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Original Article

Capsaicin reduces intrinsic apoptosis induced by hypoxia/reoxygenation in rat alveolar type II cells

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Abstract: Background: We aimed to explore whether capsaicin (Cap) suppressed apoptosis induced by hypoxia/reoxygenation (HR) of rat alveolar type II (RLE-6TN) cells and to determine the mechanism underlying this process. Method: A HR model was established by culturing RLE-6TN cells in a 1% O_2 and 5% CO_2 hypoxic chamber for 24 h, before transferring it to a 21% O_2 and 5% CO_2 incubator for 24 h. The cells were randomly allocated into three groups: (i) a control group of cells cultured under normal conditions; (ii) an HR group of cells cultured without capsaicin in a HR model; and (iii) a Cap+HR group of cells treated with capsaicin (50 μg) during hypoxia. Transmission electron microscopy and immunostaining were used to identify RLE-6TN cells. The apoptotic rate was detected by flow cytometry. Quantitative real-time polymerase chain reaction was used to test the mRNA expressions of caspases 3 and 9. Results: The RLE-6TN cells exhibited alveolar type II cell characteristics. The apoptotic rate in the HR group (47.0% ± 23.3%) was higher compared with the Cap+HR group (26.2% ± 9.3%) and was much higher compared with the control group (7.7% ± 3.5%) (both $P < 0.05$). The mRNA expressions of caspases 3 and 9 in the Cap+HR group were higher compared with the control group but lower compared with the HR group. Conclusion: Capsaicin decreased the HR-induced rate of RLE-6TN cell apoptosis, probably by inhibiting the intrinsic apoptotic pathway, which was evidenced by the reduced mRNA expressions of caspases 3 and 9.

Keywords: Alveolar epithelial cells, apoptosis, capsaicin, hypoxia, ischemia, reoxygenation

Introduction

Lung ischemia/reperfusion injury (LIRI) occurs in several situations, including lung transplantation, cardiopulmonary bypass, cardiopulmonary resuscitation, pulmonary embolism, and sepsis. LIRI increases the risk of acute rejection, adversely affects early postoperative mortality, and is the strongest risk factor for obliterative bronchiolitis [1]. Advances in understanding the mechanisms involved in LIRI have revealed a complex inflammatory process that involves activation and infiltration of innate immune cells, injury of endothelial and epithelial cells, oxidative stress, and cytokine responses [2]. Consequently, the changes may lead to pulmonary cellular necrosis [3], autophagy, or apoptosis [4, 5]. Apoptosis is triggered and modulated by intrinsic and extrinsic pathways. Caspase 3 activity has been shown to increase after ischemia/reperfusion injury [6], and there is compelling evidence that caspase 8 is a major initiator of the extrinsic pathway [7]; by contrast, caspase 9 is the only initiator caspase implicated in intrinsic apoptosis [8].

Researchers have shown that reperfusion after ischemia induces apoptosis in more than 20% of parenchymal lung cells (mainly type II pneumocytes) following lung transplantation [4], and that this might be responsible for severe organ dysfunction [9]. Moreover, 99% of the internal surface area of the lung is covered by lung alveolar epithelium, which is composed of alveolar type I (ATI) and type II (ATII) cells that are morphologically and functionally distinct. ATII cells are believed to play a pivotal role in maintaining tissue homeostasis via epithelial restoration and are capable of proliferating and transdifferentiating into ATI cells when lung injury occurs [10]. Functionally, ATII cells regulate alveolar

fluid levels and contribute to the host defense and immune response [11, 12]. These cells are also distinguished by the presence of lamellar bodies, the intracellular organelles that store and secrete surfactant protein-C (SP-C) [13]. Moreover, ATII cells have been shown to display a self-repair capacity in a rat LIRI model [14]. To clarify the mechanism of LIRI, research efforts are now increasingly focused on exploring the changes in ATII cells during hypoxia/reoxygenation (HR) injury.

