ARTIGOS

THE HEMORRHAGIC SYNDROME OF LEPTOSPIROSIS: AN EXPERIMENTAL STUDY IN GUINEA PIGS

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The hemorrhagic syndrome of leptospirosis was studied in guinea pigs. The study correlates hematological, histopathological and immunohistochemical alterations in sixty animals inoculated by the intraperitoneal route with 1ml of the culture of virulent strain of Leptospira interrogans *serovar* copenhageni. *Leptospirae antigens were detected by immunoperoxidase, chiefly in liver, kidney and heart muscle capillaries. Possible pathogenic mechanisms responsible for hemorrhagic syndrome are discussed with emphasis on toxic and anoxic attacks causing damage to endothelia, platelet depletion and alterations to hemostasia rates: prothrombin time [PT], partial thromboplastin time [PTT] and fibrinogen concentrations. The clinical-laboratory picture is compatible with the histopathological observation of disseminated intravascular coagulation [DIC] in most of the guinea pigs from day 4 of infection.*

Key-words: Leptospirosis. Hemorrhagic syndrome. Platelet depletion. Immunoperoxidase. Disseminated intravascular coagulation. DIC.

This serial study aimed to establish a dynamic physiopathological profile of the hemorrhagic phenomena that occur in leptospirosis. The construction of such a profile was made feasible by the adoption of an experimental model that could adequately reproduce the morbidity found in humans.

Our experimental approach was justified by the present lack of conclusive information in the literature, most of which is taken up with results obtained from biopsies and necropsies. Such findings do not always provide an adequate view of physiopathological phenomena[8]. Notwithstanding the pioneering experimental investigations by Arean[2] and Arean & Henry[3], and more recently by Higgins & Cousineau[16] and Alves et al[1], all of which are based on the observation of animals infected with a virulent strain of lepstospira, research on the hemorrhagic syndrome has generally been limited in its approach; hence our decision to carry out a study involving a more complete evaluation of the organic components that are most frequently damaged in leptospirosis. Our aim was to answer certain key questions relating to the human disease, which continues to be characterized by high mortality rates due to the frequent occurrence of hemorrhagic diathesis[12 23 24 25].

MATERIALS AND METHODS

The study covered hematological, histopathological and immuno-histochemical alterations related to the hemorrhagic syndrome in Guinea pigs infected with a virulent strain of *Leptospira interrogans* serovar copenhageni. Seventy animals, divided into seven groups of ten animals each, were killed after blood collection. One group (group 0 - control) was intra-peritoneally inoculated with 1ml of sterile Fletcher medium, while the six other groups (groups 1 - 6) were inoculated with 1ml of leptospira culture, i.e. Fletcher medium containing between 10^7 and 10^8 microorganisms per ml and sequentially killed from the first (group 1) to the sixth day (group 6) after inoculation.

The following lab tests were carried out on all the animals: prothrombin time [PT], partial thromboplastin time [PTT], platelet count and fibrinogen concentration. In addition, all the animals were autopsied, with removal of fragments of heart, lung, kidney, liver and gastrocnemius muscle for morphological and immunohistochemical analysis.

In our histopathological analysis the following techniques were used: hematoxylin-eosin, phosphotungstical hematoxylin, Masson's trichrome, Gomori's reticulin, PAS, Jones' silver and Warthin-Starry impregnation stain (Manual of Histologic and Special Staining Techniques, 2nd Ed. 1964 - McGraw Hill Book Company, Inc. NY).

For the detection of *L. icterohaemorrhagiae* antigen using the avidin-biotin-peroxidase method, we employed the Vectastain ABC Peroxidase (Rabbit IgG) kit from Vector Laboratories Inc. - USA (ref. nº PK-4001).

The statistical significance of our results was assessed by "t" testing at $p < 0.05$.

RESULTS

Groups 1 and 2 displayed slight alterations in their hematological and anatomopathological parameters, most notably with an increase in prothrombin time and with capillary congestion in lung, heart and gastrocnemius muscle.

