Simulated Aeromedical Evacuation Exacerbates Burn Induced Lung Injury: Targeting Mitochondrial DNA for Reversal

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Original research

Keywords: malonaldehyde, deoxyribonucleic, histopathological

Posted Date: July 9th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-38578/v1>

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Abstract

Background: Aeromedical evacuation of patients with burn trauma is an important transport method at both wartime and peacetime, which exposes patients to prolonged periods of hypobaric hypoxia. However, the effects of such exposure on burn injury, particularly on burn induced lung injury are largely unexplored. The objective of this study is to investigate the effect of hypobaric hypoxia on burn induced lung injury and to discuss the possible mechanism by using a rat burn model.

Methods: Male wistar rats inflicted with 30% total body surface area burn were exposed to hypobaric hypoxia condition (simulated 2000m altitude) or normoxia control for 24 h. Deoxyribonuclease I was systemically administrated as treatment intervention. Systemic inflammatory mediators and mitochondrial deoxyribonucleic acid level were detected. The histopathological examination, and acute lung injury score were determined. Malonaldehyde content, myeloperoxidase activity, and the nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome level in the lung tissue were measured. Data among groups were compared by using analysis of variance followed by the post hoc analysis of Tukey's test.

Results: Burn resulted in remarkably higher level of systemic inflammatory cytokines and mitochondrial deoxyribonucleic acid release, which was further heightened by hypobaric hypoxia exposure. Moreover, hypobaric hypoxia exposure gave rise to increased NLRP3 inflammasome expression, elevated malonaldehyde content and myeloperoxidase activity in the lung. Burn induced lung injury was exacerbated as shown by histopathological examination and acute lung injury score. Administration of deoxyribonuclease I markedly reduced mitochondrial deoxyribonucleic acid release and systemic inflammatory cytokines production. Furthermore, NLRP3 inflammasome level in the lung tissue was decreased and burn induced lung injury was ameliorated.

Conclusions: Our results suggested that simulated aeromedical evacuation further increased the burn induced mitochondrial deoxyribonucleic acid release and exacerbated burn induced inflammation and lung injury. Deoxyribonuclease I reduced the release of mitochondrial deoxyribonucleic acid and limited the mitochondrial deoxyribonucleic acid-induced systemic inflammation, ameliorated burn-induced acute lung injury. Intervening mitochondrial deoxyribonucleic acid level could be a potential target to protect from burn-induced lung injury during aeromedical conditions and provide with safer air evacuations for severely burned patients.

Background

Burn is a systemic damage, which can induce acute lung injury, lead to acute respiratory failure, and cause early death in severely burned patients. Even without inhalation injury, major burn patients frequently develop acute respiratory distress syndrome, eventually end up with multiple organ failure and death^{1,2}. The specific mechanism of burn induced lung injury is still not fully understood. Previous studies indicated that excessive systemic inflammatory response and the cascading release of

inflammatory mediators after burns, were among the key contributing factors to burn induced lung injury^{3,4}.

The essence of the inflammatory response after burn and trauma is the activation of innate immunity system, which identifies various danger signals through pattern recognition receptors (PRRs)⁵⁻⁷. Danger signals released from tissue damage are called damage associated molecular patterns (DAMPs), such as high mobility group protein 1, uric acid, ATP, hyaluronic acid fragment and mitochondrial DNA (mtDNA)^{8,9}. Recent studies have found that mtDNA is one of the key factors mediating innate immunity and inflammatory response after trauma. Upon tissue damage or stress, mtDNA is released into the cytoplasm, extracellular space or blood, recognized by various kinds of PRRs, activating downstream inflammatory signaling pathways, and mediating systemic inflammatory response^{10,11}. Research has shown that burn injury results in a large number of mtDNA release, thereby activates systemic inflammatory pathways and causes acute lung injury. Inhibiting the release of mtDNA is an important potential target for the treatment of burn induced lung injury¹²⁻¹⁴.

