The cause of acute lethality of mice exposed to a laser-induced shock wave to the brainstem

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Air embolism is generally considered the most common cause of death within 1 h of a blast injury. Shock lung, respiratory arrest, and circulatory failure caused by vagal reflexes contribute to fatal injuries that lead to immediate death; however, informative mechanistic data are insufficient. Here we used a laser-induced shock wave (LISW) to determine the mechanism of acute fatalities associated with blast injuries. We applied the LISW to the forehead, upper neck, and thoracic dorsum of mice and examined their vital signs. Moreover, the LISW method is well suited for creating site-specific damage. Here we show that only mice with upper neck exposure, without damage elsewhere, died more frequently compared with the other injured groups. The peripheral oxygen saturation (SpO₂) of the former mice significantly decreased for < 1 min \( p < 0.05 \) but improved within 3 min. The LISW exposure to the upper neck region was the most lethal factor, affecting the respiratory function. Protecting the upper neck region may reduce fatalities that are related to blast injuries.

Terrorist bombings continue as a major problem worldwide. Although the number of patients with injuries caused by terrorist bombs peaked in 2014, the threat of such injuries continues to threaten military and civilian populations, with ≥ 10,000 bombings each year.²³ Evidence indicates that air embolism is the most common cause of immediate death or death within 1 h of a bombing.²³ Further, other fatal injuries that can lead to immediate death include shock lung, respiratory arrest, or circulatory failure caused by vagal reflexes; however, not much is known about the underlying mechanisms.

Patients with head trauma or cerebral hemorrhage with complex lesions in the medulla oblongata and other brainstem regions die without ameliorating apnea.⁷ Furthermore, in a rat model of severe diffuse brain injury, apnea occurs for approximately 10–20 s immediately after an injury. Respiration subsequently resumes; however, the respiratory rate gradually decreases until death.⁸⁹ Our research employs shock and blast tubes to model blast injuries.⁶¹⁰ However, it is difficult to produce injuries localized to a specific site, and therefore the cause of immediate death and the responsible organs are unknown. Further, it is difficult to evaluate the response to such injuries inflicted upon each region of the brain.

Here, we used laser-induced shock waves (LISW) to determine the mechanism of acute fatalities caused by blast injuries because the LISW enables site-specific shock wave exposure. We hypothesized that respiratory failure is caused by injury involving brainstem region; therefore, LISW locally applies shock waves, which enable verification of a site-specific response.

Methods

Mice. Male C57BL/6 mice (age: 8–10 weeks, bodyweight: 23–26 g) were obtained from SLC Japan (Shizuoka, Japan). The mice were housed at 22–24 °C with a 12-h light/dark cycle and had free access to food and water. The Animal Ethics Committee of the National Defense Medical College Hospital approved the procedures for using mice (Permission No. 20004). Suffering was minimized by anesthetizing the mice and employing humane endpoints. All experiments were performed in accordance with relevant guidelines and regulations. Our reporting of research involving animals follows the recommendations of the ARRIVE guidelines.

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**Experimental procedures.** The setup for producing LISW, which is compact and easy to use and control, offers excellent safety and multifunctionality, as well as delivers site-selective shockwave application to laboratory animals at highly reproducible dose\(^5\)\(^\text{11-14}\). Furthermore, the positive pressure duration of the LISW used in this study was 0.6 s (Fig. 1), which is much shorter than that of actual explosion shock waves\(^15\). In this study, we assumed that the impulse of the shock wave, i.e., time-integrated positive pressure component, is a primary parameter to determine brain injury. The impulse of LISW was 20 Pa.s (peak pressure, 82 MPa) (Fig. 1). We applied LISW to the frontal, upper dorsal neck, and right thoracic dorsum (n = 10 per group) (Fig. 1).

**LISW method.**

1. The experimental mice were anesthetized with 1.5% isoflurane, and the hair at the experimental site, the frontal region, upper dorsal neck, and the right thoracic dorsum was removed with a depilatory cream. Anesthesia was applied until the end of the study or for ≤ 30 min.
2. The mice were placed on a plate in the supine position.
3. A light-absorbing laser target (0.5-mm thick natural black rubber disk) bonded with an optically transparent material (1.0-mm thick polyethylene terephthalate sheet) was placed on the surface of the skin of the frontal, upper dorsal neck, and right thoracic dorsum.
4. A Q-switched ruby laser (The Ruby nano Q, NIIC Co., Tokyo, Japan; 694 nm; pulse-width 20 ns) was used to deliver intense laser pulses, which were used to irradiate the laser target with the required amount of energy.
5. The laser pulse absorbed by the light-absorbing material induced a plasma, whose expansion generated shockwaves.

