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ELASTASE AND IT'S RELATIONSHIP BETWEEN LEUKOCYTES, NEUTROPHILS AND LUNG EMPHYSEMA, A RANDOMIZED STUDY IN WISTAR RATS.

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SUMMARY : Alterations in elastase, leukocytes and neutrophils are seen following the acute administration of lethal doses (LD) of endotoxin, which also effectively induces of inflammation, but there are contradictory data such as conflicting theories and hypotheses concerning to the relationship between elastase levels and leukocyte counts or neutrophils percentage.

Elastase and leukocytes (WBC) components were assessed *in vivo* at 10 minutes, 1 hour., 3 hr., 6 hr., 24 hr., and 7 days after administration of lipopolysaccharide *E. coli* endotoxin with and without a glycoprotein inhibitor (Urinastain).

Abbreviations used in this paper: LD (lethal dose), LPS (Lipopolysaccharide), U-LPS-U (Urinastatin-Lipopolysaccharide-Urinastatin), LPS-Ur (Lipopolysaccharide-Urinastatin), ARDS (acute respiratory distress syndrome).

INTRODUCTION

Septic shock is a common cause of mortality and morbidity in internal medicine, surgery, pediatrics, anesthesiology, obstetrics and gynecology[3]. Macrophages are key cellular participants in the inflammatory response and also they have been implicated as major target cells that affect the course of endotoxemia through their phagocytic and exocytic functions. Conditions which compromise or enhance macrophage function have been demonstrated to influence markedly the susceptibility of laboratory animals to circulatory shock induced by bacterial endotoxin. The objective of the present study was to test the development of lung emphysema[2] caused by the endotoxic shock. Endotoxin was administered intraperitoneally into rats to appreciate the relationship among the circulating leukocytes, neutrophis, platelets, RBC, Htc., Hb., the elastase levels and the microscopic changes in the organs.[3]

The main purpose of this study was to compare the circulating levels of elastase contained in the neutrophils[4)5)13)] among three different groups of endotoxin-induced shock, before and after using Urinastatin, a glycoprotein inhibitor[7], which prevents the elastin degradation due to the released elastase into the bloodstream[5)13)].

Septic shock affects the heart, the vascular system and almost all the organs of the body including lungs.

In response to the septic shock, the host produces macrophage migration and the C5a anaphylotoxin induces neutrophil aggregation in the compromised organs. Many vessels of the microvasculature are occluded by neutrophil microemboli,[3] the vascular endothelial cells

becomes dysfunctional due to activated complement[4], and the organ vascular bed is destroyed by capillary bed occlusion. After this, neutrophil microemboli release the leukocyte elastase responsible to many protein degradation as elastin[13], leaving to a elastin disruption and finally to the emphysema of the lungs[13].

MATERIALS AND METHODS

Endotoxin : Lipopolysaccharide B *E. coli* 0111: B4 Ld50 7.63 mg/kg (ipr-mus) lipid A 7.90% Difco laboratories Detroit Michigan U. S. A. was reconstituted with sterile water on the day of the use. In all experiments the dosage was 20 mg/kg, and shock was produced by single intraperitoneal injection.

Procedures : Adult Wistar rats from Charles River Co. Japan were used. And all the samplings were conducted under diethyl ether anesthesia from Wako Pure Chemical Industries Ltd. In each case the anesthetic was given by inhalation in a closed chamber in a dosage of 2cc. Blood samples were taken directly from the heart with a butterfly needle 27G and collected into a EDTA 2Na assay tube.

Control group : There was a control group to which the endotoxin was given to ascertain the adequate dosage to produce the death.

Laboratory : The rats were accustomed to the laboratory life for five days, before, control blood sample were taken. Then the endotoxin was injected intraperitoneally. The second sample was taken after 10 minutes, the third one at the 6 hours, the fourth one at 24 hours and the last one at the seventh day.

The sample were analyzed in a computerized analyzer for the WBC, RBC, platelet counts, Hb, and Hct.