In group 3, PT continued to be greater than in the control group, and was associated with an increase in PTT and a reduction in the platelet count and fibrinogen concentration. Capillary congestion was accentuated in lung, heart, liver, kidney and gastrocnemius muscle, and hemorrhage foci were also found, chiefly in lung, liver and gastrocnemius muscle. Intravascular coagulation foci were detected in liver, gastrocnemius muscle and kidney, and scattered foci of coagulation necrosis were observed in liver. Immunoperoxidase assays were slightly positive in liver, heart, kidney (vascular lumen and interstitium) and gastrocnemius muscle, with parasite antigen appearing mainly in a fibrillar form.

In group 4, PT and PTT values remained high, with marked platelet depletion but normalized fibrinogen concentrations. Histopathological alterations were aggravated by an intensification of the congestion, hemorrhage and intravascular coagulation already observed in heart, lung, liver, kidney and gastrocnemius muscle. Coagulation necrosis appeared to be more intense, spreading out in a multifocal form. Immunoperoxidase assays continued to be positive in heart, liver, kidney and gastrocnemius muscle.

In hematological terms, group 5 was similar to group 4, with marked platelet depletion, increased PT and PTT, and reduced fibrinogen concentrations. The histopathological picture, meanwhile, was further aggravated by increased congestion, hemorrhage and intravascular coagulation in all the tissues examined. Coagulation necrosis also increased, appearing in a multifocal form in liver. Immunoperoxidase results were intensified in liver, kidney, heart and gastrocnemius muscle, with parasite antigen appearing in filamentary and granular forms.

In group 6, the PT, PTT and fibrinogen values returned to normal, while the platelet count remained low. The histopathological profile was modified, with a reduction in intravascular coagulation; congestion and hemorrhage, however, remained intense in all the tissues examined. The immunoperoxidase assays were intensely positive, with the same appearance as in group 5.

The average values of the parameters used for hemostasis evaluation are graphically expressed in Figures 1 to 4, and histopathological alterations are shown in a comparative histogram (Figure 5).
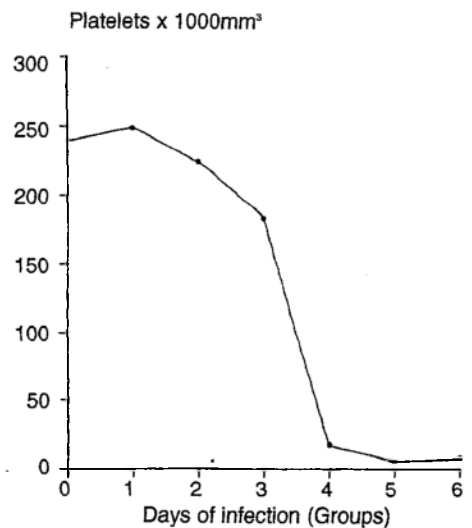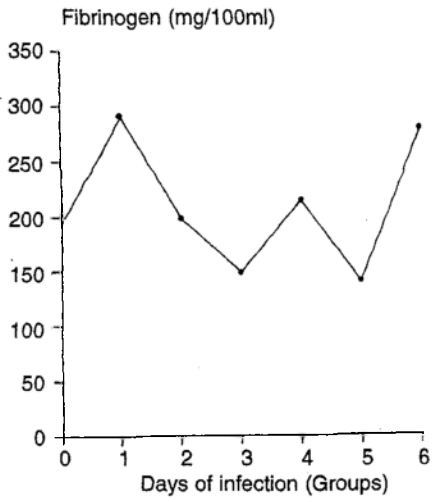

- normal Guinea pigs (Group 0)
- Guinea pigs inoculated with *Leptospira* (Groups 1 to 6)

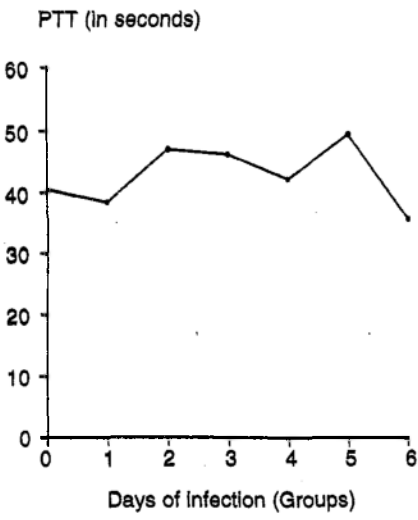*Figure 1 - Average platelet count in Guinea pigs.*