To establish a mouse model of severe tissue damage, the laser fluence of a LISW was set to 3.0 J/cm\(^2\).

**Physiological measurements.** Heart rate, mean blood pressure (MABP; MK-2000ST; Muromachi, Tokyo, Japan), peripheral oxyhaemoglobin saturation (SpO\(_2\); Mouse OX; STARR Life Science, Oakmont, PA), respiratory rate (Mouse OX; STARR Life Science, Oakmont, PA) and physiological parameters were noninvasively monitored before and after the application of LISW.

**Pathological assessment.** After the application of LISW, the mice were deeply anesthetized with an intramuscular injection of 100 mg/kg ketamine and 10 mg/kg xylazine. The brain was removed after decapitation, fixed in 4% paraformaldehyde for 24 h, and then embedded in paraffin. Sagittal sections (4-μm thick) were cut from the embedded brain and the lung coronal tissues and stained with haematoxylin–eosin (HE). To investigate the cause of lethality in the Upper neck group, Bodian staining was performed on the mice brains of this group as well as those of intact mice for pathological analysis, and the sections were observed using the BZ-X700 light microscope (Keyence Corporation, Osaka, Japan) under 2×, 10× and 100× magnification.
Statistical analysis. Data are expressed as the mean ± standard deviation (± SD). Vital signs were analyzed using one-way factorial analysis of variance with post-hoc comparisons and Tukey's test. The survival rates were analyzed using Pearson's chi-square test. Survival analysis data were compared using the Kaplan–Meier method and log-rank test, and \( p < 0.05 \) was set to indicate a significant difference. Statistical analysis was performed using Prism 8 (MDF Co., Kameido, Tokyo, Japan).

Results

Survival analysis. The survival rates of the Frontal, Upper neck, and Thoracic groups, were 80, 40 and 100%, respectively (Table 1). In the Frontal group, all deceased mice experienced respiratory arrest and loss of pulse within 3–4 min. In the Upper neck group, all deceased mice experienced respiratory arrest and loss of pulse within 1 min. With regard to survival time analysis, the upper neck group showed a significant decrease compared to the other two groups (Fig. 2).

Vital signs. Mice for which vital signs were measurable are presented in Table 2. In the group of mice in which LISW was applied to the upper neck region (Upper neck group), the SpO_2 significantly decreased for < 1 min, increased within 3 min and returned to pre-LISW levels (Table 2). All deceased mice in the Upper neck group developed apnea immediately after LISW application and exhibited marked decreases in SpO_2, heart rate, and blood pressure, with no subsequent improvement. In the group of mice in which LISW was applied to the frontal region (Frontal group), the SpO_2 was unchanged. In the group subjected to LISW to the right-back of the chest region (Thoracic group), the SpO_2 decreased to some extent and did not return to pre-LISW values.

Organ injury post-LISW treatment. In mice subjected to 3.0 J/cm² laser fluence, we observed hemorrhage on the surface of the cerebral hemisphere in the Frontal group. A similar hemorrhage was observed on the surface of the cerebellar hemisphere in the Upper neck group, and right-sided lung hemorrhage was observed in the Thoracic group (Fig. 2). No gross bleeding was observed other than at the site of application. There were no fracture lines in the skulls of all injured mice.

Pathological characteristics. No obvious changes were observed in the HE-stained brain specimens after the application of LISW in all the groups as well as at the site of injury, especially in the Frontal and Upper neck groups (Fig. 3). In the Bodian-stained mice brains of the Upper neck group, undulated axons and axonal varicosities, which are normal features of brain injury in the acute phase, were confined to the medulla oblongata (Fig. 4). The Bodian-stained brain specimen of an uninjured mouse exhibited no obvious axonal changes in the medulla oblongata. Regarding the pathological findings of the lungs, the Thoracic group exhibited alveolar hemorrhage associated with alveolar wall elongation and capillary destruction, whereas no obvious changes were noted in the other groups (Fig. 3).

