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Morphology of Fibrin and Fibrin-Platelet & Fibrin-Platelet-Leukocyte Clots

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Abstract

This article presents results of studies of the dynamics of formation and the histological structure of simple fibrin clots, fibrin clots containing platelets, and fibrin clots containing platelets and granulocytes with lymphocytes. It has been established that both the rate of formation, the rate of retraction, and the morphology of the clot depend on the number of platelets and other leukocytes contained in them. The rate of formation and the degree of retraction of the clot is higher the more platelets and other leukocytes are contained in the original plasma. The degree of density and structuring of fibrin fibers is also directly proportional to the described parameters.

Disciplinary: Veterinary Science, Biosciences.

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1 Introduction

In modern veterinary surgery, to accelerate and improve the healing of various tissue defects, preparations are widely used, prepared immediately before use, from the patient's blood [4; 6]. These are platelet-rich fibrin (Platelets Reach Fibrin (PRF)) and platelet-rich plasma (PRP - Platelets Reach Plasma). The difference between these biological preparations lies in PRP's suspension of platelets in plasma. Thus, fibrinogen is present and injected into the area of the pathological focus [1; 10]. And PRF is a fibrin clot with included platelets, which is placed directly in the area of tissue defect [4; 7]. It is believed that in both PRP and PRF, the main structural and

functional components responsible for their regenerative effect are platelets, which contain a large number of growth factors and release them into the surrounding tissues during activation [1; 7; 10].

Currently, scientific journals and especially specialized Internet sites, due to certain trends, abound in publications that present only positive and no negative effects when using PRP and PRF [6; 7; 11]. However, more and more publications are more critical about applying these methods and indicate the instability of their clinical effects [8; 9; 10]. Including indicates both weak or complete absence of a positive effect and negative cases [5; 12].

There is reason to believe that the failure of PRP and PRF may be due to both the loss or insufficient concentration of platelets during their isolation from the blood by centrifugation and the production of platelet plasma containing a significant admixture of granulocytes and lymphocytes. To a greater extent, this applies to PRF. If using PRP, its quantitative and qualitative cellular composition can be checked on a hematological analyzer before the procedure. In that case, the classical method for obtaining PRF involves forming a clot by natural coagulation from unstabilized plasma after its rapid separation from erythrocytes by centrifugation. Accordingly, it is impossible to determine the qualitative and quantitative cellular composition in an already formed clot. And with a high degree of probability, the clots obtained in this way in each case will differ from each other in cellular composition. That is, in one case, the clot may not contain platelets at all (PF – clot). The other contains a small number of platelets (PPF-clot). The third contains a large number of platelets (PRF-clot). And in the fourth, together with platelets, granulocytes with leukocytes (PRLcF-clot) may be present. Therefore, it is logical to assume that the difference in the cellular composition of the clots should be reflected in the effect these clots will have on the cells located in the zone of the pathological focus [2; 3]. This influence, and more precisely the mutual influence of the clot and the surrounding tissue of the pathological focus, should depend, inter alia, on subtle differences in the microstructure of clots with different cellular compositions. These studies (microstructure) have not been found in the available scientific literature.

The purpose of this study is to investigate the morphological features of fibrin clots depending on the presence of platelets and other leukocytes in them. Research objectives are to 1) select a method for obtaining pure fibrin clots, clots enriched only with platelets, as well as clots enriched with platelets together with lymphocytes and granulocytes; 2) investigate the macroscopic and microscopic differences in the structure of cell-free fibrin, fibrin-platelet, and fibrin-platelet-leukocyte clots.

