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Original Article

Effects of hydrocortisone and aminophylline on the aggregation of equine platelets *in vitro*

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The purpose of this study was to evaluate *in vitro* the effects of hydrocortisone and aminophylline on adenosine diphosphate (ADP)-induced platelet aggregation in horses. Blood samples from 30 healthy Thoroughbred horses were collected by via jugular venipuncture to assess platelet aggregation. Platelet-rich and platelet-poor plasma were prepared from all samples by centrifugation and divided into three different aliquots. In the first aliquot, platelet aggregation was measured after platelet activation with 1 μM and 0.5 μM ADP (Group A). In the other two aliquots, the effect of a 10 min preincubation with hydrocortisone (Group B) or aminophylline (Group C) on ADP-induced aggregation at final ADP concentrations of 1 μM and 0.5 μM was observed. Platelet aggregation, recorded by an aggregometer, was evaluated by measuring the maximum degree of platelet aggregation and the initial velocities of platelet aggregation were obtained. Our results demonstrated the inhibitory effect of hydrocortisone and the induction effect of aminophylline on equine platelet responses *in vitro*.

Keywords: adenosine diphosphate, aminophylline, horse, hydrocortisone, platelets

Introduction

Platelet cohesion, more commonly referred as platelet aggregation, may be considered to be an indirect index of platelet functionality. The efficiency of platelet adhesion and aggregation at sites of vessel wall injury is dependent on the synergistic action of various adhesive and soluble agonist receptors, with the contribution of each individual receptor determined by prevailing blood flow conditions [15]. Blood platelets are a component of the haemostatic system in which the response to an agonist can be altered by compounds formed by or stored within platelets [9]. Platelets may become activated through different pathways including ones associated with collagen and von Willebrand factor exposed to the flowing blood following vessel wall injury, adenosine diphosphate (ADP) and adenosine triphosphate released from activated platelets, or thrombin [21]. A variety of substances are important mediators of haemostasis and thrombosis, and promote platelet aggregation *in vitro*; these include ADP, adrenaline, collagen, and arachinodic acid [16,26]. The functions of these factors are commonly assessed by measuring platelet aggregation *in vitro* [36].

Evaluation of platelet response, or aggregation, to agonists *in vitro* is used to predict species-specific platelet responses *in vivo* [13,26,27]. *In vitro* platelet aggregation studies using ADP, collagene, arachidonic acid, and epinephrine have been performed in humans, camels, dogs, and calves [12,34]. Equine platelets aggregation in response to platelet activating factors and ADP has been well established [30]. Activation of platelets by ADP modifies the conformational state of receptor, enabling it to bind to the fibrinogen molecule and thus stimulating the aggregation cascade [11].

Platelet adhesion, aggregation, secretion, and survival change with atherosclerosis and thromboembolic phenomena, thus further increasing interest in anti-aggregation drugs [38]. In fact, platelet function seems to be affected by many clinical conditions in equine medicine [23,24] and by a variety of drugs. However, only few studies have comparatively evaluated the effects of some [6,17,22,32]. A few authors have investigated the modulation of platelet function by some nonsteroidal anti-inflammatory drugs such as sulfazamet, phenylbutazone, acetylsalicylic, and indomethacin [6,22,25]. Equine platelet function was also examined in relation to glucocorticoid hormones; these studies showed that although glucocorticoids can increase the number of circulating platelets, neutrophils, and red blood cells, platelet aggregation is inhibited [7]. Other researchers limited themselves to studying pharmacokinetics and bioavailability of drugs, such theophilline and amino-

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phylline, in horses [10,14] while the effects of some xanthine derivates on some hemostasis indices were only evaluated in humans [8,19]. In horses, the action of aminophylline on ADP-induced platelet aggregation has not been studied. Hydrocortisone is a main glucocorticoid hormone [1] and aminophylline is among the methylated xantines that serve as bronchodilators which are less potent and shorter-acting than theophylline [18]. Therefore, the aim of this study was to evaluate the *in vitro* effect of hydrocortisone and aminophylline on ADP-induced platelet aggregation in horses.

Materials and Methods

Thirty healthy Thoroughbreds (gelding, males and females), ranging in age from 8 to 10 years with mean body weight of 510 ± 50 kg, from the Horse Training Centre (La Pineta, Italy) were used for this study. Before the start of the study, all subjects underwent a heart exam, respiratory auscultations, and routine haematology and plasma biochemistry tests while at rest. As shown in Table 1, the average values of hematological and clotting parameters obtained from the 30 healthy horses were within the normal physiological ranges [37]. The animals were routinely fed hay and a mixture of cereals (oats and barley) three times a day (08 : 00, 12 : 00, and 20 : 00) and had access to water *ad libitum*. The animal husbandry and experimentation protocols were reviewed and approved in accordance with the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 86/609 European Economic Community.

