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Effects of Low Molecular Weight Heparin, Alone or Combined With Antithrombin III, on Mortality, Fibrin Deposits and Hemostatic Parameters in Endotoxin-Induced Disseminated Intravascular Coagulation in Rabbits

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The effect of low molecular weight heparin (LMWH) with or without antithrombin III (AT III) has been studied in a rabbit model of disseminated intravascular coagulation (DIC) induced by continuous infusion of 100 µg/kg/hr of *Escherichia coli* endotoxin for 6 hr. LMWH (5 and 10 IU/kg/hr/6 hr), alone or in combination with AT III (20 U/kg/hr/6 hr), or saline were administered simultaneously with endotoxin. Hemostatic markers at 0, 2, and 6 hr as well as kidney fibrin deposits and the mortality rate at 24 hr were determined. Rabbits receiving only endotoxin showed an impairment in hemostasis, as well as high kidney fibrin deposits and a high mortality rate. LMWH alone did not exert any effect. The simultaneous infusion of LMWH and AT III exerted a beneficial effect on the hemostatic markers and reduced the kidney fibrin deposits as well as the mortality rate in a LMWH dose-dependent manner. Fibrinogen and protein C consumption were significantly higher and renal fibrin deposits more intense in the rabbits that had died in the first 24 hr. There was also a significant positive correlation between kidney fibrin deposits and platelets, fibrinogen, and protein C consumption, taking the whole rabbit population. It is concluded that the simultaneous infusion of LMWH and AT III is useful in this DIC model and would make it possible to reduce significantly the AT III doses used when AT III is given alone. *Am. J. Hematol.* 60:6–11, 1999. © 1999 Wiley-Liss, Inc.

Key words: disseminated intravascular coagulation; low molecular weight heparin; antithrombin III; sepsis; endotoxin

INTRODUCTION

Disseminated intravascular coagulation (DIC) is a serious and frequent complication of gram-negative bacterial sepsis [1]. Despite the use of potent antibiotics and intensive supportive care, the mortality among patients with sepsis-induced DIC remains close to 60% [2].

Gram-negative bacteria include in their outer membrane a molecule called endotoxin or lipopolysaccharide. Endotoxin leads monocytes and endothelial cells to generate several cytokines that in turn activate coagulation, mainly by the extrinsic pathway [3]. As a result, higher than normal amounts of thrombin are generated. Thrombin transforms fibrinogen into fibrin and stimulates platelet aggregation, both actions leading to the formation of stable microthrombi. The exhaustion of antithrombin III

(AT III) and protein C, the main coagulation inhibitors, could perpetuate fibrin generation [4,5], thus allowing the appearance of microvascular thrombi in various organs and subsequent multiple organ failure (MOF) [6]. Therefore, treatment strategies aimed at keeping suitable levels of the coagulation inhibitors could be an interest-

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ing approach to develop more efficient therapeutic schedules.

Heparin, which accelerates coagulation inhibition by acting as a cofactor of AT III, has not been as effective as expected when used as a treatment in endotoxin-induced DIC [7]. The most likely explanation is that AT III is consumed during DIC, thus rendering useless the subsequent administration of low molecular weight heparin (LMWH). To our knowledge, there are no studies describing the effects of LMWH on endotoxin-induced DIC. However, LMWH has proved as efficient as unfractionated heparin (UFH), or better, in other thrombotic disorders [8,9]. On the other hand, although the infusion of AT III in experimental models of DIC has yielded promising results [10–14], this treatment has only been effective when suprathreshold doses have been used [15]. Therefore, a therapeutic approach that keeps plasma AT III levels at a suitable concentration to allow the action of LMWH may be of interest.

The aim of the present work has been to evaluate the effect of different doses of LMWH with or without AT III in a rabbit model of endotoxin-induced DIC. Kidney fibrin deposits and mortality have been assessed and their relationship to hemostatic parameters is reported.

MATERIALS AND METHODS

Experimental Model

Male New Zealand white rabbits (weight, 2–3 kg) were used. Animals were anesthetized by an intramuscular injection of 30 mg/kg ketamin hydrochloride and 2×10^{-3} mg/kg xylazine hydrochloride followed by intramuscular boosts of ketamin hydrochloride throughout the experiment. DIC was induced in rabbits by 100 μ g/kg/hr endotoxin (*Escherichia coli* 0111:B4, Difco Laboratories, Detroit, MI) for 6 hr in 60 ml (10 ml/hr) saline given intravenously through the marginal ear vein.