2 Materials and Methods

In the study, 20 Wistar rats were used. Blood in the amount of 1 ml was obtained from the hearts of animals previously anesthetized with sevoflurane. In addition, 3.8% sodium citrate in an amount of 0.125 ml per 1.0 ml of blood was used as an anticoagulant. Blood plasma, pure or containing platelets with leukocytes, was obtained by centrifugation at different speeds (revolutions per minute) in an OPN-3 centrifuge. The counting of cells in whole blood and variants

of the obtained plasma was carried out on an automatic hematological analyzer MEK-6550. The obtained cell-free and cellular (aligned by the number of platelets and leukocytes) plasma samples in a volume of 0.5 ml were transferred into 1.5 ml Eppendorf tubes. To each tube was added 25 µl of 10% calcium chloride to restore the function of hemocoagulation. The tubes were placed in a water bath at 37 ° C until a full-fledged clot was formed. The formed clots were removed and placed in a 10% formalin solution prepared in phosphate buffer pH 7.4. From clots, histological preparations with a thickness of 4-5 µm were prepared, some of which were stained with hematoxylin-eosin for observation microscopy, and some, according to Van Gieson, for fibrous structures. Microscopic examination was carried out at a magnification of 650 and 1500 times on a Lumam I-2 microscope equipped with a 5-megapixel digital camera. The images were viewed and digitally recorded on a personal computer with the Scope Photo Imag eSoftware Ver 3.1.386, 2009 software. The data were statistically processed on a personal computer using the BioStat Professional 2007 software. 5.1.1.

3 Result and Discussion

A priori, it is clear that unstabilized blood is not suitable for the presented research purposes, since only either a simple blood clot or a non-erythrocyte fibrin clot can be obtained from it (if the blood is quickly placed in a centrifuge immediately after collection) but with an unknown platelet-leukocyte composition. Preliminary studies have shown that plasma samples obtained from blood stabilized with heparin and trilon-B (EDTA) are unsuitable. Because even after the introduction of anticoagulant activity inhibitors such as protamine or calcium chloride. Fibrin clots or do not form at all, or macromorphologically, differ significantly from clots formed from unstabilized plasma.

According to the results obtained, the optimal source for the preparation of fibrin clots with a given platelet-leukocyte composition is citrated plasma (0.5 ml of 3.8% sodium citrate per 4.0 ml of blood). The qualitative and quantitative cellular composition of such plasma is easily determined on a hematological analyzer. The coagulation properties of the plasma are easily restored by adding 25 µl of 10% calcium chloride for every 0.5 ml of plasma. Fibrin clots obtained from such plasma are macromorphologically similar to fibrin clots prepared from blood plasma unstabilized by anticoagulants.

Routine studies on selecting optimal methods for obtaining blood plasma containing a certain cellular composition have shown the following. Completely cell-free blood plasma is obtained by centrifugation at 3000 rpm for 15 minutes.

Blood plasma containing only platelets and not containing leukocytes (or containing only trace amounts) and erythrocytes is obtained only with low-speed (1000 rpm) centrifugation for 10 minutes. In this case, all erythrocytes are deposited, leukocytes form a film on the surface of the erythrocyte sediment, and platelets remain in the supernatant plasma. It should be noted that with this method, it is possible to obtain plasma in a volume that is only 20% - 28% of the total volume of whole blood. But the number of platelets in such plasma is equal to or slightly higher than in

whole blood. Increasing the centrifugation time simultaneously increases the relative volume of plasma obtained. But it reduces the number of platelets in plasma. Increasing the speed of centrifugation also increases the volume of plasma produced. But platelet loss is even more pronounced.

A series of routine studies on selecting a method for obtaining plasma containing leukocytes showed that the best option was the one in which the erythrocyte-leukocyte sediment remaining after the isolation of platelets was thoroughly mixed and placed in tubes with a separating gel designed to obtain serum. The tubes were centrifuged at 1500 rpm for 10 minutes and additionally at 3000 rpm for 30 seconds. Additional centrifugation at 3000 rpm is required for the erythrocytes to pass through the separation gel. At lower centrifugation speeds, the erythrocyte sediment remains above the gel, making it impossible to completely and cleanly collect leukocytes. They are much heavier than platelets and are always located in a small layer at the plasma/erythrocyte sediment or plasma/separation gel interface. Plasma yield with this method is significantly lower since part of the plasma has already been taken when receiving platelets. But the quantitative and qualitative composition of leukocytes in plasma samples is close to the quantitative and qualitative composition of whole blood. However, it should be noted that in plasma obtained in this way, there is also a significant number of platelets and a certain amount of erythrocytes. And in this work, we have not found a method for obtaining leukocyte plasma without an admixture of platelets. The use of not erythrocyte sediment, but whole blood in a similar mode of centrifugation, paradoxically reduced the number of leukocytes in the plasma but slightly increased the number of platelets.