The capsaicin receptor, transient receptor potential vanilloid type 1 (TRPV1), a ligand-gated cationic channel, is a molecular integrator of multiple chemical and physical stimuli, including high temperature, capsaicin, and tissue damage [15]. Evidence has shown that pharmacological activation of TRPV1 by capsaicin conferred a cardioprotective effect against HR injury [16], while its pharmacological inhibition or genetic deletion enhanced that injury [17]. In a previous study, we demonstrated that administering the TRPV1 agonist capsaicin before ischemia attenuated LIRI in rabbits and rats [18, 19], but we did not clarify the effect of capsaicin on alveolar epithelial cell apoptosis. Therefore, we aimed to explore the effect of capsaicin on the apoptosis of ATII alveolar epithelial cells subjected to HR.

Material and methods

Materials

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) was purchased from Sigma-Aldrich (MO, USA) and dissolved in pure DMSO (Sigma). The culture media and the fetal bovine serum (FBS) were purchased from Life Technologies (NY, USA). An annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Keygen (Jiangsu, China). FBS was purchased from Gibco (Thermo Scientific, MA, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone (UT, USA). Strept-avidin-biotin complex (SABC) and diaminobenzidine (DAB) were purchased from Boster (Wuhan, China). SYBR Premix Ex Taq II (Tli RNaseH Plus) was purchased from Takara (Kusatsu, Shiga, Japan). Anti-surfactant protein-A (anti-SP-A) antibody was purchased from Abcam (ab87674, MA, USA), and anti-SP-C antibody was purchased from Santa Cruz (Sc-13979, TX, USA).

Cell culture

The rat lung tissue-derived cell line, RLE-6TN, was purchased from American Type Culture Collection (ATCC, VA, USA). Cells were cultured in DMEM supplemented with 10% FBS, and treatment with capsaicin (50 μM) was accomplished by adding it at the onset of the HR process.

Cell identification

Transmission electron microscopy and immunohistochemical (IHC) staining were used to identify the cell line.

Transmission electron microscopy

RLE-6TN cells were plated in six-well plates and incubated overnight. The cells were collected and centrifuged for 10 min at 2000 rpm. The precipitates were fixed in 4% glutaraldehyde for 10 min at 4°C and then treated with 1% osmium tetroxide for 1 h. The samples were dehydrated with increasing concentrations of ethanol and gradually infiltrated with Araldite resin. Ultrathin sections were obtained using an ultramicrotome (Leica, Mannheim, Germany). The sections were stained with uranyl acetate and lead citrate, and they were examined using a Tecnai G220 transmission electron microscope (FEI, OR, USA).

IHC assessment

Staining for IHC was performed using an SABC kit. Adherent cell sections were treated with 3 mL/L H_2O_2 in methanol for 30 min to stop endogenous peroxidase activity. The sections were blocked with normal goat serum for 30 min at room temperature and incubated with an anti-SP-A antibody (1:100) and anti-SP-C antibody (1:200) overnight at 4°C. They were then incubated for 1 h with biotinylated anti-rabbit immunoglobulin G, followed by the avidin-biotin-peroxidase complex. The color was developed in a DAB-hydrogen peroxide solution (0.1 mL/L) and counterstained with hematoxylin.

HR model

The HR model was established by culturing RLE-6TN cells in a 1% O_2 and 5% CO_2 hypoxic

chamber for 24 h, and then transferring them to a 21% O_2 and 5% CO_2 incubator for 24 h. The cells were randomly allocated to three groups (n = 8 per group): (1) a control group of cells cultured under normal conditions; (2) an HR group of cells cultured with no treatment but under the HR model; and (3) a capsaicin and HR (Cap+HR) group of cells treated with capsaicin (50 mM) during hypoxia but not during reoxygenation.

