Protective impact of landiolol against acute lung injury following hemorrhagic shock and resuscitation in rats

RISA SAKAMOTO[1], HIROKO SHIMIZU[2], RYU NAKAMURA[2], YIFU LU[3], YAQIANG LI[1], EMIKO OMORI[1], TORU TAKAHASHI[4] and HIROSHI MORIMATSU[1]

[1]Department of Anesthesiology and Resuscitology, Okayama University, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Okayama 700-8558, Japan; [2]Department of Anesthesiology and Resuscitology, Okayama University Medical School, Okayama, Okayama 700-8558, Japan; [3]Department of Human Anatomy, Shantou University Medical College, Shantou, Guangdong 515041, P.R. China; [4]Department of Anesthesiology, Okayama Saidaiji Hospital, Okayama, Okayama 704-8194, Japan

Received February 28, 2025; Accepted September 23, 2025

DOI: 10.3892/mmr.2025.13732

Abstract. Hemorrhagic shock and resuscitation (HSR) induces pulmonary inflammation, leading to acute lung injury (ALI). Notably, blocking β1 receptors can lead to organ protection through anti-inflammatory and anti-apoptotic effects. Additionally, although the β1 receptor pathway is blocked by the β1 blocker, the β2 receptor pathway may be preserved and activate the 5' adenosine monophosphate-activated protein kinase (AMPK) pathway. The present study aimed to examine whether administration of the β1 blocker landiolol could achieve lung protection in a model of HSR-ALI, alongside improvements in inflammation and apoptosis. Male Sprague-Dawley rats underwent hemorrhage keeping their mean arterial pressure at 30 mmHg for 1 h. Resuscitation by reinfusion was initiated to restore blood pressure to pre-hemorrhage levels for >15 min, followed by a 45-min stabilization period to create the HSR-ALI model. Landiolol (100 mg/kg/min) or saline was continuously administered after resuscitation. The lung tissues, which were collected for assessing inflammation and apoptosis-related damage, underwent analyses, including histological examination, neutrophil count, assessment of lung wet/dry weight ratio, detection of the mRNA levels of tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS), identification of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells, and evaluation of caspase-3 expression. In addition, phosphorylated AMPKα (pAMPKα) expression was tested via western blotting. Compared with the HSR/saline group, the HSR/landiolol group demonstrated a reduction in lung tissue damage score, and significant reductions in neutrophil count, lung wet/dry weight ratio, lung TNF-α and iNOS mRNA levels, TUNEL-positive cells and cleaved caspase-3 expression. Furthermore, landiolol administration following HSR treatment increased pAMPKα expression. No significant hypotension occurred between the HSR/landiolol and HSR/saline groups; and blood loss did not differ significantly between the groups. In conclusion, landiolol administration after HSR reduced lung inflammation and apoptosis, suggesting a potential improvement in tissue damage. Furthermore, pAMPKα activation in the HSR/landiolol group may be the mechanism underlying the pulmonary protective effects of landiolol.

Correspondence to: Dr Risa Sakamoto, Department of Anesthesiology and Resuscitology, Okayama University, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Kita, Okayama, Okayama 700-8558, Japan
E-mail: p1ar5hk6@s.okayama-u.ac.jp

Abbreviations: ALI, acute lung injury; AMPK, 5' adenosine monophosphate-activated protein kinase; ANOVA, analysis of variance; iNOS, inducible nitric oxide synthase; H&E, hematoxylin and eosin; HSR, hemorrhagic shock and resuscitation; MAP, mean arterial blood pressure; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α; IL, interleukin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; FITC, fluorescein isothiocyanate; HMGB1, high mobility group box 1; LPS, lipopolysaccharide

Key words: HSR, lung injury, landiolol, β1 blocker, inflammation, apoptosis

Introduction

Fluid and blood transfusion-based resuscitation after hemorrhagic shock (HSR) can result in damage to multiple organs (1,2). Acute lung injury (ALI) is common and severe (3,4). However, the treatment options for managing lung injury are currently limited and primarily involve ventilation volume and positional changes (5,6). Thus, effective pharmacological treatments are limited (6-8). Our previous research demonstrated an increase in inflammatory cytokines and exacerbated apoptosis in the HSR-ALI model (9,10). In patients with HSR, tissue hypoxia induces an early inflammatory response characterized by the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α). This promotes neutrophil infiltration and vascular endothelial disruption. During reperfusion, the reintroduction of oxygen leads to the generation of reactive oxygen and nitrogen species (ROS/RNS), which further exacerbate inflammation and apoptosis (11). These findings suggest that

targeting inflammation, apoptosis, and ROS/RNS might offer potential therapeutic strategies for mitigating HSR-induced lung injury.

After hemorrhagic shock (HS), patients are exposed to a hyper-catecholaminergic state characterized by enhanced sympathetic nervous system activity (12). In particular, β1 adrenergic receptor activation leads to increased heart rate and myocardial contractility, which consequently elevates metabolic demand and causes an imbalance between oxygen supply and demand. This imbalance may exacerbate ischemia-reperfusion injury and trigger systemic inflammation (13). β1-adrenergic receptor stimulation has been reported to enhance the secretion of pro-inflammatory cytokines in human monocytes and induce apoptosis when stimulated by β1-selective agonists (14,15). Therefore, β1-blockade is considered a promising strategy for suppressing these detrimental responses. Notably, the β2 receptor pathway is preserved even with the use of β1 blockers. The β2 receptor pathway includes the 5' adenosine monophosphate-activated protein kinase (AMPK) pathway, which contributes to sustaining homeostasis; reducing oxidative stress, mitigating mitochondrial dysfunction, suppressing inflammatory responses, and activating autophagy to maintain normal bodily functions (13,16,17).

Landiolol hydrochloride, a highly selective β1 blocker, has been employed to manage tachycardia and reduce the risk of atrial fibrillation during the perioperative phase and cardiac decompensation. The β1/β2 receptor selectivity ratio of landiolol (255) is substantially higher than those of commonly used β-blockers such as esmolol (33), atenolol (4.3), metoprolol (2.3), and propranolol (0.68), as shown in Table I (18,19). It also exhibits approximately eightfold greater β1-selectivity than does esmolol, with minimal activity on β2 receptors (18). Owing to this pharmacological profile, landiolol was selected in this study to evaluate its effects on lung injury following HSR. Although primarily used as an anti-arrhythmic drug, recent studies have reported that β1 blockers offer organ protection, including lung protection (20-25). These findings have been primarily observed in sepsis and ischemia-reperfusion models. In the lipopolysaccharide (LPS) model, landiolol treatment has been shown to enhance serum levels of TNF-α, interleukin (IL)-6, and high mobility group box 1 (HMGB1), along with reducing TNF-α expression in the liver (20,24). Conversely, some studies have reported no alterations in serum levels of TNF-α, IL-1β, and IL-6, or in TNF-α and IL-6 expression in the lungs, resulting in inconsistent findings (21,25).

In this study, landiolol was administered following HSR. To our knowledge, no prior research has examined the effects of landiolol on inflammatory cytokines and lung apoptosis in the HSR-ALI model. The study aimed to explore the potential of landiolol treatment after HSR for protecting lung tissue by decreasing inflammatory responses and apoptosis.

