

Diaphragmatic Function Is Preserved during Severe Hemorrhagic Shock in the Rat

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ABSTRACT

Background: Acute diaphragmatic dysfunction has been reported in septic and cardiogenic shock, but few data are available concerning the effect of hemorrhagic shock on diaphragmatic function. The authors examined the impact of a hemorrhagic shock on the diaphragm.

Methods: Four parallel groups of adult rats were submitted to hemorrhagic shock induced by controlled exsanguination targeting a mean arterial blood pressure of 30 mmHg for 1 h, followed by a 1-h fluid resuscitation with either saline or shed blood targeting a mean arterial blood pressure of 80 mmHg. Diaphragm and soleus strip contractility was measured *in vitro*. Blood flow in the muscle microcirculation was measured *in vivo* using a Laser Doppler technique. Muscle proinflammatory cytokine concentrations were also measured.

Results: Hemorrhagic shock was characterized by a decrease in mean arterial blood pressure to 34 ± 5 mmHg ($-77 \pm 4\%$; $P < 0.05$) and high plasma lactate levels (7.6 ± 0.9 mM; $P < 0.05$). Although tetanic tension of the diaphragm was not altered, hemorrhagic shock induced dramatic impairment of tetanic tension of the soleus ($-40 \pm 19\%$; $P < 0.01$), whereas proinflammatory cytokine levels were low and not different between the two muscles. Resuscitation with either blood or saline did not further modify either diaphragm or soleus performance and proinflammatory cytokine levels. The shock-induced decrease in blood flow was much more pronounced in the soleus than in the diaphragm ($-75 \pm 13\%$ vs. $-17 \pm 10\%$; $P = 0.02$), and a significant interaction was observed between shock and muscle ($P < 0.001$).

Conclusion: Diaphragm performance is preserved during hemorrhagic shock, whereas soleus performance is impaired, with no further impact of either blood or saline fluid resuscitation. (ANESTHESIOLOGY 2014; 120:425-35)

ACUTE hemorrhage is a leading cause of death, which encountered in various clinical conditions including trauma, surgery, postpartum hemorrhage, gastrointestinal tract bleeding, and complications of anticoagulants.¹⁻⁵ Even if the patient survives in the initial phase, hemorrhagic shock and massive blood transfusion may induce multiple organ failure leading to delayed mortality.^{6,7}

In hemorrhagic shock, decreased hemoglobin and circulating blood volume lead to decreased arterial oxygen transport and tissue ischemia, which in turn causes massive release of oxygen free radicals and proinflammatory cytokine production.⁸ This inflammatory response may lead to multiple organ failure, including respiratory failure.^{9,10} Among the causes of shock-induced acute respiratory failure, acute lung injury, corresponding to failure of the gas exchanger, has been extensively studied in hemorrhagic shock.¹¹ However, little is known about diaphragmatic function in hemorrhagic shock and its potential role in ventilatory pump failure and consequently respiratory failure.

What We Already Know about This Topic

- Acute diaphragmatic dysfunction can occur in septic and cardiogenic shock, but its occurrence in the more common hemorrhagic shock has not been investigated

What This Article Tells Us That Is New

- In rats undergoing hemorrhage to a mean arterial pressure of 34 mmHg for 1 h followed by resuscitation, there was an acute dysfunction of the soleus, but not diaphragm muscles, accompanied by a greater reduction in blood flow to the soleus than to the diaphragm
- These results suggest that acute diaphragmatic dysfunction may be uncommon in hemorrhagic shock

Although diaphragmatic dysfunction is a well-studied phenomenon in septic and cardiogenic shock,^{12,13} few data are available concerning the effect of hemorrhagic shock and subsequent fluid resuscitation on diaphragmatic function.^{14,15} The mechanisms involved in sepsis-induced diaphragmatic dysfunction, namely oxidative stress and

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proinflammatory cytokine production,¹⁶ are the cornerstones of hemorrhagic shock-induced multiple organ failure. It is therefore reasonable to speculate that hemorrhagic shock may induce diaphragmatic dysfunction. However, the highest blood received by the diaphragm¹⁷ approaches that for the heart¹⁸ and is clearly greater than that observed for other skeletal muscles.¹⁹ Diaphragm perfusion may thus be preserved during hemorrhagic shock in the same way as cerebral and myocardial perfusion.^{20,21} This phenomenon would counteract the deleterious effect of the inflammatory reaction to shock on diaphragmatic function.

The primary objective of this animal study was to test the hypothesis that diaphragmatic function is preserved during hemorrhagic shock, with or without fluid resuscitation with either isotonic saline or blood. As several studies in other models of shock such as sepsis have suggested that the diaphragm is more vulnerable to shock than limb muscles,^{22,23} we also studied the soleus muscle, a peripheral skeletal muscle, as a control. In addition, because the severity of organ hypoperfusion is a major determinant of ischemia, we hypothesized that the impact of hemorrhagic shock on blood perfusion would be less pronounced in the diaphragm than in the soleus. Finally, the role of proinflammatory mediators in hemorrhagic shock was assessed by measuring proinflammatory cytokines in the diaphragm and soleus muscles.

Materials and Methods

All experiments were approved by the animal research committee of the University Pierre et Marie Curie-Paris 6 (Paris, France). Care and handling of animals were in accordance with the guidelines for Institutional and Animal Care and Use Committees. Experiments were conducted in an authorized laboratory under the supervision of authorized researchers.