Flow cytometry analysis of cell apoptosis

Apoptosis was detected using a standard assay. The cells were washed once with calcium- and magnesium-free phosphate-buffered saline, harvested by trypsinization and centrifugation, and resuspended in 50 mM HEPES, pH 7.4, containing 700 mM NaCl and 12.5 mM $CaCl_2$ (annexin-binding buffer). The cells were then washed twice, centrifuged at 2000 rpm for 5 min, and resuspended in the same buffer. Aliquots of approximately 1×10^5 cells/mL were prepared, pelleted by centrifugation, and resuspended in 5 μL of FITC-annexin V (as provided in the assay kit) and 5 μL of propidium iodide (PI), and incubated at room temperature in the dark for 15 min. After 15 min, 400 μL of the binding buffer was added to the suspension. The cells were placed on ice until required for flow cytometry using a Beckman GALLIOS FACScan (CA, USA), a fluorescence-activated cell sorter, and analysis by annexin V/PI staining. A total of 10,000 events (cells) were counted per sample.

The mRNA expressions of caspases 3 and 9 in RLE-6TN cells

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to analyze the expression levels of caspases 3 and 9 in RLE-6TN cells. The qRT-PCR was performed in a Mastercycler gradient (an Applied Biosystems 7500 Sequence Detection System) using SYBR Premix Ex Taq II (Takara). Total RNA was extracted from cells using the RNAiso Plus Kit (Takara), was quantified with a spectrophotometer, and was then reverse transcribed into cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara). RT-PCR was conducted using the SYBR Two-Step qRT-PCR Kit (Takara), according to the manufacturer's instructions. The following primers were used for caspase 3: 5'-ATGCTTACTCTACCGCACCCG-3' (forward) and 5'-GAAGGACTCAAATTCCGTGGC-3' (reverse). The following primers were used for caspase 9: 5'-CCTGCCTTGGGAAACCTCAC-3' (forward) and 5'-TGCCAGAACCAATGTCCACC-3' (reverse). Rat glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene with the primers 5'-CCGTATCGGACGCCTGGTTA-3' (forward) and 5'-CCGTGGGTAGAGTCATACTGGAAC-3' (reverse). The 2-ΔΔCT method was used to analyze the relative intensities of mRNA expression.

Statistical analysis

All preparations and measurements were repeated at least three times for each sample, and at least 6 samples were collected for each group. The distributions of quantitative data sets were tested for normality by the Kolmogorov-Smirnov test. Normally distributed (parametric) variables are presented as mean ± standard deviation. Levene's test was used to evaluate the homogeneity of variances. Parametric data were analyzed by one-way ANOVA followed by Tukey's test for post hoc comparisons. A P value of < 0.05 was considered statistically significant.

Results

The RLE-6TN cell line had the typical characteristics of alveolar epithelial cells

We confirmed that the RLE-6TN cells were indeed an ATII cell line. Light microscopy showed that virtually 100% of the alveolar cell population displayed an epithelial-like monolayer morphology (**Figure 1A** and **1B**). Transmission electron microscopy also confirmed that the cells were cuboidal with numerous surfactant-containing lamellar bodies, which is the gold-standard criterion for identifying alveolar epithelial cells (**Figure 1C** and **1D**). The microvilli were also noted to be irregular. The granules of SP-A and SP-C presented as a yellow-brown reactant in the cytoplasm (positive cases), while the negative controls were without color. This suggested that the RLE-6TN cell line was capable of secreting pulmonary surfactant. After staining with hematoxylin-eosin, the nucleoli turned blue (**Figure 2A-C**).

The HR model was successfully established

The cells were exposed to prolonged hypoxia for 24 h followed by reoxygenation for 24 h to establish the HR model. Cell injury was assessed by evaluating apoptosis and changes