Materials and methods

Animals. This research received approval from the Department of Animal Resources at the Advanced Science Research Center, Okayama University (OKU-2021247 on April 1, 2021 and OKU-2023436 on April 24, 2023; Okayama, Japan) and adhered to the Guidelines for the Care and Use of Laboratory Animals based on ARRIVE (26) and the 2020 AVMA euthanasia guidelines (27). Male Sprague-Dawley rats, weighing 350-430 g (Clea Japan, Inc., Tokyo, Japan), were kept in temperature-regulated rooms (25˚C) with a 12-h light/dark cycle and had unrestricted access to water and food prior to the experiments. A total of sixty-nine rats were used in this study. Five rats were assigned to a control group without undergoing any procedures. Since there was no statistically significant difference between the control and sham groups, data of the control group were excluded from statistical analysis. Twenty-four rats were excluded from the analysis. Of these twenty-four rats, nineteen were excluded due to technical issues encountered during the HS procedure. These issues included failure to monitor blood pressure due to thrombosis, inability to achieve the target hypotensive state due to insufficient bleeding, or catheter dislodgement that prevented continuation of the experiment. All of these complications occurred during the HS procedure, and animals that could not proceed with the study were promptly euthanized. The other five rats died naturally from worsening hypotension during the HS procedure, despite attempts at resuscitation. The remaining forty rats successfully completed the experimental protocol and their data were included in the final analysis. These were divided equally into two groups: twenty in the 3-h model (n=5 each, four groups) and twenty in the 24-h model (n=5 each, four groups). All animals that successfully underwent the HSR procedure survived. The sample size was decided based on previous studies (28,29).

All rats were numbered sequentially upon arrival and housed under identical conditions in uniformly sized cages placed in the same location. All experimental procedures were consistently performed under the same environmental conditions and followed a predetermined order. Group assignment and drug preparation were carried out by H. Shimizu. R. Sakamoto was responsible for performing the HSR and sham procedures. Both R. Sakamoto and H. Shimizu jointly conducted sampling and data analysis. Information on group assignments was disclosed to R. Sakamoto only after sample collection completion. The rats were euthanized for sampling under 2-3% isoflurane anesthesia by performing a laparotomy and exsanguination via the abdominal aorta.

All rats included were confirmed to be healthy and alive before starting the experiment. Isoflurane was used throughout all procedures to provide adequate sedation and analgesia. Humane endpoints were predefined based on criteria such as ≥20% body weight loss or a marked decrease in physical activity. However, none of the animals that successfully completed the HSR procedure met these exclusion criteria during the study course. No special housing conditions were required.

Drug preparation. Prior to administration, landiolol (Landiolol Hydrochloride, Ono Pharmaceutical Co., Ltd., Osaka, Japan) was dissolved in saline and tailored to the correct dosage (100 μg/kg/min per body weight in 2 ml of saline). The dosage of landiolol used in this study was determined with reference to previously published studies (20,21,24,25).

Hemorrhagic shock and resuscitation (HSR) protocol. During the experiments, the rats were anesthetized with isoflurane (0.8-2%) and underwent either sham or HSR

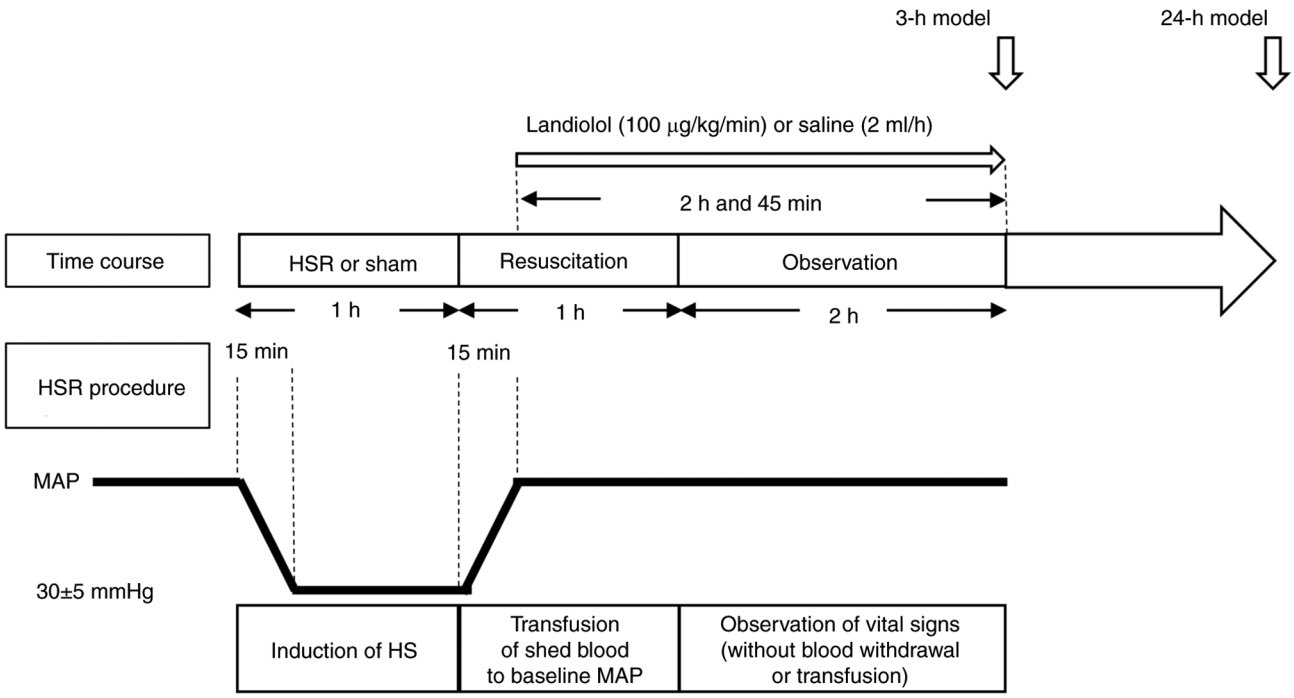

Figure 1. Experimental design. Establishment of the HSR rat model and the landiolol administration protocol. Animals were assigned to four groups (n=10 per group). Sham/saline group: administered saline after sham surgery; sham/landiolol group: administered landiolol after sham surgery; HSR/saline group: administered saline after HS procedure; HSR/landiolol group: administered landiolol after HS procedure. Rats were euthanized 3 or 24 h after the start of resuscitation; thereafter, samples were collected. Landiolol or saline was administered 15 min after the start of resuscitation and continued until 3 h after resuscitation. HS, hemorrhagic shock; HSR, hemorrhagic shock and resuscitation; MAP, mean arterial blood pressure.

surgery. Inhalational anesthesia was performed in a chamber containing isoflurane (4-5%) for induction. After confirming loss of both the righting reflex and the tail pinch reflex, anesthesia was maintained at a dosage of 0.8-2% via a face mask, in accordance with the approved animal experiment protocol (OKU2023436), with the most common concentrations being 5% for induction and 1.3 or 1% for maintenance, during which anesthetic depth was assessed by the absence of spontaneous movements. The vaporizer setting was reduced to below 1% only when marked bradycardia (approximately half of the normal heart rate) was observed. In the five rats that eventually died, this bradycardia was followed within several minutes by persistent hypotension, cardiac arrest and respiratory arrest. During these events, the isoflurane concentration was initially lowered and blood reinfusion was subsequently initiated in an attempt to stabilize the hemodynamic condition. Although euthanasia was also considered in these critical situations, it was difficult to perform because the progression from bradycardia to cardiac and respiratory arrest occurred within a very short period of time. The concentration of isoflurane was verified using a vaporizer. The HSR model was created as previously described (9,10,30,31). The left femoral artery and vein were dissected using aseptic procedures, and 22- and 24-gauge catheters were inserted, respectively. The left femoral artery catheter was used to measure blood pressure, whereas the left femoral vein catheter was used for inducing HSR. After measuring the baseline blood pressure, hemorrhage was initiated for over 15 min, aiming to maintain a mean arterial blood pressure of 30 mmHg, which was achieved by bleeding into a heparinized syringe (10 units/ml). The animals were maintained at this blood pressure (30±5 mmHg) for 45 min through additional blood withdrawal or shed blood infusion. Subsequently, resuscitation was performed for 15 min by administering all the shed blood until the pressure was restored to baseline levels. The rats were kept under anesthesia for 2 h, during which only the blood pressure and pulse rate were monitored (Fig. 1). The sham group underwent identical procedures, except for bleeding. All rats maintained spontaneous breathing throughout the experiment. All procedures were performed on a heating pad, with continuous monitoring and regulation to maintain the rectal temperature within the physiological range.

