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## Experimentally-Induced Acute Lung Injury: the Protective Effect of Hydroxyethyl Starch

Alessandro Di Filippo,<sup>1</sup> Marco Ciapetti,<sup>1</sup> Dolores Prencipe,<sup>1</sup> Laura Tini,<sup>1</sup> Andrea Casucci,<sup>2</sup> Riccardo Ciuti,<sup>3</sup> Daniela Messeri,<sup>4</sup> Stefano Falchi,<sup>5</sup> and Carlo Dani<sup>1</sup>

<sup>1</sup>Department of Critical Care, University of Florence; <sup>2</sup>Department of Oto-Neuro-Ophthalmological Surgical Sciences, University of Florence; <sup>3</sup>Laboratory of Pharmacotoxicology, Careggi University Hospital of Florence; <sup>4</sup>Unit of Infectious Disease, 3rd Local Health Service, Pistoia; and <sup>5</sup>A.S.F. Firenze, IOT Day Surgery, Florence; Italy

**Abstract.** The objective of this study was to evaluate the effects of hydroxyethyl starch, (130/0.4) 6%, compared to Ringer's acetate and modified gelatin on hypoxemia, inflammatory response, and oxidative stress in an experimental model of acute lung injury (ALI). The ALI/Adult Respiratory Distress Syndrome (ARDS) experimental model was produced by a bronchoalveolar saline lavage. Mature New Zealand white rabbits were anesthetized, provided with a tracheostomy and vascular catheters, and randomized to receive 25 ml/kg/hr of Ringer's acetate (group R, n = 7), 25 ml/kg/hr of modified gelatin (group G, n = 7), or 25 ml/kg/hr of hydroxyethyl starch (group S, n = 7). All of the rabbits received mechanical ventilation to maintain the PaCO<sub>2</sub> between 35 and 45 mm Hg. Blood gas levels and hemodynamic values were recorded before induction of lung injury (T0) and 10 (T10), 120 (T120) and 240 (T240) min following induction of lung injury. At the same time-points, blood samples were collected to measure the plasma levels of TNF $\alpha$  (tumor necrosis factor-alpha) and TBARS (thiobarbituric acid-reactive substances). The experiment yielded the following results: The blood PaO<sub>2</sub>/FiO<sub>2</sub> ratio was higher in group S than in groups R and G at T10, T120, and T240 (p <0.05). In group S, the plasma TNF $\alpha$  and TBARS concentrations were lower than in groups R and G at T120 and T240 (p <0.05). In conclusion, rabbits treated with hydroxyethyl starch, (130/0.4) 6%, demonstrated reductions of hypoxemia, inflammatory response, and oxidative lung damage, compared to rabbits treated with Ringer's acetate or modified gelatin.

**Keywords:** acute lung injury, respiratory failure, inflammation, lung disease, bronchoalveolar lavage, hydroxyethyl starch

### Introduction

Acute Lung Injury (ALI)/Adult Respiratory Distress Syndrome (ARDS) is characterized by an alteration of pulmonary capillary permeability that facilitates the passage of fluids and activated inflammatory cells into the interstitial tissues and ultimately into the alveoli themselves [1,2]. Interstitial edema and

inflammatory parenchymal damage cause severe hypoxemia, which is often refractory to oxygen therapy. Pulmonary injuries are followed by fibroblast proliferation, collagen deposition, and fibrosis, which impair the respiratory exchange in advanced stages of the disease [1].

Recently, experimental evidence has suggested a beneficial effect of hydroxyethyl starch in reducing interstitial edema due to systemic inflammatory response, through various mechanisms: fluid reabsorption in the interstitium [3,4]; down-regulation of inflammatory mediators [5-9] sustained by suppression of NF-kappaB activation

Address correspondence to Alessandro Di Filippo M.D., Department of Critical Care, University of Florence, c/o A.U.O.C. Careggi, Viale Morgagni 85, Firenze 50124, Italy; tel 39 055 427 7498; fax 39 055 430 393; e-mail [aledanibateba@hotmail.com](mailto:aledanibateba@hotmail.com).