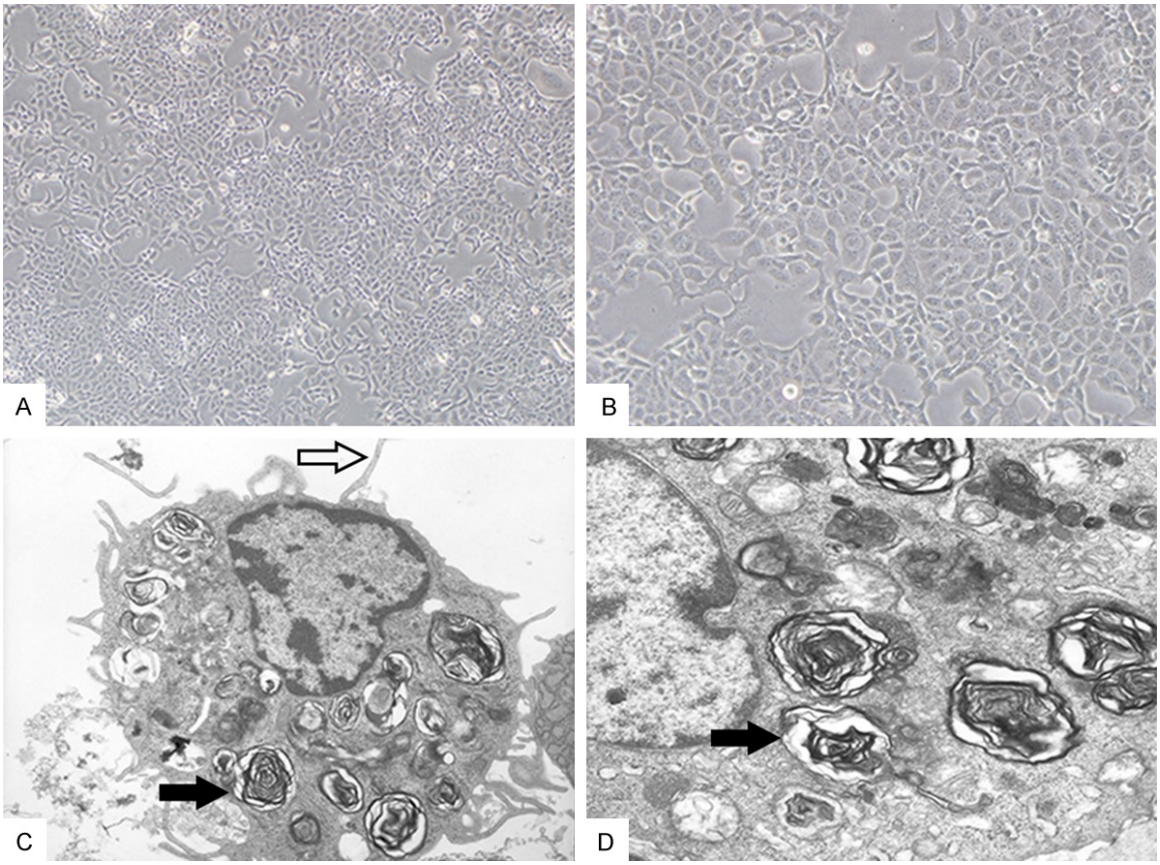

Figure 1. Optical microscopy and electron microscopy of RLE-6TN cells. Optical microscopy of RLE-6TN cells at × 100 (A) and × 200 (B). Electron microscopy of RLE-6TN cells: low-power electron microscopy illustrating RLE-6TN cells with apical microvilli and lamellar bodies (open and black arrows, respectively; C); and high-power electron microscopy showing lamellar bodies (black arrows; D).

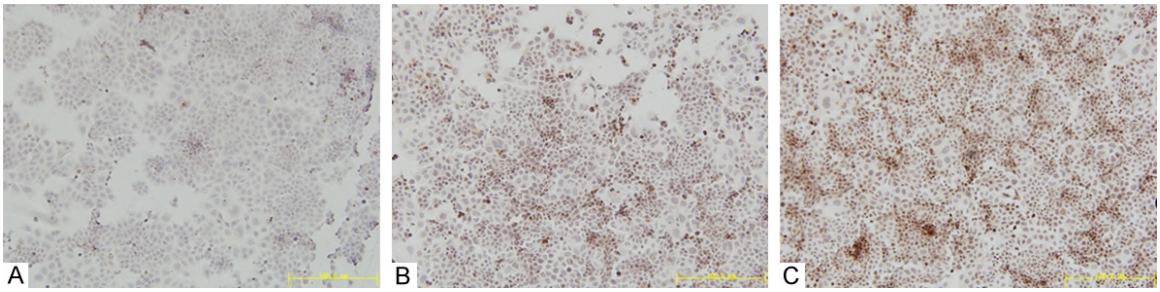

Figure 2. Immunohistochemical staining of surfactant proteins A and C in RLE-6TN cells. (A) Phosphate-buffered saline, (B) anti-surfactant protein-A antibody at a dilution of 1:100, and (C) anti-surfactant protein-C antibody at a dilution of 1:200.

