



Protective effect and mechanism of hydrogen treatment on lung epithelial barrier dysfunction in rats with sepsis

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ABSTRACT. This study aimed to explore the protective effect of hydrogen and to investigate the underlying mechanism of its preliminary effect on the alveolar epithelial barrier function in septic rats. Forty-five male Sprague-Dawley rats were divided randomly into three groups (N = 15): control [saline injection (intraperitoneal, *ip*), air drawing; SA], acute lung injury group [lipopolysaccharide (LPS) injection (*ip*, 15 mg/kg), air drawing; LA], and acute lung injury combined with hydrogen drawing group [LPS injection (*ip*, 15 mg/kg), 2% hydrogen drawing; LH]. The rats were euthanized after 6 h of treatment, and the extravascular lung water (EVLW), pulmonary alveolar-arterial oxygen pressure (A-aDO₂), and respiratory index (RI) of each group were measured. The aquaporin-1 (AQP-1) protein expression in the lung tissues was detected using immunohistochemistry and western blotting, and the correlation between the EVLW and AQP-1 was analyzed. The lung morphology was observed with light and electron microscopy. In the LA group, EVLW (0.87 ± 0.17), A-aDO₂ (113.21 ± 13.92), RI (0.65 ± 0.26), and AQP-1 expression increased. Additionally, thickened alveolar walls, significant invasion of inflammatory cells around the vessels, capillary ectasia, hyperemia/hemorrhage in the alveolar space, significantly swollen mitochondria, and increased vacuolar degeneration were observed. A significant negative correlation between AQP-1 expression and EVLW was observed ($R^2 = 0.8806$). Compared with the LA

group, EVLW (0.71 ± 0.19), A-aDO₂ (132.42 ± 17.39), RI (0.75 ± 0.24), and inflammatory reaction decreased and AQP-1 expression increased in the LH group. The damage to pulmonary epithelial cells improved after hydrogen treatment in rats with sepsis; hydrogen could protect the pulmonary epithelial barrier function by acting on AQP-1.

Key words: Hydrogen; Lung; Barrier function; Sepsis; Rat; AQP-1

INTRODUCTION

Acute lung injury is the leading cause of death of critical patients with septic shock. An uncontrolled and amplified inflammation response plays an important role in the pathogenesis of sepsis and acute lung injury (Hotchkiss and Karl, 2003). Although great progress on treatment has been made, mortality is still as high as 35 to 40% (Flamm, 1981). Many clinical trials have reported that early fluid resuscitation can improve the prognosis of patients with septic shock complicated with acute lung injury (Jones et al., 2007; Trzeciak et al., 2008), but the retention of large amounts of fluid in the tissue spaces may be aggravated in acute lung injury in patients with septic shock (Mitchell et al., 1992; Schrier, 2010). Patients with sepsis complicated with acute lung injury could have water barrier dysfunction of the alveolar epithelium, and transepithelial transportation of liquids and reabsorption of alveolar edema fluid could occur, which disrupt the fluid balance and lead to pulmonary edema and dysfunction (Martin et al., 2003; Strohmaier et al., 2005). Thus, the protection of barrier function of the pulmonary epithelial cells may be the key to treatment of septic shock complicated with acute lung injury.

Hydrogen, with anti-oxidation, anti-inflammation, and anti-apoptosis properties (Hayashida et al., 2008; Ohsawa et al., 2008), is one of the most important physiological regulators, since it can selectively reduce hydroxyl radical and the peracetic nitrosyl anion (Ohsawa et al., 2007). In the course of anti-oxidative stress, hydrogen reduces the level of malondialdehyde and myeloperoxidase activity significantly in ischemia-reperfusion tissue injury, while inhibiting apoptosis. Related research shows that hydrogen acts simultaneously on many ischemia-reperfusion injury steps, including oxidative stress, neutrophil infiltration, apoptosis, inflammatory mediators; therefore, it reduces tissue and organ injury caused by ischemia-reperfusion significantly (Ji et al., 2011). It has been reported that treatment with hydrogen reduces acute lung injury in rats with septic shock (Ohta, 2012). Therefore, we sought to determine whether hydrogen reduces lung damage by protecting the alveolar epithelial barrier.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley (SD) rats, weighing between 250 and 280 g, were purchased from the Experimental Animals Department of China Medical University (Shenyang, China), and were housed in a specific pathogen-free environment.