Air evacuation of severely burned patients is a frequently seen scenario at both peacetime and wartime. The main effect of air evacuation environment on the body is hypobaric hypoxia. The lungs are among the most direct target organs exposing to the hypobaric hypoxia environment. However, how the air environment influences burn induced lung injury is unclear. There is a lot of literature showed that hypoxia is an independent contributing factor to acute lung injury. Moreover, hypoxia can induce the body's inflammatory response, and inflammation can lead to tissue hypoxia, the two are intertwined, and influence each other, causing acute lung injury¹⁵⁻¹⁸. Hypoxia environment has great influence on the function of mitochondria. Lack of oxygen makes mitochondrial function disorder, produces excess mitochondrial reactive oxygen species, causes mitochondrial damage, and subsequently results in the release of mtDNA and systemic inflammatory reaction^{19,20}. Therefore, mtDNA mediated systemic inflammatory response may be a common pathway for both burn induced lung injury and hypoxia induced lung injury. Intervention of mtDNA mediated inflammatory signaling pathways may be a potential therapeutic target to ameliorate burn induced lung injury under the air evacuation environment.

Mitochondrial DNA can activate diverse PRRs such as cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), the nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome and toll like receptor 9 (TLR9), etc^{11,21}. Because of its downstream signal molecules are too numerous to intervene, reducing the release of mtDNA from the source should be a most direct and effective intervention measure. So, we tested the effect of deoxyribonuclease I (DNase I), a specific endonuclease, on burn induced mtDNA release and consequent lung injury in simulated aeromedical evacuation condition. We found in this study that simulated aeromedical evacuation further increased the burn induced mtDNA release and exacerbated burn induced inflammation and lung injury. Deoxyribonuclease I reduced the release of mtDNA and limited the mtDNA-induced systemic inflammation, ameliorated burn induced acute lung injury.

Methods

Experimental animals

The research protocol was approved by the Committee of Scientific Research of Air Force General Hospital of PLA, China. Male Wistar rats weighing 200–250 g were housed conventionally in an animal room with constant temperature (25°C) and humidity (50–60%), and free access to food and water. Animals were housed for at least 7 days prior to start the experiments. Using NCSS-PASS 11.0.4 software (NCSS, LLC, Kaysville, UT) and assuming a power of test 0.9 and a significance level alpha of 0.05, we calculated that 10 animals per group would be required for these experiments.

Burn model and animal grouping

Rats were anesthetized with intraperitoneal injection of 50 mg/kg body weight pentobarbital sodium (Sigma-Aldrich, St. Louis, MO). The hairs of the dorsal area were removed and a 30% total body surface area (TBSA) full-thickness burn injury was established by placing the animal in a template exposing 30% TBSA to 100°C heated water for 10 seconds. Rats were then resuscitated by intraperitoneal injection of physiological saline (40mL/kg) and placed in individual cages. The wounds were topically applied with povidone iodine solution, once daily. Sham burned rats were treated in the same manner except for that they were immersed in room temperature water. Immediately after burn injury was produced, rats in the treatment group were intravenously injected with 8mg/kg DNase α . Rats in the control group received equal amount of saline solution. To simulate the aeromedical evacuation condition, the rats were placed in a hypobaric hypoxic chamber (simulating 2000 m above sea level) 4 hours after burn injury for 4 hours. Right after the exposure, animals were euthanized, and blood or tissue samples were collected.

Detection of plasma mtDNA

mtDNA was isolated from plasma by using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). All procedures were performed following the manufacturer's instructions. Briefly, 50 μ l of PBS was added to 50 μ L of plasma sample and the mixture was centrifuged at 16,000g for 15 min at 4°C. The supernatant was obtained for next measurement. Plasma levels of mtDNA were determined by using SYBR Green real-time fluorescent quantitative PCR kits. Rat MT-ND2 gene were analyzed using primers: forward (5'-AAGGAGAGTGGAAGGATGT) and reverse (5'-ATTAGCAGCAGCAGATGG). The results were calculated by using the $2^{-\Delta\Delta CT}$ method, where CT represents cycle threshold value.

Detection of NLRP3 protein level by western blot

Western blot analysis was performed as described previously²². Membranes were incubated with anti-NLRP3 antibody (Santa Cruz Biotechnology, Inc., Dallas, Texas) and second antibody. The relative optical density was analyzed.

Detection of inflammatory cytokines in serum

The concentrations of inflammatory mediators (IL-1 β and IL-6) in serum were measured using specific enzyme-linked immunosorbent assay systems (Thermo Scientific, Waltham, MA) with accordance to the manufacturer's instructions.

Assessment of the lung injury

Lung tissues were fixed with paraformaldehyde and stained with hematoxylin and eosin (H&E) before the examination. Five fields were randomly selected in each slice and observed by two independent pathological specialists who is blinded to the experimental groups. The lung injury scoring system is used as previously described²³. Alveolar wall thickness, cellular infiltration and hemorrhage were each scored from 0 (no injury) to 4 (maximal injury). Counts of each score were summed up and the results were recorded as acute lung injury (ALI) score.