in morphology. The cells manifested shrinkage, detachment, and fragmentation after exposure to HR (**Figure 3**), but capsaicin treatment (Cap+HR) efficiently protected cells.

Capsaicin suppressed HR-induced apoptosis

This study used flow cytometric analysis by FITC-annexin V and PI staining to quantify HR-induced apoptosis (**Figure 4A**). The cells were pretreated with or without 50 μM capsaicin during HR. **Figure 4B** shows that HR treatment sharply increased the rate of apoptosis in RLE-6TN cells (HR group, 47.0% ± 23.3%; control group, 7.7% ± 3.5%; $P < 0.05$). Moreover, the rate of apoptosis in the Cap+HR group (26.2% ± 9.3%) was lower compared with that in the

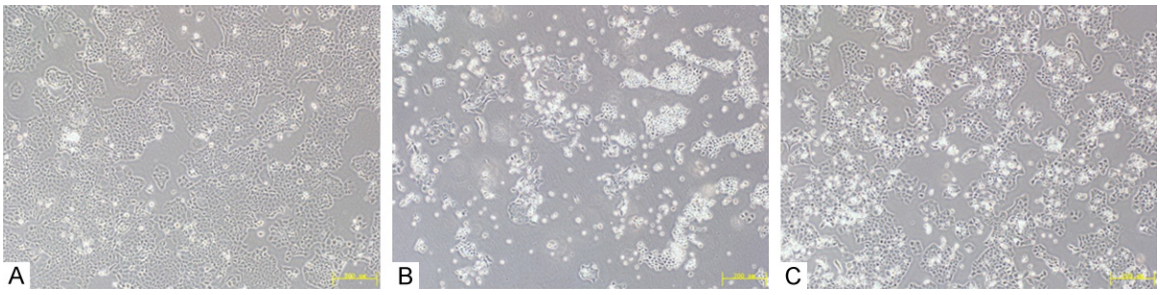

Figure 3. Morphological changes in different groups of RLE-6TN cells (× 100). The HR group and the Cap+HR groups showed more suspension cells compared with the control group. The Cap+HR group had fewer suspension cells compared with the HR group, and the cells tended to be normally shaped. Abbreviations: HR, hypoxia/reoxygenation; Cap, capsaicin.