Hydrogen

Pure hydrogen (>99.999%) was generated using a hydrogen generator JK-300H (JingKe RuiDa Technology Co., Ltd., Beijing, China).

Rat model of sepsis with acute lung injury and grouping

Forty-five male SD rats were divided randomly into three groups using a random number table. After 10% chloral hydrate [300 mg/kg, intraperitoneal (*ip*)] anesthesia, a tracheotomy was performed on the rats, and they were connected to a ventilator with a respiratory rate of 100 breaths/min and a tidal volume of 10 mL/kg. A femoral artery puncture and a cannulation nursing intervention were performed, and the heart rate and mean arterial pressure of rats were monitored. Rats were fit with an indwelling catheter via the femoral vein puncture, and the body temperature was maintained with an electric heater. After maintaining a stable state for 30 min, rats were divided randomly into the control group [saline injection (*ip*) and air drawing, SA group], the acute lung injury group [lipopolysaccharide (LPS) injection (*ip*) and air drawing, LA group], and the acute lung injury combined with hydrogen drawing group [LPS injection (*ip*) and 2% hydrogen drawing, LH group]. The LA and LH groups were injected (*ip*) with 15 mg/kg LPS; the SA was injected (*ip*) with an equivalent volume of saline.

Lung tissue sample treatment

The trachea and chest of rats were exposed and opened after being euthanized. Then, the lower lobe of the right lung was harvested immediately, and the extravascular lung water (EVLW) was measured by the gravity method (Schindler et al., 2000). The lung tissue was weighed without the bronchus, distilled water at the same weight as the EVLW was added, and then the tissue was stirred fully into homogenates with an agitator and weighed. The homogenates were centrifuged at 5000 *g* and then placed at 4°C for 1 h. The supernatant was collected. The arterial blood was extracted and collected. The concentration of hemoglobin (Hb) was measured in the supernatant and blood, separately. Then, the tissue homogenates, blood and supernatant were dried for more than 72 h. The water content (%) was determined and the EVLW content was calculated according to the following formula: EVLW = total water content of lung (TPW) - water weight of the blood, where, homogenate Hb = supernatant Hb x (water content of the homogenate %/water content of the supernatant %); blood weight = homogenate weight x homogenate Hb / blood Hb; water weight of the blood = blood weight x water content of the blood %; TPW = water content of the homogenate % x homogenate weight - supplementary water.

The left lung was harvested and fixed in 4% paraformaldehyde for the paraffin sections. A 1-cm section of the upper lobe of the right lung was obtained, cut into a 1-mm³ sample, and fixed in 2.5% glutaraldehyde for electron microscopy.

Pulmonary gas exchange measurement

The blood gas was collected at various time points in order to estimate pulmonary gas exchange. The pulmonary alveolar-arterial oxygen pressure (A-aDO₂) was calculated, and the pulmonary gas exchange function was evaluated according to following formula:

A-aDO₂ and respiratory index (RI) $aDO_2 = (FiO_2 \times 713 - 5 / 4 \times PaCO_2) - PaO_2$, where FiO₂ (fraction of inspiration O₂); PaCO₂ (partial arterial carbon dioxide tension); PaO₂ (partial arterial oxygen tension).

Immunohistochemistry and detection of aquaporin 1 (AQP-1) expression in lung tissue

After being fixed in 40 g/L formaldehyde, all samples were paraffin-embedded and series-

sectioned (4-mm thickness). Each sample was prepared for hematoxylin-eosin (H&E) staining and immunohistochemistry staining. For immunohistochemistry, the s-p method (streptavidin-peroxidase method) was used to label AQP-1, and all steps followed the instruction (Histostain-Plus IHC Kit, mouse, Thermo Fisher Scientific Inc., Shanghai, China). The nucleus was stained with DAPI and counterstained with hematoxylin. For the negative control, phosphate-buffered saline was used instead of the primary antibody. Staining was observed by optical microscopy.