Elastase : To measure the elastase levels, the serum of a 2cc blood sample was frozen for 2 days before the use and then warmed in a Yamato waterbath incubator model BT-23 from Yamato scientific Co. Ltd. Tokyo Japan for 30 minutes at 25°C, then the elastase levels were assayed with the PMN Elastase Merck immunoassay 15689 from E. Merck, Frankfurter StraBe 250, D-6100 Darmstad 1 ; and then measured in a Shimadsu double beam spectrophotometer, model VV-150-02 from Seisakusho Ltd. Kyoto Japan at a length wave of 405nm.

Histopathological examination : After the rats died, specimens of the lungs, liver, bowel and Kidneys were taken and stored in 20% formalin for 5 days, then all of the specimens were sliced and stained with eosine-hematoxylin, to read with a light microscope under blinded fashion.

Statistics : The data were presented as mean ±. Parametric results were analyzed by modified Wilcoxon *test.*

Glycoprotein inhibitor : The Urinastatin from Mochida Pharmaceutical Co. Japan was given 50000 IU intraperitoneally 10 minutes before and 10 minutes after the endotoxin injection a half dose each time ; To another group only once the total dose was administered 10 minutes after the endotoxin injection.

RESULTS

The experiments were successful in 47 of 65 experiments. Three groups were classified as follows : Group I of eleven rats were used to study the changs after LPS injection (LPS group); Group II of seventeen rats to study the changes caused by Urinastatin usage before and after LPS adminisration (U-LPS-U group) ; and Group III of nineteen rats for Urinastatin administration after LPS injection (LPS-UR group). Survival rates within seven days were studied in all the rats.

Survival : (**Table 1**) The period of the experiment was seven days and the survival rates were as follows ; from the first LPS group 2 rats died between 7 and 9 hours (81.9% survival rate), 6 rats between 9 and 10 hours (54.5% survival rate) and 3 rats between 10 and 11 hours (0% survival rate) ; from the U-LPS-U group 2 rats died on the third experimental day (88.2% survival rate) 2 rats on the fourth day (76.4% survival rate), and 1 on the fifth day (70.5% survival rate) and from the LPS-Ur group none of the rate died (100% survival rate). (**Fig. 1**)

Elastase dosage : (**Table 2**) The control value of elastase was 89.3 ± 14.8 ug/m*l.* Ten minutes after LPS injection all samplings reached a twice-fold level without exception. However, the LPS-Ur group throughout experimental period has had lesser levels of elastase comparing with

Table 1. Comparative survival rates between all the groups at the experiment period.

PERIOD	LPS	U-LPS-U	LPS-UR
Pre-exper	11 ratr (10%)	17 rats (100%)	19 rats (100%)
7 hr	9 rats (81.9)	17 rats (100%)	≠
9 hr	3 rats (54.5%)	17 rats (100%)	≠
11 hr	all die (0%)	17 rats (100%)	≠
24 hr	+	17 rats (100%)	≠
3 day	+	15 rats (88.2%)	≠
4 day	+	13 rats (76.4%)	≠
5 day	+	12 rats (70.5%)	≠
6 day	+	≠	≠
7 day	+	≠	≠

+ dead rats.
≠ no variation, same as above.

Table 2. Comparative values of elastase between all the groups at the experiment period.

PERIOD	LPS	U-LPS-U	LPS-UR
Basal	89.3±14.8	89.3±14.8	89.3±14.8
10 min	203.5±68.3	212.2±19.6	176.3±34.7
1 hr.	191.2±44.7	214.0± 8.5	181.0±11.0
3 hrs.	200.0±10.6	212.1±24.7	138.0±10.5
6 hrs.	201.0±10.5	210.0±25.8	165.8±13.9
24 hrs.	+	192.2± 7.6	157.0±12.8
7 days.	+	202.5±30.9	165.4±46.8

Values are ug/ml.
+ Rat died before this sampling.