Detection of Malonaldehyde (MDA) content and myeloperoxidase (MPO) activity

Assays were performed according to a commercial kit and the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as previously described²⁴.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 6.01 software. Data are presented as means and standard deviations. The data among groups were compared by using analysis of variance, while the post hoc analysis was performed by Tukey's test. Values of $p < 0.05$ were considered statistically significant.

Results

mtDNA level in serum

mtDNA level in serum of burned rats were significantly higher than those of sham burned rats. After the exposure to hypobaric hypoxia, mtDNA level were further elevated, nearly twice as compared to the normoxia group ($p \leq 0.01$). DNase I treatment significantly reduced the increase of mtDNA level in serum of burned rats under hypobaric hypoxia environment ($p \leq 0.01$, Figure 1).

Level of inflammatory cytokines in serum

Burn resulted in markedly higher level of IL-1 β and IL-6 production in serum. These inflammatory cytokines production were further elevated when burned rats were exposed to hypobaric hypoxic environment. DNase I treatment significantly decreased the IL-1 β and IL-6 production in serum of burned rats under hypobaric hypoxia condition ($p \leq 0.01$, Figure 2).

Level of MDA content and MPO activity in lung tissue

In order to evaluate the oxidative damage and inflammatory infiltration in the lung tissue, we conducted experiments to detect the level of MDA content and MPO activity in lung tissue. As shown in Figure 3, burn resulted in significantly elevated level of MDA content and MPO activity in the lung tissue, which was further heightened by the exposure to hypobaric hypoxia. Moreover, these increases were restored by DNase I treatment ($p \leq 0.01$).

Histopathologic examination and assessment of the lung injury

To observe the severity of lung injury, we performed H&E staining and evaluated the lung injury according to the lung injury score system. As shown in Figure 4, the image of the sham burn group displayed normal structure of the lung tissue (A) while that of the burn group showed excessive inflammatory cells infiltration and edema of alveolar walls (B). Furthermore, after burned rats were exposed to hypoxic condition, there were more inflammatory infiltration and more severe damage of pulmonary alveoli showed in their lungs (C). Finally, the DNase I treatment attenuated inflammatory infiltration, restored edema of alveolar walls and ameliorated lung injury as shown in Figure 4 D. Determination of ALI score demonstrated that hypoxia group had higher score than normoxia group ($p \leq 0.01$), while the score of DNase I treatment group was significantly lower than that of hypoxia group ($p \leq 0.01$, Figure 4 E).

NLRP3 protein level in the lung tissue

Because the NLRP3 inflammasome is an important downstream signaling to mtDNA, and recent studies have demonstrated its key contributing role to acute lung injury²⁵. We designed experiments to determine the level of NLRP3 protein in lung tissue by using western blot analysis. As shown in Figure 5, the NLRP3 protein level in burn group was significantly higher than that of sham group. Hypoxia exposure further increased the NLRP3 protein level in rat lung tissue. Moreover, the elevated level of NLRP3 was remarkably reduced by DNase I treatment ($p \leq 0.01$).

Discussion

Aeromedical evacuation is a rapid and effective way to evacuate patients at both peacetime and wartime. But little is known about the possible effects of aeromedical evacuation on patients with severe burn injury, particularly on burn induced acute lung injury, which is still one of the leading causes of early death in severely burned patients. In this study, we firstly observed the effect of simulated aeromedical evacuation on burn induced lung injury, and secondly explored the possible role of mtDNA induced inflammation in the pathogenesis of burn induced lung injury under hypobaric hypoxia environment. The results of this study demonstrated that simulated aeromedical evacuation exacerbated burn induced lung injury. mtDNA mediated systemic inflammation possibly played an important role in the exacerbation of lung injury. Targeting mtDNA by DNase I can ameliorate burn induced lung injury under hypobaric hypoxia environment.

There have been a lot of literatures discussing about the effect of aeromedical evacuation on some types of injuries, such as traumatic brain injury, hemorrhagic shock and blast injury^{26–30}. However, as for the

effect of aeromedical evacuation on burn injury related pathophysiology, little is known. The most prominent influence of aeromedical evacuation on the human body is hypobaric hypoxia ³¹. Previous studies concerning the traumatic brain injury or blast injury have demonstrated that hypobaric hypoxia exacerbated the inflammatory response to these injuries and thus worsened these types of injuries ^{28,32}. In the present study, we found the same results as these above studies. Burn induced systemic inflammation and remote lung injury was also exacerbated by hypobaric hypoxia exposure. These results conform to the notion that hypoxia can induce inflammation and cause acute lung injury ^{15,18}.

