Interpretation of Platelets in The Horse

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Abstract: Currently we can consider that, in addition to its role in hemostasis, platelets also participate in other important processes such as thrombosis, inflammation, tissue remodeling and the innate defense mechanisms. The hemostatic activity of platelets includes different events to stop bleeding. Within these functions we can mention the adhesion to the endothelium of the affected blood vessel, the activation, the aggregation, and the release of substances that initiate hemostatic events, and also the providing a phospholipid surface for activation of numerous coagulation factors. Similarly, platelets release multiple growth factors responsible for regulating the growth and division of endothelial cells and fibroblasts. In this way, among other things, angiogenesis and tissue regeneration are favored. Platelets also participate in inflammatory processes by the release of factors that initiate the inflammatory cascade and favor the chemotaxis of neutrophils, monocytes, macrophages, acute phase proteins and target cell signaling. Finally, platelets participate in the immune response by interacting with the complement system and immunoglobulins.

Keywords: Hematology, Horse, Platelet, Thrombocytopenia.

1. INTRODUCTION

Platelets play an essential role in hemostasis and in the modulation of inflammatory and immunological reactions. The basis of primary hemostasis is the interaction between platelets and the activated endothelium and sub endothelial collagen. Thus, platelets are the first responders to vessel damage through two differents pathways. The first one involves the adhesión to P-selecting on activated endothelium. The second way, also the most often, is called the Von Willebrand´s factor (vWF), which forms a bridge between the sub endothelial collagen and platelet glycoprotein Ib. When platelets adhere, normally they tend to collapse to form a monolayer which is responsible for stopping blood loss. If the damage in the blood vessel is important, followed by the aggregation of the platelets, the release of the granules occurs. This series of events causes subsequent activation of secondary haemostasis. As a consequence of this stimulation, a change in the conformation of the plasma membrane is induced allowing the expression of the glycoprotein IIb-IIa, which then binds to the fibrinogen, resulting in the aggregation of the platelets. This situation allows the consolidation of the platelet plug. With platelet aggregation, the granules release their contents by further amplifying platelet aggregation. As a result the platelet plug is formed resulting in hemostasis [1].

In addition, platelets intervene in the inflammatory response by releasing vasoactive substances. These substances, such as prostaglandins, cationic proteins, collagenase, elastase, histamine, serotonin and chemotactinogen, trigger or maintain the inflammatory response and influence repair of tissues after injury. This function is determined by the release of platelet activating factor, produced especially neutrophils and macrophages [2]. On the other hand, platelets have phagocytic capacity of microorganisms. In cases of bacterial endotoxemia caused by *E. coli*, it intervenes by eliminating the bacterial polysaccharides present in the circulatory system [3].

Recent reports have demonstrated the ability of platelets to induce changes in the phenotype of cancer cells that acquire invasiveness, thus increasing their metastatic potential. In addition, platelet aggregates promote the dissemination of tumor cells in the blood, favor the adhesion to the vascular endothelium surface and allow extra vascular formation of neoplasms. A hypothesis launched in this regard, argues that the targeting of platelets may represent a novel strategy to prevent the development and progression of cancer [4]. To the present, to the knowledge of the authors, there is no evidence on this activity in the horse.

2. PHYSIOLOGICAL CHARACTERISTICS OF PLATELETS IN HORSE

In the equine the platelets are round, oval or elongated, measure 2.5-3.5μm in diameter and have clear blue cytoplasm with fine azurophilegranules [5].

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E-ISSN: 2312-5411/17

Normally the survival time of equine platelets in the blood circulation is 5-9 days [5]. Equine platelets stain very lightly with Wright-Giemsa and therefore can be difficult to identify in the blood smears. However, platelets are stained well with Diff-Quick®. Giant platelets are morphologically larger than the diameter of a RBC and are associated with accelerated thrombocytopoiesis. Automated platelet counts artificially decrease if there are platelet aggregates [7]. Pseudopodia can be observed on activated platelets [8].