Animal Preparation

This randomized, controlled *in vitro* study was performed on 15-week-old adult male Wistar rats (Janvier, Le Genest Saint-Isle, France). Rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally) and tracheotomized. During the entire experiment, animals were ventilated with a volume-driven small animal ventilator (Model 665A; Harvard Apparatus, Holliston, MA). The tidal volume was set at 0.5 ml/100 g body weight, and respiratory rate was set at 55–60 breaths/min. Breathing air was humidified and enriched with oxygen. Ventilator settings and oxygen flow were adjusted to maintain arterial partial pressure of carbon dioxide (P_{aCO_2}) between 35 and 40 mmHg and P_{aO_2} between 80 and 100 mmHg. Airway pressure recording was continuously monitored to ascertain complete relaxation of the diaphragm under mechanical ventilation (DP 15–32; Validyne, Northridge, CA). Additional anesthesia (pentobarbital, 5 mg) was performed if spontaneous breathing activity was detected. The right

carotid artery was cannulated and connected to a pressure transducer to monitor arterial blood pressure and heart rate (blood pressure transducer TD104A; Biopac Systems, Santa Barbara, CA). During the experiments, continuous infusion of heparin (10 units/h; Sanofi-Synthelabo, Paris, France) was administered *via* the carotid artery using a pump (Pilote A2; Fresenius Kabi, Bad Homburg, Germany). The tail vein was cannulated for continuous infusion of isotonic saline at a rate of 1 ml/h (Baxter, Deerfield, IL). Body temperature was maintained at 37°C throughout the experiment by external warming with a homeothermic blanket system (Harvard Apparatus).

Experimental Protocol

Animals were divided into four parallel groups (fig. 1): (1) a “control” group ($n = 8$), which was ventilated for 120 min with mean arterial blood pressure (MAP) monitoring but without bleeding; (2) a “shock” group ($n = 8$), in which the animals were bled *via* the right carotid artery catheter until a MAP of 30–40 mmHg was reached. This controlled hypotension was maintained for 60 min by reinfusing or withdrawing blood as required. This “shock” group was designed to assess the specific impact of shock and was only studied for 1 h, as the mortality of the animals increased considerably after the first hour; (3) a “shock-saline” group ($n = 8$), in which the 60-min controlled hypotension (30–40 mmHg) period was followed by resuscitation with 0.9% saline targeting a MAP of 80 mmHg for 60 min; (4) a “shock-blood” group ($n = 8$), in which the 60-min controlled hypotension (30–40 mmHg) was followed by resuscitation with shed blood and, if necessary, 0.9% saline to target a MAP of 80 mmHg for the next 60 min. It is of notice that, with respect to the high shock-induced mortality (37%), experimentations were performed on a total of 41 animals.

Blood samples were taken from the carotid artery at baseline (t_0), after hemorrhagic shock (t_{60}), and after resuscitation (t_{120}) and were used for immediate determination of arterial blood gases, plasma lactate, and hemoglobin levels (GEM Premier 3000 Critical Care Analyser; Instrumentation Laboratory, Saint-Mandé, France). A muscle strip from the ventral part of the costal diaphragm and a muscle strip from the soleus muscle were carefully dissected *in situ* to study contractile function. Part of the remaining muscles was frozen in liquid nitrogen to examine cytokine expression. Blood sample was also drawn and centrifuged, and plasma was frozen for further cytokine measurements.

Contractile Performance of Diaphragm and Soleus Muscles

Each muscle strip was rapidly mounted in a tissue chamber containing a Krebs–Henseleit solution: NaCl, 118 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4 , 1.1 mM; $NaHCO_3$, 24 mM; $CaCl_2$, 2.5 mM; and glucose, 4.5 mM. The solution was bubbled with a gas mixture of 95% O_2 –5% CO_2 and maintained at 27°C and pH 7.4 using a thermostatic water

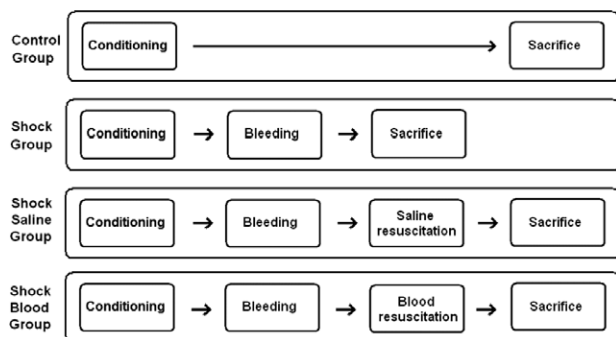


Fig. 1. Study design. Animals were divided into four groups: (1) a “control” group, which was ventilated for 120 min without bleeding; (2) a “shock” group, in which the animals were bled via the right carotid artery catheter until reaching a mean arterial blood pressure of 30–40 mmHg that was maintained for 60 min by reinfusing or withdrawing blood; (3) a “shock-saline” group, in which the 60-min controlled hypotension period was followed by resuscitation with 0.9% saline for 60 min; and (4) a “shock-blood” group, in which the 60-min controlled hypotension was followed by resuscitation with shed blood and, when necessary, 0.9% saline.

circulator. Muscle extremities were held in spring clips and attached to an electromagnetic force transducer, as previously described.²⁴

