brains as compared to respective Con and DM brains. Ex-4 (IRE) and PEx-4 (IRPE) treatment significantly reduced IR-enhanced Caspase-1, LC3B and Caspase-3 fluorescence in the brains of control and DM rats. Data are expressed as mean ± SEM in each group (n = 6) using the single values. * P<0.05 compared with Con
Figure 7

Effect of Ex-4 and PEx-4 on the voiding function in response to stroke injury (IR) among the eight groups of rats. Representative traces of continuous cystometryograms in urethane anesthetized rats of Control (A) or DM groups (B). The normal voiding pattern and parameter are demonstrated in Control (Con), whereas
a hypersensitive underactive bladder with an increased ICI is indicated in DM bladders. IR caused the urine incontinence, depressed ICI, Am, increased MD and BP in Con and DM bladders. The treatment of 20 µg Ex-4 (IRE) or 20 µg PEx-4 (IRPE) seems to play a role in improving IR or DMIR-induced voiding dysfunction. Data are expressed as mean ± SEM in each group (n = 10) using the single values. C: ICI, intercontraction interval; D: Am, amplitude; E: MD, micturition duration; F: BP, baseline bladder pressure. * P<0.05 compared with sham control (Con). # P<0.05 compared with sham DM group. a P<0.05 compared with respective IR group. b P<0.05 compared with respective IRE group.

Figure 8

The lamina propria layers by H&E stain (indicated by yellow arrows) in DM bladders were thicker than those in control bladders (A). Stroke injury (IR) had no effect on the thicken lamina propria layers. Blue collagen fibers (indicated by yellow arrows) stained by Masson’s stain were increased in DM bladder compared with Con bladder (B). The use of ImageJ is applied to analyze blue collagen accumulation (C). The blue collagen stain intensity was significantly higher in the DM bladders than that in Con bladders. IR and the treatment of 20 µg Ex-4 (IRE) or PEx-4 (IRPE) had no significant effect on DM-enhanced blue collagen accumulation (D). Data are expressed as mean ± SEM in each group (n = 6) using the single values. * P<0.05 compared with control (Con). Scale bar (white line) = 200 µm.
Figure 9

The effect of Ex-4 and PEx-4 treatment on ER stress, apoptosis, pyroptosis and autophagy associated proteins in Con or DM rat bladders with or without IR injury (A). The levels of ER stress associated proteins including pIRE-1 (B), cleaved Caspase-12 expression (C), pJNK (D), CHOP (E), pyroptosis associated proteins including IL1-β (F), Caspase-1 (G), autophagy associated protein LC3B (H), Beclin-1 (I), tATG (J), and apoptosis associated proteins including cleaved Caspase-3 (K) and PARP (L) are up-regulated in DM as compared to Con. IR further enhanced pIRE-1, cleaved Caspase-12, pJNK, CHOP, IL1-β, Caspase-1, LC3B, Beclin-1, Caspase-3 and PARP in ConIR as compared to Con. IR significantly enhanced CHOP, Caspase-1, Caspase-3 in DM as compared to Con. Ex-4 (IRE) or PEx-4 (IRPE) treatment significantly down-regulated ER stress, pyroptosis, autophagy and apoptosis in the Con and DM rats of IRE and IRPE groups. Data are expressed as mean ± SEM in each group (n = 6) using the single values. * P<0.05 compared with sham control. # P<0.05 compared with sham DM. a P<0.05 compared with respective IR group. b P<0.05 compared with respective IRE group.
Figure 10

Effect of Ex-4 and PEx-4 treatment on the bladder expression of pyroptosis, autophagy and apoptosis in control and DM rats subjected to IR injury. A: the level of green fluorescent intensity of Caspase-1 mediated pyroptosis, LC3B mediated autophagy and Caspase-3 mediated apoptosis is determined with an immunofluorescent stain in the rat bladders. DM significantly enhanced bladder Caspase-1 (B), LC3B (C) and Caspase-3 (D) stains indicated by green fluorescence, whereas IR further and significantly increased Caspase-1, LC3B and Caspase-3 stains in the IR bladders of CON and DM rats as compared to respective CON and DM bladders. Ex-4 (IRE) and PEx-4 (IRPE) treatment significantly reduced IR-enhanced Caspase-1, LC3B and Caspase-3 fluorescence in the bladders of CON and DM rats. Data are expressed as mean ± SEM in each group (n = 6) using the single values. * P<0.05 compared with Sham CON group. # P<0.05 compared with DMIR. a P<0.05 compared with respective IR group. b P<0.05 compared with respective IRE group. scale bar = 200 μm.