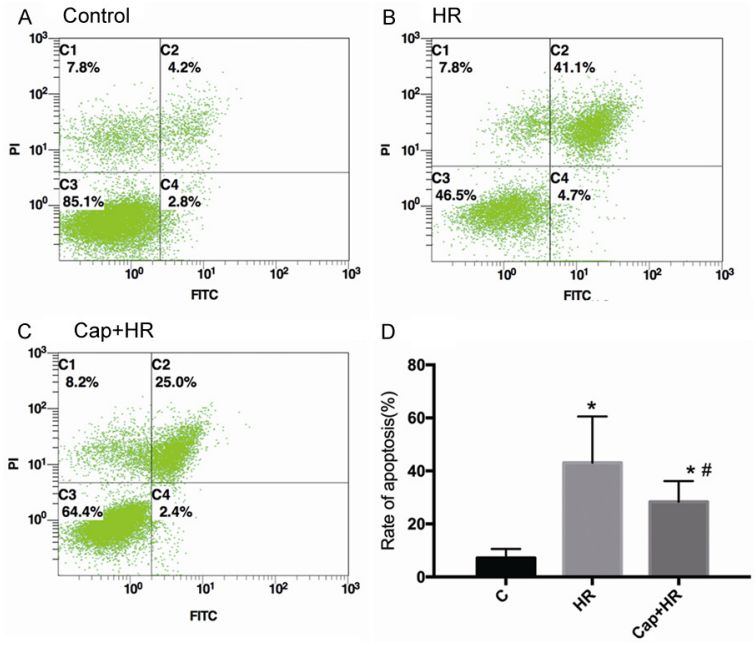

Figure 4. Capsaicin induced RLE-6TN cell apoptosis. (A-C) Typical flow cytometry results from control group (A), HR group (B), Cap+HR group (C) showing that apoptosis was unequal between groups. The percentage of live (lower left), early apoptotic (lower right), and late apoptotic (upper right) populations are shown. (D) Comparisons of the apoptosis rates between the three groups (n = 8 per group). Data are expressed as mean ± SEM. *P < 0.05 versus control group, #P < 0.05 versus HR group. Abbreviations: HR, hypoxia/reoxygenation; Cap, capsaicin.

HR group (P < 0.05, **Figure 4B**), implying that capsaicin could significantly suppress HR-induced apoptosis in RLE-6TN cells.

Capsaicin downregulated the mRNA expression of caspases 3 and 9

As shown in **Figure 5**, the mRNA expression of caspases 3 and 9 in RLE-6TN cells increased significantly in the HR group compared with the control group (P < 0.05). However, it was lower in the Cap+HR group compared with the HR group (P < 0.05).

Discussion

Although capsaicin has previously been shown to reduce LIRI in vivo, its impact on alveolar epithelial cells was unclear. In the present study, we found that capsaicin decreased the rate of RLE-6TN cell apoptosis after HR injury. Capsaicin also reduced the mRNA expressions of caspases 3 and 9, indicating that it probably exerted its protective effect by downregulating the intrinsic apoptotic pathway.

Our findings provide a novel insight into the role of capsaicin in RLE-6TN cells that are subjected to HR injury. Most in vitro research involving lung protection has tended to use the A549 cell line derived from lung cancer [20, 21]. In contrast to this, we used the RLE-6TN cell line derived from ATII cells, which are believed to play a much more pivotal role than ATI cells in maintaining tissue homeostasis via epithelium restoration [22]. It is widely known that ATII cells are distinguished by the presence of lamellar bodies, the intracellular organelles that store and secrete SP-C [12]. By demonstrating the presence of lamellar bodies using microscopy and the presence of SP-A and SP-C

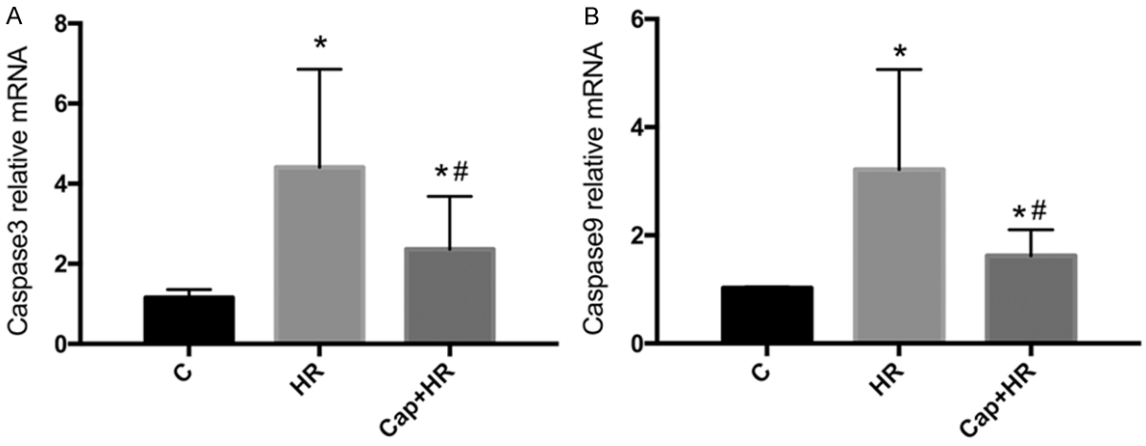

Figure 5. Relative mRNA expression levels of caspases 3 and 9 in the three groups. *P < 0.05 versus control group, #P < 0.05 versus HR group. Abbreviations: HR, hypoxia/reoxygenation; Cap, capsaicin.

using immunohistochemistry, we were able to confirm that the RLE-6TN cells were a valid ATII cell line.