Table 1. Comparison of post-LISW survival rates. Survival rates: Frontal group, 80%; Upper neck group, 40%; Thoracic group, 100%.

| Number of mice | Frontal | Upper neck | Thoracic |
|----------------|---------|------------|----------|
| Survival       | 8       | 4          | 10       |
| Mortality      | 2       | 6          | 0        |
| Total          | 10      | 10         | 10       |

Figure 2. Site-specific characteristics after LISW application. (a) Survival of the three groups: Frontal group: All mice died within 3–4 min; Upper neck group: All mice died within 1 min; Thoracic group: No deaths. *\( p < 0.05 \) (log-rank test). (b, c) Extent of macroscopic damage after application of the LISW. (b) Frontal group: Hemorrhage on the surface of the cerebral hemisphere. (c) Upper neck group: Hemorrhage on the surface of the cerebellar hemisphere. (d) Thoracic group: Hemorrhage in the right lung.
Table 2. Comparison of post-LISW vital signs. n: number of animals excluding dead animals in each group. *p < 0.01, vs. Frontal and Thoracic groups. *p < 0.05, vs. Frontal and Thoracic groups. #: Immediately before LISW application.

| Minute | Peripheral oxyhemoglobin saturation (%) | Pulse rate (beats per minute) | Mean blood pressure (mmHg) | Respiratory rate (breathes per minute) |
|--------|----------------------------------------|-------------------------------|---------------------------|---------------------------------------|
|        | Frontal (n) | Upper neck (n) | Thoracic (n) | Frontal (n) | Upper neck (n) | Thoracic (n) | Frontal (n) | Upper neck (n) | Thoracic (n) |
| #      | 97.1 ± 1.4 (10) | 97.1 ± 0.8 (10) | 96.4 ± 1.4 (10) | 389.9 ± 66.7 (10) | 413.4 ± 66.1 (10) | 355.8 ± 63.6 (10) | 91.5 ± 17.2 (10) | 97.7 ± 27.6 (10) | 91.5 ± 18.5 (10) | 110.1 ± 30.3 (10) | 112.3 ± 26.4 (10) | 141.2 ± 43.7 (10) |
| 0.5    | 96.6 ± 1.8 (10) | 59.9 ± 18.8** (8) | 90.1 ± 7.41 (10) | 372.2 ± 74.8 (10) | 270.7 ± 57.4* (8) | 365.7 ± 61.8 (10) | 80.7 ± 27.2 (10) | 82.5 ± 10.9 (4) | 77.5 ± 20.0 (4) | 123.3 ± 51.6 (8) | 100.6 ± 26.7 (8) | 152.1 ± 51.7 (10) |
| 1      | 96.7 ± 2.7 (10) | 75.5 ± 23.7 (4) | 92.2 ± 6.1 (10) | 374.3 ± 58.8 (10) | 347.0 ± 82 (4) | 354.5 ± 66.4 (10) | 80.7 ± 27.2 (10) | 82.5 ± 10.9 (4) | 77.5 ± 20.0 (4) | 123.3 ± 51.6 (8) | 100.6 ± 26.7 (8) | 152.1 ± 51.7 (10) |
| 1.5    | 97.6 ± 0.9 (10) | 81.3 ± 27.8 (4) | 93.6 ± 4.9 (10) | 358.3 ± 65.5 (10) | 329.0 ± 67.9 (4) | 359.8 ± 73.2 (10) | 104.6 ± 62.0 (10) | 106.5 ± 60.7 (4) | 150.1 ± 52.5 (10) | 137.3 ± 88.0 (4) | 96.3 ± 67.1 (4) | 157.7 ± 58.4 (10) |
| 2      | 97.5 ± 1.3 (10) | 88.6 ± 15 (4) | 92.5 ± 6.7 (10) | 338.2 ± 78.8 (10) | 374.5 ± 119.0 (4) | 356.9 ± 76.0 (10) | 76.0 ± 21.2 (10) | 90.8 ± 47.7 (4) | 89.2 ± 36.5 (10) | 106.6 ± 42.9 (10) | 139.5 ± 60.4 (4) | 114.5 ± 49.5 (10) |
| 2.5    | 96.7 ± 3.7 (10) | 85.1 ± 20.1 (4) | 92.4 ± 6.4 (10) | 341.8 ± 89.9 (10) | 344.8 ± 69.9 (4) | 352.1 ± 74.1 (10) | 106.6 ± 42.9 (10) | 139.5 ± 60.4 (4) | 114.5 ± 49.5 (10) | 115.2 ± 35.2 (10) | 84.5 ± 42.4 (4) | 142.8 ± 51.2 (10) |
| 3      | 97.0 ± 2.4 (10) | 95.8 ± 2.1 (4) | 91.7 ± 7.5 (10) | 337.6 ± 84.1 (10) | 335.0 ± 51.7 (4) | 362.8 ± 83.3 (10) | 84.4 ± 18.3 (10) | 83.0 ± 28.8 (10) | 85.5 ± 23.2 (10) | 110.8 ± 35.3 (4) | 148.8 ± 88.4 (4) | 117.6 ± 36.6 (10) |
| 3.5    | 97.0 ± 1.8 (9) | 96.7 ± 0.7 (4) | 91.6 ± 7.5 (10) | 311.7 ± 49.6 (9) | 357.0 ± 65.0 (4) | 354.9 ± 81.3 (10) | 110.8 ± 35.3 (9) | 148.8 ± 88.4 (4) | 117.6 ± 36.6 (10) | 102.3 ± 34.7 (9) | 177.8 ± 133.0 (9) | 127.0 ± 44.3 (10) |
| 4      | 97.3 ± 1.0 (9) | 96.8 ± 0.6 (4) | 93.1 ± 5.6 (10) | 321.7 ± 60.9 (9) | 385.0 ± 119.6 (4) | 356.7 ± 77.6 (10) | 80.1 ± 23.1 (9) | 96.3 ± 7.9 (4) | 87.6 ± 13.5 (9) | 102.3 ± 34.7 (9) | 177.8 ± 133.0 (9) | 127.0 ± 44.3 (10) |