Table I. β1/β2 receptor selectivity ratios of commonly used β-blockers.

β-blocker	β1/β2 selectivity ratio
Landiolol	255
Esmolol	33
Atenolol	4.3
Metoprolol	2.3
Propranolol	0.68

Values are expressed as β1/β2 receptor selectivity ratios. Data adapted from references (18,19).

Experimental design. To examine the effects of landiolol administration on HSR-induced lung injury, the rats were randomly divided into the following four groups: sham/saline (n=10),

sham/landiolol (n=10), HSR/saline (n=10), and HSR/landiolol (n=10). Landiolol (100 μg/kg/min) or vehicle (saline 2 ml/h) was injected into the tail vein after HSR induction or sham surgery (Fig. 1). Continuous infusion was initiated 15 min after starting resuscitation and continued for 2 h and 45 min. The duration of administration was the same in both the 3 and 24-h models. At specific time points (3 h or 24 h) after resuscitation, the animals were euthanized by phlebotomy under isoflurane inhalation (2-3%). In our previous study, we found that TNF-α mRNA expression peaked at 3 h after resuscitation in the rat HSR-ALI model, supporting this time point for evaluating early inflammatory gene expression (31). Meanwhile, previous studies have shown that histological lung injury, lung wet/dry ratio, and apoptosis markers such as cleaved caspase-3 are most prominent at 24 h post-insult in related ischemia-reperfusion injury models (32,33). Therefore, the 24-h time point was selected for assessing lung tissue damage and apoptosis. The left lung was removed to determine the pulmonary wet-to-dry weight ratio. The right upper lung was excised, quickly and gently rinsed with saline, fixed in formalin, and stained for histological analysis. Regarding the preparation of RNA and proteins, the right middle and lower lungs were frozen immediately in liquid nitrogen and stored at -80˚C until further use.

Measurement of hemodynamic parameters (blood pressure and heart rate). To record the arterial pressure, an arterial catheter was connected to a pressure transducer (Meritrans DTSPlus® SCK-7874, Merit Medical Japan K.K, Shinjuku-ku, Tokyo, Japan) attached to a bridge amplifier (LifeScopeI® BSM-2303, Nihon Kohden Corporation, Shinjuku-ku, Tokyo, Japan). The blood pressure and heart rate were continuously recorded. The data were measured every 5 min and saved.

Histopathological examination. After 24 h of resuscitation, the rats were euthanized using the aforementioned method. Subsequently, the right upper lobe of the lungs was excised and fixed in 10% neutral buffered formalin, followed by paraffin embedding and sectioning at a thickness of 5 μm for histological examinations. Following deparaffinization and dehydration, the sections were stained with hematoxylin and eosin (H&E) staining. The lung histological changes were evaluated using blinded evaluation by five observers using a light microscope in accordance with previously described methods (34-36). In each rat, ten regions of lung parenchyma were assessed on a scale from 0 (normal) to 3 (severe) for four parameters: intravascular congestion, pulmonary edema, inflammatory cell infiltration, and intra-alveolar hemorrhage. The final results were reported as the median total score across these parameters, with a maximum score of 30 for each parameter and 120 for the combined lung injury score.

Neutrophils in the lungs were stained using a naphthol AS-D chloroacetate esterase staining kit (Sigma Diagnostics, St. Louis, MO, USA) on sections adjacent to those used for the histopathological analysis (30). An observer, blinded to the groups, counted the positively stained cells in five nonconsecutive sections per rat at x400 magnification.

Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein isothiocyanate nick-end labeling staining. Transferase-mediated dUTP-fluorescein isothiocyanate (FITC) nick-end labeling (TUNEL) staining was conducted using the MEBSTAIN Apoptosis TUNEL Kit Direct (No. 8445; MBL, Nagano, Japan) following the manufacturer's instructions. The sections were briefly incubated with terminal deoxynucleotidyl transferase and FITC-labeled dUTP, then counterstained with 0.5 μg/ml propidium iodide. TUNEL-positive cells were counted in five non-consecutive sections per rat at x400 magnification by a blinded observer using a Zeiss LSM510 confocal laser scanning microscope (Zeiss, Jena, Germany).

Lung wet weight to dry weight (wet/dry) ratio. The left lung tissue samples were collected 24 h after resuscitation, weighed for their wet weight, and then dried at 110˚C for 24 h to obtain the dry weight. The wet-to-dry weight ratio, calculated by dividing the wet weight by the dry weight, was used as an indicator of pulmonary edema (9,31,37).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was isolated from the lung tissues at 3 h after HSR using TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, OH, USA) following the manufacturer's instructions. The total RNA was purified using the RNeasy® Mini kit (Qiagen Sciences, Germantown, MD, USA). After removing potentially contaminating DNA with DNase I (RNase-Free DNase set; Qiagen GmbH, Hilden, Germany), reverse transcription of the total RNA was performed using a QuantiTect® Reverse Transcription Kit (Qiagen GmbH) to generate first-strand cDNA. The PCR mixture was prepared using the SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan). PCR was performed using StepOnePlus (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. The primer sequences for TNF-α, inducible nitric oxide synthase (iNOS), and β-actin were as follows: 5'-GCCCTG GTATGAGCCCATGTA-3' and 5'-CCTCACAGAGCAATG ACTCCAAAG-3' for TNF-α; 5'-CAAACTGTGTGCCTG GAGGTTC-3' and 5'-AAGTAGGTGAGGGCTTGCCTG A-3' for iNOS; and 5'-AACCCTAAGGCCAACCGTGAA-3' and 5'-CAGGGACAACACAGCCTGGA-3' for β-actin, respectively. PCR specificity was confirmed using melting curve analysis and DNA sequencing. Quantification of gene expression was performed using a standard curve method, as previously described (38,39). The mRNA levels of TNF-α and iNOS were normalized to the mRNA level of β-actin.

Protein extraction. The total proteins were extracted from a portion of the right lung lobe collected 24 h after establishing the HSR model, using Tissue Protein Extraction Reagent (T-PER) (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. Lung tissue was briefly homogenized in T-PER containing 5 mM dithiothreitol, 5 mM ethylenediaminetetraacetic acid, a protease inhibitor (cOmplete; Roche Diagnostics GmbH, Sigma-Aldrich, St. Louis, MO, USA), and phosphatase inhibitors (PhosSTOP; Roche Diagnostics GmbH, Sigma-Aldrich, St. Louis, MO, USA). The sample was centrifuged at 10,000 x g for 30 min at 4˚C, after which the supernatants were collected and stored for later analysis. Protein concentrations in the lung homogenates were