Diaphragm and soleus muscle strips were electrically stimulated by means of two platinum electrodes positioned parallel to the muscle and delivering electrical stimulation of 1 ms duration. After a 30-min stabilization period, muscle strips recovered their optimal mechanical performance. Mechanical variables were measured at the apex of the length-active isometric force curve (L_{max}). Maximum tetanic isometric tension was achieved at a stimulation frequency of 100 Hz with a train duration of 250 ms for the diaphragm and 500 ms for the soleus. At the end of the experiment, each muscle cross-sectional area (in millimeter square) was calculated from the ratio of muscle weight to muscle length at L_{max} , assuming a muscle density of 1.06. All analyses were performed from digital records of tension and length obtained with a computer. Conventional mechanical variables at L_{max} were calculated from three tetanic contractions. The first contraction was isotonic and was loaded with the preload corresponding to L_{max} . The second contraction was abruptly clamped to zero load just after the electrical stimulus with critical damping. The third contraction was fully isometric at L_{max} . We determined the maximum isometric total tension normalized per cross-sectional area and the peak of the positive tension derivatives ($+dF/dt$) of the isometric contraction and the maximum unloaded shortening velocity (V_{max}) using the zero-load technique.

Measurement of Muscle Microvascular Blood Flow

In a separate set of animals, diaphragm and soleus muscle microvascular blood flow was measured continuously using

a Laser Doppler Flowmeter probe and device (Periflux 5000; Perimed, Craponne, France) during shock. Recordings were performed continuously during the experiments using a data acquisition system (MP36R; Biopac Systems, Paris, France). Technically, laser light was guided from the source to the tissue by an optical fiber, and the back-scattered light was transmitted to a detector. The detected light was processed and converted to a continuous voltage output, from which the volumetric flow of erythrocytes was computed and converted to perfusion units.

The diaphragm was exposed *via* a midline laparotomy, and the Laser Doppler probe was applied perpendicularly against the dorsal part of the diaphragm, along the falciform ligament. The soleus was exposed after careful dissection of the rat leg. The probe was then applied perpendicularly against the soleus muscle. The immobility of the probe in the diaphragm and soleus was ensured by the proximity between the various muscles and organs and was subsequently confirmed by the stability of the signal. Diaphragmatic signal analysis was restricted to the end-expiratory phase to avoid any influence of diaphragm motion on the recordings.

RNA Extraction and Real-time Quantitative Polymerase Chain Reaction for Messenger RNA Levels. Total RNA was extracted from diaphragm and soleus samples using TRIZOL reagent (Invitrogen, Carlsbad, CA). The isolated RNA was treated with DNase I (Ambion, Austin, TX). Quantification of nucleic acid samples was realized using a microvolume spectrophotometry method (Thermo Scientific NanoDrop Products, Wilmington, DE). A total of 2 μ g of RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Life Technologies, Saint Aubin, France) and random primers. Real-time polymerase chain reaction was performed by using Mx3005P quantitative polymerase chain reaction system (Agilent Technologies Inc., Santa Clara, CA). Specific primers were designed to detect the expression of rat genes. *RPLP0* gene coding for large ribosomal protein was selected as the housekeeping gene. Relative messenger RNA level quantifications of target genes were determined using the cycle threshold method, and the data were expressed as fold change compared with the control group.

Evaluation of Cytokine Expression Levels

Frozen diaphragm and soleus samples (100 mg) were homogenized with Triton-HEPES buffer [1% Triton X-100, 50 mM HEPES [pH 8]; 150 mM NaCl, 10% glycerol, 2 mM EDTA; 1.5 mM $MgCl_2$, 10 U/ml antiprotease cocktail (Sigma Aldrich, St. Louis, MO)]. Total protein was determined using a bicinchoninic acid (BCA) assay method (Bio-Rad Laboratories, Hercules, CA). Tumor necrosis factor- α , interleukin-1 α , and interleukin-6 levels in the diaphragm and soleus were determined by enzyme-linked immunosorbent assay kits (HS Quantikine; R&D Systems, Minneapolis, MN).

Statistical Analysis

Data are expressed as mean \pm SD and median [25–75 interquartile range] for nonnormally distributed variables (Kolmogorov–Smirnov test). The primary outcome was the *in vitro* total tension of the diaphragm and soleus. Between-group comparisons were performed with one-way ANOVA followed, when significant, by a *post hoc* Tukey test in normally distributed data and a Kruskal–Wallis test followed by a Dunn *post hoc* test in nonnormally distributed data. The impact of shock on muscle microvascular blood flow was evaluated using two-way ANOVA (shock effect and muscle-type effect), and the relationship between MAP and muscle microvascular blood flow was investigated in both muscles using logarithmic regression and Spearman correlation. Comparisons between diaphragm and soleus cytokines were performed using two-way ANOVA (muscle effect and group effect). All statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL). All *P* values were two-tailed, and a *P* value of less than 0.05 was considered significant.

Results

Fluid Balance, Arterial Pressure, and Blood Lactate

The mean volume of shed blood was 12 ± 3 ml and was not significantly different between groups ($P = 0.92$). The targeted controlled hypotension was reached for all animals. The amount of additional pentobarbital provided was 21 ± 3 mg and was not significantly different between groups.