[10-13], and sealing of the microcirculatory endothelial lesions, which seems to reduce the extravascular leak of fluids and inflammatory cells [14,15]. A number of studies have demonstrated that treatment with hydroxyethyl starch is associated with decreased capillary permeability in an animal model of fecal peritonitis [3], in neonatal cardiopulmonary bypass [4], and in trauma [16]. These considerations support our hypothesis that administration of hydroxyethyl starch could be more effective in reducing oxidative stress in acutely injured lungs, compared to administration of Ringer's acetate or modified gelatin. To test this hypothesis we performed a prospective study in an animal model of respiratory failure treated with hydroxyethyl starch (130/0.4) 6%, Ringer's acetate, or modified gelatin. Plasma concentrations of TNF $\alpha$  (Tumor Necrosis Factor alpha) and TBARS (Thiobarbituric Acid Reactants) were measured as indicators of inflammatory response and oxidative stress during in the initial stage of the syndrome.

## Materials and Methods

The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals and was approved by the Animal Care Committee of the University of Florence. The study was conducted on 21 New Zealand albino rabbits, weighing 2.2 to 3.5 kg. General anesthesia was induced with ketamine (10 mg/kg, im) and midazolam (1 mg/kg, im) and maintained by continuous iv infusion of propofol (3 mg/kg/hr) and ketamine (10 mg/kg/hr). A catheter was placed in an ear vein for hydration fluid and drug administration during the experiment. The rabbits were preoxygenated during spontaneous breathing with 100% oxygen by facial mask.

The anterior neck was dissected and a tracheotomy was performed in order to position a neonatal cuffless tracheostomy tube (size 3, internal diameter 3.0 mm, Shiley, Irvine, CA, USA). Once the tracheal cannula was inserted, muscle paralysis was induced by pancuronium bromide (0.2 mg/kg, iv) and maintained with 0.1 mg/kg doses every 30 min. The rabbits were mechanically ventilated using a neonatal pressure-controlled ventilator (Bear Cub 750 psv, Life Medical Equipment Inc., Miami, FL, USA) at peak pressure (PP) 10 cm H<sub>2</sub>O, respiratory rate (RR) 40 breaths/min, positive end-expiratory pressure (PEEP) 2 cm H<sub>2</sub>O, and FiO<sub>2</sub> 0.7. Mechanical ventilation lasted 240  $\pm$  10 min. During the procedure, the ECG was monitored continuously and arterial blood pressure was recorded using a catheter inserted in the carotid artery and connected to a transducer. After the initial blood gas analysis, the minute volume was set to maintain PaCO<sub>2</sub> between 35 and 45 mm Hg by modifying the peak

pressure value to between 10 and 20 cm H<sub>2</sub>O. The other ventilator parameters were unchanged.

Lung injury was induced by performing repeated pulmonary lavages with 0.9% saline solution (20 ml/kg) instilled in the tracheostomy tube through a small catheter, as described by Lachmann et al [17]. The following parameters were monitored every 30 min with a blood gas analyzer (Radiometer ABL 610, Copenhagen, Denmark): PaCO<sub>2</sub> (mm Hg), PaO<sub>2</sub> (mm Hg), BE (mmol/m), bicarbonate (mmol/l), pH, and hemoglobin (g/dl). PaO<sub>2</sub>/FiO<sub>2</sub> values were computed using the ratio between the PaO<sub>2</sub> value from the blood gas analysis and the FiO<sub>2</sub> setting on the ventilator. Hemodynamic parameters were recorded before the induction of lung injury (T0), and at 10 (T10), 120 (T120), and 240 (T240) min after induction of lung injury. During the experiment, the rabbits were treated with ethylephedrine in case of severe hypotension (<60 mm Hg), atropine in case of bradycardia (0.01 mg), and sodium bicarbonate to correct metabolic acidosis (blood pH <7.25). At the end of the experiment, the rabbits were killed with a lethal dose of potassium chloride.

