Research article

Regulatory effect of heat shock protein 70 in stress-induced rat intestinal epithelial barrier dysfunction

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Background: Psychological stress is one of the factors associated with many human diseases; the mechanisms need to be further understood. **Methods:** Rats were subjected to chronic water avoid stress. Intestinal epithelial heat shock protein (HSP) 70 was evaluated. The intestinal epithelial permeability was examined with Ussing chamber technique. **Results:** HSP70 was detected in normal intestinal epithelial cells. Psychological stress decreased HSP70 in the intestinal epithelial cells that correlated with the stress-induced intestinal epithelial hyperpermeability. Pretreatment with HSP70 abrogated stress-induced intestinal barrier dysfunction. **Conclusions**: Chronic stress inhibits HSP70 activity in rat intestinal epithelial layer that is associated with intestinal epithelial barrier dysfunction, which can be prevented by pretreatment with HSP70 protein. (Yang PC, Tu YH, Perdue MH, Oluwole C, Struiksma S. Regulatory effect of heat shock protein 70 in stress-induced rat intestinal epithelial barrier dysfunction. **North Am J Med Sci** 2009; 1: 9-15).

Key words. Intestine; Epithelium; Heat shock protein 70; Stress.

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Introduction

Psychological stress is a common event in daily life. Human being may benefit from a mild stress because it promotes or improves physiological functions in the body, such as a mild increase in noradrenalin in the blood improves circulation; a little more secretions of thyrotrophic releasing hormone help basic metabolism in the body. However, severe stress or sustained stress may cause detrimental effect in some organs or tissues in the body [1]. There is evidence that hypertension associates with long-term stress [2]. Hyperthyroidism may relate to sustained stress [3]. It is well accepted that psychological stress can induce peptic ulcer [4], etc. The mechanism of stress inducing pathology and phathophysiology in the body remains unclear. The growing evidence implicates that over-secretion of some hormones such as corticotrophin releasing hormone from the hypothalamus may be involved in stress-induced disorders in the body [5].

The intestine is one of the common targets of stress. Cumulative reports correlate stress to the pathogenesis of inflammatory bowel disease, such as ulcerative colitis and Crohn's disease [6, 7]. We also have found that stress may be involved in intestinal sensitization (Yang, et al. Unpublished data). Previous work indicates that stress can induce intestinal luminal bacterial relocation [8]. Other intestinal diseases such as eosinophilic intestinal inflammation and intestinal tumor, etc may have an association with stress [9, 10].

Intestinal barrier consists of a single layer of epithelial cells. The tight junctions connect the epithelial cells each other that form a barrier between internal environment and external environment [11]. The intestinal epithelial barrier allows nutrients and water to be absorbed and prevents noxious substances from absorption. Our previous work indicate that psychological stress is one of the factors in causing intestinal barrier dysfunction by showing intact protein to be absorbed into intestinal tissue after exposure to chronic psychological stress [12-14].

Heat shock proteins (HSPs), also called stress proteins, are a group of proteins that are present in all cells in all life forms [15]. They are induced when a cell undergoes various types of environmental stresses like heat, cold and oxygen deprivation. HSPs are also present in cells under perfectly normal conditions. They act like 'chaperones,' making sure that the cell's proteins are in the right shape and in the right place at the right time. For example, HSPs help new or distorted proteins fold into shape, which is essential for their function. They also shuttle proteins from one compartment to another inside the cell, and transport old proteins to 'garbage disposals' inside the cell. Heat shock proteins are also believed to play a role in the presentation of pieces of proteins (or peptides) on the cell surface to help the immune system recognize diseased cells [16, 17].

Based on the information above, we hypothesized that HSPs in the intestinal epithelium can be affected by psychological stress that associated with intestinal epithelial barrier dysfunction. In an animal model, we aimed to investigate that (i) HSP70 levels

in the normal intestinal epithelium; (ii) dynamic alteration of HSP70 levels in the intestinal epithelial cells treated by chronic stress; (iii) abrogation of stress-induced intestinal epithelial barrier dysfunction. The results showed that intestinal epithelium contained HSP70; the levels of HSP70 were decreased in the intestinal epithelium after treatment with water-avoid stress; pretreatment with HSP70 could efficiently prevent rat intestinal epithelium from stress-induced epithelial barrier dysfunction.