As compared with baseline value, MAP decreased significantly in the “shock” ($-77 \pm 4\%$), “shock-saline” ($-73 \pm 8\%$), and “shock-blood” ($-77 \pm 3\%$; all $P < 0.05$)

groups, whereas no significant decrease was observed in the control group (fig. 2). By t_{60} the blood lactate level increased significantly in the “shock,” “shock-saline,” and “shock-blood” (all $P < 0.05$) groups, it but remained unchanged in the control group (fig. 3A). Hemorrhagic shock induced metabolic acidosis, as indicated by reductions in arterial pH and bicarbonate (table 1). Hemoglobin decreased significantly in the “shock,” “shock-saline,” and “shock-blood” groups (all $P < 0.05$), whereas it remained unchanged in the control group (fig. 3B).

In the “shock-saline” group, none of the animals reached the targeted resuscitation MAP despite infusion with 47 ± 9 ml of saline. By t_{120} MAP increased by $+70 \pm 24\%$ as compared with t_{60} ($P < 0.01$). Blood lactate level remained increased ($-15 \pm 38\%$ as compared with t_{60} , not significant), acidosis remained marked, whereas hemoglobin level further decreased ($-34 \pm 10\%$ as compared with t_{60} ; $P < 0.001$).

In the “shock-blood” group, all but one animal reached the targeted resuscitation MAP. Overall, infusion of 11 ± 3 ml of blood followed by 8 ± 7 ml of saline allowed an increase of MAP by $+182 \pm 39\%$ as compared with t_{60} ($P < 0.01$). Concomitantly, blood lactate level decreased by $-61 \pm 15\%$ ($P < 0.001$), and bicarbonate and hemoglobin levels increased ($10 \pm 31\%$; $P < 0.01$).

Diaphragm and Soleus Contractility

Shock was not associated with a significant change in total tension or any other variables of diaphragmatic contractility (table 2). Resuscitation with either saline or blood did not significantly modify diaphragmatic total tension ($-12 \pm 16\%$ and $+9 \pm 22\%$, respectively, not significant).

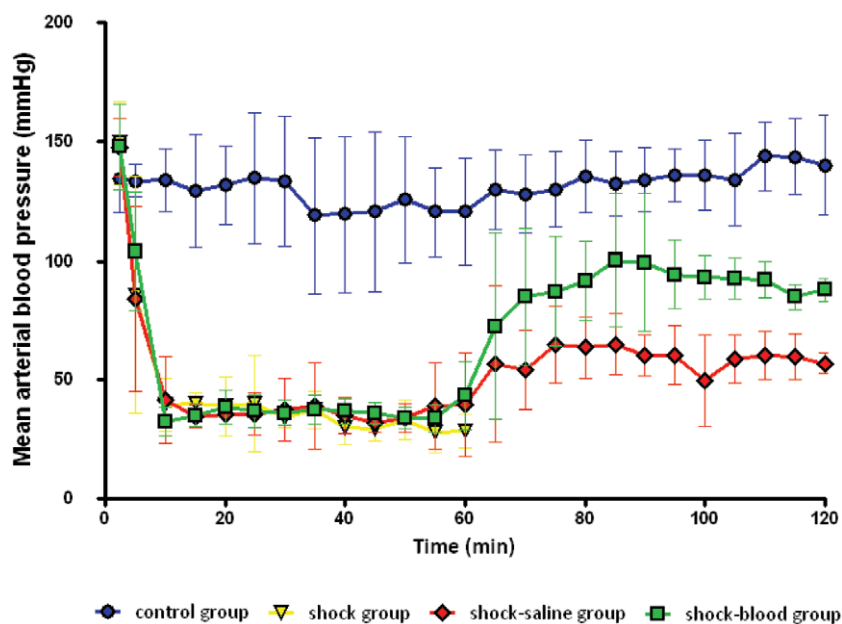


Fig. 2. Time course of mean arterial blood pressure in the four groups during the two different phases of the protocol ($n = 8$ in each group). Hemorrhagic shock from t_0 to t_{60} and fluid or blood resuscitation from t_{60} to t_{120} . Data are expressed as mean \pm SD.