The rabbits were randomly assigned to 3 experimental groups: Group R (n = 7), infusion of Ringer's acetate at 25 ml/kg/hr; Group G (n = 7), infusion of modified gelatin at 25 ml/kg/hr; and Group S (n = 7): infusion of hydroxyethyl starch, (130/0.4) 6%, at 25 ml/kg/hr. Arterial blood samples (4 ml) from each rabbit at T0, T10, T120, and T240 were drawn in Vacutainer tubes containing EDTA and centrifuged at 4,000 x g for 10 min. The plasma was removed and stored at -80°C for measurements of TNF $\alpha$  and TBARS.

Plasma TNF $\alpha$  was assayed with the Hybridomus solid-phase ELISA kit (Patricell Ltd., Nottingham, UK) and a microtiter plate spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). A standard curve was prepared by plotting the absorbance vs concentration of TNF $\alpha$  (pg/ml) in standards. Plasma TNF $\alpha$  levels were determined by comparing the absorbances of unknown samples to the standard curve.

Plasma thiobarbituric acid reactant substances (TBARS) were measured using a MDA kit (Sobioda, Grenoble, France). Malondialdehyde (MDA) was reacted with thiobarbituric acid (TBA) to form a red MDA-TBA adduct [19], which was extracted into butanol and measured by spectrophotometry at 546 nm.

**Statistics.** Heart rate, arterial pressure, blood hemoglobin, pH, bicarbonate, PaO<sub>2</sub>, PaCO<sub>2</sub>, TNF $\alpha$ , and TBARS values at T0, T10, T120, and T240 in the 3 experimental groups were expressed as means  $\pm$  SD. Comparison of the means of the 3 groups at each time-point was performed by ANOVA for 3 independent samples (p <0.05 was deemed significant).

## Results

The mean Systolic arterial Pressure (SaP) was lower (p <0.05) in group R compared to groups G and S at 10 and 120 min of ventilatory support after lung injury (Table 1); the mean Diastolic arterial Pressure (DaP) was significantly lower in group R at 10 min

Table 1. Hemodynamic and blood hemoglobin data in the R, G, and S groups at 0, 10, 120, and 240 min after the induction of lung injury (means  $\pm$  SD; ns = not significant).

Parameter and time	Group R (Ringer's acetate)	Group G (modified gelatin)	Group S hydroxyethyl starch	p by ANOVA
HR (beats/min)				
T0	216 $\pm$ 16	266 $\pm$ 40	242 $\pm$ 61	ns
T10	213 $\pm$ 14	290 $\pm$ 36	249 $\pm$ 28	ns
T120	229 $\pm$ 27	250 $\pm$ 29	233 $\pm$ 42	ns
T240	202 $\pm$ 30	235 $\pm$ 38	228 $\pm$ 37	ns
SaP (mm Hg)				
T0	98 $\pm$ 18	113 $\pm$ 8	108 $\pm$ 22	ns
T10	89 $\pm$ 19	111 $\pm$ 11	120 $\pm$ 19	<0.05
T120	92 $\pm$ 16	119 $\pm$ 12	113 $\pm$ 16	<0.05
T240	105 $\pm$ 20	133 $\pm$ 32	110 $\pm$ 22	ns
DaP (mm Hg)				
T0	75 $\pm$ 13	85 $\pm$ 8	77 $\pm$ 18	ns
T10	67 $\pm$ 19	86 $\pm$ 7	90 $\pm$ 11	<0.05
T120	71 $\pm$ 11	87 $\pm$ 12	81 $\pm$ 16	ns
T240	82 $\pm$ 21	83 $\pm$ 7	82 $\pm$ 19	ns
Hb (g/dl)				
T0	12.0 $\pm$ 2.2	10.7 $\pm$ 1.8	10.2 $\pm$ 2.9	ns
T10	11.0 $\pm$ 2.0	10.0 $\pm$ 1.5	9.0 $\pm$ 2.7	ns
T120	11.0 $\pm$ 2.8	8.4 $\pm$ 1.4	7.1 $\pm$ 2.1	<0.05
T240	9.3 $\pm$ 5.3	8.1 $\pm$ 2.1	7.1 $\pm$ 1.8	ns

compared to the other 2 groups. At 120 min after lung injury, blood hemoglobin (Hb) concentration (Table 1) was significantly higher in group R, compared to the other 2 groups.