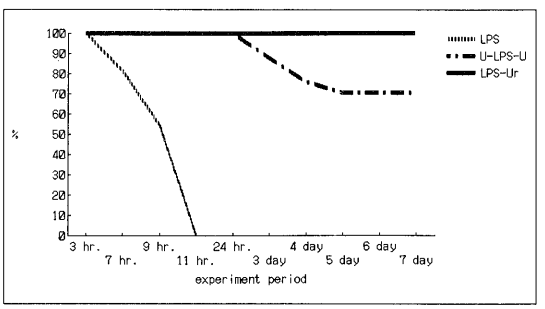

Fig. 1. Comparative graph of the survival percentages among the three groups. The LPS group had survival rate no longer than 11 hours.

the other groups, showing a clear difference at tha third hour sampling when it fell down to 138 ± 10.5 μg/m*l* with a significant difference of mean ($p<0.005$), comparded with the LPS group (200.0 ± 10.6 μg/m*l*) and the U-LPS-U group (212.1 ± 24.7 μg/m*l*), although there was no difference between the LPS and the U-LPS-U groups. At the sixth hour sampling the LPS-Ur group (165.8 ± 13.9 μg/ml) shows a significant difference ($P<0.005$) between the LPS and U-LPS-U groups (201.1 ± 10.5 and 210.5 ± 25.8 μg/m*l* respectively). The sixth hour sampling was the last one for the LPS group, because the death of rats begun at the seventh hour post LPS endotoxin injection. The last rat from the LPS group died during the twelve hour period. The twenty-fourth hour sampling of the other two groups showed a significant difference ($p<0.005$) between the LPS-Ur group (157.0 ± 12.8 μg/m*l*) and the U-LPS-U group (192.2 ± 7.6μg/m*l*). At the last sampling day there was no difference between the U-LPS-U groups (202.5 ± 30.9 and 165.4 ± 46.8 μg/m*l* respectively). Comparative graph is shown (**Fig. 2**).

WBC counts: (**Table 3**) In regards to the WBC counts (8) at the 10 minutes of experiment, the LPS group (10350 ± 139/cu mm), and the U-LPS-U group (8200 ± 75/cu mm) showed a difference ($P<0.005$) among the LPS-Ur group

Table 3. Comparative WBC values between all the groups at the experiment period.

PERIOD	LPS	U-LPS-U	LPS-UR
Basal	12771±3914	11875± 4962	11511±4754
10 min	10350± 139	8200± 750	18300± 800
1 hr.	10800± 810	10400± 750	15800± 700
3 hrs.	7200± 810	10300± 130	8855± 393
6 hrs.	21100± 810	13100± 750	9611± 780
24 hrs.	+	18525± 3875	16062± 417
7 day.	+	22287±10448	20966±3101

Values are /cu mm.
+ Rat died before this sampling.

Table 4. Comparative values of neutrophils between all the groups at the experiment period.

PERIOD	LPS	U-LPS-U	LPS-UR
Basal	24.0±6.0	24.0± 6.0	24.0± 6.0
10 min	21.0±0.1	36.0± 2.5	27.0± 0.1
1 hr.	20.0±0.6	82.0± 1.4	49.0± 0.7
3 hrs.	82.6±4.0	87.7± 4.8	48.3± 4.8
6 hrs.	78.0±1.8	87.6± 1.4	95.0± 2.1
24 hrs.	+	81.6± 1.2	77.2± 1.2
7 day.	+	34.8±10.0	37.6±11.0

Values are /cu mm.
+ Rat died before this sampling.

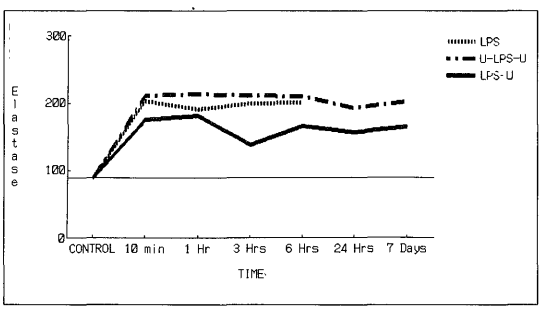

Fig. 2. Comparative graph of the elastase values (ug/ml) among the three groups, showing the lowest values for the LPS-Ur group.