3. REFERENCE VALUES OF PLATELET PARAMETERS IN HORSES

Compared witn other mammals equine platelet concentrations are some of the lowest reported. Platelet concentration in a normal horse is in average around 6 to 10 platelets in oil immersion field (100x objective). Normal values of equine platelets variates between 100 and 350.000/μL [8]. Some of the platelet parameters include mean platelet volume (MPV; fL), platelet distribution with (PDW; %) and plaquetocrit (PCT; %). MPV is the mean platelet volume. The increase in MPV usually coincides with a greater release of large or giant platelets and is indicative of a platelet regenerative response. The value of PDW in combination with that of MPV can indicate if there is an increased number of smaller and / or larger platelets. PCT is a better indicator of total functional platelet function compared to platelet count. This fact is the result of the greater functional capacity of the larger platelets. In our laboratory (Laboratory of Clinical Analysis, Veterinary Hospital, Cardenal Herrera University) we have obtained reference values ranging from 5.3 to 7.8fL, 26 to 74% and 0.05 to 0.22% for MPV, PDW and PCT, respectively. However, these parameters can be altered by the activation of platelets [9].

4. PHYSIOLOGICAL FACTORS INFLUENCING PLATELETS IN HORSES

4.1. Blood Sample Collection, Anticoagulant and Analytical Time

In order to make a diagnosis of true thrombocytopenia in horse is important the accurate collection of blood samples for the adequate platelet count. Repeated venipuncture, changes in blood flow, or delay in performing the analysis alter the platelet count. Platelet agglutination is suggestive of platelet activation and aggregation during blood collection. As a consequence, we may have erroneously low platelet concentrations [8]. Also the temperature as well as the storage conditions has an important correlation and significant effects in the platelet aggregation [10]. Due to platelet aggregation it has been reported in a Thoroughbred gelding [11], a platelets clumping or EDTA-dependent pseudothrombocytopenia. However, the severity of pseudothrombocytopenia can be reduces by pre-warming EDTA blood samples to 37ºC prior to hematological analysis [12]. In the blood tubeltoccurs a pseudothrombocytopenia due to EDTA-induced unmasking of platelet antigens *Via* EDTA binding with natural antibodies and the subsequent platelet aggregation. Therefore is advisable to perform the analysis within the first 2 hrs after collection, due to MPV can be altered if the EDTA-samples are kept refrigerated. On the other hand, prolonged sample storage can result in pseudothrombocytosis due to misclassification with the Advia 120 (Bayer Corp., Tarrytown, NY, USA) of ghost RBCs as platelets [13]. For that reason could be interesting to use sodium citrate as anticoagulant to measure platelet size [14, 15]. Using low-molecular-weight heparin as an anticoagulant [16], platelet cumpling and thrombocytopenia also have described in equine blood samples. Horses treated with heparin may result in anemia from erythrocyte agglutination and also should be monitored for increased bleeding tendency.

4.2. Age and Breed

Related to the breed, Jeffcott [17], found that the number of platelets in Quarter Horses was higher compared with other equine breeds. An explanation for this result is unclear, although in this case others factors than the breed should be taken into consideration. According to the age, platelet numbers in foals do not change during the first year of life but age determines a progressive decrease [18, 19]. However, despite this findings, a previous reprot in horses is not agree with these results [20].

4.3. Exercise and Training

The intensity of the exercise has an important effect on the platelet parameters in the equine. Both brief and maximal exercise produces significant increase in platelet count. However, moderate exercise does not appear to alter the number of platelets. In addition, in response to high intensity exercise, the studies of Piccione *et al*, [21] reported a reduction in platelet aggregation capacity, although this affirmation is not shared by other authors [16]. Also the platelet activity in response to exercise has been related to changes in

blood pH and hemoconcentration, environmental conditions, changes in ionized calcium concentrations [22]. In addition, Andriichuk and Tkachenko [23] showed a significant decrease in MVP after exercise in mares.

4.4. Reproductive Status

Satué *et al,* [24, 25] described a progressive decline in platelet numbers during pregnancy in Carthusian broodmares. This situation could be attributed possibly to the combined effect of stress and increased release of estrogen, progesterone and other steroid hormones. However, different authors have reported that calving [26] and lactation have no influence on the number of circulating platelets in mares [24, 26].