Studies on obtaining clots from citrated plasma have shown that the time during which coagulation and retraction occur significantly differs depending on whether there are platelets and leukocytes in the plasma. Accordingly, the minimum velocity and the maximum time of clot formation and retraction are observed in completely cell-free plasma. Conversely, the maximum speed and, accordingly, the minimum time of formation and retraction of clots is observed in plasma rich in platelets and leukocytes (Table 1).

Table 1: Time of formation of a clot from recalcified blood plasma, depending on the quantitative and qualitative composition of leukocytes

Object of study	Approximate time of the beginning of coagulation, min	Approximate time of clot formation, min	Approximate clot retraction time, min
Cell-free plasma	Difficult to define *	138.00±81,06	absent
Low platelet plasma	18.00±5.70	47.00±16.04	retraction is poorly expressed
(≤10 × 103 / μL)	4.80±1.92	12.60±7.83	53.20±9.20
Plasma with a high platelet count	1.32±0.30	5.80±2.80	42.00±11.51

* - difficult to determine because the clot formation time is very long.

Fibrin, fibrin-platelet, fibrin-platelet-leukocyte clots differ both in appearance and in the degree of retraction. The highest degree of retraction and, accordingly, the smallest size after it was observed in fibrin-platelet-leukocyte clots. Acellular fibrin clots were practically not retracted. In fibrin-platelet clots, the degree of retraction was intermediate (Figure 1).

Microscopic examination of histological preparations prepared from clots showed the following.

1. In the acellular fibrin clot, fibrin filaments are located mostly unstructured, forming a coarse-mesh network (Figure 2).

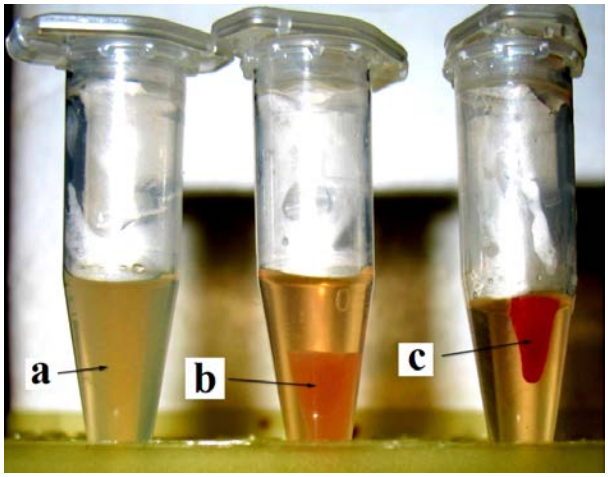

Figure 1: Macromorphology of fibrin clots with different platelet-leukocyte compositions. a - acellular fibrin clot. The retraction of the clot is not pronounced; b - fibrin-platelet clot. Clear retraction of the clot; c - fibrin-platelet-leukocyte clot. Very strong retraction of the clot.