Western blotting for detection of the expression of AQP-1 in the lung tissue

The protein sample (10 mg) from each group was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on a 5% stacking gel and 12.5% separating gel and transferred to a polyvinylidene fluoride membrane using the Trans-Blot instrument (Bio-Rad Laboratories Inc., USA). Following the transfer, membranes were incubated with the AQP-1 antibody (1:3000, Proteintech Group Inc. Wuhan, China), and the antigen-antibody complexes were detected using an enhanced chemiluminescence reagent. All the proteins were analyzed with the Quantity One software.

Statistical analysis

Data are reported as means \pm standard deviation and were analyzed statistically using one-way ANOVA and the chi-square test. Bivariate analysis used linear regression and relative analysis methods. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Change in barrier function of rat pulmonary epithelium

EVLW measurement

The EVLW values of the LA and LH groups increased significantly compared with the SA group ($P < 0.05$) and this increase was more significant in the LA group than that the LH group ($P < 0.05$) (Table 1).

Table 1. Extravascular lung water measurement (EVLW, %) (means \pm s, N = 15).

	SA	LA	LH
EVLW %	0.44 \pm 0.12	0.87 \pm 0.17 ^a	0.71 \pm 0.19 ^{ab}

^aCompared with SH group ($P < 0.05$). ^bCompared with LA group ($P < 0.05$). SA = saline injection (intraperitoneal, *ip*), air drawing; LA = acute lung injury group [lipopolysaccharide (LPS) injection (*ip*, 15 mg/kg), air drawing]; LH = acute lung injury combined with hydrogen drawing group [LPS injection (*ip*, 15 mg/kg), 2% hydrogen drawing].

A-aOD2 and RI

The A-aOD2 of the LA and LH groups decreased significantly ($P < 0.05$); the A-aOD2 of the LH group increased significantly compared with that of the LA group ($P < 0.05$) (Table 2).

The RI of the LA and LH groups decreased significantly compared with the SA group ($P < 0.05$); the RI of the LH group increased significantly compared with the LA group ($P < 0.05$) (Table 2).

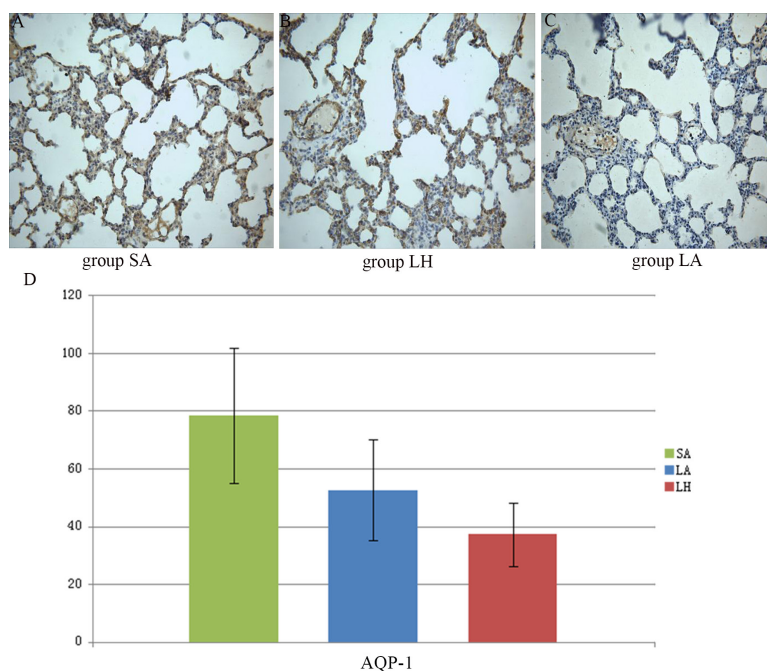
Table 2. Alveolar-arterial oxygen pressure (A-aOD₂) and respiratory index (RI) of rats (means \pm SD, N = 15).