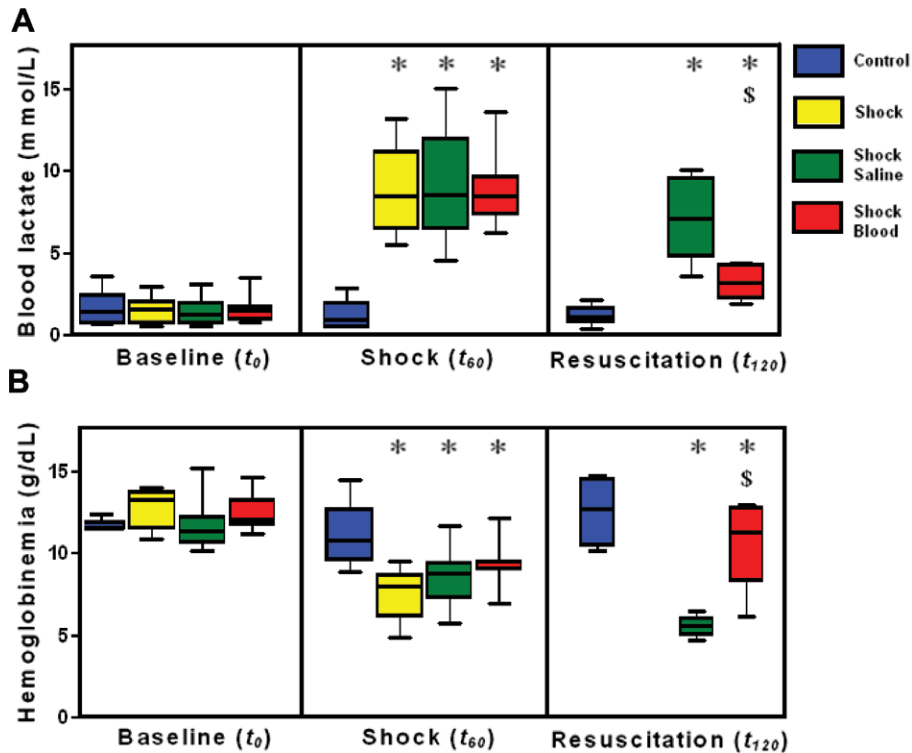


Fig. 3. Hemoglobin (A) and blood lactate (B) levels in the four groups at each time (i.e., t_0 , t_{60} , and t_{120}). Results are presented in the form of box plots. Boxes are drawn between the first and third quartiles of the distribution, black bars indicate the median, and whiskers indicate the minimum and maximum values. * $P < 0.05$ versus control group; \$ $P < 0.05$ versus shock-saline group.

Conversely, in the soleus, shock was followed by a marked decrease in both total tension ($-40 \pm 19\%$; $P < 0.01$) and $+dF/dt_{\max}$ ($-35 \pm 21\%$; $P < 0.01$) with no significant change in V_{\max} (table 2). No further significant change in contractile function of soleus muscle was noted after resuscitation with either saline or blood.

Diaphragm and Soleus Blood Flow

In both diaphragm and soleus, hemorrhagic shock lead to a decrease in muscle blood flow as compared with baseline (fig. 4). The correlation between MAP and muscle blood flow was significant in both diaphragm ($R^2 = 0.50$; $P < 0.001$) and soleus ($R^2 = 0.85$; $P < 0.001$). The relationship between MAP and diaphragmatic blood flow fitted with a logarithmic regression curve (fig. 5). Finally, the shock-induced decrease in blood flow was much more pronounced in the soleus than in the diaphragm ($-66 \pm 18\%$ vs. $-38 \pm 10\%$; $P < 0.001$), and a significant interaction was observed between shock and muscle ($F = 33$; $P < 0.001$).

Proinflammatory Cytokines

Proinflammatory cytokine gene expression and production in the diaphragm and soleus muscles are shown in figure 6 and table 3. Compared with the control group, hemorrhagic shock and resuscitation with either saline or blood did not induce any significant increase

in proinflammatory cytokine levels in the diaphragm and soleus.

Discussion

The major findings of this study can be summarized as follows: (1) hemorrhagic shock did not alter diaphragmatic contractility, whereas soleus contractility was severely impaired; (2) hemorrhagic shock was associated with a dramatic decrease in soleus perfusion, whereas the diaphragm perfusion was only slightly decreased; (3) resuscitation with either isotonic saline or blood did not further modify the function of either muscles; and (4) neither shock nor resuscitation was associated with upregulation of cytokine expression in the diaphragm and soleus.

A first and major finding of our study was that no significant diaphragmatic dysfunction was observed despite the severity of the hemorrhagic shock induced in our experimental model, as reflected by low MAP and increased blood lactate level.⁷ In patients with trauma, such shock would correspond to a loss of approximately 40% of the circulating blood volume resulting in stage IV hemorrhagic shock, the most severe form of the American College of Surgeons classification.^{25,26} Simultaneously, the performance of the soleus was severely impaired, which contrasts with previous observations in septic shock, in which the diaphragm was more vulnerable to shock than

Table 1. Arterial Blood Gases in the Four Groups at Each Time Point

	Control (n = 8)	Shock (n = 8)	Shock-saline (n = 8)	Shock-blood (n = 8)
Baseline (t_0)				
pH	7.34±0.13	7.37±0.06	7.39±0.07	7.36±0.07
PaO ₂ (mmHg)	92±37	108±36	103±26	120±57
Paco ₂ (mmHg)	37±15	37±11	39±10	42±11
HCO ₃ ⁻ (mM/l)	21±5	22±7	24±5	26±5
SaO ₂ (%)	96±5	97±3	96±3	96±7
Hemorrhagic shock (t_{60})				
pH	7.34±0.12	7.18±0.17	7.19±0.13	7.23±0.09
PaO ₂ (mmHg)	115±45	116±29	115±31	115±31
Paco ₂ (mmHg)	32±12	25±11	33±11	32±7
HCO ₃ ⁻ (mM/l)	19±6	10±5*	11±4*	13±3*
SaO ₂ (%)	96±2	97±3	96±2	97±2
Resuscitation (t_{120})				
pH	7.36±0.12	NA	7.04±0.19	7.23±0.12
PaO ₂ (mmHg)	85±16	NA	129±68	127±74
Paco ₂ (mmHg)	41±14	NA	34±14	38±8
HCO ₃ ⁻ (mM/l)	23±6	NA	9±3*	17±5*†
SaO ₂ (%)	95±3	NA	90±8	96±3

Data are mean ± SD.

* $P < 0.05$ vs. values of control group. † $P < 0.05$ vs. shock-saline group.

HCO₃⁻ = bicarbonates; NA = not available; PaCO₂ = arterial partial pressure of carbon dioxide; PaO₂ = arterial partial pressure of oxygen; SaO₂ = arterial oxygen saturation.