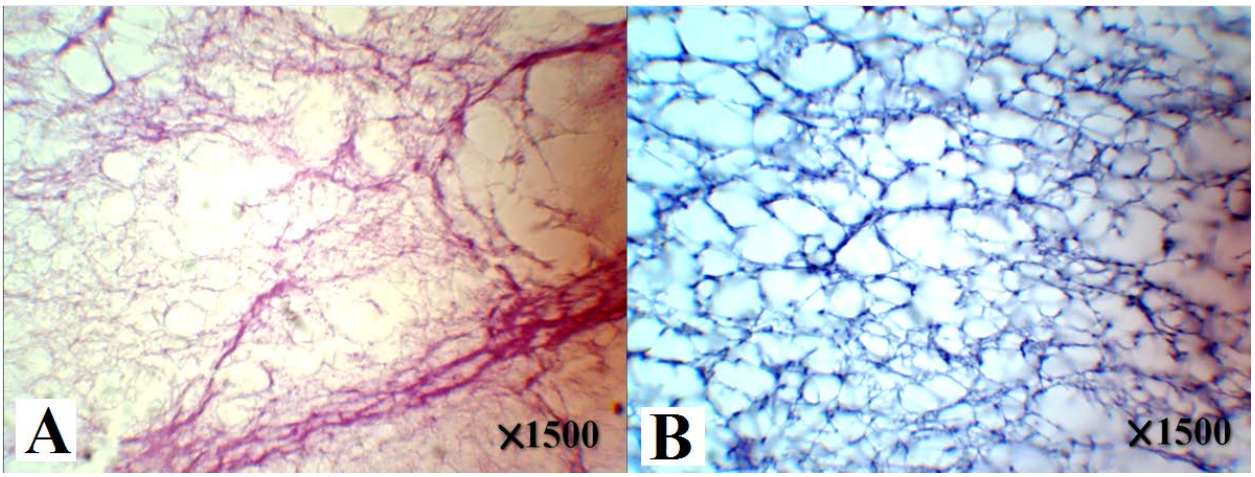

Figure 2: Structure of acellular fibrin clot. A - dyeing for fibers according to Van Gieson; B - hematoxylin-eosin stain.

2. The structure of a clot containing single platelets is heteromorphic. Among the unstructured coarse-meshed network of fibrin fibers, areas resembling a cobweb are visualized, in the center and the nodal junctions of platelets' accumulations (Figure 3).

Among the unstructured coarse-mesh network of fibrin fibers (1), areas resembling a cobweb are visualized (2), in the center and in the nodal junctions of which there are accumulations of platelets (3).

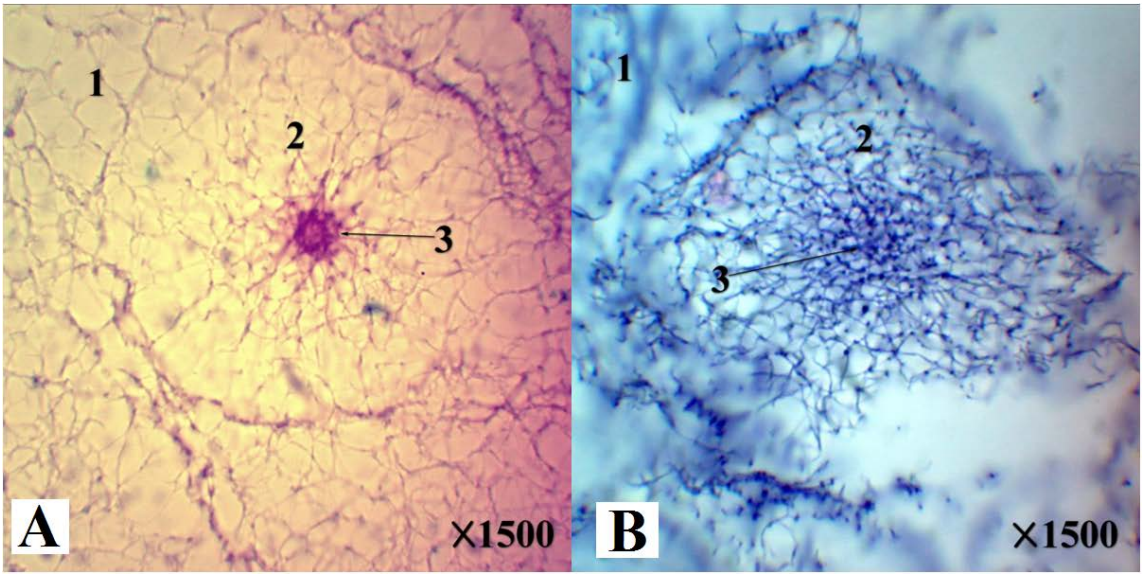

Figure 3: Structure of a clot containing single platelets. A - dyeing for fibers according to Van Gieson; B - hematoxylin-eosin stain.