5. CUANTITATIVE ALTERATIONS IN PLATELETS

5.1. Thrombocytosis

The high number of platelets in blood or thrombocytosis can occur in both physiological and pathological conditions. Mild physiological thrombocytosis may occur by platelet mobilization during and immediately after exercise or excitation. The major cause is due to splenic contraction and the release of sequestered platelets into the peripheral circulation [27, 28]. Pathological thrombocytosis may be related to increased bone marrow megakaryocyte production in response to primary myeloproliferative disorder in association with other neoplastic conditions. Such situation includespolycythemia vera, which is also called primary polycythemia or erythremia. The most common form, the reactive thrombocytosis, is related to acute or chronic inflammatory disorders (eg, peritonitis, pleuritis), or liver disease [29, 30]. It is also associated with infections of *Rhodococcusequi* or type 1 herpesvirus in response to acute hemorrhages as in fractures and neoplasms [28, 31]. In these cases, reactive thrombocytosis is not usually associated with an increased risk of thrombosis.

5.2. Thrombocytopenia

At present, most laboratories define thrombocytopenia as a platelet count below 100.000/μl. Because accumulation of platelets is common in horses, the blood smear should always be evaluated to confirm the actual decrease in platelets and to prevent the existence of pseudothrombocytopenia. The decrease in the number of circulating platelets may also be the result of decreased bone marrow production, increased destruction, or increased platelet use during coagulation.

Thrombocytopenia may be due to a variety of mechanisms such as reduction of thrombopoiesis, increased peripheral destruction of platelets, sequestration of the spleen and loss of platelets by idiopathic origin [28, 32].

5.2.1. Reduction of Thrombopoiesis

Thrombocytopenia can be caused by altering the functionality of the bone marrow due to any pathology and / or myelosuppresive drugs as phenylbutazone, chloramphenicol, estrogens as well as irradiation. Also this pathology in some cases is accompanied by anemia and leucopenia. The medullary pathologies that can occur with thrombocytopenia in horses are myeloptisis, myelofibrosis, myeloproliferative disease, myelodysplasia and idiopathic medullary aplasias with pancytopenia.

Under these situations megakaryocytes may be absent or present in low numbers, even through adequate numbers present in the intact marrow. Possibly this is due because of trapping of these cells within the sub endothelial layer of marrow sinuses. A bone marrow biopsy would be adequate to assessing megakaryocytes on the horse. Bone marrow core biopsies are technically more difficult to obtain than aspirates. However, they are essential for confirmation of suspected generalized bone marrow suppression, hypocellularity, myelofibrosis, or a plastic anemia [33].

Several cases has been reported about platelet alteration in horses. A case of a 5-year-old Quarter Horse with a bone marrow myeloid-to-erythroid ratio of 30.5:1, absence of megakaryocytes and severe clotting disorders was presented by Brumbaugh *et al,* [34]. Also, Bienzle *et al*, [35] described absolute megakaryocytic hypoplasia, erythroid hypoplasia, depletion of granulocytic reserve, predominance of immature blast-like leukocytes and a myeloid-to-erythroid ratio of 50:1. Kohn *et al*, [36] identificates a familial megakaryocytic hypoplasia in Standardbred trotters. In the same way, Morris *et al*, [37] reported the case of a 10-month-old Standardbred colt, with edema and hemorrhagic diathesis. The colts can had severe thrombocytopenia, anemia, mild hypoproteinemia and marked eosinophilia, with immature or atypical circulating eosinophils. Bone marrow aspirate showed atypical eosinophil precursors, with few erythroid precursors, and no megakaryocytes. Epistaxis is a common clinical sign of myeloma in horses [38, 39]. The primary cause of bleeding seems to be thrombocytopenia, although some factors such as paraproteinmediated functional inhibition of platelets and coagulation has been reported [40].

In those cases to assess platelet production flow cytometric enumeration of thiazole orange-positive platelets in peripheral blood may be useful [41].