Materials and Methods

Animals.

Male Wistar Kyoto (WKY) rats, 250 to 300 g body weight, were purchased from Charles River and were housed at controlled room temperature (22–25 °C) on a 12-h light/12-h dark cycle. Standard commercial rodent diet was supplied to all animals and tap water was provided ad libitum. Experimental procedures were conducted in accordance with the Guiding Principles in the Animal Care Committee at McMaster University.

Water avoid stress protocol

Rats were handled daily by the same investigator for 2 weeks before the study and then submitted to water avoidance stress daily for 10 days. The procedure involved placing the rat on a round platform (8 cm in diameter) in the middle of a plastic container (56 × 50 cm) filled with warm water (25°C) to 1 cm below the height of the platform. Rats avoided the aversive stimulus (water) by remaining on the platform for 1 h. Control rats were placed on the same platform above a waterless container for 1 h. Body weight changes, as an index of growth, and food intake were measured (g/day) just before the stress or sham protocol. All experimental procedures were performed between 8:00 and 10:00 AM to minimize the effect of circadian rhythm.

HSP70 mRNA expression in the intestinal epithelium

The expression of mRNA and protein of HSP70 in the intestinal epithelium was measured with the samples from each experimental rat. Primers of HSP70 mRNA were designed by us. We found the HSP70 gene sequence in the PubMed (NM_001004257), designed the primers with software Primer3. Forward: gttccagaggctgttcaagc; reverse: tcttgctctggacacattgc. Specificity of the primers was proved using software Blast. Enterocytes were isolated from the small intestine by previously reported methods ([18, 19] with modifications. The scraped intestinal epithelium was incubated in RPMI 1640 media (Invitrogen, Carlsbad, CA) containing 1 mM DTT (Sigma) for 15 min at room temperature to remove mucus. The isolated cells were collected and washed in RPMI (as above without DTT), following with filtration through nylon mesh (Nytex, Tetko, Elmsford, NY). Epithelial cells were purified by density gradient centrifugation on a Percoll gradient (Amersham Pharmacia Biotech). Intestinal epithelial cells were collected, washed, and resuspended in RPMI. The viability of enterocytes (trypan blue negative) was >95%. The estimated purity of epithelial cells was determined to be ~90% by flow cytometry using cytokeratin as the epithelial cell marker [33, 35]. RNA was extracted from the isolated enterocytes using the RNeasy Mini kit (Qiagen). The DNA thermal cycler (Teche PHC-3; Mandel Scientific Guelph, ON, Canada) was programmed to perform a protocol as follows: 94°C for 3 min for 1 cycle; 94°C for 1.5 min (denaturation), 60°C for 2 min (annealing), and 72°C for 3 min (extension) for 35 cycles; and 72°C for 7 min for final extension. Negative controls were performed with samples lacking cDNA or samples with mRNA that were not reverse transcribed. RT-PCR products were then electrophoresed in a 0.8% agarose gel in the presence of 0.5 μg/ml ethidium bromide, visualized with an ultraviolet transilluminator, and photographs were taken. Molecular weight markers, Ready load {varphi}-X174 RF DNA/HaeIII fragments (Invitrogen) were used. The intensity of the DNA bands was analyzed using a densitometer with software from Kodak Digital Science 1D (GIBCO, Rockville, MD).