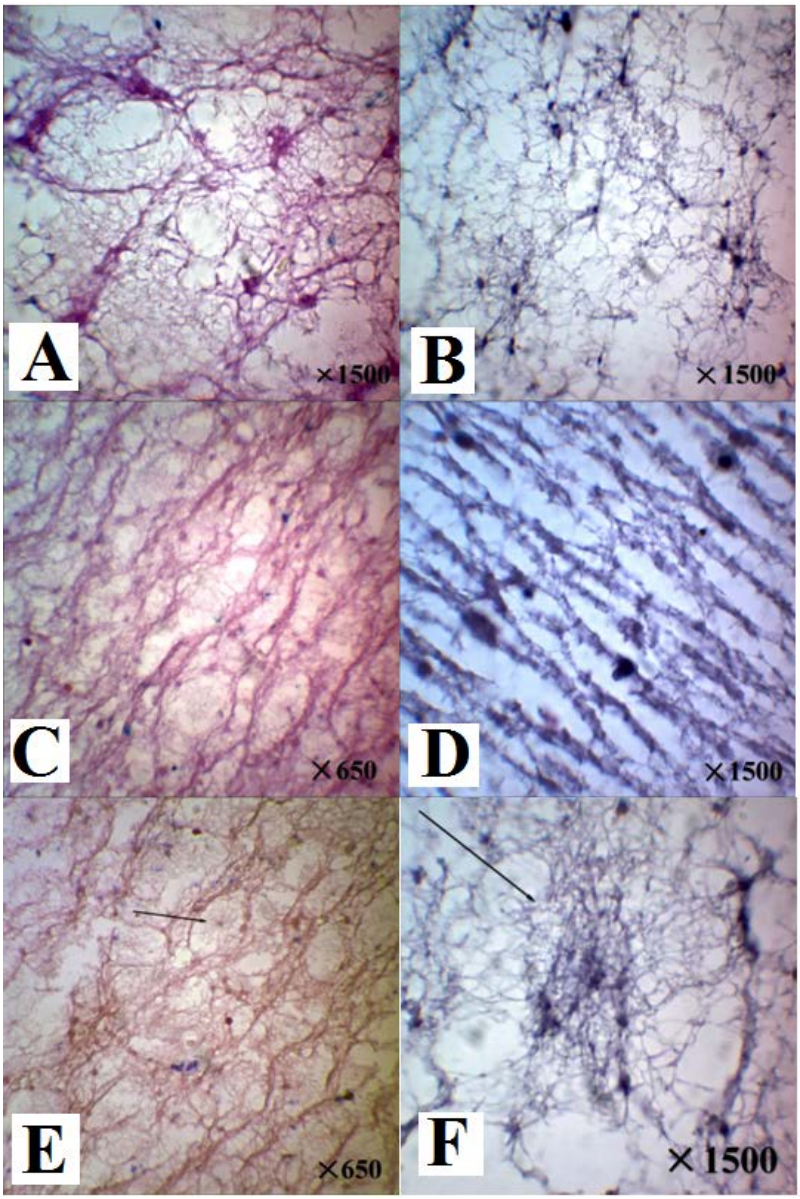

Figure 4: Heteromorphic structure of a fibrin-platelet clot. A, C, E - dyeing for fibers according to Van Gieson; B, D, F - hematoxylin-eosin stain.

3. The structure of a fibrin-platelet clot containing many platelets is characterized by the fact that it is even more heteromorphic. Fibrin filaments in it are located more compactly, forming both an unstructured network (Figure 4 (A; B), and a network with linearly elongated structures located parallel to each other (Figure 4 (C; D). In addition, platelets are in close contact with filamentous structures. In some areas of the preparation, visualize structures resembling a cobweb, in the center and in the nodal junctions of which there are accumulations of platelets (Figure 4 (D; E).

In all of the above variants of clots, along the periphery of histosections, areas of a structureless dense accumulation of fibrin are visualized. However, in all likelihood, this is an artifact caused by the influence of formalin during material fixation.

Unstructured fibrin filament network (A, B). Linearly elongated fibrous structures parallel to each other (C, D). Areas of the preparation in which structures resembling a cobweb are visualized (indicated by arrows), in the center, and the nodal junctions of platelets accumulate (E, F). In all areas of the preparations, single platelets and their conglomerates are visualized in close contact with fibrin filaments.

4. In the fibrin-platelet-leukocyte clot, the fibrin filaments are arranged so tightly that they are visualized not as fibers but as a continuous homogeneous mass (Figure 5 (1). Platelets are not visualized, which may be because they are screened by densely packed fibrin. Or with the fact that in this type of clot, platelet membranes are destroyed. Leukocytes retain their usual cellular shape (Figure 5 (2; 3). And the picture of the clot as a whole is somewhat reminiscent of the red pulp of the spleen.