Table 1. Summary of haematological and clotting parameters of 30 Thoroughbred horses with the corresponding reference values

Parameters	Mean values*	Reference values [2,37]
Red blood cell (M/μL)	8.79 ± 0.80	8.7 ~ 11.4
White blood cell (K/μL)	6.83 ± 1.32	6.8 ~ 11.1
Haemoglobin (g/dL)	14.51 ± 1.33	14.1 ~ 18.0
Packed cell volume (%)	34.33 ± 8.02	32 ~ 53
Mean corpuscular volume (fL)	47.50 ± 2.40	39.6 ~ 47.2
Mean corpuscular haemoglobin (pg)	16.18 ± 0.90	14.7 ~ 17.5
Mean corpuscular haemoglobin concentration (g/dL)	36.35 ± 1.20	35.8 ~ 38.0
Platelets (g/dL)	161.29 ± 25.35	120 ~ 400
Mean platelet volume (g/dL)	9.18 ± 1.04	6 ~ 11
Prothrombin time (sec)	12.65 ± 1.10	10 ~ 14
Activated partial thromboplastin time (sec)	46.90 ± 3.03	25 ~ 50
Fibrinogen concentration (mg/dL)	140.60 ± 10.12	100 ~ 400

*Data are expressed as the means ± SD.

Blood samples were collected from all horses via jugular venipuncture into 3.6 mL tubes containing 3.8% sodium citrate (1 part citrate: 9 parts blood) to assess platelet aggregation. From all blood samples, platelet-rich and platelet-poor plasma were collected by centrifugation and divided into three different aliquots. Platelet-rich plasma (PRP) was obtained by centrifuging the blood at 300 × g for 20 min within 15 min of collection. The upper 2/3 of the PRP layer was carefully removed using a plastic transfer pipette and transferred into plastic laboratory containers. The remainder of the blood samples was centrifuged at 3,000 × g for 10 min to obtain the platelet-poor plasma, which was also removed and transferred into plastic containers.

Platelet aggregation in each sample was studied using a previously described method [28]. To the first aliquot (285 μL) of PRP, 15 μL of ADP were added to evaluate ADP-induced platelet aggregation (Group A). To the other two aliquots (285 μL) of PRP, 15 μL of ADP and 15 μL of diluted hydrocortisone (Group B), or 15 μL of ADP and 10 μL of diluted aminophylline (Group C) were added. 10 μL of hydrocortisone (Flebocortid Richter, 500 mg/5 mL; Sanofi-Aventis, Italy) were added to 4.99 mL of isotonic saline and 15 μL of this solution were added to 285 μL of PRP in order to obtain a final corticoid concentration of 10 μL/mL, and 30 μL of aminophylline (Aminomal, 240 mg/10 mL; Malesci, Italy) were added to 970 μL of 0.9% sodium chloride and 10 μL of this solution were added to 285 μL of PRP in order to obtain a final aminophylline concentration of 20 μg/mL. Using this protocol, the effect of a 10-min pre-incubation with hydrocortisone and with aminophylline on ADP-induced aggregation was determined. The final concentrations of the aggregating agent, ADP, were 1.0 and 0.5 μM. Platelet aggregation was recorded for at least 4 min using an aggregometer (CLOT2; SEAC, Italy).

Platelet aggregation responses were evaluated according to two parameters: the maximum degree of aggregation and the initial velocity of aggregation. The maximum degree of aggregation, expressed as a percentage, was determined by measuring the maximum height of the aggregation wave over a 4-min period beginning at the onset of platelet aggregation. The initial velocity of aggregation was determined by drawing a line tangent to the steepest linear portion of the aggregation tracing, and determining the slope from one point along the curve. The slope of this tangent was expressed as percent per minute.

All results were expressed as the mean ± SD, and showed the maximum degree of platelet aggregation along with the slope of platelet aggregation. All data were normally