Prothrombin time (in seconds)
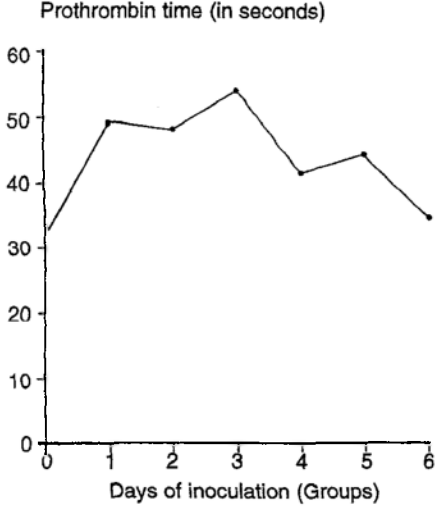
- normal Guinea pigs (Group 0)
- Guinea pigs inoculated with *Leptospira* (Groups 1 to 6)

Figure 2 - Average prothrombin time in Guinea pigs.

Fibrinogen (mg/100ml)

- normal Guinea pigs (Group 0)
- Guinea pigs inoculated with *Leptospira* (Groups 1 to 6)

Figure 4 - Average fibrinogen concentration in Guinea pigs.

PTT (in seconds)

- normal Guinea pigs (Group 0)
- Guinea pigs inoculated with *Leptospira* (Groups 1 to 6)

Figure 3 - Average PTT in Guinea pigs.

Intensity of alterations

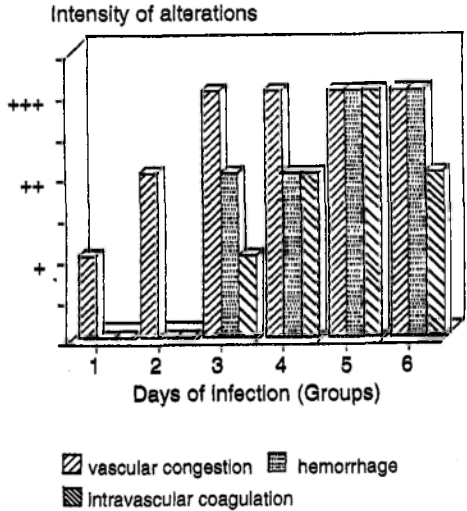▨ vascular congestion ▦ hemorrhage
▧ intravascular coagulation

Figure 5 - Evolution of histopathological alterations in Guinea pigs inoculated with Leptospira.
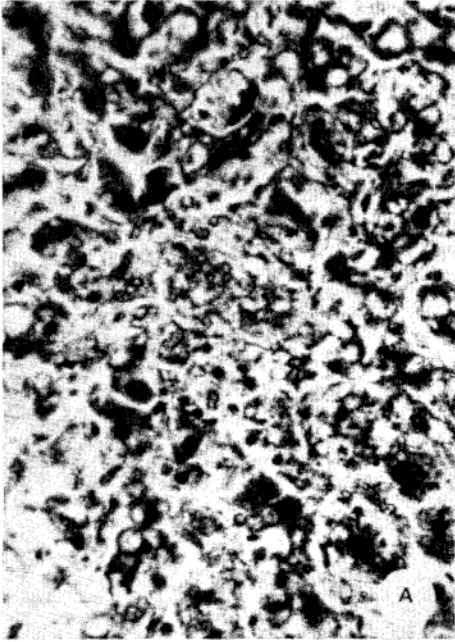
Figura 6A - Central area of coagulation necrosis. Masson's trichrome. 400 X.