Treatments were started simultaneously with endotoxin infusion through the contralateral marginal ear vein. Six different groups were established: 1. Low-dose LMWH group (n = 18) were given five anti-factor Xa international units (IU)/kg/hr Dalteparin (Pharmacia & Upjohn, Sweden) for 6 hr in 60 ml (10 ml/hr) of saline; 2. High-dose LMWH group (n = 16) were given 10 anti-factor Xa IU/kg/hr Dalteparin for 6 hr in 60 ml (10 ml/hr) of saline; 3. Low-dose LMWH plus AT III group (n = 10) were given the lower Dalteparin dose (5 IU/kg/hr/6 hr) plus 20 U/kg/hr/6 hr AT III (Kybernin-P, Centeon SA, Marburg, Germany) in 60 ml (10 ml/hr) of saline; 4. High-dose LMWH plus AT III group (n = 10) were given the higher Dalteparin dose (10 IU/kg/hr/6 hr) plus 20 U/kg/hr/6 hr AT III in 60 ml (10 ml/hr) of saline; 5. Endotoxin control group (n = 10) was infused with saline as placebo (10 ml/hr) for 6 hr; and 6. Ten rabbits that were given neither endotoxin nor treatment were

infused with saline (10 ml/hr by both marginal ear veins for 6 hr) as an additional control group.

Surviving rabbits were sacrificed 24 hr after the start by intravenous injection of 60 mg/kg Nembutal (Abbot Laboratories, Abbot Park, IL). Kidneys were extracted from all animals (survivors and nonsurvivors) for subsequent histological studies.

Laboratory Methods

Blood samples were taken through a catheter inserted into a femoral artery before starting the endotoxin infusion, and 2 and 6 hr afterward. Blood samples (9 vol.) were collected in 3.2% citrate (1 vol.). Blood samples were kept on ice no longer than 2 hr to determine fibrinogen, AT III and protein C activity. Platelet-poor plasma was obtained by centrifugation at 1,600 g for 20 min at 4°C in a Hermle Z 382 K centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany) and stored at –70°C until assay. Tubes containing K₃-EDTA were used to collect blood for platelet counts.

Platelets were determined in a Counter STKS automatic analyzer (Coulter Corp., Hialeah, FL). Fibrinogen was measured following the Clauss method [16]. AT III activity was measured by an amidolytic assay (Coamatic Antithrombin III, Chromogenix, Stockholm, Sweden) [17]. Protein C activity was determined using a commercially available assay (Coamatic Protein C, Chromogenix) [18].

Histological Examination

Kidney sections were fixed in formalin, embedded in paraffin, stained with Masson's trichrome and examined for the presence of fibrin microthrombi by a pathologist unaware of the experimental design. Tissue sections were scored on a scale from 0 to 4 as previously described [14]. Briefly: (0) no fibrin; (1) partial fibrin deposits in some glomeruli; (2) partial deposits in all glomeruli; (3) large quantities of fibrin in all glomeruli; and (4) fibrin thrombi in glomerular capillaries and in noncapillary vessels.

Data Analysis

Results at 2 and 6 hr were converted to percentages assuming a value of 100% for basal data, and expressed as mean \pm SEM. The Student's *t*-test was used to look for differences in hemostatic parameters between endotoxin control and saline control groups. One-way ANOVA for multiple comparisons followed by the Tukey B test was applied to compare the hemostatic parameters between endotoxin control and treatment groups. The Kruskal-Wallis test followed by the Mann-Whitney U test was used to compare fibrin deposits between endotoxin control and treatment groups. Differences in fibrin deposits between survivor and nonsurvivor animals were assessed by the Mann-Whitney U test. Differences in mortality

TABLE I. Hemostatic Parameters at Baseline in the Six Groups of Rabbits*

Group	Platelets ($\times 10^9/l$)	Fibrinogen (mg/dl)	AT III (%)	Protein C (%)
Saline	473.0 \pm 47.1	201.3 \pm 16.7	99.4 \pm 3.2	106.1 \pm 6.4
Endotoxin control	454.2 \pm 40.8	247.5 \pm 31.4	117.9 \pm 3.0	112.4 \pm 5.0
LMWH (5 IU/kg/hr)	501.4 \pm 35.9	217.5 \pm 29.6	101.4 \pm 6.5	85.8 \pm 6.8
LMWH (10 IU/kg/hr)	481.4 \pm 34.4	200.1 \pm 12.8	118.9 \pm 7.4	98.7 \pm 5.3
LMWH (5 IU/kg/hr) + AT III (20 U/kg/hr)	407.5 \pm 21.3	224.0 \pm 25.1	113.7 \pm 3.7	112.6 \pm 10.4
LMWH (10 IU/kg/hr) + AT III (20 U/kg/hr)	435.0 \pm 29.2	229.5 \pm 18.0	90.9 \pm 10.9	105.6 \pm 11.3

*Data shown as mean \pm SEM. AT III, antithrombin III; LMWH, low molecular weight heparin.