Figure 3. HE-stained brain and lung after application of the LISW. (a, d) HE-stained brain specimen after application of the LISW in the Frontal group. (b, e) HE-stained brain specimen after application of the LISW in the Upper neck group. (c) HE-stained brain specimen after application of the LISW in the Thoracic group. (a–e) No obvious pathological changes were observed. (f, i) HE-stained lung specimen after application of the LISW in the Thoracic group. There were alveolar hemorrhage associated with alveolar wall elongation and capillary destruction. (g) HE-stained lung specimen after application of the LISW in the Frontal group. (g, h) HE-stained lung specimen after application of the LISW in the Upper neck group. (g, h) No obvious pathological changes were observed. (a–i) scale 200 μm.
Discussion

Past evidence indicates that air embolism causes immediate or shortly delayed death in response to a blast injury. Thus, lung damage from an explosion likely causes an embolism. We first expected that a fatal injury would occur when LISW was applied to the lungs, not the head. However, all mice in the Thoracic group survived, although with decreased SpO2, in contrast with the mice that survived in the Upper neck group. In addition, the mortality rate was higher in the Upper neck group, suggesting that damage to the upper neck caused respiratory and cardiac arrests. In this study, we similarly demonstrated that respiratory arrest was caused by the disruption of a central nervous mechanism and that injury to this region, including the medulla oblongata, was most likely fatal.

Marmarou et al. reported that respiratory depression and subsequent hypotension are the main causes of death in a rat model of severe diffuse brain injury and that respiratory depression may be associated with the central vs the peripheral nervous system. Others reported that direct cranial severe blast injury causes immediate death, which may be explained by primary brainstem failure; however, a neuropathological analysis was not performed. Here we similarly show that respiratory arrest was caused by the disruption of a central nervous mechanism and that the injury to the region, including the medulla oblongata, was most likely fatal. Further, our results suggest that the damage to the upper neck region caused respiratory and cardiac arrest.

Models of severe brain injury that directly impacted the dura mater exhibited significant hypertension immediately after injury. However, models of severe, diffuse brain injury, such as the weight-drop model, caused hypotension immediately after the injury. Our present results demonstrate that blood pressure decreased after the application of the LISW to the head region. Cernak et al. found that, in a rat model of moderate diffuse brain injury, there was a transient increase in the blood pressure for 5 s after the injury, followed by a significant reduction 1 min after the injury.
Apnea, bradycardia and hypotension occur in thoracic blast injuries, and pulmonary C fibers may be involved\(^{40-42}\). Further, hypotension is caused by bradycardia but not by efferent stimulation of the vagal nerve\(^{20-22}\), which were not apparent here. Moreover, we expected that the vagal reflex may be induced by unilateral stimulation, such as the application of a right-dorsal LISW. In a rat model of blast-induced traumatic brain injury, bradycardia is observed immediately after injury because of the vagal reflex\(^{35}\). Here, we found that the heart rate decreased when LISW was applied to the frontal and upper neck areas. Thus, certain neural reflexes may have caused circulatory changes when the head was exposed to shock waves.