Table 2. Diaphragm and Soleus Contractile Properties in the Four Groups

Group	Control (n = 8)	Shock (n = 8)	Shock-saline (n = 8)	Shock-blood (n = 8)
Diaphragm				
Total tension, 100 Hz (mN/mm ²)	93±18	111±22	84±16	104±18
V _{max} (L _{max} /s)	5.0±0.9	4.6±0.5	4.9±1.1	4.2±2
+dF/dt _{max} (mN/s)	782±345	920±346	607±126	916±396
Soleus				
Total tension, 100 Hz (mN/mm)	98±16	60±17*	48±32*	49±22*
V _{max} (L _{max} /s)	3.2±0.6	3.1±0.6	2.3±1.1	2.4±0.6
+dF/dt _{max} (mN/s)	609±72	396±129*	316±267*	283±155*

Total tension is measured during a tetanic isometric contraction achieved at a stimulation frequency of 100 Hz. Data are mean ± SD.

* $P < 0.05$ vs. values of control group.

+dF/dt_{max} = positive peak of the tension derivative in the fully isometric contraction; V_{max} = maximal unloaded shortening velocity.

the soleus.^{16,22} To elucidate this difference and in view of contradictory reports on the impact of hypotension on diaphragmatic function,^{27,28} muscle microcirculatory blood flow was quantified using a Laser Doppler technique. This approach provides accurate determination of blood flow within capillaries, arterioles, and venules. During hemorrhagic shock, the reduction of microcirculatory blood flow was fourfold higher in the soleus than in the diaphragm, which is reminiscent of the classic notion that cardiac output is preferentially directed toward vital organs. Myocardial and cerebral perfusions are indeed maintained, whereas perfusion of the liver, intestines, kidneys, and muscles is significantly altered.^{20,29} In this respect, as the diaphragm constitutes a vital organ, diaphragmatic blood flow would be preserved.^{30,31} This phenomenon is not observed in other types of shock. In septic shock, the

capillary perfusion of the diaphragm is clearly impaired, and in cardiogenic shock, blood flow to the respiratory and limb muscles is equally reduced.^{17,21} It is noteworthy that our results reinforce the idea that, in these two types of shock (septic and cardiogenic), diaphragmatic dysfunction is not only the consequence of arterial hypotension but also involves many other mechanisms.

Another key finding of our study is that reperfusion with either saline or blood had no further impact on either diaphragmatic or soleus function. The fact that fluid resuscitation with saline failed to restore soleus muscle function was somewhat unexpected. As illustrated by our results, saline infusion was associated with significant hemodilution, which decreases the capacity of blood to carry oxygen.³² Blood lactate therefore remained high, suggesting persistent impairment of global perfusion. In addition, previous

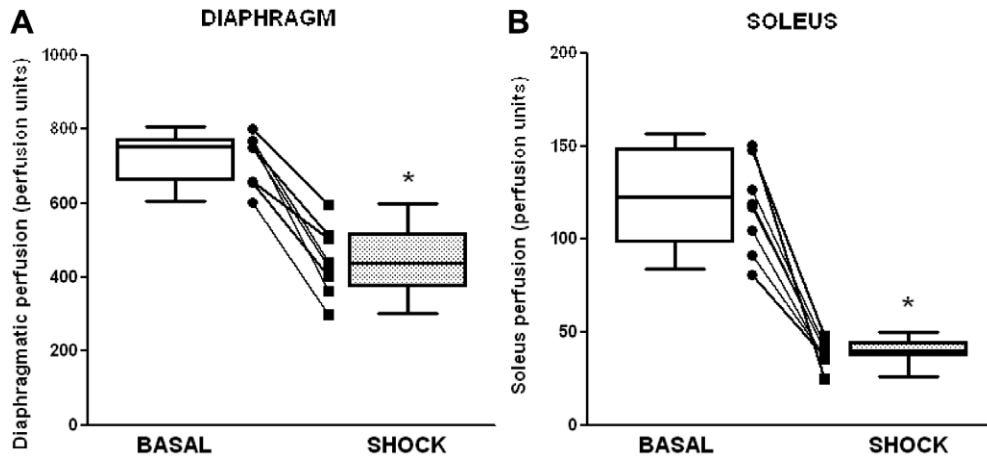


Fig. 4. Microvascular blood flow in the diaphragm (A) and soleus (B) at baseline (Base) and after hemorrhagic shock (Shock). Results are presented in the form of box plots as well as individual values. Boxes are drawn between the first and third quartiles of the distribution, black bars indicate the median, and whiskers indicate the minimum and maximum values. * $P < 0.05$ versus baseline (t_0).