Figura 6C - Coagulation necrosis and hemorrhage within cardiac muscle fibers. Hematoxylin-Eosin, 400 X.
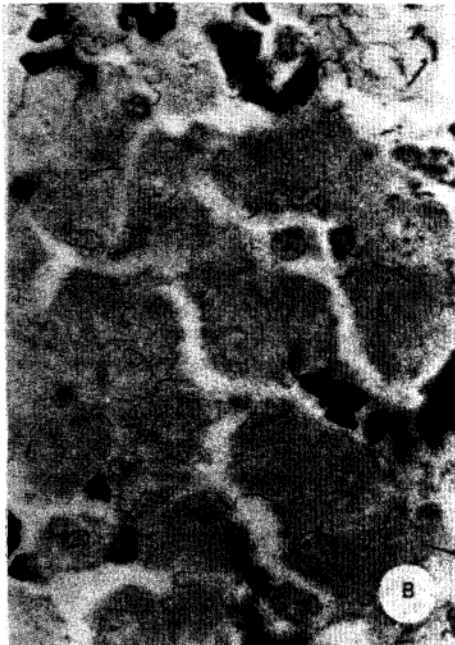

Figura 6B - Large numbers of leptospirae permeating sinusoids. Warthin-Starry, 1000 X.
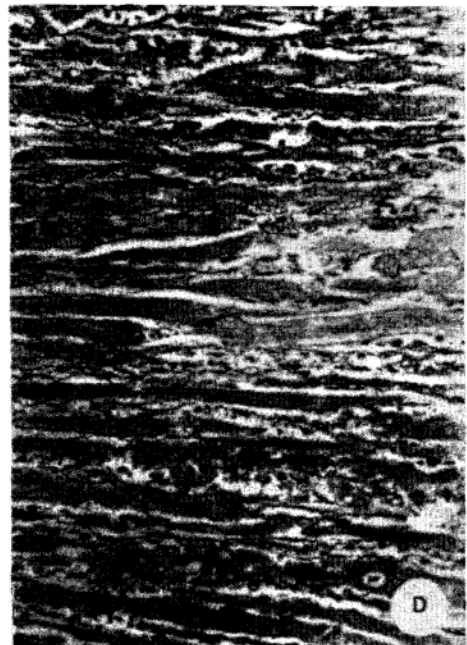

Figura 6D - Extensive areas of necrosis of gastrocnemius muscle fibers. Masson's trichrome, 200 X.
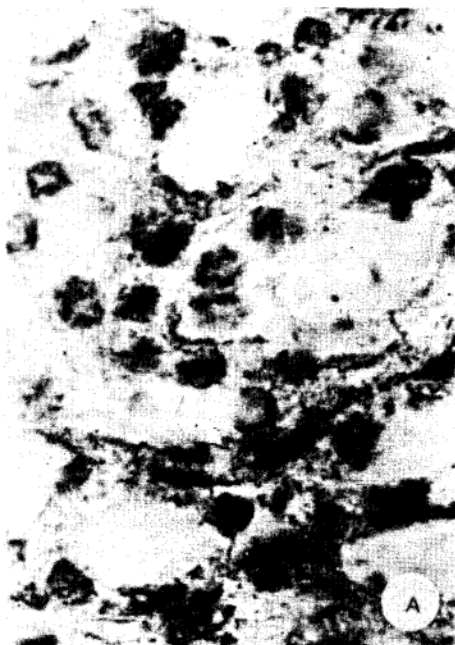
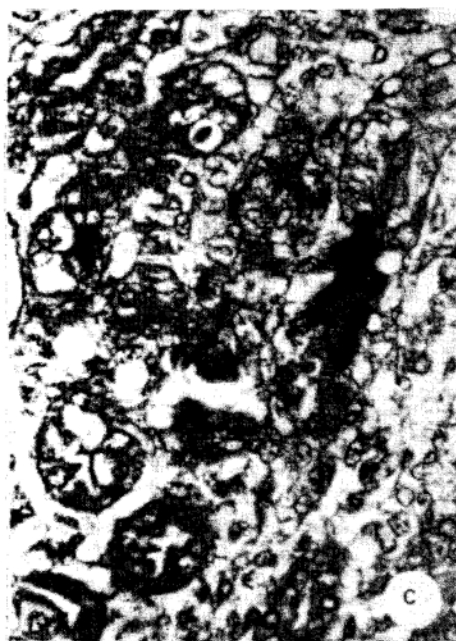Figura 7A - Positive antileptospira response in peritubular capillary. Avidin Biotin Peroxidase. 1000 X

Figura 7C - Fibrin thrombus in peritubular capillaries. Masson's trichrome, 400 X.