HSP protein expression in the intestinal epithelium

Rat intestinal epithelial cells were prepared as described above. The collected cells were lysed with ice-cold lysis buffer containing (in mM except where specified) 25 Tris•HCl, pH 7.4, 25 NaCl, 25 NaF, 25 sodium pyrophosphate, 1 sodium vanadate, 2.5 EDTA, 2.5 EGTA, 0.05% (wt/vol) Triton X-100, 0.5% (wt/vol) SDS, 0.5% (wt/vol) deoxycholate, 0.5% (wt/vol) Nonidet P-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 PMSF. The lysates were centrifuged at 45,000 g for 1 h at 4°C to yield the whole cell extract. The protein concentration was determined by the BCA reagents according to the manufacturer's instructions. Samples from these supernatant fractions (30 μg protein) were denatured and subjected to SDS-PAGE using a 10% (wt/vol) running gel. Proteins were transferred to nitrocellulose membrane, and the membrane was incubated successively at room temperature with 5% (wt/vol) BSA in 50 mM Tris•HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5 (TTBS) for 1 h. The HSP70 was identified and quantified by Western blot analysis using HSP70 antibody according to the recommendation of the manufacturer. Briefly, membranes were incubated overnight at 4°C with anti-HSP70 antibody used at a dilution of 1:1,000 in TTBS. Membranes were washed with TTBS four times for 5 min each and incubated with a 1:2,000 dilution of anti-rabbit horseradish peroxidase antibody for 1 h. After being incubated, the membrane was washed extensively with TTBS. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL (Amersham International). HSP70 protein in the intestinal epithelial cells was also assessed with ELISA. The antibodies and dilution used in ELISA were the same as those described in Western blot.

Intestinal epithelial barrier function

Epithelial permeability is an important indicator of intestinal barrier function. In the present study, we used Ussing chamber technique to measure the intestinal epithelial permeability of the rats exposed to chronic stress. The intestine of each rat was excised and rinsed in PBS of pH 7.4 and avoiding Peyer's patches, experimental segments were obtained. The first 10 cm of the top of small intestine was cut away; the next 10 cm was used as the jejunum and used to do the experiments. The underlying muscularis was removed and four intestinal segments were mounted in 4 Ussing chambers respectively in which a surface area of 0.6
cm2 was exposed. 8 ml Kreb's solution was added to each side of Ussing chambers with a supplement of 10 mM mannitol in luminal side and 10mM glucose in serosal side. Each side of the chamber was bubbled with a mixture of 95% O2 and 5% CO2 in order to mix each solution and to maintain the viability of the membrane. The temperature was maintained at 37 °C during the experiment by a circulating water bath. Every five minutes, a pulse of 1 mV was passed through the tissue, and by measuring the change in Isc, the conductance was calculated by Ohm's law. Baseline values for Isc and conductance were recorded at equilibrium, 20 minutes after mounting of the tissues. Segments with signs of poor viability---that is, unstable Isc and/or conductance---were excluded from the study.

Intestinal epithelial permeability was assessed by measuring flux of horseradish peroxidase (HRP), a model protein macromolecule from mucosal to serosal transport. Fifteen minutes after mounting the tissues, HRP (type II, Sigma Chemical Co., St Louis, Missouri, USA) was added to the luminal buffer at a final concentration of 10 µM and allowed to equilibrate for 30 minutes. Serosal samples (0.5 ml) were obtained at 30 minute intervals for 90 min and replaced by 0.5 ml of appropriate buffer solution. HRP activity was determined by a modified Worthington method, as previously described [20]. The mucosal to serosal flux of HRP was reported as the average value of two consecutive stable flux periods (between 30 and 90 minutes), and expressed as pmol/h/cm2.

Intestinal tissues were obtained from Ussing chambers90 min after addition of HRP into the luminal compartment. Tissues were immediately fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for two hours at 22°C, rinsed for 18 hours (4°C) with 0.05 Tris buffer (pH 7.6), and washed three times, five minutes each time. Methods for HRP product identification have been described previously [20]. Quantitative analysis of HRP uptake in intracellular endosomes and paracellular HRP transport were performed on coded high magnification photomicrographs, 12 per rat (6 rats/group). The total area of HRP containing endosomes within enterocytes was determined in an area of 300 µm2 in the apical region of the cells, using a computerized image analysis system (Kontron Mop Videoplan; Kontron, Eching, Germany).