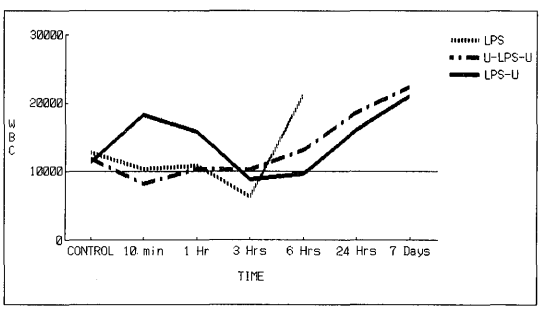

Fig. 3. Comparative graph of the WBC counts among the three groups. The highest counts were at the 10 minutes sampling for the LPS-U group.

(18300 ± 80/cu mm). The LPS-Ur group was 1.76 folds of the LPS groups and 2.23 folds of the U-LPS-U group. At the first hour sampling WBC counts were higher in the LPS-Ur group (15800 ± 70/cu mm) than in the LPS and the U-LPS-U groups (10800 ± 281 and 10400 ± 175/cu mm respectively); at the third hour sampling the WBC counts did not show difference among all the groups. The sixth hour sampling values of all the groups showed a significant difference, 1.6 fold between Group LPS (21100 ± 81/cu mm) and Group U-LPS-U (13100±75/cu mm); and a 2.1 fold ($p<0.005$) between Group LPS and Grouop LPS-Ur (9611 ± 78/cu mm). The other two samples showed slightly different values between the U-LPS-U and the LPS-Ur groups, without statistical significance, lesser for the latter. The comparative graph is shown (**Fig. 3**).

Neutrophils: (**Table 4**) The U-LPS-U group

shows higher percentages of neutrophil (36.6 ± 2.5%) than the other groups. At the 10 minutes sampling the LPS-Ur groups rose 2.2 fold towards the first hour sample (82.0 ± 1.4%). After this time the values did not show significant differences until the seventh day sampling where it approached the 10 minutes period values (34.8 ± 10.0%); The LPS group began with the lowest values (21.0 ± 0.1%) of neutrophils, and continue the same at the ten minutes as the first hour percentages. At the third hour sampling the LPS group (82.6 ± 4.0%) rose to 1.7 folds of the LPS-Ur group (48.3 ± 4.8%) ($p<0.005$) without significant difference compared with the U-LPS-U group (87.7 ± 4.8%). At the sixth hour sampling the remainder of the test did not show difference between U-LPS and LPS-Ur groups.

After this the LPS group rats died (between 7 and 11 hours postendotoxin). The comparative graph is shown (**Fig. 4**).

Hb and Hct: There was no difference among all groups concerning this matter. It was tested to see if any change occur due to the sampling methods. The Hb. concentrations ranged from 10.5 to 17.8 gr/cu cc, and Hct range from 31.4 to 51.4 ge/cu cc without any relation with other tests.

Platelet counts: There was no significant change in platelet counts, some dimishment of values was found in the LPS-Ur group, 125,000/cu mm. At the third hour sampling the LPS group and the U-LPS-U group had 415,000/cu mm and 543,000/cu mm respectively.

Clinical findings: After LPS endotoxin injection to the rats we could observe piloerection, diarrhea, tachypnea and mucosal bleeding[9]

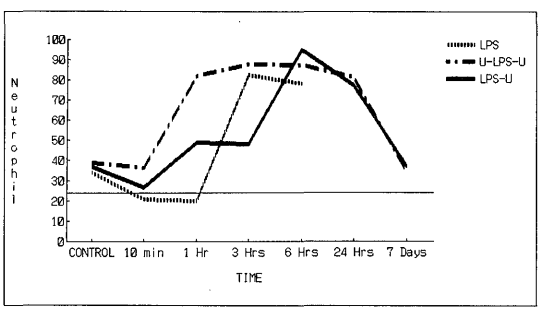

Fig. 4. Comparative graph of the neutrophils among the three groups.