TABLE II. Hemostatic Parameters at Two and Six Hr After Endotoxin Infusion in the Endotoxin Control and Treatment Groups[†]

Group		Platelets (%)	Fibrinogen (%)	AT III (%)	Protein C (%)
Endotoxin control	2 hr	48.8 \pm 4.6	95.5 \pm 3.9	87.7 \pm 3.9	58.6 \pm 5.2
	6 hr	20.3 \pm 3.5	58.2 \pm 5.1	73.1 \pm 5.8	15.1 \pm 3.3
LMWH (5 IU/kg/hr)	2 hr	70.9 \pm 5.0*	89.0 \pm 7.0	94.6 \pm 7.8	83.6 \pm 11.3
	6 hr	40.6 \pm 4.8*	65.5 \pm 7.5	72.7 \pm 4.7	27.4 \pm 6.4
LMWH (10 IU/kg/hr)	2 hr	65.7 \pm 3.9	82.5 \pm 4.1	85.2 \pm 5.2	70.4 \pm 3.3
	6 hr	41.0 \pm 7.2*	59.0 \pm 4.8	76.9 \pm 5.2	18.2 \pm 5.7
LMWH (5 IU/kg/hr) + AT III (20 U/kg/hr)	2 hr	62.0 \pm 5.4	92.5 \pm 5.1	102.0 \pm 3.2**	72.2 \pm 4.2
	6 hr	44.1 \pm 5.4**	83.5 \pm 5.7**	101.7 \pm 5.5**	40.7 \pm 6.2*
LMWH (10 IU/kg/hr) + AT III (20 U/kg/hr)	2 hr	61.4 \pm 3.8	85.5 \pm 6.9	187.3 \pm 74.2*	85.6 \pm 3.6**
	6 hr	47.6 \pm 4.7	76.1 \pm 7.4	227.6 \pm 84.4**	60.9 \pm 6.0**

[†]Data shown as mean \pm SEM percent of the basal value. AT III, antithrombin III; LMWH, low molecular weight heparin.

* $P < 0.05$.

** $P < 0.01$ as compared with the endotoxin control group.

rate at 24 hr were assessed by Fisher's exact test. Differences in hemostatic parameters between surviving and nonsurviving animals were determined by the Student's *t*-test. Possible correlations between kidney fibrin deposits and hemostatic parameters were analyzed by the Spearman rank correlation test.

RESULTS

Effects of LMWH and LMWH Plus AT III on Endotoxin-Induced Changes in Coagulation

Table I shows the baseline plasma levels of the different coagulation parameters in the saline control, endotoxin control, and treated groups. In the control group of rabbits without endotoxin, no changes in the analyzed parameters were observed (not shown).

Table II shows the plasma levels, expressed in percentages with respect to the basal value which is assumed to be 100%, of the different coagulation parameters analyzed throughout the experiment in the endotoxin control and treated groups.

Infusion of endotoxin into rabbits caused a significant decrease, with respect to the saline group, in platelets and protein C at 2 hr ($P < 0.01$) and in platelets, fibrinogen, protein C ($P < 0.001$) and AT III ($P < 0.05$) at 6 hr.

Infusion of both low and high LMWH doses had little effect on coagulation parameters, showing only a lower

decrease in platelets at 2 and 6 hr in the low-dose LMWH group ($P < 0.05$).

However, the simultaneous infusion of LMWH and AT III remarkably improved the hemostatic profile in the rabbits that received this treatment with respect to the endotoxin control group; in addition to an improvement in platelets and fibrinogen, the AT III levels were kept at a level similar to baseline when low-dose LMWH was used and significantly increased when this treatment was applied with high-dose LMWH (Fig. 1A). Moreover, a remarkable reduction in the protein C consumption was assessed (Fig. 1B), such effect being more marked again when LMWH was given at high dose ($P < 0.01$ at 2 and 6 hr) than when low dose was used ($P < 0.05$ at 6 hr).