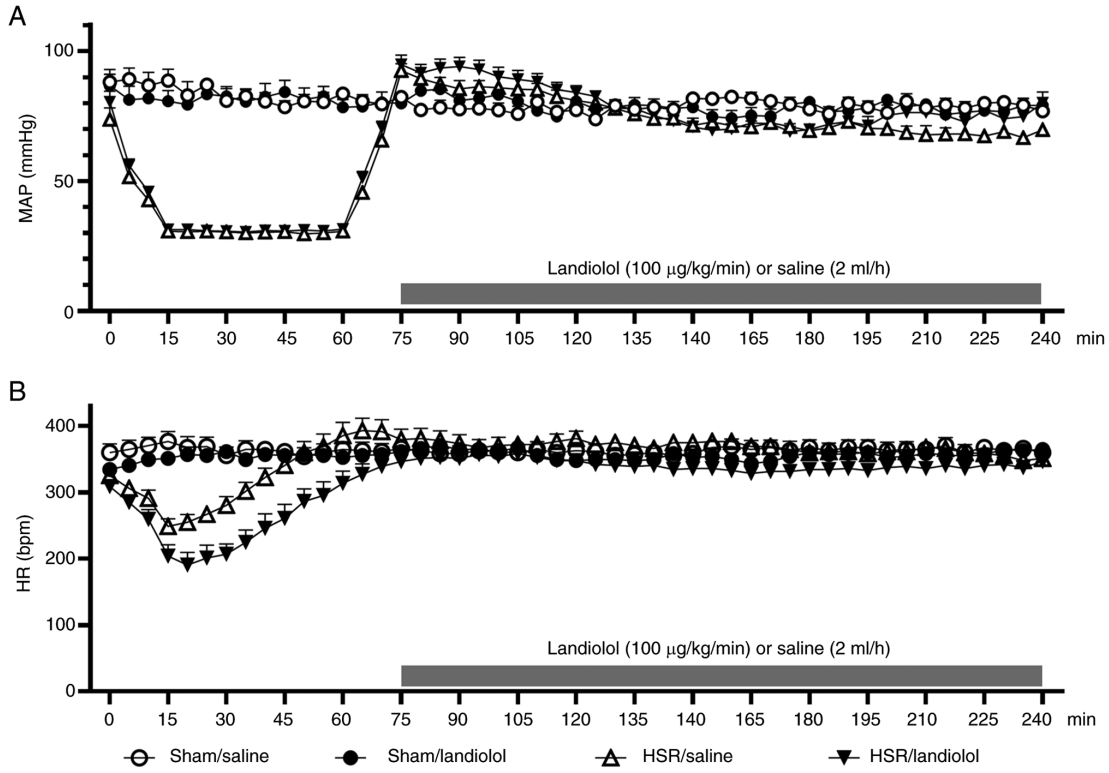

Figure 2. Landiolol administration did not affect vital signs during or after HSR. Landiolol or vehicle (normal saline) was administered through the tail vein immediately following resuscitation. The MAP and HR were recorded at 5-min intervals during and after the HSR procedure. (A) MAP measured at 5-min intervals throughout and following HSR. The x-axis represents the time elapsed since the onset of hemorrhagic shock while the y-axis shows the MAP values. The gray band at the bottom of the graph represents the duration of drug or vehicle administration (B) HR recorded at 5-min intervals during and after HSR. The x-axis shows the time from the start of hemorrhagic shock, and the y-axis displays the heart rate. The gray band at the bottom of the graph represents the duration of drug or vehicle administration. Data are presented as mean ± standard error of the mean (n=10 per group).HR, heart rate; HSR, hemorrhagic shock and resuscitation; MAP, mean arterial blood pressure.

measured using the Pierce BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions, with readings taken on a Nivo 5 Multimode Microplate Reader (PerkinElmer, Shelton, CT, USA).

Western blot analysis. Samples with approximately 50 μg of protein were loaded onto sodium dodecyl sulfate-polyacrylamide gels (10, 12, or 15% concentration) for electrophoresis, followed by the transfer of proteins onto Amersham Hybond-PVDF membranes (GE Healthcare Life Sciences, Chicago, IL, USA). The membranes were blocked at 25˚C for 1 h using 4% (w/v) BlockAce (DS Pharma Biomedical Co., Ltd., Osaka, Japan) or Blocking One-P (NACALAI TESQUE, Inc., Kyoto, Japan). Subsequently, the membranes were incubated overnight at 4˚C with primary antibodies: cleaved caspase-3 [rabbit anti-cleaved caspase-3 (Asp175), Cell Signaling, #9661, 1:500], Caspase-3 (rabbit anti-caspase-3, Cell Signaling, #9662, 1:1,000), GAPDH [rabbit anti-GAPDH (FL 335), Santa Cruz, sc-25778, 1:5,000], pAMPKα [rabbit anti-phosphorylated-AMPKα (Thr172) (40H9), Cell Signaling, #2535, 1:1,000], and AMPKα (rabbit anti-AMPKα, Cell Signaling, #2532 1:1,000). After washing in Tris-buffered saline with Tween 20, the membranes were incubated with secondary antibodies (goat anti-rabbit IgG-HRP, Abcam, ab6721, 1:20,000 and anti-rabbit IgG-HRP conjugate, Promega, W401B, 1:20,000) for 1 h at 25˚C. The membranes were then treated with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) following the manufacturer's guidelines. Imaging was done with the ChemiDoc XRS Plus System (Bio-Rad), with automatic exposure settings, and densitometry analysis was conducted using Image Lab Version 5.0 software (Bio-Rad).

Statistical analysis. The data are presented as the mean ± standard error of the mean (SEM) or as the median (interquartile range), as appropriate. Statistical analysis was performed using unpaired Student's t-test, one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons, or Kruskal-Wallis test followed by Dunn's post hoc test for non-parametric data, as appropriate. A two-sided P-value of <0.05 was considered statistically significant. All analyses were performed using GraphPad Prism 10 (GraphPad Software Inc., San Diego, CA, USA).

Results

Landiolol administration does not adversely affect the hemodynamic status after HSR. During the HS period, the mean arterial blood pressure in the HSR groups was maintained within the target range (30±5 mmHg) (Fig. 1). During the subsequent resuscitation period, the heart rate and mean blood pressure did not differ between the sham and HSR groups (Fig. 2A and B). However, during the observation period after resuscitation, the HSR/saline group exhibited a tendency