Histological finding: Specimens of the lungs, kidneys and liver were examined histologically. The LPS group showed a marked thickening of the alveolar wall and congestive small vessels, neutrophil infiltration in the parenchyma surrounding the alveoli (**Fig. 5a**). The U-LPS-U group showed thickening of the alveolar wall with neutrophil infiltration. Intraalveolar congestion and edema[2] was also observed (**Fig. 5b**).

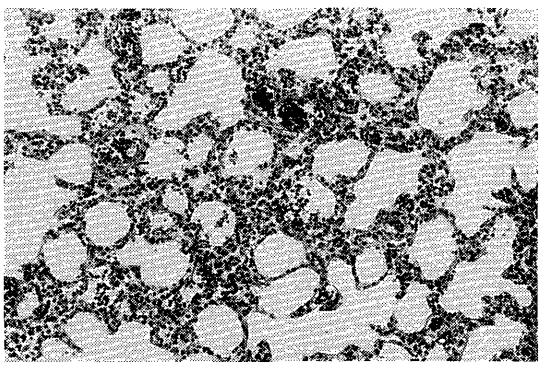

Fig. 5a.

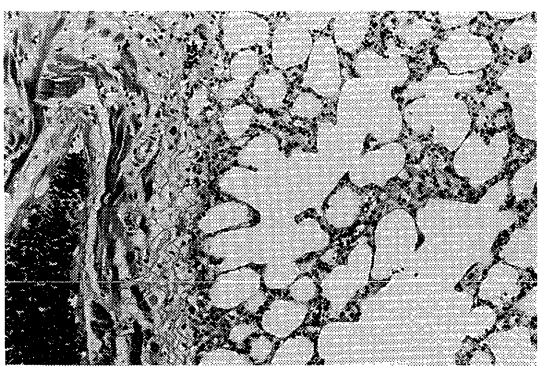

Fig. 5b.

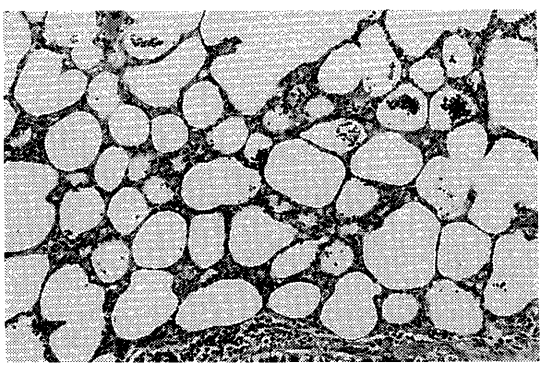

Fig. 5c.

The LPS-Ur group did not show significant changes near the alveoli but we could see neutrophil peribronchiolar infiltration. The parenchyma had no pathological changes, except some focal infiltration (**Fig. 5c**).

The liver histological slices of the LPS group showed, sinusoidal congestion and hepatic extravasation without inflammatory signs (**Fig. 6a**). The U-LPS-U and the LPS-Ur groups did not show sinusoidal congestion but perivasculitis in the latter (**Fig. 6b** and **6c** respectively).