There were no significant differences in blood pH, BE, bicarbonate, and PaCO<sub>2</sub> among the 3 groups during the experimental period (times 0, 10, 120, 240 min), but the PaO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub> ratio decreased after the lung injury (Table 2). PaO<sub>2</sub> was significantly higher in group S compared to groups R and G at 120 and 240 min. The PaO<sub>2</sub>/FiO<sub>2</sub> ratio (Table 2, Fig. 1) was significantly higher in group S compared to the other 2 groups at 10, 120, and 240 min. After bronchoalveolar lavage, the plasma TNF $\alpha$  and TBARS concentrations increased in all groups (Table 3, Figs. 2 and 3), but were significantly lower in group S compared to groups R and G at 120 and 240 min.

## Discussion

The major pathophysiologic mechanisms of ALI/ARDS are: (a) parenchymal lung damage inducing inadequate pulmonary gas exchange, hypoxemia, reduction of lung compliance, and increased airway

resistance, and (b) pulmonary aggregation of inflammatory cells and release of mediators that cause further endothelial and parenchymal damage [18]. Based on these considerations, we postulated that an animal model of ALI/ARDS with hypoxemia and hemodynamic instability might be a suitable model for this investigation.

Lachmann et al [17] observed severe disturbance of pulmonary function parameters, decrease of dynamic compliance, and increases of both inspiratory and expiratory pulmonary resistance in an experimental model of ALI/ARDS that was produced by bronchoalveolar lavage with saline solution, which depletes the alveolar surfactant and causes lesions similar to ARDS in the pulmonary parenchyma and in the microcirculation. Microscopic study of histologic lung sections showed focal and diffuse atelectasis, desquamation of bronchial and bronchiolar epithelial cells, and presence of hyaline membranes in the preterminal airways [17]. In addition, signs of peribronchial inflammation with edema and infiltration of leukocytes and eosinophils were observed. This resemblance between clinical and experimentally-induced ARDS indicates the validity of reproducing

Table 2. Arterial blood gas data and Pa<sub>2</sub>(Fi)<sub>2</sub> ratio in the R, G, and S groups at 0, 10, 120, and 240 min after the induction of lung injury (means ± SD; ns = not significant).

Parameter and time	Group R (Ringer's acetate)	Group G (modified gelatin)	Group S hydroxyethyl starch	p by ANOVA
pH				
T0	7.35±0.12	7.34±0.02	7.35±0.09	ns
T10	7.16±0.11	7.25±0.10	7.27±0.09	ns
T120	7.23±0.09	7.31±0.08	7.24±0.10	ns
T240	7.21±0.16	7.25±0.09	7.32±0.06	ns
PaO <sub>2</sub> (mm Hg)				
T0	427.6±147.9	377.1±78.9	373.7±134.4	ns
T10	118.3±88.5	78.3±18.4	187.0±130.4	ns
T120	74.1±27.1	67.5±26.0	168.8±102.1	<0.05
T240	103.4±68.9	94.3±48.7	228.9±109.5	<0.05
PaCO <sub>2</sub> (mm Hg)				
T0	26.5±8.9	38.2±3.8	31.2±7.5	ns
T10	44.7±19.8	51.8±8.3	41.8±11.3	ns
T120	50.7±4.5	44.2±9.7	44.4±10.1	ns
T240	45.4±10.7	47.7±10.4	39.2±4.9	ns
PaO <sub>2</sub> /FiO <sub>2</sub>				
T0	503±104	501±65	463±142	ns
T10	141±117	109±34	274±155	<0.05
T120	90±49	96±38	233±132	<0.05
T240	108±79	120±69	312±129	<0.05
HCO <sub>3</sub> (mmol/L)				
T0	13.45±3.60	20.34±4.28	17.02±2.37	ns
T10	21.10±5.55	21.38±3.32	18.99±2.94	ns
T120	21.18±5.38	21.37±3.54	19.58±2.45	ns
T240	18.24±5.73	20.54±2.06	19.99±2.09	ns
BE (mmol/L)				
T0	-11.44±5.91	-4.38±4.38	-7.53±2.73	ns
T10	-8.51±5.82	-4.96±4.36	-6.68±3.58	ns
T120	-5.23±5.80	-4.01±3.92	-6.80±2.36	ns
T240	-8.26±8.11	-5.62±2.89	-4.35±4.25	ns

Table 3. Plasma levels of TNFα and TBARS in the R, G, and S groups at 0, 10, 120, and 240 min after the induction of lung injury (means ± SD; ns = not significant).