5.2.2. Increased Peripheral Destruction of Platelets

It occurs in immune-mediated thrombocytopenia, (IMT) primary or secondary.

In the primary immunomediated thrombocytopenia or autoimmune disease (Systemic lupus erythematosus), antibodies to membrane antigens are produced. So the platelets are removed by the action of the mononuclear phagocytic system in liver, spleen and bone marrow [42-44]. Neonatal alloimmune thrombocytopenia has been previously reported in foals of several equine breeds [45, 46].

Secondary immunomediated thrombocytopenia is associated with differents agents. Virus (herpes virus, influenza, equine infectious anemia-EIA), bacterial infections (endotoxaemia, neonatal septicemia, *Anaplasmaphagocytophilum*), neoplasms (lymphosarcoma), immune-mediated hemolytic anemia (IMHA), glomerulonephritis, drugs (penicillin or trimethoprim-sulfadoxine), vasculitis and toxins are the principals described [1, 47, 48]. Another possibility are horses receiving heparin therapy wich may also develop thrombocytopenia [49].

The hallmarks of immune-mediated thrombocytopenia are: 1) enhancement of platelet destruction which leads to a decrease in the number of peripheral platelets and 2) the presence of antibodies associated to platelets. The antibodies, bind to the surface of the platelets resulting in premature destruction made by splenic macrophages and hepatic Kupffer´s cells remove antibody-coated platelets from blood. In horses, IMHA and IMT are caused by the antibody-mediated destruction of red blood cells and can occur independently or concurrently. The flow cytometry used to evaluate antibodies associated with the surface of blood cells and red blood cells (IgG and IgM) or complement has been adapted for equine erythrocytes and platelets in order to detect membrane-bound antibodies in cases of IMHA and IMT. Tests with isotope antibodies to equine immunoglobulins can elucidate the class of antibody bound to cells [50, 51].

Bloodsucking insects can transmit the retrovirus wich cause the Equine Infectious Anemia (EIA). This disease produce thrombocytopenia during acute episodes and is closely correlates with fever and viremia [28, 52]. Also in EIA, there is immune complex consisting of retrovirus and antibodies, which deposit on the platelets [53]. Although megakaryocytes are not altered during acute disease, if antibodies against platelet membranes cross-react with megakaryocyte membrane antigens, megakaryocytes may be decreased or absent in the bone marrow. However, foals with another affection such as severe combined immunodeficiency (SCID), develop the same degree of thrombocytopenia as immunocompetent foals after EIA infection. SCID-bearing colts lack functional T and B-lymphocytes and cell-mediated or antigen-specific antibody responses. In addition, platelet production is significantly reduced both in SCID and in foals immunocompetent for EIA infection. Besides, platelets are activated but are hypo functional in acutely infected horses. In this way, platelet aggregates can be formed and removed from circulation. These mechanisms represent the non-immune destruction of platelets in horses infected with EIA. On the other hand, infection of endothelial cells could lead to thrombocytopenia by promoting the adhesion and aggregation of platelets. Finally, altered cytokine production in the bone marrow may also lead to decreased platelet production [47, 48].

Equine Granulocytic Ehrlichiosis (EGE) is an infectious, noncontagious, seasonal disease produced by *Anaplasmaphagocytophilum*. Horses with this infection have abnormal hematology wich includes thrombocytopenia, leukopenia and mild anemia [28]. Diagnosis of EGE is based on finding of intracitoplasmatic inclusions (morulae) within granulocytes in the peripheral blood, and detection of the DNA of *A. phagocytophilum* using specific polymerase chain reaction assays [54]. Anaplasmosis infection may be diagnosed by the presence of morulae within the neutrophil and eosinophil cytoplasm in severe cases of the disease [55]. Morulaes appear as pleomorphic, blue-gray to dark blue spoke-wheel shapes. Inclusion bodies represent a cluster of coco bacillary organisms, varying in size from 0.2 to 5μm in diameter, within cytoplasmic inclusión membrane-bound vacuoles. The appearance of cytoplasmic inclusion bodies correlates closely with the onset of fever, and they remain visible for approximately 10 days [56].