It has been reported that dysfunction associated with brainstem injury may present as abnormal brainstem reflexes, impaired consciousness, respiratory failure, and autonomic failure. Ataxic respiration (irregular pauses and periods of apnea) and central apnea have been observed, particularly in rostral–ventral medullary cord injuries, and they are associated with poor prognoses\(^{36}\). Cardiac arrhythmias also occur frequently following brainstem injuries and are associated with increased mortality\(^{27}\). A well-known brainstem reflex is the trigeminocardiac reflex (TCR), a unique brainstem reflex that manifests as a sudden decrease in heart rate and mean blood pressure, cardiac arrhythmia, cardiac arrest, apnea, and other typical hemodynamic perturbations, among other autonomic responses\(^{38}\). Hence, venous air embolism and TCR should be suspected when rapid bradycardia and hypotension are present with head effects\(^{37}\); however, a study of hemodynamic changes after massive pulmonary air embolism in dogs identified no prominent decrease in heart rate and blood pressure or cardiac arrest even after 5 min of model creation\(^{39}\). The present results suggest that apnea and the prominent decrease in heart rate and blood pressure were most likely caused by damage to the brainstem, including the medulla oblongata, causing a severe TCR.

Widespread axonal swelling and degeneration characterize traumatic brain injury\(^{31}\). Axonal undulations are the first morphological signs of axonal injury, followed by the formation of axonal varicosities\(^{32}\). The direct mechanical destruction of microtubules occurs at the axonal swelling and axonal varicosity sites, which is associated with a marked decrease in axonal transport. These comprise the typical features of acute axonal injury\(^{33}\).

Thus, the application of LISW to the upper neck may significantly affect only the region surrounding the medulla oblongata. Further, mice in the Upper neck group experienced respiratory impairment or death, suggesting that pathology was related to impaired axonal transport in the medulla oblongata.

The results of numerous attempts to examine organ-specific responses, as mentioned above, are inconsistent; for example, hypertension occurs in one experimental model and hypotension in another even when the same organ (the brain) is analyzed. This may be explained by the difficulty in specifically applying the shock wave to the chest so as not to affect the head. Thus, in the blast experiment, the application of the shockwaves to a single organ without affecting other organs is technically difficult\(^{34-36}\). Two important points should be considered regarding animal studies using rodents to elucidate the mechanisms of traumatic injury attributable to explosions. First, the effects of acceleration attributable to a blast wind, which is known as the tertiary mechanism, should be minimized because many traumatic injuries resulting from explosions, especially Traumatic brain injuries, are known to occur without this mechanism. Thus, it is important to examine the primary mechanism (the effects of the shock wave itself). Second, although actual traumatic injury from explosions is a systemic injury in general, it is often too complex to analyze the outcome of whole-body exposure. Thus, the effects of exposure on the brain and other tissues and organs need to be separated. Because LISW is not accompanied by wind (dynamic pressure) and its energy is spatially confined, both the effects of wind and exposure of other organs (the brain) is analyzed. This may be explained by the difficulty in specifically applying the shock wave to the chest so as not to affect the head. Thus, in the blast experiment, the application of the shockwaves to a single organ without affecting other organs is technically difficult\(^{34-36}\).

The difference in the effects of pressure wave reflection at the brain–skull boundary is crucial. The size of the mouse brain is roughly one-twentieth of that of the human brain\(^ {39}\). On the basis of the scaling law, we believed that the appropriate shock wave duration to be applied to the mouse brain would be one-twentieth of the aforementioned durations, ranging from 12.5 μs to several hundred microseconds\(^ {38}\). In this study, experiments were conducted according to the hypothesis that the force product is the most important parameter in determining damage\(^ {37}\).

We are currently studying the possibility of extending the time range, and we need to test the hypothesis with LISW using an optimal time range in the future.

In conclusion, damage to organs such as the brain stem, including the medulla oblongata and others located in the upper dorsal neck, may cause immediate death. Moreover, damage to the brainstem is likely fatal, suggesting that it is critically important to develop equipment that protects the brainstem.

**Data availability**

The data generated and analyzed in this study are available from the corresponding author upon reasonable request.

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Author contributions
All authors were involved in conceptualizing and designing the study. K.Y., N. K. performed research. K.Y. analyzed the data and prepared the manuscript. All authors have reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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