Parameter and time	Group R (Ringer's acetate)	Group G (modified gelatin)	Group S hydroxyethyl starch	p by ANOVA
TNFα (pg/ml)				
T0	13.52±5.7	10.04±5.0	10.52±5.4	ns
T10	27.02±9.9	23.09±15.3	14.46±5.4	ns
T120	29.04±12.5	22.90±17.8	11.69±2.4	<0.05
T240	26.33±10.8	21.87±10.8	12.58±5.8	<0.05
TBARS (μmol/L)				
T0	0.78±0.3	0.69±0.3	0.49±0.4	ns
T10	1.12±0.5	0.85±0.3	0.52±0.3	ns
T120	1.75±0.5	1.05±0.2	0.69±0.69	<0.05
T240	1.65±0.5	1.14±0.3	0.75±0.4	<0.05

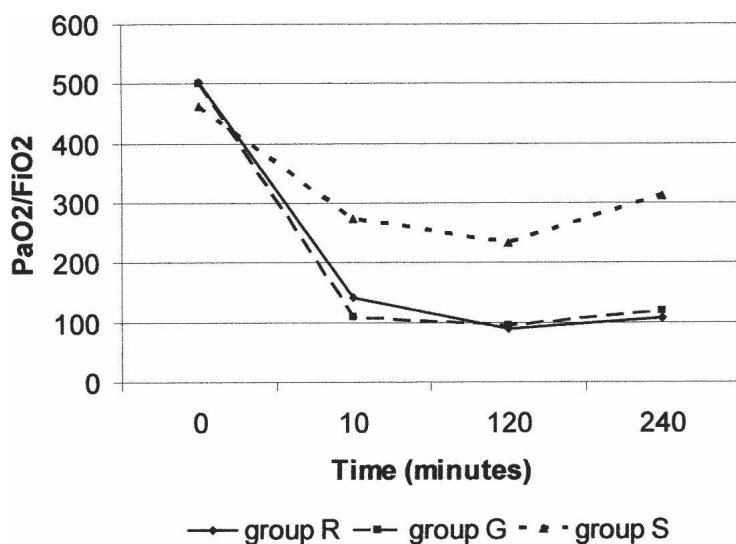


Fig. 1. Values of PaO<sub>2</sub>/FiO<sub>2</sub> ratio in the experimental model of ALI/ARDS obtained with bronchoalveolar lavage. Group R: rabbits treated with Ringer's acetate. Group G: rabbits treated with modified gelatin. Group S: rabbits treated with hydroxyethyl starch (130/0.4) 6%.

\* =  $p < 0.05$  by ANOVA.