Thrombocytopenia can also be produced by *Theileriaequi* and *Babesiacaballi*. These microorganisms cause immune-mediated destruction, splenic sequestration, and/or excess of platelets consumption as is observed in disseminated intravascular coagulation (DIC). Infected erythrocytes with *Babesiacaballi*can cause the formation of microthrombi within small vessels, leading to venous stasis and

vasculitis [57]. IMT has also been documented in horses with lymphosarcoma [58] and on idiopathic disorder [32].

Platelet counts of less than 30.000/μL, usually is associated with clinical bleeding due IMT in the horse include petechial and ecchymotic hemorrhages of mucosal membranes, epistaxis, increased bleeding after venipuncture, melena or hyphema [28].

5.2.3. Increased Consumption and Loss of Platelets

It is associated with hemorrhage, DIC and localized activation of coagulative and fibrinolytic processes, such as vasculitis (equine hemorrhagic purpura), vascular neoplasms (disseminated hemangiosarcoma), renal pathologies (hemolytic-uremic syndrome), gastrointestinal or inflammatory diseases [49]. In sepsis and endotoxemiasucha as acute toxic colitis, intestinal strangulating obstruction, neonatal septicemia, abnormalities of hemostasis and fibrinolytic pathways may lead to arterial thrombosis. Either excessive consumption or loss through the GI tract in protein-losing enteropathy causes thrombocytopenia and deficiencies in antithrombin III, have been observed in affected patients. Laboratory diagnosis of DIC include thrombocytopenia (usually < 100.000/μL), prolonged PT, aPTT, low fibrinogen (< 150mg/dL), low ATIII activity and increased FDP sor D-dimers [1]. In horses hemolytic uremic syndrome is produced by acute renal failure with microangiopathicintravasculary hemolysis and disseminated or renal intravascular coagulation [59, 60].

Thrombocytopenia related with hemorrhage or trauma is usually mild to moderate and rapidly reversible [28].

5.2.4. Platelet Sequestration in the Spleen

In human medicine and in small animals splenic pathologies are associated with thrombocytopenia because of the storage capacity of platelets in the spleen and the production of anti-megakaryocyte antibodies. This circumstance is much less important in horses [28].

6. PLATELET FUNCTION DEFECTS AND VON WILLEBRAND DISEASE IN HORSE

Platelet dysfunction may be hereditary or acquired and may stem from an intrinsic platelet defect or from an extrinsic factor that alters the function of normal platelets. Hereditary Glanzmann's thrombasthenia is a disorder resulting in poor platelet aggregation and clot retraction caused by one or more defects in quantity or quality of platelet surface glycoprotein IIb/IIIa [61]. Glanzmann's thrombasthenia have been showed in a Peruvian Paso mare of 17 years old [62] and an 18-month-old Oldenbourg filly [63, 64].

Von Willebrand disease is due to a qualitative or quantitative deficiency of the multimeric protein vWF, which is required for platelet adhesion. Von Willebrand disease is due to a deficiency in high-molecular-weight multimers of vWF. It has been reported in Quarter Horses in consistent with type 2 von Willebrand disease in other species [65, 66]. Administration of some drugs can cause acquired defects in platelet function. Drugs that impair platelet function include aspirin, phenylbutazone, sulfonamides, estrogens, chlorpromazine, halothane, nonsteroidal anti-inflammatory drugs (NSAIDs), and phenothiazines. Uremia and liver disease may also adversely affect platelet function. The production of elevated levels of immunoglobulins, specifically IgM in monoclonal gammopathies, impairs platelet function by protein coating of platelet surfaces [67].

CONCLUSION

The quantitative analysis and the assessment of peripheral blood smear of platelets are one of the most important tool to diagnose hematological and systemic consequences of many diseases in horses. Due to the role of platelets in hemostasis, the inflammatory and immunological response, the differential diagnosis of thrombocytopenia should always be taken into consideration.

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Received on 06-06-17 Accepted on 28-08-17 Published on 30-12-2016

DOI: http://dx.doi.org/10.12974/2312-5411.2017.04.3