To further dissect why hypobaric hypoxia exposure exacerbated burn induced systemic inflammation and lung injury, we tested the possible role of mtDNA on the pathogenesis of burn induced lung injury under hypobaric hypoxia environment. mtDNA is released after tissue injuries and plays a crucial role in the development of inflammation after injury. Studies have shown that mtDNA can induce inflammatory responses and cause lung injury in thermal injury murine models ^{13,14}. Furthermore, mitochondrion is among the most sensitive target organs when exposing to hypoxia. Hypoxia leads to mitochondrial dysfunction and induces the release of a variety of DAMPs such as nuclear high mobility group box 1 and mtDNA ^{33,34}. Therefore, it is reasonable that hypobaric hypoxia will increase the burn injury induced mtDNA release, which is testified by the results of the present study.

mtDNA induces inflammatory response through various receptors and related downstream signaling pathways, including cGAS, NLRP3 and TLR9, etc. We hypothesized that giving external DNase I, a nuclease responsible for degrading extracellular DNA, can reduce the mtDNA release from the source, and thus abrogate mtDNA induced inflammation and ameliorate remote lung injury. The results of our study showed that a single dose of 8 mg/kg DNase I significantly reduced the mtDNA level in serum, suppressed NLRP3 inflammasome level in the lung tissue and ameliorated lung injury in this rat burn model. These results are of certain significance because the recombinant human DNase I has been applied in clinic for patients with some pulmonary diseases such as cystic fibrosis and pneumonia, and has shown excellent safety and efficacy ^{35,36}. But the mechanisms of these protective effects are likely different from its actions reported herein. It is interesting to infer if the above mentioned effects are partially through antagonizing mtDNA signaling. This speculation is to be further explored.

Conclusions

The results in this study suggested that simulated aeromedical evacuation further increased the burn induced mtDNA release and exacerbated burn induced inflammation and lung injury. Deoxyribonuclease I reduced the release of mtDNA and limited the mtDNA-induced systemic inflammation, ameliorated burn injury-induced acute lung injury. Intervening mitochondrial DNA level could be a potential therapy target to protect from burn-induced lung injury during aeromedical conditions and provide with safer air evacuations for severely burned patients.

Declarations

- Ethics approval and consent to participate

The research protocol was approved by the Committee of Scientific Research of Air Force General Hospital of PLA, China.

- Consent for publication

Not applicable

- Availability of data and materials

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

- Competing interests

The authors declare that they have no competing interests

- Funding

This study is funded by Major Applied Basic Research Project from the Logistics Security Ministry of the Central Military Commission (Project number: AKJ15J001)

- Authors' contributions

BZ designed the study. MJX, XFZ and BL carried out the experiments. SJW and BL coordinated the experiments. BLL and SJW performed the data preparation and statistical analysis. MJX drafted the manuscript. XFZ and BZ participated in the revision of the manuscript. All authors read and approved the final manuscript.

- Acknowledgements

Not applicable

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Figures

Figure 1

mtDNA level in serum was detected by quantitative PCR and calculated by using the $2^{-\Delta\Delta CT}$ method, where CT represents cycle threshold value. Results are presented as means $\pm$ SD; n=10 rats/group. **p < 0.01.

Figure 2

Level of inflammatory cytokines in serum was detected by ELISA. Results are presented as means $\pm$ SD; n=10 rats/group. **p<0.01.

Figure 3

Level of MDA content and MPO activity in lung tissue were detected according to a commercial kit and the manufacturer's instructions. Results are presented as means±SD; n=10 rats/group. **p<0.01, *p<0.05.

Figure 4

Histopathologic examination and assessment of the lung injury. A-D, representative images of H&E staining of lung tissue in sham, burn normoxia, burn hypoxia and DNase I group, respectively. scale bar =

500um. E: ALI score was calculated and compared among groups. Results are presented as means±SD; n=10 rats/group. **p<0.01.

Figure 5

NLRP3 protein level in the lung tissue was detected by western blot. Results are presented as means±SD; n=10 rats/group. **p<0.01.