Concerning the histological slices of the kidneys the LPS group showed massive acute tubular necrosis[9], and the glomerular portion congestion without necrosis (**Fig. 7a**). The U-LPS-U group showed slight tubular necrosis and clear congestion in the glomerular portion (**Fig. 7b**). The LPS-Ur group showed slightly

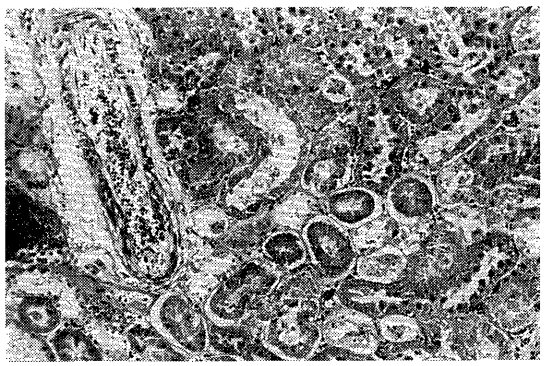

Fig. 6a.

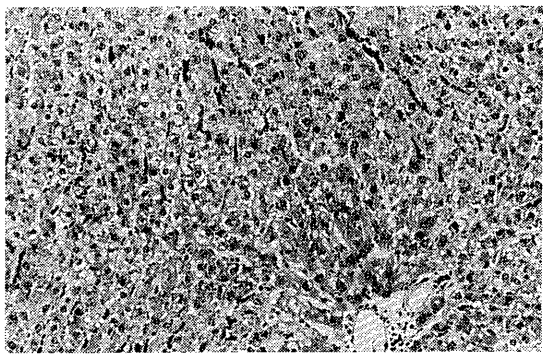

Fig. 7a.

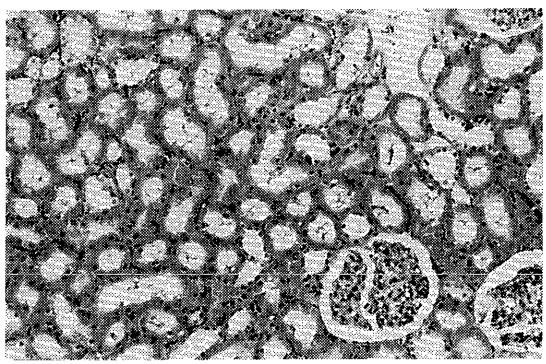

Fig. 6b.

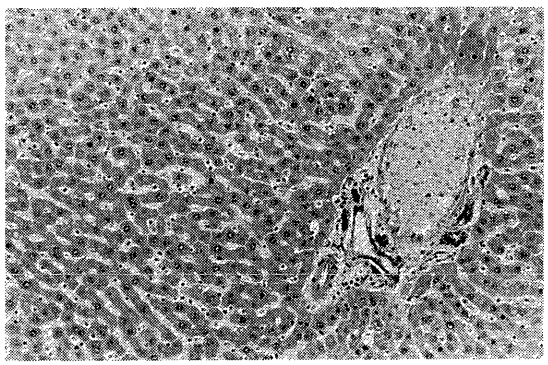

Fig. 7b.

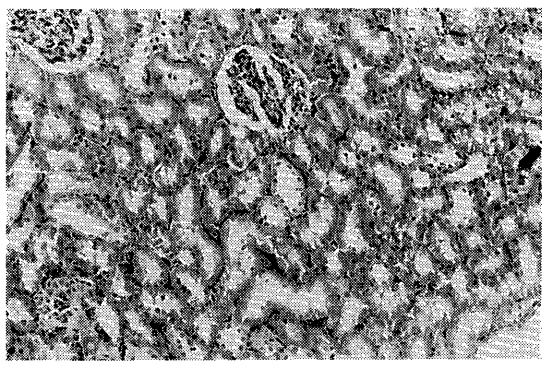

Fig. 6c.

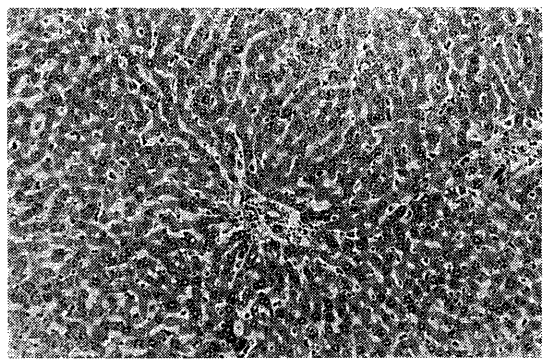

Fig. 7c.

tubular necrosis as glomerular congestion (**Fig. 7c**).