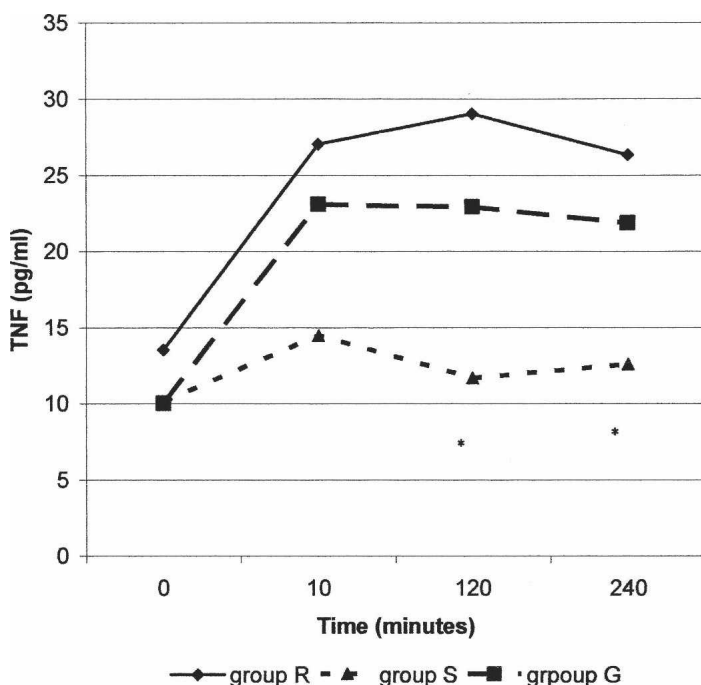


Fig. 2. Plasma TNFα levels (pg/ml) of rabbits with ALI/ARDS produced by bronchoalveolar lavage. Group R: rabbits treated with Ringer's acetate. Group G: rabbits treated with modified gelatin. Group S: rabbits treated with hydroxyethyl starch (130/0.4) 6%.

\* =  $p < 0.05$  by ANOVA.

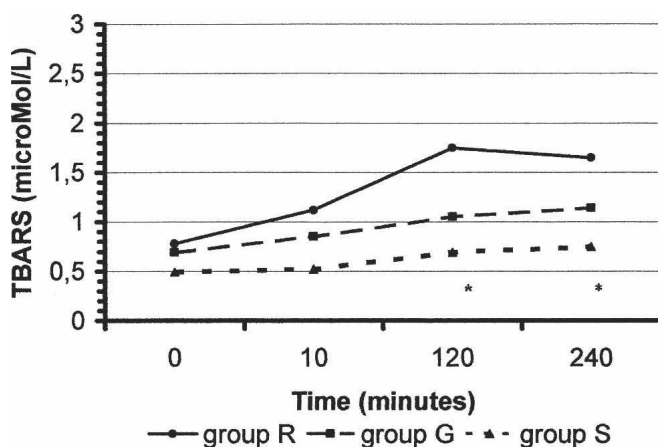


Fig. 3. Plasma TBARS levels (μmol/L) of rabbits with ALI/ARDS produced by bronchoalveolar lavage. Group R: rabbits treated with Ringer's acetate. Group G: rabbits treated with modified gelatin. Group S: rabbits treated with hydroxyethyl starch (130/0.4) 6%.

\* =  $p < 0.05$  by ANOVA.



modifications in blood gas parameters, pulmonary function, and histopathological changes [20].

We choose the model proposed by Lachmann et al [17] because, compared to other models, it has the following advantages: ease of application and repetition and an ability to reproduce the alterations of ventilation/perfusion ratio that are characteristic of ALI/ARDS. Hydroxyethyl starch has been shown to reduce the inflammatory response and interstitial edema caused by increased capillary permeability in various clinical and experimental studies [3-16]. The mechanism is not completely known, but Zikria et al [15] suggested a biophysical mechanism of action that they described as a sealing effect; ie, macromolecules of adequate form and dimension could help in the repair of capillary endothelium by sealing the pores between cellular junctions.

Hoffmann and coworkers [21], analyzing the rolling and adherence of leukocytes and the microhemodynamics in postcapillary venules by intravital fluorescence microscopy, observed a relevant protective effect of hydroxyethyl starch on endotoxin-induced microcirculatory disorder in vivo. They reported that hydroxyethyl starch, (130/0.4) 6%, was more effective than saline in preventing LPS-induced leukocyte adherence, capillary perfusion failure, and reducing LPS-induced macromolecular leakage [21]. Marx et al [22] tested the effects of hydroxyethyl starch (HES 130 kD) and Ringer's solution (RS) on plasma volume (PV) maintenance in a porcine model of intraperitoneal septic shock. In this model, HES 130 kD, but not RS, was effective in maintaining PV and preserving systemic hemodynamics and oxygenation [22].