	A-aDO ₂ (mmHg)	RI
Group SA	161.54 \pm 19.12	0.78 \pm 0.27
Group LA	113.21 \pm 13.92 ^a	0.65 \pm 0.26 ^a
Group LH	132.42 \pm 17.39 ^{ab}	0.75 \pm 0.24 ^{ab}

^aCompared with SH group ($P < 0.05$). ^bCompared with LA group ($P < 0.05$). SA = saline injection (intraperitoneal, *ip*), air drawing; LA = acute lung injury group [lipopolysaccharide (LPS) injection (*ip*, 15 mg/kg), air drawing]; LH = acute lung injury combined with hydrogen drawing group [LPS injection (*ip*, 15 mg/kg), 2% hydrogen drawing].

Expression and content of alveolar epithelial AQP-1

The immunohistochemistry results show high protein expression of AQP-1 in the lung tissue and brown-stained granules in the endothelial cells of the capillaries around the airway and alveolus in the SA group (Figure 1A). AQP-1 protein expression decreased gradually in the LA and LH groups (Figure 1B and 1C); the AQP-1 expression of the LA group was lower than that of the LH group, and the visible AQP-1 staining was lower than that of the control group (Figure 1D).

**Figure 1.** Protein expression of aquaporin-1 (AQP-1) in each group.

The western blotting results show that AQP-1 protein expression presented a high-density zone in the SA group. AQP-1 protein expression, activity, and integral optical density decreased significantly in both LH and LA groups ($P < 0.05$); compared with the SA group, the density zone was much lower and the decrease in protein present was greater in the LA group ($P < 0.05$) (Figure 2).

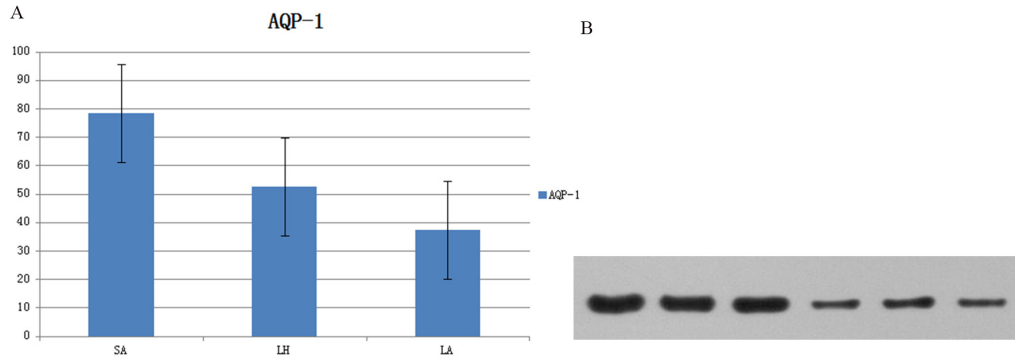


Figure 2. Western blotting results of aquaporin-1 (AQP-1) of each group.

Observation of lung tissue H&E staining by light microscopy

In the lung tissues of the rats in the SA group, complete structures, clear alveolar spaces, no edema in the alveolar septa, slight capillary ectasia, and slight inflammatory cell invasion were observed (Figure 3A). In the LH group, increased lung exudation, slightly thickened alveolar walls, invasive inflammatory cells around the vessel, and capillary ectasia were observed (Figure 3B). In the LA group, significantly thickened alveolar walls, hyperemia/hemorrhage in the alveolar internal spaces, and significant invasion of inflammatory cells were observed (Figure 3C).

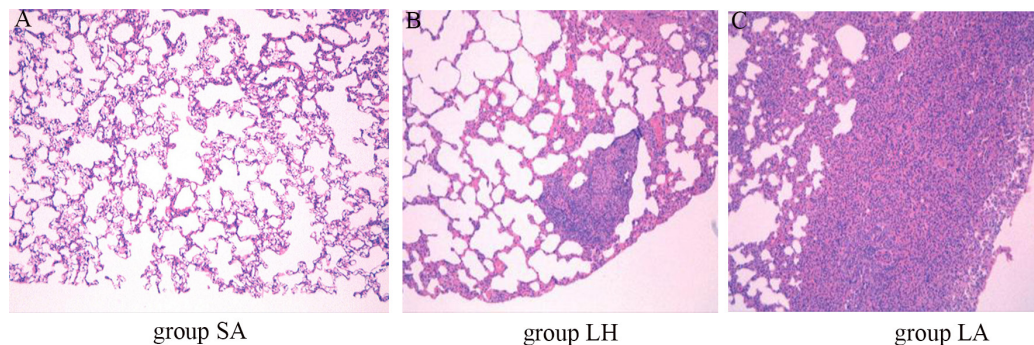


Figure 3. Hematoxylin and eosin staining of lung tissue obtained from each group.