Figura 7B - Enlargement of interalveolar septa by congestion and extravasation of red blood cells from the capillaries. Masson's trichrome, 400 X.

DISCUSSION

Our systematic observation of guinea pigs sequentialy killed from the first to the sixth day after inoculation with a virulent strain of leptospira, provided us with a clear picture of the kinetics of this disease.

On the first two days (groups 1 and 2), the animals did not exhibit a significant clinical picture, a finding that was compatible with our observations of only slight visceral impairment in the form of vascular congestion, mainly in lung, heart and gastrocnemius muscle.

The attack on the cappilary system begins by causing mild disturbances in terms of permeability, and finally culminates in endothelial necrosis[5,6]. This process, Alves et al[1] suggest, probably results from the direct action either of the entire leptospira, or of its degradation products. Thomas and Higbie[30] confirm the direct involvement of pathogenic leptospirae on the basis of their capacity to attach themselves to the cells, thereby damaging them. More recently, De Brito et al[7], employing immunoelectromicroscopy techniques, have demonstrated the affinity of leptospira antigenic material for the host cellular membrane, suggesting a probable

interaction with cellular surface proteins prior to attack.

The third day after inoculation (group 3) represents a dividing line between the first stage (involving mild and relatively non-specific manifestations) and the second stage (characterized by important clinical, pathological and hematological alterations). The platelet count begins to decline, accompanied by an increase in PT and PTT and a reduction in fibrinogen levels. Although clinically without hemorrhage, in histopathological tests the animals exhibited accentuated vascular congestion, extravasated red blood cells and slight deposits of fibrin in capillary blood vessels in liver, kidney and muscle.

Among these histopathological phenomena, congestion was the most constant, mainly in liver, kidney and lung. We believe that this congestion precedes and is associated with the vascular damage that leads to extravasation of red blood cells to the interstitium[15]. Other authors, such as Higgins and Cousineau[16] and more recently Venugopal and Ratnam[30], have emphasized this histopathological pattern, linking it to the hemorrhagic diathesis that is characteristic of serious forms of human leptospirosis[10 23].

Another significant finding was the detection (via immunoperoxidase testing) of leptospira antigens with a filamentary or granular aspect in liver sinusoids, kidney interstitium and tubules, heart tissue and gastrocnemius muscle. This finding opens new perspectives for understanding the physiopathogenic pathways of the disease, an area that is still far from being clarified[1 6 11]. The predominance of these antigens in capillaries reinforces the idea that they play an important role in the causation of hemorrhagic phenomena.

In summary, the platelet depletion, the reduced fibrinogen levels and the increases in PT and PTT, all of which are associated with a clinical picture compatible with obstruction of microcirculation[28], lend weight to the hypothesis that DIC is probably responsible for the hemorrhagic phenomena which increasingly occur as the disease runs its course.

On the other hand, Laing[20] describes acute platelet depletion associated with microangiopathy and slight alterations to coagulation factors, a condition that he identifies as thrombotic thrombocytopenic purpura.

The essential fact is that all these phenomena lead to thrombocytopenia, which appears to be a constant feature of leptospirosis and which plays a central role in the causation of hemorrhagic syndrome[14 16 21].

We have chosen to focus on this syndrome not only because of its intensity and constancy in the human disease, but principally because of the possibilities of its reversal through therapy.

From an anatomopathological point of view, a significant finding in our study was the occurrence of coagulation necrosis, mainly of liver tissue. In our opinion, the intensification of this necrosis from the 3rd day (group 3) conforms with the toxic-anoxic picture that is also characteristic of serious forms of the human disease[24 26]. The coagulation necrosis observed in liver (which is different from the descriptions in the literature of isolated necrosis) destroys tissue to a variable extent, keeping a close relationship with the presence of fibrin thrombi, and of fibrillar and granular-formed parasite antigen.