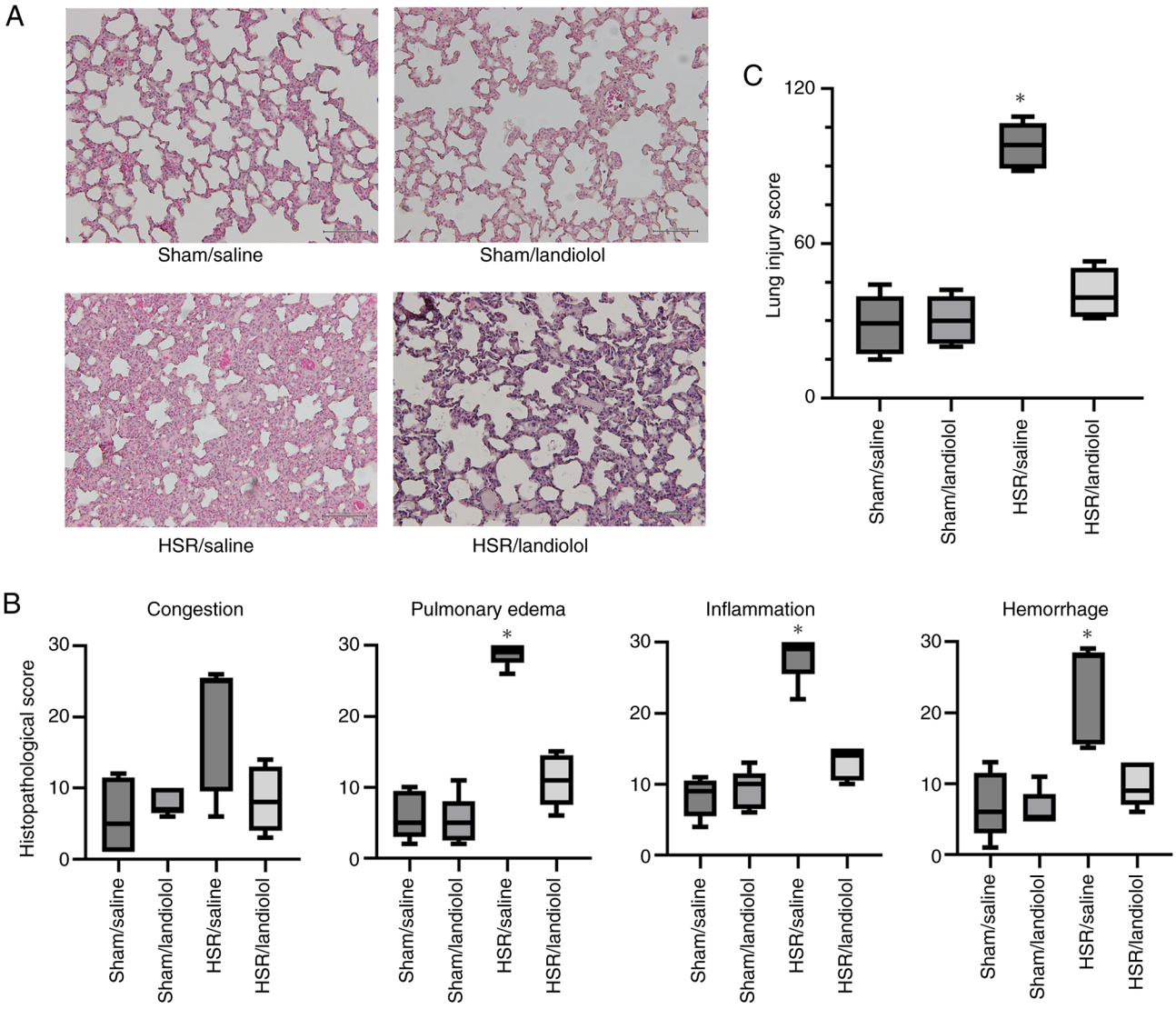

Figure 3. Histological assessment of lung injury following HSR. Lungs were collected from HSR model rats, treated with or without landiolol, 24 h after resuscitation for histological analysis. (A) Representative images from four independent experiments (H&E staining; original magnification, x200; scale bar, 100 μm). (B) The severity of histopathological alterations in the lungs was scored for congestion, edema, inflammation, and hemorrhage. Ten areas of lung parenchyma per rat were rated on a scale from 0 (normal) to 3 (severe) for each parameter, resulting in a maximum of 30 points per parameter. Scoring was performed independently by five blinded observers, and data are presented as median (interquartile range) and analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. (C) The total histopathological score was calculated by summing scores across the four parameters, with a maximum possible score of 120. Again, scoring was performed by five blinded observers, and the median values (interquartile range) were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. The sum score reflects the extent of lung tissue injury for each group. *P<0.05 vs. sham/saline. Sham/saline and sham/landiolol groups received sham surgery with saline or landiolol; HSR/saline and HSR/landiolol groups underwent HSR with saline or landiolol administration. HSR, hemorrhagic shock and resuscitation.

toward decreased mean blood pressure compared with that by the sham/saline group. In contrast, the HSR/landiolol group showed no significant difference or tendency toward lower blood pressure compared with that in the HSR/saline group. This observation suggests that the HSR procedure could cause hypotension after the procedure; however, landiolol administration did not induce harmful blood pressure reduction. Regarding the heart rate, no significant differences were observed among the four groups after landiolol administration. No significant difference in blood loss was observed between the HSR/saline and HSR/landiolol groups (Fig. S1).

Effect of landiolol on HSR-Induced lung histological damage. In this study, we examined the effect of landiolol treatment on lung injury induced by HSR following HS. Histopathological analysis showed that lung sections from the sham group appeared approximately normal. In contrast, the HSR/saline group displayed interstitial edema, marked by noticeable alveolar septal thickening and inflammatory cell infiltration, 24 h after HSR (Fig. 3A). Although there was no statistically significant difference between the HSR/saline and HSR/landiolol groups, the histopathological score in the HSR/landiolol group was reduced to a level comparable with that of the sham/saline group. Landiolol treatment after HSR tended to attenuate these pathological changes, such as edema, inflammation, and hemorrhage (Fig. 3A and B). The impact of landiolol was further validated through histopathological scoring by

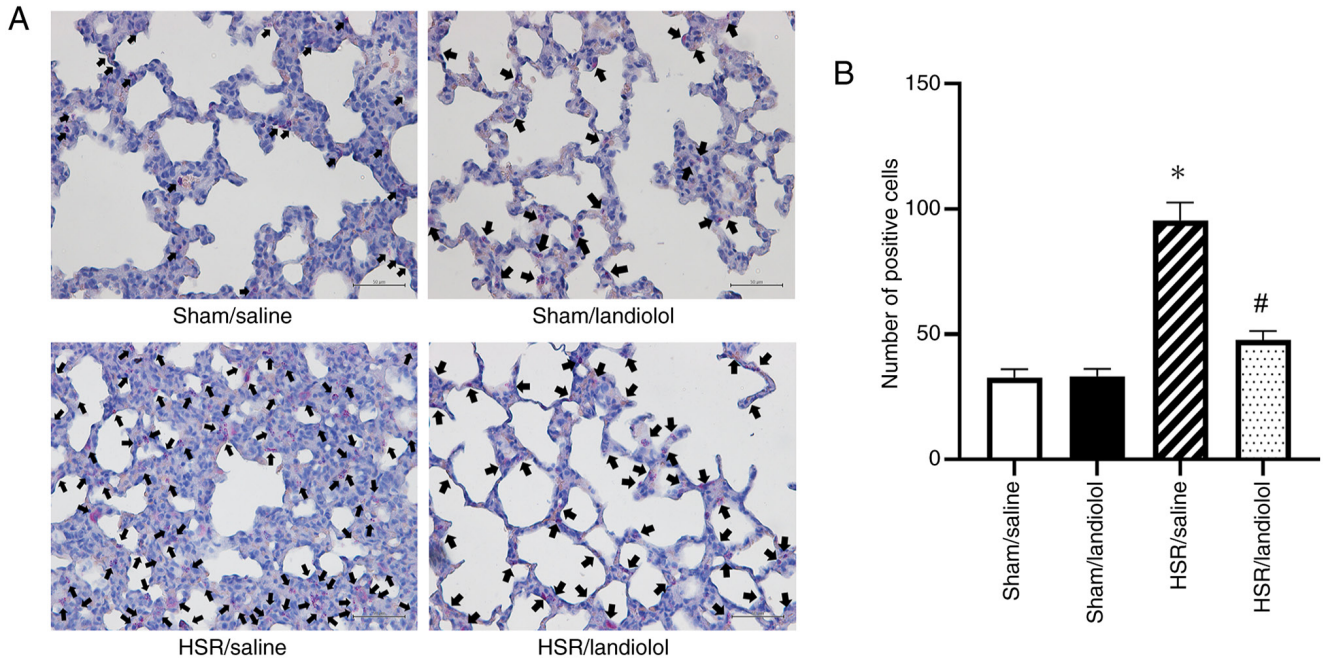

Figure 4. Neutrophil accumulation in the lungs following HSR. Lungs from HSR model rats treated with or without landiolol were collected 24 h post-resuscitation. Neutrophils were stained using a naphthol AS-D chloroacetate esterase assay. (A) Representative images from four independent experiments. Black arrows point to neutrophils stained positively (original magnification, x400; scale bar, 50 μm). (B) Neutrophil counts in five non-consecutive lung sections per rat at x400 magnification (n=5 per group). Data are shown as mean ± standard error of the mean and analyzed using analysis of variance followed by the Tukey-Kramer test. *P<0.0001 vs. sham/saline; #P<0.0001 vs. HSR/saline. Sham/saline and sham/landiolol groups received sham surgery with saline or landiolol; HSR/saline and HSR/landiolol groups underwent HSR with saline or landiolol administration. HSR, hemorrhagic shock and resuscitation.