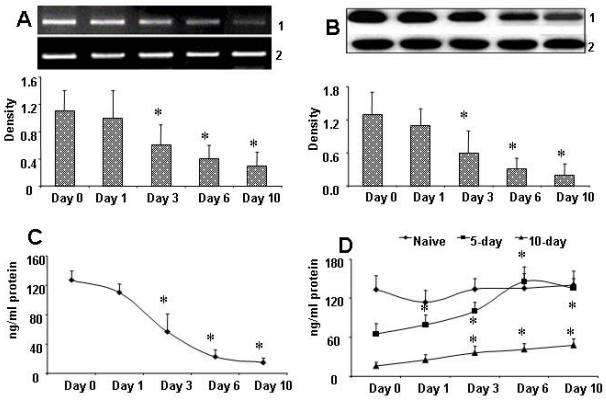

Fig 1. Intestinal epithelial cells express HSP70. Rats were treated with chronic stress (water-avoided stress) for 1 to 10 days. The rats were sacrificed on designated time points. Intestinal epithelial cells were purified from the small intestinal segments. A. HSP70 mRNA was determined with RT-PCR (gel 1). The band density was normalized with internal actin controls (gel 2). B. HSP70 protein was determined with Western blot (gel 1). Density of the bands was normalized with internal actin controls (gel 2). C. ELISA was used to quantify HSP70 expression. D. HSP70 recover time after treatment with chronic stress. Each group consists of 6 rats. Data were expressed as mean ± SD. *, $p<0.05$, compared with Day 0 group (naïve controls).

Results

Chronic stress decreases HSP70 expression in the intestinal epithelial cells.

The HSP70 expression in the intestinal epithelial cells was assessed with RT-PCR, ELISA and Western blot. The naïve rat intestinal epithelial cells clearly express HSP70 mRNA. Chronic stress clearly reduced HSP70 mRNA expression in a time-dependent manner (Fig 1A). Western blot results showed that intestinal epithelium expressed HSP70 at protein level. The HSP protein expression was also repressed by chronic stress (Fig 1B). ELISA was used to quantify HSP70 protein expression in the intestinal epithelial cells that showed HSP70 protein in the stress treated intestinal epithelial cells was decreased in consistent with the stress treatment time (Fig 1C). Another three groups of rats were used to observe the recover time of HSP70 in the intestinal epithelial cells after the treatment with chronic stress. The results showed that the amount of HSP70 in the intestinal epithelial cells was recovered automatically after the chronic stress treatment. The group treated with chronic stress for 5 days showed HSP70 recovered gradually and reached the level of naïve control in 6 days. The group treated with chronic for 10 days showed a slow recovery of HSP70 in the intestinal epithelial cells, it only reached 34% of the naïve control group on day 10 after treatment with chronic stress (Fig 1D).

Chronic stress impaired intestinal epithelial barrier function that was parallel with the alternation of intestinal epithelial HSP70 levels.

Jejunal segments were excised from each experimental rat after the treatment with chronic stress. Epithelial ion secretion of the epithelial layer was recorded in Ussing chambers. Epithelial conductance was recorded at the same time. The results showed that chronic stress increased short circuit current (Isc) and up regulated conductance in the intestinal epithelial layer (Fig 2). Correlation analysis was performed with the results of Isc and HSP70 expression of the intestinal epithelial cells. The results showed that a significant correlation existed between the amount of HSP70 and Isc of the intestinal epithelial cells (r=0.86, p<0.001).

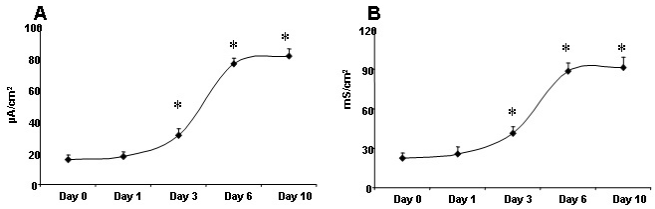

Fig 2. Determination of gut barrier function. Intestinal epithelial layers were mounted on Ussing chambers. Isc and conductance were recorded. Each time point consists of 6 rats. Data are expressed as mean ± SD. *, p<0.05, compared with Day 0 group (naïve control).

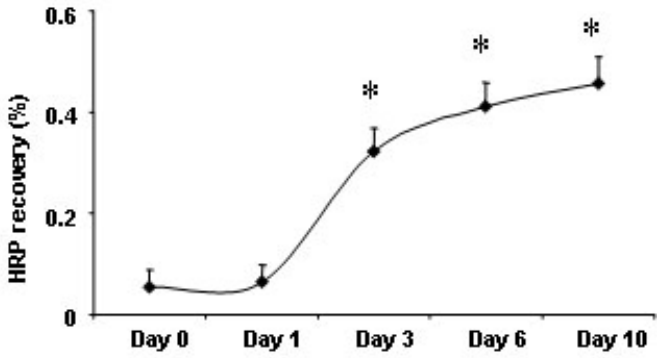

Fig 3. Assessing gut epithelial permeability. HRP flux. Intestinal barrier functions were determined with HRP flux in Ussing chambers. Data are expressed as mean □ SD from 24 measurements of 6 separated experiments. The Y axis represent HRP in serosal side that is divided by the added HRP in luminal side. *, p<0.05, compared with Day 0 group (naïve controls).