Observation of lung tissue with transmission electron microscopy

In the SA group, the lung tissue ultrastructure was almost normal and vascular endothelial cell morphology and structure were normal. In the LA group, the lung tissue ultrastructure was damaged significantly, the blood-air barrier structure was fuzzy, edematous, and thickened, part of the alveolar epithelial cells were damaged, the basement membrane of the capillary endothelium was broken, type-II microvillus epithelial cells were sparse, the mitochondria structure was damaged and vacuolar, and lamellar bodies were empty. The ultrastructure of the lung tissue was less damaged in the LH group compared to the LA group (data not shown).

Relationship between the change of protein AQP-1 expression in lung tissue and barrier function of pulmonary epithelial cells

The relationship between the AQP-1 protein expression in the lung tissue and the extravascular water content of the rat pulmonary epithelium was analyzed. There was a significant negative correlation between AQP-1 protein expression and the EVLW in the lung tissue (Figure 4).

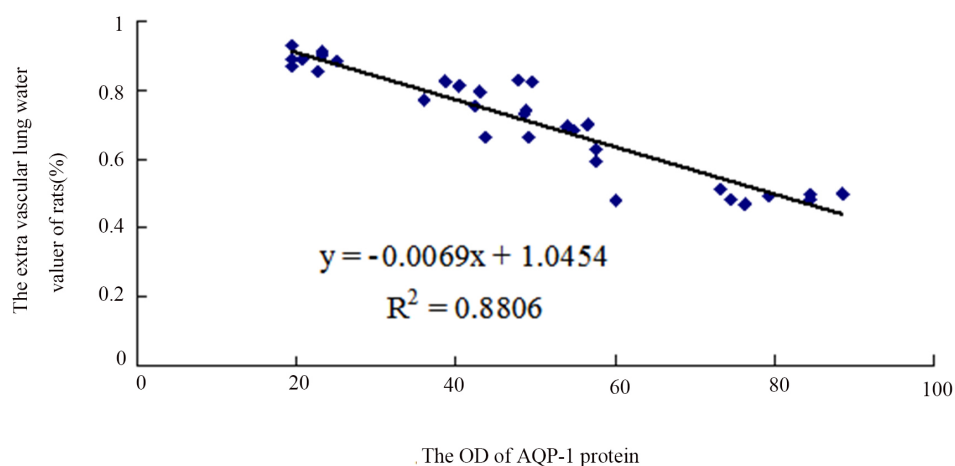


Figure 4. Value relationship between aquaporin-1 (AQP-1) protein expression and the extravascular lung water (EVLW) in the lung tissue.

DISCUSSION

Pulmonary edema after acute lung injury is the major pathological factor leading to respiratory failure. In physiological conditions, the alveolar epithelium serves as an important barrier to prevent pulmonary edema. Whether the alveolar epithelium can clear alveolar edema fluid is both a sign of lung damage and a key to treatment and prognosis of acute lung injury. By measuring the EVLW content, the change of water permeability in lung tissue can be assessed at an early stage of injury (Martin et al., 2005). Our experiment showed that EVLW increased in the lungs of rats with sepsis, indicating that acute lung injury caused by sepsis could lead to hyper-permeability by damaging the alveolar epithelial barrier function.

A-aDO₂ mainly reflects the diffusing capacity of the lung, with increasing values representing some obstacles to diffusion. RI is the ratio of the alveolar-arterial oxygen partial pressure difference and the arterial oxygen pressure (A-aDO₂/PaO₂) and is another index to assess the normal oxygenation of the body, being influenced by the ventilation perfusion ratio, lung diffusing capacity, and aeration condition. By eliminating the influence of FiO₂, RI is more reliable than PaO₂, which reflects patient's function state accurately, and is helpful to reveal the effect of mechanical ventilation of postoperative acute lung damage. There is a significant correlation between postoperative RI and lung function status. Our experiment showed that A-aDO₂ and RI both increased in rats with sepsis, indicating that acute lung damage caused by sepsis could lead to decreased lung diffusing capacity and lung damage by alveolar epithelial edema.