This type of necrosis, which has not been previously observed in leptospirosis, exacerbates the endothelial damage, possibly playing a secondary role in relation to the direct action of the parasite or its toxic products. The latter, besides causing vascular obstruction, could trigger the DIC process[9 17 22].

The animals' morbidity on the 4th day (group 4) was patently more advanced than in previous groups, with more serious clinical pathological findings, mainly in the form of increased hemorrhagic phenomena in lung, kidney and gastrocnemius muscle, accompanied by intravascular coagulation in lung, liver, kidney and gastrocnemius muscle. Coagulation necrosis was intensified, appearing in a multifocal form in liver. These lesions, which do not always contain parasites, suggest the participation of a leptospira-linked toxic factor in the view of Knight et al[19], Chaperon et al[4], Navarro and Kociba[22] and Vinh et al[31]. Hematological alterations are also more pronounced, with accentuated platelet depletion, high PT and PTT values and normalization of fibrinogen concentrations.

Despite the apparently paradoxical behavior of the fibrinogen, the overall pattern of alterations strengthens the hypothesis of DIC. The increase in, and subsequent normalization of fibrinogen levels may be due to a compensating mechanism that allows synthesis of fibrinogen, thereby offsetting its destruction; alternatively, it may be due to the mobilization of a tissue pool in response to the severe systemic attacks[27].

The 5th and 6th days (Groups 5 and 6) were characterized by the acute onset of the disease, with the guinea pigs displaying serious anatomopathological symptoms, such as tubular necrosis, intersticial nephritis, miocarditis, miositis, coagulation necrosis and intense hemorrhage in virtually all organs and tissues. A notable finding was the aggravation of coagulation necrosis in liver, with multifocal manifestations in about 80% of the animals. The overall clinical picture could be attributable to an obstruction of microcirculation, with all the accompanying symptoms observed in Weil's syndrome[10 24 25].

In group 5, platelet depletion was accentuated, accompanied by increases in PT and PTT and reductions in fibrinogen levels, a pattern fully compatible with DIC. On the sixth day (group 6), the platelet depletion persisted, but PT and PTT returned to normal, and fibrinogen concentrations increased. This pattern is consistent with the reduction in histopathological evidence of intravascular coagulation.

Our analysis of this complex array of lesions reveals a clinical picture in which hemorrhagic phenomena not only predominate, but also seem to be largely responsible for the onset of acute systemic impairment. Attacks on endothelia (probably of a toxic-anoxic nature) are provoked by the parasite or its products, acting either directly on host cells or indirectly via immunological pathways. In our view, any of these alternative mechanisms could be responsible for the triggering of the DIC process, whose central role has been clearly demonstrated in this study.

RESUMO

Os autores estudam a síndrome hemorrágica da leptospirose em cobaios inoculados com amostra virulenta de Leptospira interrogans *sorovar* copenhageni. *São abordados aspectos hematológicos, histopatológicos e imuno-histoquímicos. Aumento do tempo de protrombina (PT) e do tempo parcial tromboplastina (PTT), bem como redução do número de plaquetas e do fibrinogênio acompanharam-se de congestão vascular, hemorragias e coagulação intravascular no fígado, rim, coração, músculo esquelético e pulmão. O antígeno de* Leptospira *foi detectado principalmente em tecido hepático, renal e cardíaco. São discutidos possíveis mecanismos patogênicos responsáveis pela síndrome hemorrágica, com ênfase na agressão tóxico-anóxica do endotélio vascular, que propiciaria a instalação de coagulação intravascular disseminada (CIVD) observada, sobretudo, nos cobaios dos grupos 4, 5 e 6.*

Palavras-chaves: Leptospirose. Síndrome hemorrágica. Plaquetopenia. Coagulação intravascular disseminada. CIVD. Imunoperoxidase.

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