DISCUSSION

It is well known that tissue and intravascular elastase activity increase after endotoxin administration and under surgical stresses[7]. Increased activity of the elatase was attributed to the neutrophil migration to the lungs and increased pulmonary vascular resistance[10][11]. The present study demonstrated that the serum collected from the endotoxin treated rats in different intervals between 10 minutes and 7 days after endotoxin injection varied by the influence of glycoprotein inhibitor Urinastain. As shown in the results of the LPS-Ur group, it was had lower activity than the other two groups, but reached the nadir at the third hour samping ($p<0.005$). Recently Strieter[16] reported that endothelial cell gene expression of a neutrophil chemotactic factor was enhanced by TNF-alpha, LPS, IL-1. Based on this fact, endotoxic cell gene expression may be facilitated and elastase activity will be greatly activated in endotoxic shock.

The data indicated that the neutrophils can be activatied by endotoxin[5] and produce elastase and other mediators which are responsible to the elastin degradation in many organs consisting elastic fibers.

The experiment was continued until rats died spontaneously. We had chosen the elastase activities as an indicator of elastin degradation and shock severity.

The data of the present experiment allowed us to observe that values such as 190 $\mu g/ml$ and higher are hazardous. The LPS group rats died during this elastase activity phase. Furthermore, the WBC counts and the neutrophil percentage were compared with elastase activity to find some relationships. Pulmonary edema was seen as the result from the complement activation through the anaphylatoxin generation which produces high vascular permeability[12].

Elastase activity increased in the organs such as the lung and the kidney caused damages, it is a consequence of generation of chemotactic factors that result in migration PMN cells into an area of inflammatory response[15] which serves to protect the host from invasion, or injure the host by causing extensive destruction of normal tissue[9]. These changes have been observed in the histological specimens. When these cells break down they release lysosomal enzymes that are proteolytic and destroy tissues[12], specifically the elastase released from the neutrophis.

The results obtained in this study lead us to think about the possible advantages of the use of Urinastatin.

The metabolic pathway of this glycoprotein inhibitor remains unclear[14], but some hypothese could be that the Urinastatin plays a role in protecting neutrophil walls during the acute phase of the stress without permitting the elastase releasing from the neutrophils until the stress period becomes stable. The stress period means the twelve hours from the administration of the LPS endotoxin. None of the rats of the LPS group remained alive during this period due to the endotoxic shock. The other two groups survived for at least three days, but some of the rats in the U-LPS-U group died later as observed in the results of this experiment. It means 70.6% survival for this group compared with the 100% survival of the LPS-Ur groups. This significant difference was caused by the different method of Urinastatin administration to the U-LPS-U group as recommended by the manufacturer to give. The total dose of 50000 IU was injected in two divided doses, 10 minutes before and 10 minutes after the endotoxin administration (25000 IU at each time). To the LPS-Ur group the total dose of 50000 I. U. was given at one time. It means that the half dose prior to the LPS endotoxin administration is not enough to give a "neutrophilic wall protection" such as one whole dose as in the LPS-Ur group. As Haniuda et al. suggested, it could be administered after major surgery, and shock[7].

As a consequence of this hypothesized "neutrophil wall protection" elastase was released to the organs where the neutrophil migration has happened (in this case, to the lungs) and the adequate protection was corroborated by the pathologic findings.

The Elastase levels could be used as a parameter of disease severity[4] and lungs compromise such as alveoli wall thickening,

edema emphysema, and finally to the well-known ARDS. Therefore, this the therapeutic use of Urinastatin glycoprotein inhibitor is of great value to avoid tranferring ARDS by post-stress administration as soon as possible after shock and surgery, specifically lung surgery.

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