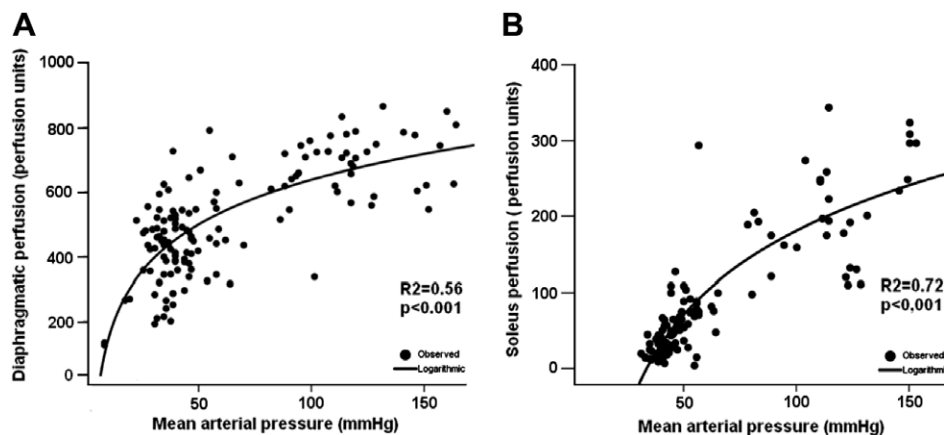


Fig. 5. Relationship between mean arterial blood pressure and microvascular blood flow in the diaphragm (A) and the soleus (B) during hemorrhagic shock. The experiment lasted 90 min: a 30-min control period was followed by a 60-min controlled hypotension (30–40 mmHg) period. Microvascular blood flow and mean arterial blood pressure were recorded every 5 min for a total of 18 points per muscle and animal ($n = 8$ animals). The dots indicate individual values and the full lines indicate the logarithmic regression curve fit.

authors have strongly suggested that hemodilution could increase shunting within organs.^{32,33} The “shock-saline” group failed to achieve the targeted MAP, and it could therefore be argued that this group was under-resuscitated. However, the fluid volume infused to animals was similar to that reported in previous studies³² and MAP actually reached 59 ± 10 mmHg. This is consistent with the target of limited resuscitation supposed to maintain a sufficient level of organ perfusion while avoiding the deleterious effects of more aggressive resuscitation, namely inflammation and organ failure.^{34,35}

Because blood transfusion both restores blood volume and maintains the oxygen-carrying capacity of circulating blood, we evaluated to what extent early blood transfusion could improve hemorrhage-induced soleus dysfunction.

Although blood transfusion improved global perfusion, as suggested by the decrease in blood lactate level, it did not improve soleus muscle performance. A possible explanation is that ischemia may have caused irreversible or slowly reversible damage to the soleus muscle. Another possible explanation is that blood transfusion may have caused muscle ischemia. Ischemic defects after blood transfusion have been described in various organs and might be related to reduced erythrocyte deformability.^{32,36–38} Third, resuscitation may have caused ischemia–reperfusion injuries to the soleus. Resuscitation is indeed a double-edged sword. Although reperfusion restores blood flow and subsequent oxygen transport to organs, it is also associated with massive release of proinflammatory mediators, including oxygen free radicals and proinflammatory mediators,³⁹ which

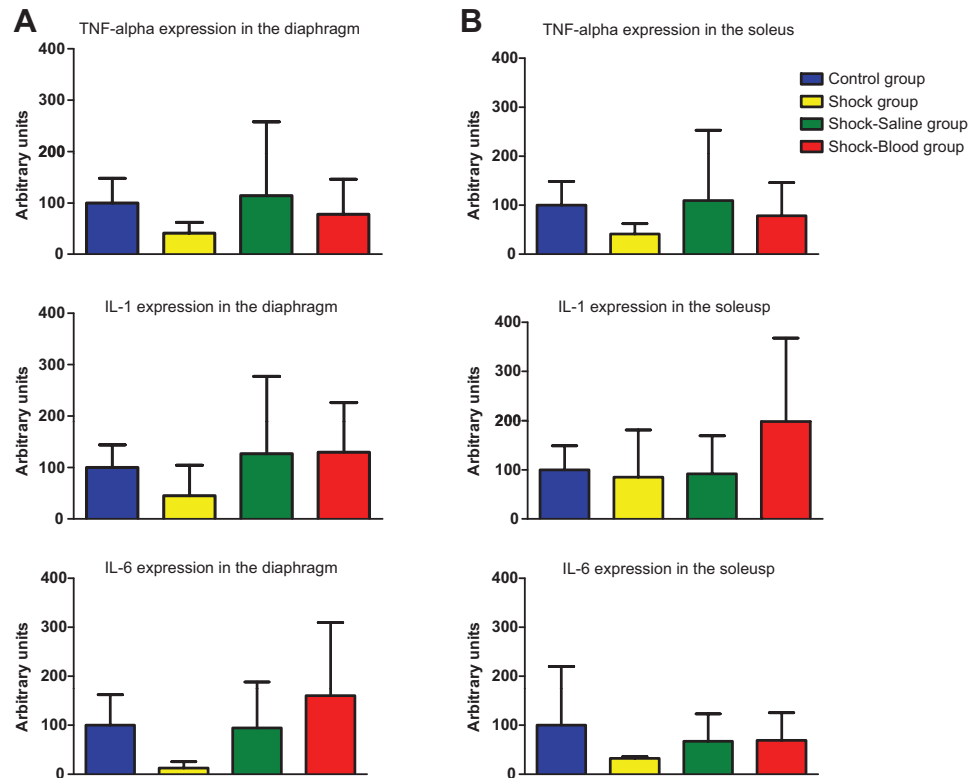


Fig. 6. Proinflammatory cytokine gene expression levels in the diaphragm (A) and in the soleus muscles (B) in the four groups. Data are normalized to the *RPLP0* gene coding for large ribosomal protein housekeeping gene and expressed as a fraction of the mean value (arbitrarily defined as 100) obtained in the control group. All data are group means \pm SD ($n = 6$ animals per group). No significant difference between groups. IL = interleukin; TNF = tumor necrosis factor.