Histological Findings

Intense fibrin deposits were detected in the endotoxin control group, even in noncapillary vessels. The average value according to the scale described above was 2.61 ± 0.34 , which is significantly higher ($P = 0.0078$) than the value obtained in rabbits given saline without endotoxin (score 0). Low-dose LMWH plus AT III significantly reduced fibrin deposits in kidneys (score 0.65 ± 0.36 , $P = 0.005$). At high-dose LMWH plus AT III a dramatic improvement in renal deposits was found, and no fibrin was detected in any rabbits (score 0, $P < 0.001$). How-

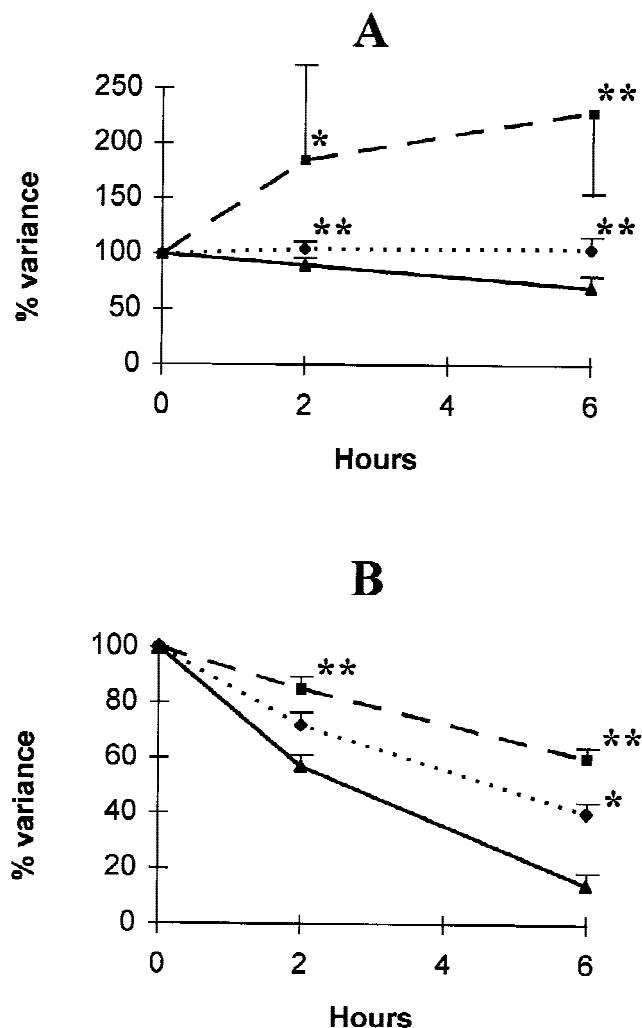


Fig. 1. A: AT III activity in plasma before and 2 and 6 hr after endotoxin infusion in endotoxin control rabbits (▲), and in rabbits receiving low (◆) and high (■) LMWH plus AT III. Values are expressed as the mean \pm SEM percent of the initial value before endotoxin infusion. * $P < 0.05$ and ** $P < 0.01$ as compared with the endotoxin control group. B: Protein C activity in plasma before, and 2 and 6 hr after, endotoxin infusion in endotoxin control rabbits (▲), and in rabbits receiving low (◆) and high (■) LMWH plus AT III. Values are expressed as the mean \pm SEM percent of the initial value before endotoxin infusion. * $P < 0.05$ and ** $P < 0.01$ as compared with the endotoxin control group.

ever, neither low nor high LMWH doses without AT III had any significant effect on the extent of fibrin deposition (score 2.02 ± 0.34 and 1.71 ± 0.31) (Fig. 2).

Taking all the animals together except the saline control group ($n = 64$), a significant positive correlation between protein C consumption at 2 and 6 hr and kidney fibrin deposition was observed ($r = 0.29$, $P < 0.05$ and $r = 0.56$, $P < 0.001$, respectively). There was also a positive correlation between kidney fibrin deposits and the decrease in platelets and fibrinogen at 6 hr ($r = 0.33$ and $r = 0.36$, $P < 0.001$).

Mortality Rate

Seven of 10 rabbits (70%) died in the first 24 hr following endotoxin infusion in the endotoxin control group, whereas none of the saline control group animals died. Both LMWH doses infused alone did not remarkably reduce the mortality rate: Nine of 18 rabbits (50%) died at low dose and seven of 16 rabbits (44%) died at high dose; however, when AT III and LMWH were simultaneously infused, mortality decreased to 30% (three of 10 rabbits) at low LMWH dose and 20% (two of 10 rabbits) at high LMWH dose, the latter not reaching statistical significance with respect to the endotoxin control group ($P = 0.06$), probably due to the relatively low number of animals studied.