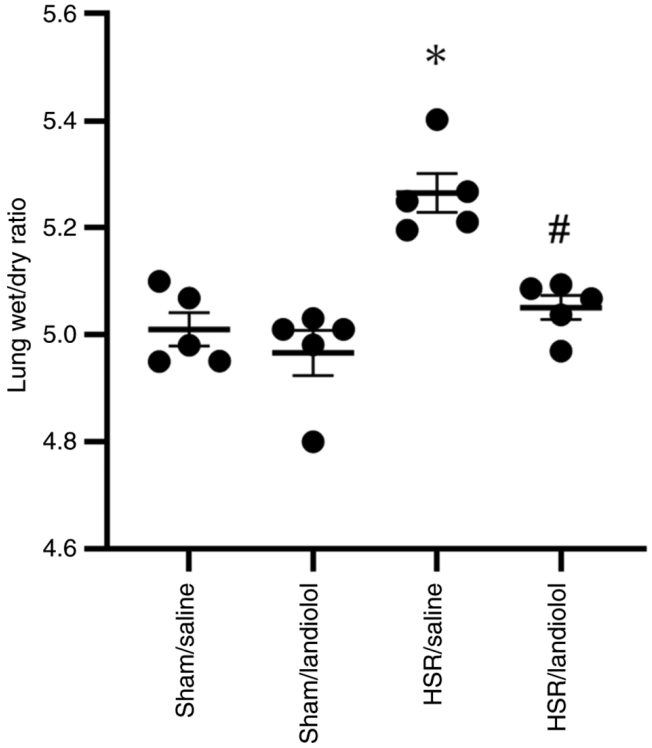

Figure 5. Lung wet/dry weight ratio following HSR. Lungs from HSR model rats, treated with or without landiolol, were collected 24 h post-resuscitation, and the wet/dry weight ratio was measured. Data are presented as mean ± standard error of the mean (n=5 per group). Statistical analysis was conducted using analysis of variance, followed by the Tukey-Kramer test. *P<0.0001 vs. sham/saline; #P<0.005 vs. HSR/saline. Sham/saline and sham/landiolol groups received sham surgery with saline or landiolol; HSR/saline and HSR/landiolol groups underwent HSR with saline or landiolol administration. HSR, hemorrhagic shock and resuscitation.

an independent, blinded researcher, showing a decrease in the histopathological score (Fig. 3C).

Effect of landiolol administration on neutrophil accumulation in HSR-induced lung injury. To provide additional visual and quantitative evidence of lung inflammation, neutrophils in the lung tissue were stained and counted (Fig. 4A). Neutrophil infiltration remained mild in the sham group, without notable difference between the sham/saline and sham/landiolol groups. However, in the HSR/saline group, the number of infiltrating neutrophils was significantly higher 24 h after HSR compared with the sham/saline and sham/landiolol groups. In contrast, neutrophil infiltration was substantially lower in the HSR/landiolol group than in the HSR/saline group (Fig. 4B). These neutrophil staining results indicated that landiolol administration alleviated tissue injury and decreased neutrophil infiltration.

Effect of landiolol on lung wet/dry weight ratio in HSR model rats. The lung wet/dry ratio, a parameter of lung edema, was assessed in the 24 h models. Compared with those in the sham groups, no differences were observed; the wet/dry ratio in the sham/saline group was 5.01±0.03 and 4.97±0.04 in the sham/landiolol group. The lung wet/dry ratio in the HSR/saline group significantly increased compared with that in the sham groups (5.27±0.04). However, landiolol administration significantly attenuated HSR-induced lung edema, and the wet/dry ratio of HSR/landiolol was statistically comparable to the sham groups (5.05±0.02) (Fig. 5). These results suggest that landiolol administration could reduce lung edema in HSR model rats.

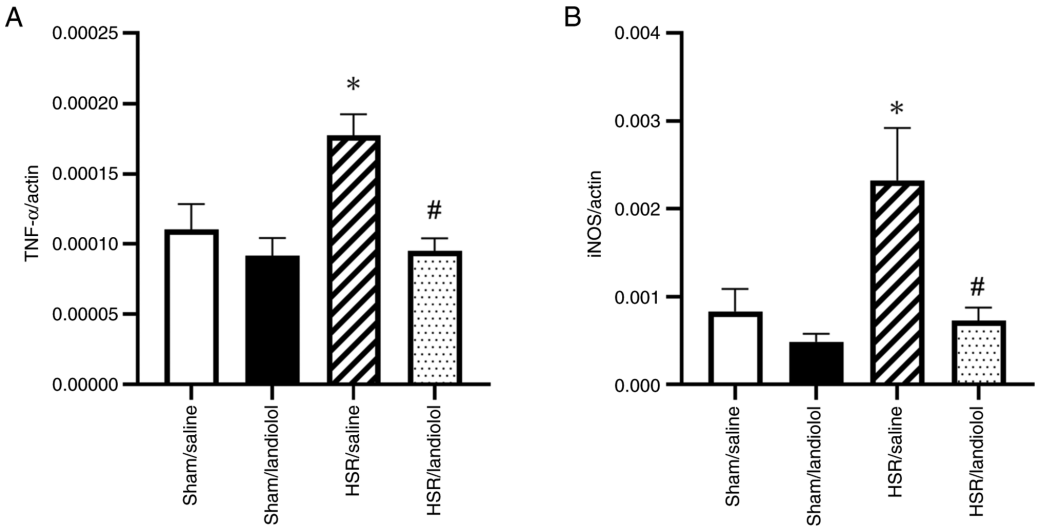

Figure 6. mRNA levels of inflammatory mediators in the lungs following HSR, measured by RT-qPCR. Lungs from HSR model rats treated with either saline or landiolol were collected 3 h post-resuscitation, and mRNA levels of (A) TNF-α and (B) iNOS were measured by RT-qPCR. Data are presented as mean ± standard error of the mean and analyzed using analysis of variance followed by the Tukey-Kramer test (n=5 per group). *P<0.05 vs. sham/saline; #P<0.05 vs. HSR/saline. Sham/saline and sham/landiolol groups received sham surgery with saline or landiolol; HSR/saline and HSR/landiolol groups underwent HSR with saline or landiolol administration. HSR, hemorrhagic shock and resuscitation; RT-qPCR,reverse transcription-quantitative polymerase chain reaction; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase.