Acute lung damage is still one of the usual complications of septic shock. An inflammation response caused by sepsis is characterized by the damage of vascular endothelial cells and the increase in microvascular permeability, leading to pathological changes in the pulmonary vasculature and pulmonary parenchyma, which decrease lung compliance, increase intrapulmonary shunt, and cause hypoxemia. Our study showed thickened alveolar wall, significant invasion of inflammatory cells around vessels, capillary ectasia, hyperemia/hemorrhage in the alveolar internal space, and inflammatory cells in the LA group. The mitochondria swelled significantly as observed using electron microscopy, and the vacuolar degeneration increased in the LH group and LA group.

Comparing the septic rats administered hydrogen (group LH) and those administered oxygen (group LA), both the lung EVLW and the diffusing capacity improved in LH group and LA group (Table 1 and Table 2), while the damage of rat lung tissue observed by HE staining and the ultrastructure under the electron microscope was relieved slightly in LH group and LA group (Figure 2), indicating that hydrogen had a protective function against the acute lung damage of rats with sepsis by protecting the lung barrier function. Representing EVLW as an index of pulmonary epithelial barrier function, a linear regression and correlation analysis between EVLW and AQP-1 was calculated. Our results showed that there was a significant negative correlation between AQP-1 expression and EVLW in the lung tissue (Figure 4), indicating that as the degree of pulmonary edema is aggravated, the AQP-1 protein and mRNA expression level decreases, which may relate to the damage of the alveolar-capillary membrane barrier. Alveolar and vascular endothelial cells are destroyed during the process of lung damage, thus decreasing the total AQP-1 protein level.

AQP is a family of cell membrane transport proteins related to water permeability, whose discovery provided insight into the molecular mechanism of membrane water permeability in various tissues. Recent discoveries have shown that the water permeability of the alveolar air space and capillary spaces in AQP-1-knockout mice decreases ten-fold compared with that of the wild-type mice (Bai et al., 1999; Song et al., 2000), while a 14- to 16-fold decrease is observed in AQP-1/AQP-4-knockout mice (Song et al., 2000). King et al. (2002) discovered that after intravenous infusion of 3 L normal saline and measurement of the tracheal wall changes before and after infusion by scanning with high resolution CT, the pulmonary vasculature of both the individuals with congenital AQP-1 gene deficiency and normal individuals increased in thickness by 20%, but the airway wall of normal individuals increased in thickness by 50% whereas the airway wall of congenital AQP-1 gene-deficient individuals showed no change. These results revealed that AQP-1 has an important role in pulmonary vascular permeability. Umenishi et al. (1996) discovered that the AQP-1 expression level in rabbits increased rapidly from postnatal day one, and alveolar water permeability increased. AQP expression is closely related to its function. The important moment was the transforming period that the large liquid was absorbed in fetal lung and the alveolar space was turned into gas exchange room, which might be important to the recovery of normal respiratory function. Our results are consistent with those of the previous study. We also found that after hydrogen treatment the AQP-1 protein level increased in the lungs of rats with sepsis (Figure 3), indicating that hydrogen could protect the barrier function of pulmonary epithelial cells by acting on AQP-1, but further studies are required to investigate this.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