Taking again all the animals together except the saline control group, a significantly more pronounced decrease was found in plasma protein C activity at 6 hr in animals that died ($n = 28$) with respect to survivors ($n = 36$) (22.0 ± 4.3 vs. 37.3 ± 4.5 , $P < 0.05$); platelet drop and fibrinogen were also higher at 6 hr in nonsurviving rabbits (32.5 ± 3.5 vs. 44.5 ± 3.8 , $P < 0.05$ and 55.0 ± 2.9 vs. 76.9 ± 4.4 , $P < 0.001$, respectively); as expected, nonsurvivors showed significantly more intense renal fibrin deposits (2.75 ± 0.36 vs. 1.59 ± 0.29 , $P < 0.001$).

DISCUSSION

AT III consumption in patients with shock and DIC seems to play an important pathological role, and the benefits of a substitution therapy with AT III in these patients have been accurately reviewed [19]. In this study we report the effect of LMWH given alone or together with AT III on endotoxin-induced DIC in rabbits. Administration of endotoxin in our model resulted in severe DIC as shown by the marked decrease in platelets, fibrinogen, AT III, and protein C, which is in agreement with previous clinical and experimental studies. Moreover, intense renal fibrin deposition and a high mortality rate were assessed as a result of the endotoxin infusion.

LMWHs, which are mixtures of structurally and functionally heterogeneous polysaccharide chains, are anticoagulants broadly used in different human clinical conditions related to thrombotic phenomena, which exert their action by enhancing the inhibitory capacity of AT III against factor Xa and thrombin [20]. When LMWH was infused without AT III there were no beneficial effects either on hemostatic parameters or on renal fibrin deposition or mortality. These results concur with those of previous studies showing that UFH alone is useless in the same model of endotoxin-induced DIC [14]. The marked AT III decrease, mainly due to consumption during complex formation with activated clotting factors but also to inactivation by proteolytic enzymes from polymorphonuclear granulocytes (e.g., PMN-elastase), could explain

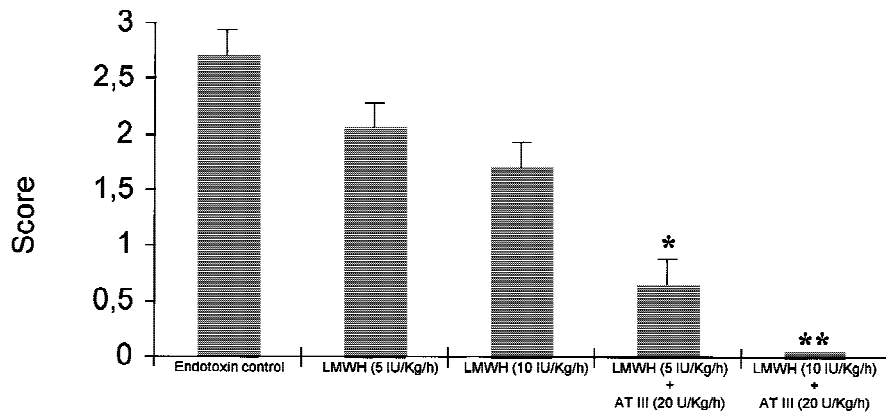


Fig. 2. Score of fibrin deposition in kidneys in endotoxin control group and in rabbits receiving LMWH (low and high dose) with and without AT III. Score values are expressed as mean \pm SEM. * $P < 0.01$ and ** $P < 0.001$ as compared with the endotoxin control group.

the uselessness of this treatment [21,22]. The results presented in a randomized study that compared the efficacy of heparin vs. AT III in patients with DIC due to sepsis or septic shock [23] would support our hypothesis, since these authors obtained a much better outcome in the patients who were treated with AT III, especially in the most severe cases, thus drawing attention to the uselessness of heparins when AT III stocks are exhausted.