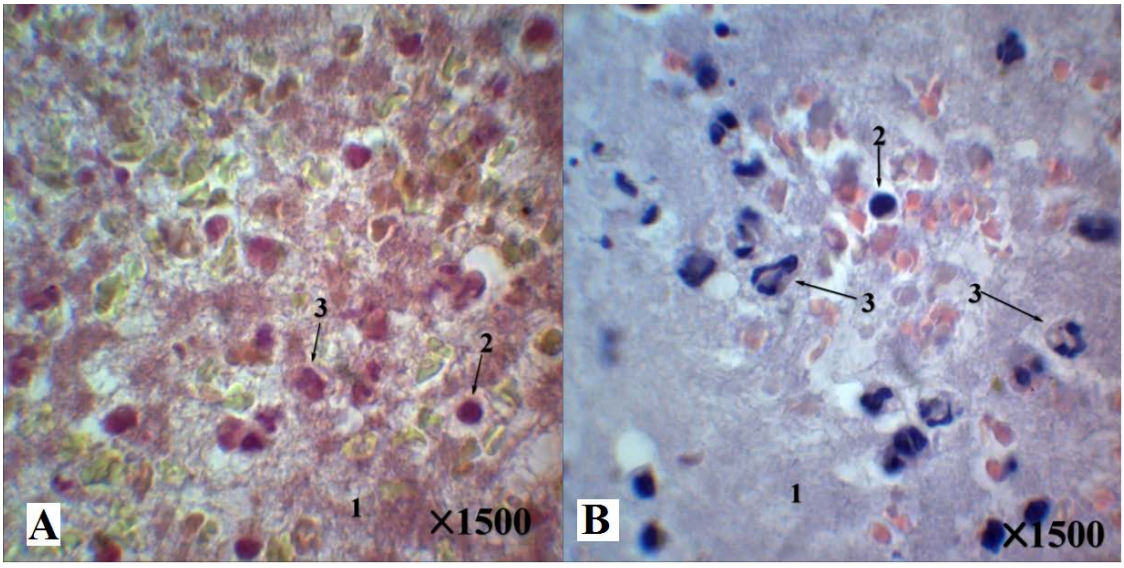

Figure 5: Structure of a fibrin-platelet-leukocyte clot. A - Van Gieson stain; B – hematoxylin-eosin stain.

Fibrin filaments are densely arranged and are visualized not as fibers but as a continuous homogeneous mass (1). Platelets are not visualized. Lymphocytes (2) and neutrophils (3) retain their usual cellular form.

4 Conclusion

This study finds that the rate of formation and rate of retraction of the clot depends on both the quantitative and qualitative cellular composition. Also, the rate of clot formation is higher, the more platelets are contained in the initial plasma. Further, the rate of formation and rate of retraction of the clot is influenced not only by platelets but also leukocytes. In the presence of leukocytes, the clot forms faster, and its retraction is faster and more pronounced. Furthermore, the architectonics of the network organization of fibrin fibers in the fibrin-platelet clot depends on the number of platelets and the presence of other leukocytes. The density of such a network is directly proportional to the number of platelets in the plasma and increases even more in the presence of lymphocytes and granulocytes. Leukocytes in the fibrin-platelet-leukocyte clot completely retain their morphological characteristics. Moreover, structural differences in acellular fibrin, fibrin-platelet, and phyrin-platelet-leukocyte clots indicate that their effect on the wound process will also be different.

Thus, this confirms the initial hypothesis that different fibrin clots, which differ qualitatively and quantitatively in platelet-leukocyte composition, can have different effects on the processes of inflammation and regeneration. Further research requires the development of new methods of cell separation when obtaining blood plasma to simplify and standardize this operation. In addition, it will allow to go beyond the laboratory experiment and introduce them into clinical practice.

5 Availability of Data and Material

Data can be made available by contacting the corresponding author.

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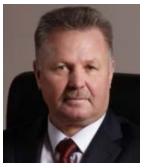

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