- Bai C, Fukuda N, Song Y, Ma T, et al. (1999). Lung fluid transport in aquaporin-1 and aquaporin-4 knockout mice. *J. Clin. Invest.* 103: 555-561. <http://dx.doi.org/10.1172/JCI4138>
- Flamm ES (1981). Suction decompression of aneurysms. Technical note. *J. Neurosurg.* 54: 275-276. <http://dx.doi.org/10.3171/jns.1981.54.2.0275>
- Hayashida K, Sano M, Ohsawa I, Shinmura K, et al. (2008). Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochem. Biophys. Res. Commun.* 373: 30-35. <http://dx.doi.org/10.1016/j.bbrc.2008.05.165>
- Hotchkiss RS and Karl IE (2003). The pathophysiology and treatment of sepsis. *N. Engl. J. Med.* 348: 138-150. <http://dx.doi.org/10.1056/NEJMra021333>
- Ji Q, Hui K, Zhang L, Sun X, et al. (2011). The effect of hydrogen-rich saline on the brain of rats with transient ischemia. *J. Surg. Res.* 168: e95-e101. <http://dx.doi.org/10.1016/j.jss.2011.01.057>
- Jones AE, Focht A, Horton JM and Kline JA (2007). Prospective external validation of the clinical effectiveness of an emergency department-based early goal-directed therapy protocol for severe sepsis and septic shock. *Chest* 132: 425-432. <http://dx.doi.org/10.1378/chest.07-0234>
- King LS, Nielsen S, Agre P and Brown RH (2002). Decreased pulmonary vascular permeability in aquaporin-1-null humans. *Proc. Natl. Acad. Sci. USA* 99: 1059-1063. <http://dx.doi.org/10.1073/pnas.022626499>
- Martin GS, Eaton S, Mealer M and Moss M (2005). Extravascular lung water in patients with severe sepsis: a prospective cohort study. *Crit. Care* 9: R74-R82. <http://dx.doi.org/10.1186/cc3025>
- Mitchell JP, Schuller D, Calandrino FS and Schuster DP (1992). Improved outcome based on fluid in ill patients requiring pulmonary artery catheterization. *Am. Rev. Respir. Dis.* 145: 990-998. <http://dx.doi.org/10.1164/ajrccm/145.5.990>
- Martin TR, Nakamura M and Matute-Bello G (2003). The role of apoptosis in acute lung injury. *Crit. Care Med.* 31 (Suppl): S184-S188. <http://dx.doi.org/10.1097/01.CCM.0000057841.33876.B1>
- Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, et al. (2007). Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat. Med.* 13: 688-694. <http://dx.doi.org/10.1038/nm1577>
- Ohsawa I, Nishimaki K, Yamagata K, Ishikawa M, et al. (2008). Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice. *Biochem. Biophys. Res. Commun.* 377: 1195-1198. <http://dx.doi.org/10.1016/j.bbrc.2008.10.156>
- Ohta S (2012). Molecular hydrogen is a novel antioxidant to efficiently reduce oxidative stress with potential for the improvement of mitochondrial diseases. *Biochim. Biophys. Acta* 1820: 586-594. <http://dx.doi.org/10.1016/j.bbagen.2011.05.006>
- Schindler T, Bornmann W, Pellicena P, Miller WT, et al. (2000). Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science* 289: 1938-1942. <http://dx.doi.org/10.1126/science.289.5486.1938>
- Schrier RW (2010). Fluid administration in critically ill patients with acute kidney injury. *Clin. J. Am. Soc. Nephrol.* 5: 733-739. <http://dx.doi.org/10.2215/CJN.00060110>
- Song Y, Ma T, Matthay MA and Verkman AS (2000). Role of aquaporin-4 in airspace-to-capillary water permeability in intact mouse lung measured by a novel gravimetric method. *J. Gen. Physiol.* 115: 17-27. <http://dx.doi.org/10.1085/jgp.115.1.17>
- Strohmaier W, Trupka A, Pfeiler C, Thurnher M, et al. (2005). Bilateral lavage with diluted surfactant function after unilateral contusion in pigs. *Crit. Care Med.* 33: 2286-2293. <http://dx.doi.org/10.1097/01.CCM.0000182819.11807.16>
- Trzeciak S, McCoy JV, Phillip Dellinger R, Arnold RC, et al.; Microcirculatory Alterations in Resuscitation and Shock (MARS) investigators (2008). Early increases in microcirculatory perfusion during protocol-directed resuscitation are associated with reduced multi-organ failure at 24 h in patients with sepsis. *Intensive Care Med.* 34: 2210-2217. <http://dx.doi.org/10.1007/s00134-008-1193-6>
- Umenishi F, Carter EP, Yang B, Oliver B, et al. (1996). Sharp increase in rat lung water channel expression in the perinatal period. *Am. J. Respir. Cell Mol. Biol.* 15: 673-679. <http://dx.doi.org/10.1165/ajrcmb.15.5.8918374>