Capsaicin is the spicy component of pepper and can bind to TRPV1 located on unmyelinated nociceptive C-fibers and thin A-myelinated fibers [23]. In a previous study, we proved that the administration of capsaicin attenuated LIRI in rabbits and rats [18, 19], but we did not explore the direct effect of capsaicin on alveolar epithelial cells directly exposed to LIRI. By conducting the present research, we showed that capsaicin did in fact decrease the ATII cell apoptosis induced by HR injury. This finding is consistent with previous reports showing that capsaicin has antioxidative and anti-apoptotic activities in endothelial cells and macrophages [24, 25], spermatogenic cells [25], and bone marrow mesenchymal stem cells [26]. However, some studies have indicated that capsaicin exacerbated HR-induced apoptosis in H9C2 cells [27]. Additionally, it has been reported that capsaicin has an antitumor effect, inducing apoptosis in cancer cells [28-31].

The opposing effects of capsaicin shown in previous reports have been attributed to the use of different cells line, different capsaicin doses, and different HR periods, among other factors. In the present study, however, we investigated the effect on the normal alveolar cell line while most previous studies utilized tumor cell lines expressing very different metabolisms. Exposure to high doses of capsaicin (> 100 mg capsaicin per kg of body weight) for a prolonged time is also known to cause injury [32, 33], whereas low doses are known to exert a protective effect on organs. Indeed, studies have consistently shown that capsaicin did not damage cells at concentrations of < 100 mM, including in human lung carcinoma cells (A549) [34, 35], human KB cancer cells [36], human bronchiolar epithelial cells (BEAS-2B), and human hepatoma (HepG2) cells [35]. Thus, our design sought to mitigate the previous issues in the literature, providing a novel insight into the pathology of HR injury.

Apoptosis, as spontaneous programmed cell death, can be triggered by a series of downward cascade processes. Morphologically, it presents as cell shrinkage, chromatin condensation, and DNA fragmentation without compromising the plasma membrane or the integrity of intracellular organelles [3]. This was observed in both the HR group and in the Cap+HR group. Apoptosis can be activated by two major pathways. One is the extrinsic pathway, which is initiated by ligation of the death receptor on the membrane and is followed by cleavage of caspase 8 with subsequent activation of the downstream caspase cascade [5, 37]. The other the intrinsic pathway, which begins with the activation of proapoptotic proteins leading to mitochondrial dysfunction, resulting in caspase 9 cleavage and activation [38]. The cleavage and activation of caspase 3 then allows cells to undergo the final stages of apoptosis by triggering the common executive caspase of both the extrinsic and the intrinsic pathways [38].

Apoptosis is initiated in response to HR [3], and it has also been verified that hypoxia can activate caspases 3, 8, and 9 via Ca^{2+}-induced activation of other proteases. Therefore, caspase accumulation was measured to identify the step at which capsaicin intervention occurred. Our data showed that capsaicin suppressed the mRNA expressions of caspases 3 and 9. As a representative caspase of the intrinsic apoptosis pathway, caspase 9 plays a critical role in influencing subsequent steps. Thus, capsaicin probably suppressed HR-induced apoptosis by inhibiting the intrinsic apoptotic pathway, though we lack the data to conclude that capsaicin can decrease apoptosis at the protein level. This latter point requires further investigation.

In conclusion, this study showed that treatment with capsaicin can reduce the HR-induced apoptosis of RLE-6TN cells in vitro. The reduced expression of caspases 3 and 9 implies that capsaicin probably suppresses apoptosis by inhibiting the intrinsic pathway.

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Disclosure of conflict of interest

None.

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