Intestinal epithelial permeability increased after chronic stress that correlated with the changes of HSP70 expression

Intestinal epithelial layer of the rats treated with chronic stress was measured with HRP flux in Ussing chamber. HRP is a macromolecular protein with a limited amount to be transported across the intestinal epithelial layer under normal physiological condition. After treatment with chronic stress, the permeability HRP of the intestinal epithelial layer was significantly increased in a time-dependent manner. Correlation analysis results showed that the HRP amount in the serosal side was significantly correlated with the amount of decreased HSP70 in the intestinal epithelial cells (Fig 3, r=0.75, p<0.001).

The epithelial layer was fixed and processed to be observed with electron microscopy. HRP containing endosomes were observed in the epithelial cells. The naïve intestinal cells contained HRP endosomes with limited numbers and small in size (Fig 4A). The epithelial cells from the rats treated with chronic stress showed increase in HRP endosome number and enlarged size (Fig 4B). The HRP endosome area was measured with an image process system. The average HRP endosome area was presented in Fig 4C. Correlation analysis results showed a significant correlation between HRP endosome area in the intestinal epithelial cells and the amount of HSP70 (r=0.77, p<0.001).

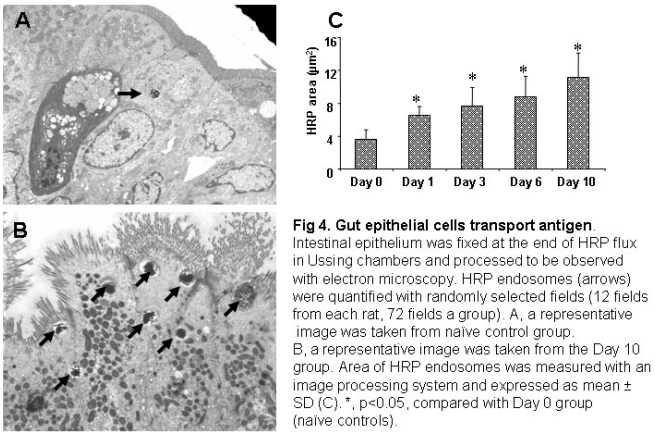

Fig 4. Gut epithelial cells transport antigen. Intestinal epithelium was fixed at the end of HRP flux in Ussing chambers and processed to be observed with electron microscopy. HRP endosomes (arrows) were quantified with randomly selected fields (12 fields from each rat, 72 fields a group). A, a representative image was taken from naïve control group. B, a representative image was taken from the Day 10 group. Area of HRP endosomes was measured with an image processing system and expressed as mean ± SD (C). *, p<0.05, compared with Day 0 group (naïve controls).

Pretreatment with HSP70 abrogated chronic stress induced intestinal hyperpermeability

A group of rats were ip injected with 10 g/rat 30 min before each stress session. A control group of rats was injected with water instead. Both groups of rats were subjected to chronic stress for 10 days. After the 10-day stress treatment, the rats were sacrificed, intestinal segments were mounted on Ussing chambers and HRP flux was performed. The results showed HRP flux was significantly decreased in the HSP70 pretreated rats compared with the saline group (Fig 5).

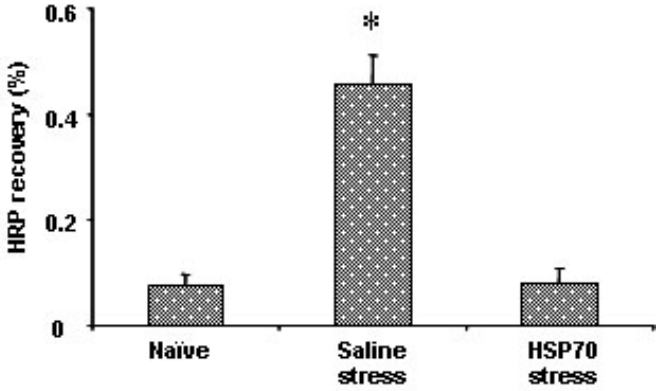

Fig 5. HSP70 protects gut barrier function. HRP flux was performed with the small intestinal epithelial layer from the experiments in which the rats were pretreated with HSP70. Data are expressed as mean ± SD. *, p<0.05, compared with naïve controls.