Table 3. Cytokine Protein Expression Levels in the Diaphragm and in the Soleus Muscle

	Control (n = 6)	Shock (n = 6)	Shock-saline (n = 6)	Shock-blood (n = 6)
Diaphragm				
TNF- α (pg/ μ g)	87 [19–158]	110 [17–115]	68 [23–127]	143 [113–152]
IL-1 β (pg/ μ g)	29 [17–56]	28 [15–41]	71 [54–100]	150 [106–165]
IL-6 (pg/ μ g)	360 [191–852]	170 [105–215]	430 [308–1,169]	872 [642–1,435]
Soleus				
TNF- α (pg/ μ g)	149 [93–192]	145 [105–190]	148 [106–171]	148 [112–161]
IL-1 β (pg/ μ g)	11 [5–15]	4 [0–14]	0 [0–10]	3 [1–12]
IL-6 (pg/ μ g)	373 [215–546]	195 [11–240]	261 [56–301]	295 [124–460]

Data are median [25–75 interquartile range]. No significant difference between groups.

Control = mechanical ventilation without bleeding; IL = interleukin; Shock = hemorrhagic shock; Shock-blood = hemorrhagic shock followed by resuscitation with blood; Shock-saline = hemorrhagic shock followed by resuscitation with saline; TNF = tumor necrosis factor.

contribute to multiple organ failure.⁴⁰ In a model of lower torso ischemia–reperfusion, 30 min of infrarenal cross-clamping followed by 2 h of revascularization resulted in a significant diaphragmatic dysfunction.¹⁵ In models of hemorrhagic shock, myocardial dysfunction is observed after 2–6 h of resuscitation.^{41,42} Nonetheless, our model of hemorrhagic shock differs from the models used in these studies by two points: first, in all these studies, reperfusion lasted longer than in the current study; which is why we may have failed to observe reperfusion-induced

diaphragmatic dysfunction in our study; second, resuscitation was not associated with upregulation of proinflammatory cytokines in the diaphragm and soleus. Our results contrast with the vigorous inflammatory response observed during septic shock, in which inflammation parallels muscle dysfunction.^{16,22} Proinflammatory cytokine upregulation has also been described in the myocardium after hemorrhagic shock and has been considered to be part of the ischemia–reperfusion phenomenon.^{41,42} In the current case, tissue proinflammatory cytokine upregulation

was not observed in the muscles, which makes inflammation an unlikely cause for the severe contractile dysfunction observed in the soleus.

This study has several limitations. First, the anesthetic regimen used in this study may have affected the microcirculatory response to hypovolemia. However, the need to prevent any suffering in the animals made anesthesia mandatory and drugs that are known to alter muscle contractile properties, such as benzodiazepines were avoided.⁴³ Second, differences in the fiber-type composition may partly explain some of the differences observed after induction of hemorrhagic shock, as the diaphragm is more richly endowed in type II fibers than the soleus.⁴⁴ However, previous studies have suggested that the muscle response to ischemia and reperfusion primarily affects type II fibers,^{45,46} which represent about one half of diaphragmatic muscle fibers, but less than 20% of soleus fibers.⁴⁷ It is noteworthy that our results showed that diaphragmatic contractility was less severely altered than soleus contractility. Third, our hemorrhagic shock model may not have been sufficiently severe to generate diaphragmatic ischemia. However, the important volume of shed blood and high blood lactate levels reached strongly suggest that our model induced severe hemorrhagic shock with global ischemia. In preliminary studies, our attempts to design models either targeting lower MAP or lasting more than 1 h resulted in high animal mortality. Fourth, animals were only exposed to a 1-h shock and were followed for 1 h after resuscitation, which may not have been sufficiently long to induce muscle ischemia–reperfusion injury, as suggested by previous studies on myocardium.^{41,42} However, longer-lasting shock would expose animals to a risk of bacterial translocation from the intestine, which would introduce confounding factors. Fifth, animals were mechanically ventilated. Although it has recently been shown that mechanical ventilation reduces blood flow to the diaphragm but not to the soleus, this phenomenon is not observed after 30 min but occurs after 6 h of mechanical ventilation.⁴⁶ In addition, control and shock animals were equally exposed to mechanical ventilation. Our results are therefore not likely to be influenced by mechanical ventilation. Sixth, acidosis has been shown to cause diaphragm dysfunction,⁴⁸ which was not evidenced in the current study. However, the impact of acidosis has been observed *in vivo* whereas our study was *in vitro*. It is therefore possible that our diaphragm strips were buffered during the 30-min stabilization phase. In addition, diaphragm dysfunction has been described as a consequence of respiratory acidosis⁴⁸ while animals exhibited metabolic acidosis in our study. Finally, controlled hemorrhage is a particular model that may not strictly reflect hemorrhagic shock in clinical conditions. Moreover, in the shock-saline group, we failed to reach the targeted MAP. This is not surprising, as similar results have been reported in previous studies with either a comparably

high MAP target during resuscitation or a comparably low MAP during hemorrhagic shock.^{49,50}

In conclusion, diaphragmatic contractility was preserved during hemorrhagic shock, whereas soleus performance was impaired. This finding may be explained by preservation of diaphragmatic microcirculation even for low levels of MAP, which is reminiscent of the preserved heart and brain perfusion observed in similar circumstances. Furthermore, low levels of proinflammatory cytokines in the muscles of resuscitated animals suggest that an inflammatory component in the observed soleus muscle dysfunction is not likely. Future studies should be conducted to elucidate the mechanisms underlying preservation of diaphragmatic microcirculation during hemorrhagic shock. This could be useful to develop therapies designed to prevent diaphragmatic ischemia in various other conditions.

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Competing Interests

The authors declare no competing interests.

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