However, the simultaneous addition of AT III and LMWH improved the hemostatic profile (platelets, fibrinogen, protein C, AT III) as well as the renal fibrin deposits in a LMWH dose-dependent manner. An earlier study performed in our laboratory in the same model showed that AT III given alone at the dose used in the current study (20 U/Kg/hr/6 hr) had no beneficial effect on renal fibrin deposition [14]. Nevertheless, the same study showed that a much higher AT III dose did succeed in reducing the intensity of kidney fibrin deposits, in agreement with other models of endotoxin-induced DIC that have reported a beneficial effect of AT III alone, again at much higher doses than ours [10–13]. Therefore, the improvement observed in the current study seems to be due to the simultaneous infusion of both LMWH and AT III. On the other hand, the treatment with UFH (10 IU/Kg/hr/6 hr) and AT III (20 U/Kg/hr/6 hr) in the same earlier study [14] did not improve either the platelets or the fibrinogen and, although a decrease in the kidney fibrin deposits was achieved in such conditions (score 0.9 ± 0.8), this reduction was less pronounced than the one currently being assessed when LMWH and AT III are used at the same doses (score 0). Moreover, it must be remembered that the end point in the UFH and AT III experiments was at 8 hr instead of at 24 hr. UFH and AT III also decreased mortality, although again the endpoint was at 8 hr. From these data, LMWH seems to be more efficient than UFH in this model.

When AT III was given with the low-dose LMWH the plasma AT III levels did not decrease with respect to baseline. Furthermore, when this treatment was administered at high-dose LMWH the AT III levels were continuously increasing through the experiment. On the

other hand, the lower protein C consumption could be explained in the light of the diminished thrombin formation; the lower amount of thrombin-thrombomodulin complexes would prevent the rapid protein C activation and subsequent exhaustion observed in the nontreated rabbits. It seems to be clear that the reported decrease in the kidney fibrin deposits, with the remarkable total absence of fibrin in the group of high dose LMWH and AT III, is a consequence of the improvement in the hemostatic profile. On one hand, this finding would be compatible with our previous studies [14] in which both the simultaneous infusion of UFH and AT III and the administration of a suprathreshold dose of AT III succeeded in reducing the kidney fibrin deposition, presumably as a consequence of maintaining suitable AT III levels. A recent study in a guinea pig DIC model would also support this idea, although it must be noted that the causal agents were gram-positive bacteria [24]. On the other hand, the strong correlation obtained between protein C and renal fibrin deposition would suggest an important pathophysiological role for this inhibitor. A recent study in our laboratory infusing r-hirudin in endotoxin-induced rabbits also showed a strong positive correlation between protein C consumption and renal fibrin deposits [25]. Moreover, an acquired protein C deficiency correlated positively with the extent of the skin lesion, in turn reflecting the severity of microvascular thrombosis, has been reported in human septic patients [26]. Taking these findings together, it is tempting to speculate that protein C plays an etiologic role in microthrombosis development, although the possibility that this molecule is merely a sensitive and reliable DIC marker cannot be excluded.

Mortality decreased in rabbits treated simultaneously with LMWH and AT III in a LMWH dose-dependent manner with respect to control animals. The fact that this decrease was not statistically significant could be explained by the relatively low number of animals included in the study. Nevertheless, the decrease from 70% to 20% shows a tendency to an improved survival in the treated rabbits.

When all the rabbits were divided into two groups, survivors at 24 hr and nonsurvivors, significantly more intense renal fibrin deposits as well as a significantly higher consumption of platelets, fibrinogen, and protein C in the nonsurvivor animals were reported. Among the latter, the difference assessed in the protein C levels must be underlined once more.

Finally, we want to say that the benefits of the simultaneous infusion of LMWH and AT III seem to be similar to those obtained after the administration of r-hirudin in the same rabbit DIC model [25].

CONCLUSIONS

In conclusion, simultaneous infusion of AT III and LMWH improves the hemostatic profile and decreases the renal fibrin deposits and mortality rate in this model of endotoxin-induced DIC in rabbits. The fact that similar doses of LMWH infused alone do not have beneficial effects shows that, in our model, LMWH needs an exogenous support of AT III to exert its action. On the other hand, given that infusion of AT III alone at the current dose had previously proven to be ineffective, it seems to be clear that the inhibitor, when given at such dose, needs an exogenous support of its cofactor to succeed efficiently in improving the hemostatic profile. Moving to the human therapeutic field, we suggest that the simultaneous infusion of LMWH and AT III would reduce the amount of the inhibitor needed to be efficient, which is interesting from the cost benefit point of view. Finally, the observed correlations between fibrin deposits and hemostatic parameters as well as the comparison of such parameter between nonsurvivor and survivor rabbits suggest that protein C could play an important role in the pathogenesis of endotoxin-induced DIC, although further studies are required to assess this point.

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