Discussion

The present findings indicate that the normal rat intestinal epithelial cells express HRP70. Chronic stress can decrease the amount of HSP70 in the intestinal epithelial cells. Decreased amount of HRP70 correlates with the time of stress treatment. Pretreatment with HSP70 can efficiently abrogate chronic stress induced intestinal epithelial layer hyperpermeability.

HSP70 is one of the members of the chaperone family. Chaperones are a functionally related group of proteins that assist protein folding in bacteria, plant and animal cells under physiological and stress conditions. In addition to their role in protein folding, chaperones facilitate translocation of proteins across membranes, help assemble and disassemble protein complexes, help present substrates for degradation, and suppress protein aggregation [21, 22]. HSP70 is an important subgroup of highly evolutionarily conserved chaperones that is the ATP-dependent HSPs, which share the ability to recognize and bind nascent and unfolded proteins, thus preventing aggregation, and facilitating correct protein folding [23; 24]. HSP70 are currently being considered for the potential treatment of diseases involving protein aggregation and misfolding from neurodegenerative diseases [25] to cancer [26]. The cell has a complex system for maintaining proper protein folding, which begins with facilitation of folding of nascent proteins, monitoring for the presence of unfolded proteins in different intracellular compartments, and targeting of misfolded or abnormal proteins for degradation. Many aspects of protein–protein interactions are also specifically regulated by chaperones. The accumulation of unfolded proteins in the endoplasmic reticulum lumen can trigger the unfolded protein response, which is implicated in the shutdown of protein synthesis that is a hallmark of the response to ischemia and other severe cellular stresses [27].

One of the pathological changes induced by chronic stress is intestinal epithelial cell apoptosis. We have found chronic induced intestinal epithelial cell apoptosis in a previous study [28]. Correlation between apoptosis and stress in other tissues has been reported in previous studies [29]. Recent work has highlighted the ability of Hsp70 to suppress multiple types of cell death including necrotic death, classical apoptosis, and other programmed cell death pathways that are independent of caspases and not blocked by Bcl-2 [30, 31]. These studies provide an explanation to the present study that HSP70 inhibited chronic stress induced intestinal barrier dysfunction may be via prevent stress induced intestinal epithelial cell apoptosis. There are also a large number of studies demonstrating neuroprotection by the chaperone Hsp70 [32-34].

We detected that the chronic stress inhibited intestinal epithelial cell HSP70 expression at both mRNA and protein levels. This can be evidence in supporting the concept that chronic stress is involved in many chronic disorders such as inflammatory bowel diseases (IBD). Cumulative evidence demonstrates that apoptosis of intestinal epithelial cell play a role in pathogenesis of IBD [35, 36]. A large body of publications indicates that chronic stress may induce or exacerbate IBD but short of direct evidence [7, 37]. The present study provides indirect supporting evidence that chronic stress detriments intestinal epithelial barrier functions via down regulate HSP70 expression, increases its permeability that may develop to IBD eventually if without medical intervention. Studies performed in cell lines and immune cells have shown that HSP70 can block apoptosis at both early [38] and late [31] steps in the cascade. We did not examine epithelial cell apoptosis in this study, because we have shown the data previously [28].

Pretreatment with HSP70 significantly protect the intestinal epithelial barrier function impaired by chronic stress as shown by present study. The protection may be mediated by one or more of the many activities ascribed to HSP70, including refolding denatured proteins and preventing unfolded and damaged proteins from aggregating, or by a direct anti-apoptotic mechanism. We have presented evidence for a correlation between decreased HSP70 and impaired intestinal epithelial barrier dysfunction in the present study. Others have shown that over-expression or transgenic over-expression of HSP70 can efficiently protect ischemic stress induced tissue damage [25, 32] that is consistent with the present results.

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