Many studies have demonstrated essential roles of inflammatory cytokines in modulating the clinical findings of ALI/ARDS. Experimental infusion of TNF $\alpha$  in animals causes intense margination and migration of neutrophils through the walls of the pulmonary vessels, thrombotic occlusion of large arteries, significant increase of pulmonary permeability, hypoxemia, pulmonary hypertension, and decrease of dynamic compliance [23]. Furthermore, TNF $\alpha$  increases neutrophil degranulation and superoxide production, which causes tissue injury [24]. Free radical-induced oxid-

ation of membrane lipids leads to the formation of cytotoxic aldehydes, which are degradation products of lipid hydroperoxides. These aldehydes are relatively stable yet biologically active, and may therefore be considered as cytotoxic second messengers. Since they originate from free radical-induced reactions with cellular lipids, the presence of these aldehydes is considered a marker of a recent free radical-mediated reaction; consequently, an increase in cytotoxic aldehyde concentrations implies that oxidative stress occurred.

Among the analytical approaches for the estimation of oxygen radical-mediated damage in biological systems, determination of malondialdehyde (MDA) has been employed most commonly. In biological matrices, MDA exists both free and bound to SH and NH<sub>2</sub> groups of proteins and nucleic acids [25]. Usually the total (free and bound) MDA is evaluated by assay of thiobarbituric acid-reactant substances (TBARS) [26].

Several authors have reported that hydroxyethyl starch inhibits tissue NF-kappaB activation during sepsis induced by administration of lipopolysaccharide in rats [10-13] or in the model of sepsis induced by cecal ligation and puncture [14]. The inhibition of NF-kappaB activation results in lower plasma TNF $\alpha$  concentration, less pulmonary capillary permeability, and reduced free radical production.

Since interstitial edema and increased pulmonary capillary permeability represent pathogenic situations that are essential to the development of ALI/ARDS, in the present study we used the model of bronchoalveolar saline lavage to evaluate the efficacy of hydroxyethyl starch, (130/0.4) 6%, in the initial phase of acute respiratory syndrome, and its ability to limit progression toward ARDS. Hydroxyethyl starch was compared with Ringer's acetate and another commonly used colloid (ie, modified gelatin) with the aim of establishing whether the therapeutic efficacies of these oncotic agents were significantly different. Koustova et al [27] demonstrated that Ringer's lactate increased the production of oxygen reactive species by neutrophils and the expression of leukocytes involved in the inflammatory response, cellular migration, and apoptosis. For this reason, in the

present study, we infused the rabbits with Ringer's acetate instead of Ringer's lactate.

Protective effects of hydroxyethyl starch against hypoxemia were shown in our study by the improved  $\text{PaO}_2/\text{FiO}_2$  ratio, the lower plasma  $\text{TNF}\alpha$  level, and the lower plasma TBARS level in group S, compared to groups R and G. The hemodynamic parameters overlapped in the 3 groups, with the exception of systolic pressure, which was lower in the Ringer's acetate group at T10 and T120 compared to the other groups. This probably was due to the effect of the greater plasma volume conferred by the two oncotic agents, which was also indicated by a lower diastolic pressure at T10.

Blood hemoglobin level was significantly higher in group R at T120 compared with the other groups. This result may be related to the oncotic effect of hydroxyethyl starch and gelatin. At all time-points, blood hemoglobin was lower in group S than group G, but these differences were not significant. The blood pH, BE, bicarbonate and  $\text{PaCO}_2$  values in the 3 groups were not significantly different at any of the observation times.

The blood  $\text{PaO}_2/\text{FiO}_2$  ratio was higher in the hydroxyethyl starch-treated group compared to the Ringer's acetate- or modified gelatin-treated groups. The results for  $\text{TNF}\alpha$  and oxygen free radical production demonstrate a major phlogistic reaction in rabbits treated with Ringer's acetate and modified gelatin compared to the rabbits treated with hydroxyethyl starch.

Our results confirm an anti-inflammatory effect of hydroxyethyl starch during experimental ALI/ARDS, which has been noted by other authors in various experimental model of sepsis, in comparison to crystalloids and gelatin. The use of hydroxyethyl starch, (130/0.4) 6%, in the initial phases of respiratory insufficiency appears to protect against the progression of the syndrome, by ameliorating hypoxemia and the inflammatory response to pulmonary insult.

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