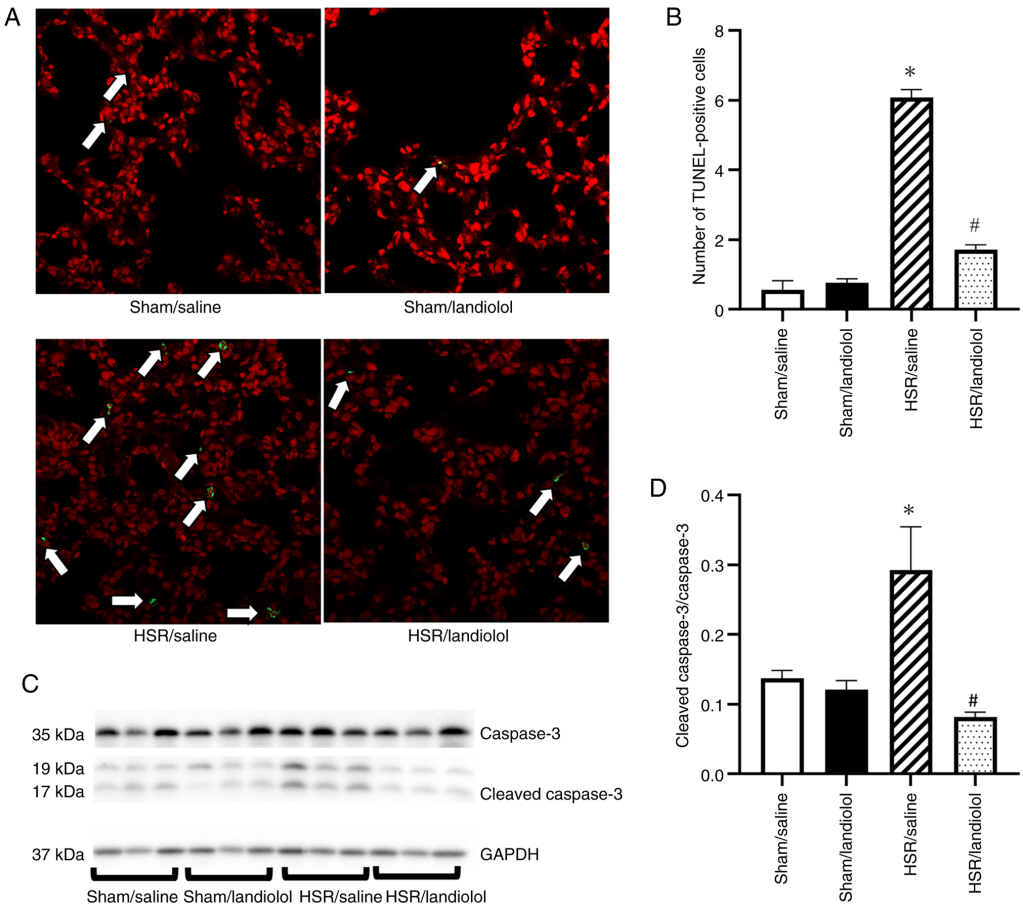

Figure 7. Apoptotic cell death in the lungs following HSR. Lung tissue was collected 24 h post-resuscitation and assessed for apoptosis using the TUNEL assay and cleaved caspase-3 protein expression via western blot. (A) Representative TUNEL-stained images of lung sections 24 h after HSR (original magnification, x400). White arrows point to TUNEL-positive cells. (B) Quantification of TUNEL-positive cells in lung sections at 24 h post-HSR. Data are presented as mean ± SEM (n=5 per group). (C) Western blot analysis for caspase-3 and cleaved caspase-3, with GAPDH as the loading control. (D) Densitometric analysis of cleaved caspase-3 expression, normalized to caspase-3 levels. Data are presented as mean ± SEM (n=3 per group). Data were analyzed with analysis of variance followed by the Tukey-Kramer test. *P<0.05 vs. sham/saline; #P<0.05 vs. HSR/saline. Sham/saline and sham/landiolol groups received sham surgery with saline or landiolol; HSR/saline and HSR/landiolol groups underwent HSR with saline or landiolol administration. HSR, hemorrhagic shock and resuscitation; SEM, standard error of the mean; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

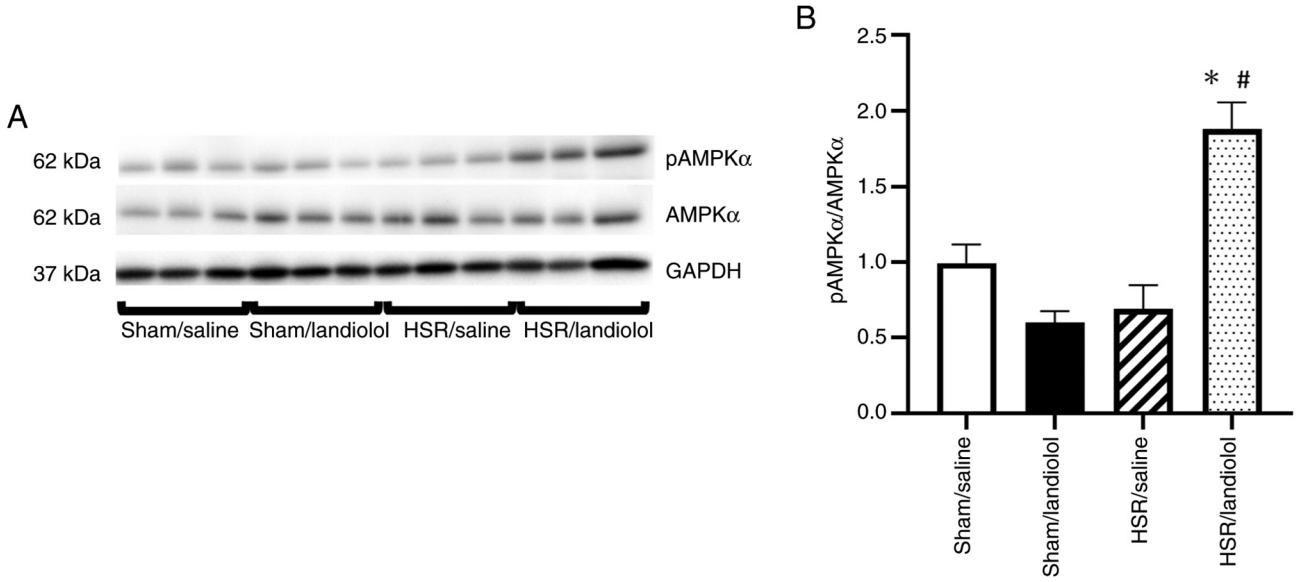

Figure 8. Expression of AMPK in the lungs following HSR. Lung samples were collected 24 h post-resuscitation and analyzed for AMPK expression by western blotting. (A) Western blot analysis using an antibody for pAMPKα and AMPKα with GAPDH as the loading control. (B) Densitometric analysis of pAMPKα levels, normalized to AMPKα. Data are shown as mean ± SEM and analyzed using analysis of variance followed by the Tukey-Kramer test (n=3 per group). *P<0.005 vs. sham/landiolol; #P<0.005 vs. HSR/saline. Sham/saline and sham/landiolol groups received sham surgery with saline or landiolol; HSR/saline and HSR/landiolol groups underwent HSR with saline or landiolol administration. AMPK, 5' adenosine monophosphate-activated protein kinase; HSR, hemorrhagic shock and resuscitation; pAMPK, phosphorylated AMPK.

Effect of landiolol on gene expressions of inflammatory mediators in HSR-induced lung injury. To assess the anti-inflammatory effects of landiolol, we analyzed the mRNA expression of TNF-α and iNOS in the lungs 3 h after HSR using RT-qPCR. There were no statistically significant differences in the levels of these inflammatory markers between the sham groups. However, the HSR/saline group demonstrated a significant increase in the TNF-α and iNOS expression compared with that in the sham group. In contrast, landiolol administration reduced the mRNA levels of TNF-α and iNOS by roughly 50 and 30%, respectively, bringing them close to the levels observed in the sham groups (Fig. 6A and B). These findings indicate that landiolol administration lowered inflammatory mRNA expression in the lungs of HSR model rats.

Effect of landiolol on apoptotic cell death in HSR-induced lung injury. To assess apoptosis, we analyzed the TUNEL-positive cells and cleaved caspase-3 expression in the lungs 24 h after HSR using western blotting. The lung sections from the sham group had very few TUNEL-positive cells, but their numbers increased following the HSR procedure. In contrast, landiolol treatment significantly reduced the number of TUNEL-positive cells compared with the HSR/saline group (Fig. 7A and B). Similarly, cleaved caspase-3 expression was minimally detectable in the sham group but increased in the HSR group. However, landiolol treatment effectively inhibited the increase in cleaved caspase-3 (Fig. 7C and D).

Effect of landiolol on the AMPK pathway in the lungs following HSR. We examined pAMPKα via western blotting. pAMPKα is a protein involved in mitochondrial biogenesis, autophagy, and related processes. Regarding pAMPKα, we found no significant differences between the sham/saline and sham/landiolol groups. The HSR/saline group also showed no differences compared with the sham group. However, landiolol treatment was found to increase pAMPKα expression compared with the other three groups (Fig. 8A and B).

