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# Apoptosis and death receptor signaling in diaphragm of burnt rats

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ABSTRACT

*Background:* Respiratory dysfunction is a frequent complication after severe burn injury. Respiratory muscle atrophy may induce respiratory dysfunction due to insufficient inspiratory motive power. Accumulated evidence suggests that apoptosis is very important in skeletal muscle atrophy in multiple pathologic conditions. Therefore, we hypothesize that myonuclear apoptosis contributes to diaphragm atrophy induced by burn injury, and death receptor signaling activation plays a role in this process.

*Methods:* Wistar rats in the burn-injured group were subjected to a full-thickness scald injury around 40% of total body surface area. Diaphragm samples were examined for myonuclear apoptosis by transmission electron microscope, terminal deoxynucleotidyl transferase-mediated nick end labeling assay, and immunohistochemistry for caspase-3. Serum level of apoptotic ligands were assessed by ELISA. Activation of death receptor signaling was examined by Western blotting.

*Results:* Burn injury resulted in significant reductions of diaphragm muscle mass and myofiber cross-section area. Apoptosis in diaphragm appeared from day 1 and peaked on day 4 after injury. The level of soluble TNF-related apoptosis-inducing ligand and the ratio of Fas ligand to soluble Fas in serum significantly increased after burn injury. In diaphragm of burnt animals, the expressions of proapoptotic proteins, such as cleaved caspase-8, cleaved caspase-3, and Bax-to-Bcl-2 ratio were upregulated, whereas expression of pAkt, an antiapoptotic protein, was downregulated. Immunohistochemistry revealed that the most of the caspase-3 was expressed in myofiber nuclei and their surrounding cytoplasm area in tissue sections.

*Conclusions:* Severe burn injury induces myonuclear apoptosis in diaphragm, which could be a contributor to diaphragm muscle atrophy. Activation of death receptor signaling may be a mechanism of apoptosis in diaphragm.



## Introduction

Respiratory dysfunction is a frequent complication after severe burn injury, especially combined with inhalation injury, which further leads to high morbidity and mortality.[1] Inflammation, edema, exudates, infection, and airway obstruction are the frequent contributors to pulmonary dysfunction after severe burn injury. However, impairment of

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lung and airway function only explains one aspect of the respiratory dysfunction.[2] As the motive power of external respiration, respiratory muscle atrophy is another important contributor to respiratory dysfunction, which may lead to weakened breathing, insufficient ventilation, cough reflex suppression, increased rate of pulmonary infection, difficulty in withdrawing from mechanical respirators,[3] and even respiratory failure.

The diaphragm muscle is the primary muscle for inspiration and provides 60%–80% of the inspiratory power.[4] It is well established that diaphragm atrophy contributes to chronic obstructive pulmonary disease, critical conditions with mechanical ventilation, and respiratory complications with aging.[2,3,5] But it is unknown whether there is diaphragm muscle atrophy and its potential contribution to respiratory dysfunction after severe burn injury.

Evidence accumulated over the last 20 y has shown that apoptosis is a significant contributor to skeletal muscle atrophy associated with burns, aging, denervation, and chronic pulmonary and cardiac dysfunction.[6–11] Apoptosis and oxidative stress are also responsible for diaphragm muscle atrophy in chronic obstructive pulmonary disease, mechanical ventilation, shock, hyperglycemia, and endotoxemia.[3,12–14] Similar to other skeletal muscle fibers, diaphragm myofibers are multinucleated. So, its apoptosis is not whole cell apoptosis as that in mononucleated cells but myonuclear apoptosis in which apoptotic myonuclei are eliminated from myofibers.[15] Apoptosis can be induced by several signaling pathways, including death receptor–mediated, mitochondrial, and endoplasmatic reticulum pathways. Death receptor-mediated apoptotic signaling pathways are activated by apoptotic ligands binding to death receptors on the plasma membrane, followed by death-inducing signaling complex formation, leading to initiation of the caspase cascade through caspase-8 and finally inducing the damage of genomic DNA.[16] But apoptosis and its mechanisms in diaphragm muscle after burn injury remain to be elucidated.

According to our previous study, severe burn injury induces increased myonuclear apoptosis in extremity skeletal muscle.[8] Also, major burn patients are always accompanied by systemic inflammatory response syndrome that results in significant increase of serum proapoptotic factors including tumor necrosis factor (TNF)-$\alpha$, Fas ligand (FasL), and C-reactive protein, which possibly contributes to apoptosis.[17] Therefore, we hypothesize that myonuclear apoptosis contributes to the development of diaphragm muscle atrophy induced by severe burn injury, and activation of death receptor–mediated apoptotic signaling plays a role in this process.

## Materials and methods

### *Burnt rat model and time points of experiments*

The animal experiment procedures were consistent with the International Guiding Principles for Biomedical Research Involving Animals and were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Chinese PLA General Hospital. Seven-week-old male Wistar rats (body weight, 240–260 g) from Chinese Medical Scientific Institute (Beijing, China) were used for experiment. After receiving analgesia (0.05 mg/kg buprenorphine, subcutaneous injection) as described by Al-Mousawi *et al.*,[18] the rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 $mg \cdot kg^{-1}$ body weight). The hair of back and abdomen was clipped. Animals were then sustained a scald injury of 40% of total body surface area by immersing the dorsal and ventral skin of the trunk in 80°C water for 15 and 8 s, respectively. This procedure was verified to be effective to cause full-thickness thermal injury exclusive to skin.[8] Fluid resuscitation of 40 mL/kg of intraperitoneal compound sodium chloride injection was given to all animals immediately after thermal injury. Animals were then kept warm until they were completely recovered from anesthesia. Both scald- and sham-injured areas were treated as previously described.[8] Analgesia was given after burn injury or if necessary in the following days.[18] Sham-burned rats in the control group were treated in the same procedure as that in the burned group, except for scald injury.

Arterial blood and diaphragm samples were collected at 0, 1, 4, 7, and 10 post-injury days after rts were euthanized. Wet weight of completely excised diaphragm muscles was determined. The ventral and left dorsal part of the diaphragm was used for Western blot analysis, and the other sample was used for immunohistochemistry (IHC) and transmission electron microscopy (TEM) evaluation.

### *Transmission electron microscopy evaluation*

The diaphragmatic tissue samples used for TEM evaluation were prepared as previously described.[8] Briefly, 80 nm–thick sections were cut followed by uranyl acetate and lead citrate staining according to standard procedures. Transmission electron microscope JEM 1200EX II (JEOL) was used for examination.

### *Terminal deoxynucleotidyl transferase-mediated nick end labeling, immunohistochemistry assay, and myofiber cross-section area measurement*

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) is a widely accepted method to identify apoptosis *in situ* in cardiac[19] and skeletal muscle myocytes.[20] In the present study, In Situ Cell Death Detection Kit (Roche Applied Science, Germany) was used for detecting myonuclear apoptosis in diaphragm according to the instructions of manufacturer. Briefly, diaphragm muscles were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin as previously described.[8] After being treated with proteinase K and subsequent 0.1% Triton X-100, the tissue on the slides were further interacted with TUNEL mixture at 37°C for 30 min. TUNEL-positive nuclei were stained as brown. Thereafter, the slides underwent double staining with incubation of rabbit anti-laminin antibody (1:100, Boster, Wuhan, China), which is selectively bound to the basal lamina. Another set of slides were incubated with primary antibody of rabbit anti-caspase-3 (1:50, Cell Signaling Technology). 3,3' Diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC) were used for staining of caspase-3 and laminin, respectively.