Discussion

This study showed that intravenous administration of landiolol at a dose of 100 μg/kg/min following the HSR procedure substantially alleviated lung injury induced by HSR. This finding was supported by a trend toward reductions in histological alterations, and was further substantiated by significant reductions in neutrophil infiltration and lung edema. Furthermore, landiolol administration markedly lowered the mRNA expression of inflammatory mediators, including TNF-α and iNOS. Additionally, landiolol administration reduced apoptotic cell death, as shown by a decrease in the TUNEL-positive cells and cleaved caspase-3 expression. We investigated the pAMPKα expression as a mechanism for the anti-inflammatory and anti-apoptotic effects of landiolol and found by western blotting that pAMPKα expression was increased when landiolol was administered after HSR procedures. In addition, landiolol administration following the HSR procedure had no discernible effect on the mean arterial blood pressure.

TNF-α promotes neutrophil infiltration and increases vascular permeability, leading to endothelial injury. Neutrophils act on vascular endothelial cells and produce superoxides and other ROS, which cause cellular injury and edema (40). This endothelial disruption induces the expression of iNOS, which generates reactive nitrogen species such as nitric oxide, thereby exacerbating oxidative injury

during reperfusion (41). Furthermore, endothelial damage results in vascular leakage, which exacerbates tissue edema in the lungs (11). Targeting upstream inflammatory mediators such as TNF-α, iNOS-induced vascular injury, and neutrophil-mediated permeability enhancement may help attenuate lung damage. For this reason, these factors were selected as therapeutic targets in our study.

Landiolol is a drug used for treating arrhythmia; however, previous studies have shown that landiolol treatment has lung-protective effects. In a rat LPS model, Hagiwara *et al* reported that landiolol administration reduced HMGB1 expression in the lungs and serum and alleviated histological lung injury (20). Matsuishi *et al* reported that landiolol ameliorated histological findings and PaO_2 levels in a rat ALI model of early sepsis by suppressing elevated levels of pulmonary endothelin-1 (25). Notably, our research is the first to suggest that landiolol administration may play a protective role against ALI in a non-septic HSR model. Considering our findings and those of other studies (20,25), landiolol appears to be highly effective in protecting the lungs from ALI.

When investigating the mechanisms underlying the protective effects of landiolol on ALI following HSR, we observed that landiolol administration suppressed HSR-induced expression of inflammatory genes. This finding aligns with those of previous reports. Hagiwara *et al* showed that landiolol administration reduces TNF-α and IL-6 levels in the serum of the LPS rat model (20). Yoshino showed that landiolol administration to the LPS rat model improves TNF-α expression levels in the liver (24). Furthermore, Ackland *et al* reported that the β1 adrenergic receptor blocker metoprolol suppresses the expression of IFN-γ, IL-1β, IL-6, TNF-α, IL-18, and MCP-1 in the liver when administered to the LPS rat model (42). The findings of our study also indicated that landiolol administration decreased the expression of inflammatory genes in the HSR-induced ALI model. Thus, landiolol exhibits anti-inflammatory effects, representing a key mechanism in its protective action against lung injury.

Our study demonstrated that landiolol inhibited apoptosis in HSR-induced lung injury. Previous reports have also shown that β1-blockers may have anti-apoptotic effects. Zaugg *et al* reported that a β1 blocker atenolol suppressed TUNEL-positive cells and increased the expression of the anti-apoptotic protein Bcl2 in catecholamine-induced apoptosis (15). Hashemi *et al* reported that atenolol administration increases MEF2 transcriptional activity, which is involved in the anti-apoptotic effects of catecholamine-induced apoptosis in rat cardiomyocytes (43). Additionally, Taha *et al* reported that the administration of atenolol suppressed the gene expression of the pro-apoptosis protein caspase 1 and increases the expression of Bcl2 in a rat intestinal ischemia-reperfusion model (44). These reports and our experimental results suggest that β1-blocker has anti-apoptotic effects. Although the precise mechanism underlying the anti-apoptotic effects of landiolol could not be fully elucidated in this study, increased p-AMPKα expression observed in the HSR/Landiolol group suggests a potential role of AMPK pathway activation. AMPK is known to suppress oxidative stress, promote autophagy, and preserve mitochondrial function, all of which may contribute to the observed reduction in apoptosis (16,17). In addition, the AMPK/Nrf2 signaling pathway has been reported as a potential therapeutic target for treating ALI. Activation of this pathway may upregulate downstream effectors such as heme oxygenase-1 and modulate macrophage activity, thereby attenuating inflammation (45). Further investigation into the upstream and downstream signaling pathways of AMPK is warranted in future studies. Additionally, *in vitro* studies have reported that landiolol can scavenge multiple types of free radicals, which may also contribute to its anti-apoptotic properties (46).

Our study had some limitations. First, our model does not comply with the Berlin definition regarding the disuse of positive end-expiratory pressure and image evaluations, oxygenation index, pulmonary function indicator (47). However, conforming to this definition for animal experiments is challenging. Therefore, we performed the experiments according to the ALI definition for animal experiments (48). The severity of ALI for the animals was approximated using H&E staining, neutrophil staining, and the wet/dry ratio. Second, this HSR model is difficult to establish, with a mortality rate of approximately 11% during the HS procedure, and several animals were excluded due to complications. Third, we lacked survival rate data. Although the mortality during the HS procedure was relatively high, all rats that successfully underwent HSR treatment survived, indicating that this represents a mild lung injury model. Forth, the primary aim of this study was to assess the lung-protective effects of landiolol administration; however, the detailed mechanisms underlying these effects were not fully elucidated. Previous studies have demonstrated the organ-protective role of AMPK using Compound C, a specific AMPK inhibitor (49-51). In the future, we plan to investigate whether the AMPK pathway contributes to the protective effects observed in this study by conducting validation experiments using Compound C. This study was conducted using only male rats to ensure consistency with previous experiments (9,10,28-31). The potential influence of sex differences on the outcomes was not assessed and remains a subject for future investigation.

In conclusion, landiolol administration after HSR improved HSR-induced ALI through its anti-inflammatory and anti-apoptotic effects, at least in part, mediated by the activation of the AMPK pathway without severe hypotension. Further research is required to elucidate the exact mechanisms and pharmacological characteristics of landiolol. Thus, landiolol administration may be a therapeutic approach for acute lung injury following HSR.

Acknowledgements

The StepOnePlus and Zeiss LSM510 devices used in this study were obtained from the Central Research Laboratory, Okayama University Medical School (Okayama, Japan). The authors would also like to thank Mr. Kosuke Iguchi, Ms. Misako Yanagita and Ms. Shukuko Wani (medical students at Okayama University, Okayama, Japan) for their technical support.

Funding

This work was supported by a Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (grant nos. JP19K09381 and JP23K08360).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

RS, HS and TT designed the study. RS wrote the first draft of the manuscript. HS and HM critically revised the manuscript for intellectual content. RS, HS, YLu and EO performed the experiments. RS and HS confirm the authenticity of all the raw data. RS, HS, YLi, RN and EO performed histological scoring, which was conducted in a blinded manner. RS, HS, TT and HM analyzed and interpreted the data. RS and HS performed the statistical analyses. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experimental procedures in the present study were approved by the Department of Animal Resources, Advanced Science Research Center, Okayama University (approval no. OKU-2021247 on April 1, 2021 and approval no. OKU-2023436 on April 24, 2023).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, AI tools (ChatGPT and Consensus) were used to improve the readability and language of the manuscript, and subsequently, the authors revised and edited the content produced by the AI tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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