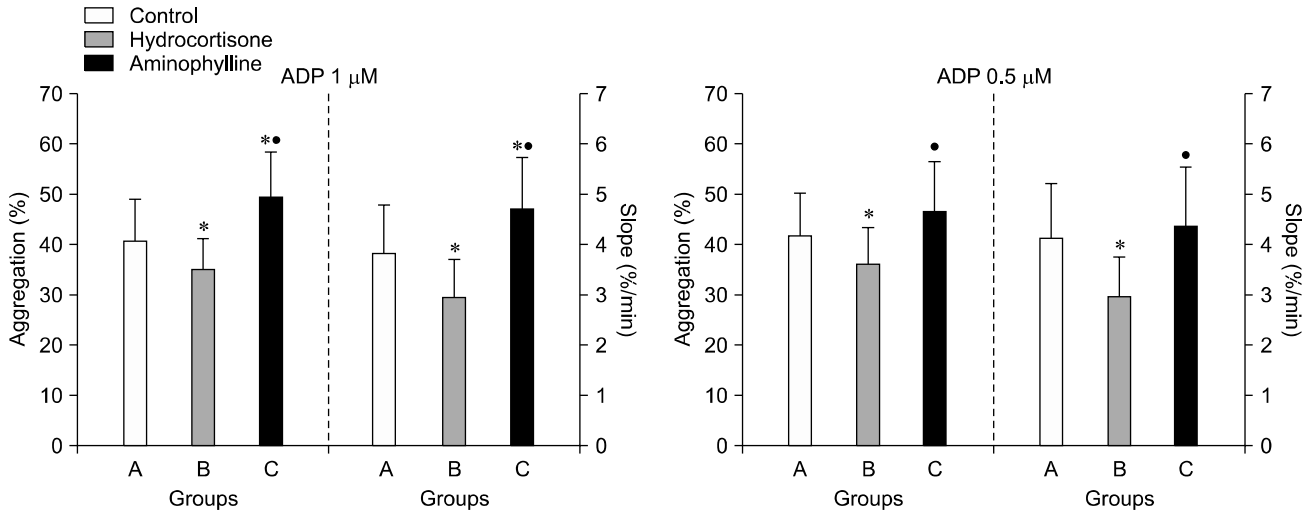

Fig. 1. Average values of the maximum degree of platelet aggregation expressed as a percentage and the initial velocity of platelet aggregation expressed as the percentage per 1 min for samples from Group A (adenosine diphosphate [ADP]-induced platelet aggregation), Group B (ADP-induced platelet aggregation with hydrocortisone pre-incubation), and Group C (ADP-induced platelet aggregation with aminophylline pre-incubation). Values were measured in the presence of final ADP concentrations of 1 μM and 0.5 μM. *$vs.$ Group A ($p < 0.05$), • $vs.$ Group B ($p < 0.001$).

distributed ($p < 0.05$, Kolmogorov-Smirnov's test) and a one-way ANOVA was used to determine significant differences between all groups. A p value < 0.05 was considered to be statistically significant. Bonferroni's multiple comparison test was used for post hoc comparison. Data were analyzed using software (Statistica 7.5; StastSoft, USA).

Results

Fig. 1 shows average values of the maximum degree of platelet aggregation and the slope of platelet aggregation expressed in with the conventional unit of measurement. Results of the one-way ANOVA showed statistically significant differences between the groups treated with hydrocortisone or aminophylline. At a final ADP concentration of 1 μM, the maximum degree of aggregation and slope of aggregation were $F_{2,89} = 25.56$ ($p < 0.0001$) and $F_{2,89} = 27.10$ ($p < 0.0001$), respectively. At final a ADP concentration of 0.5 μM, the maximum degree of aggregation and the slope of aggregation were $F_{2,89} = 11.61$ ($p < 0.0001$) and $F_{2,89} = 15.92$ ($p < 0.0001$), respectively. The mean maximum degree of platelet aggregation expressed as a percentage and the initial velocity of platelet aggregation expressed as a percentage per 1 min with final ADP concentrations of 1 μM and 0.5 μM are shown in Fig. 1.

Discussion

The results from this study showed statistically significant differences for the maximum degree and slope of equine platelet aggregation between the samples pre-incubated with hydrocortisone or aminophylline. These findings demonstrated decreases in the parameters measured in our study following the addition of hydrocortisone. These values were increased after the addition of aminophylline.

Contrary to previous findings which showed that hydrocortisone does not affect major platelet receptors [31] and has no effect on prostaglandin synthesis in platelets [29,35], our research found that glucocorticoid inhibits platelet aggregation. Hydrocortisone probably affects the interaction of ADP with its receptors. Furthermore, the stimulation of phospholipase A_2, activation of phospholipase C by tromboxane and platelet activating factor, formation of the second messengers inositol trisphosphate and diacylglycerol, and platelet degranulation (release of Ca^{+2}, ADP, and serotonin) may also be impacted since these stages are directly related to the structure and function of the platelet membrane [33]. Hydrocortisone seems to inhibit prostaglandin synthesis by intact cells and its effect seems to be caused by inhibition of phospholipase A_2 that catalyzes the hydrolysis of arachidonic acid by membrane phospholipids [3]. Additionally, glucocorticoids inhibit vasodilatation that accompanies the synthesis of prostaglandins by the vessel wall, normally associated with transient vasoconstriction, followed by vessel relaxation [20]. It has been demonstrated that the vessel relaxation after constriction is promoted by prostaglandins released from the vessel wall in response to injury, and that the inhibition of prostaglandin synthesis leads to a sustained vasoconstriction [3].

Unlike previous findings in which methyl xantines were

found to markedly inhibit platelet aggregation [5], our results showed that methylxantine increased equine platelet aggregation. Methylated xantines such as caffeine and theophylline inhibit human platelet aggregation and are thought to inhibit platelet aggregation by promoting the accumulation of cyclic AMP in platelets through the inhibition of phosphodiesterase [5]. However, if we assume that the effects of methylxantines are secondary to the inhibition of phosphodiesterase activity, this may indicate that cyclic AMP plays a regulatory role in many important platelet functions. Moreover, it has been demonstrated that aminophylline does not affect the platelet release reaction [4]. These discrepancies may be due to differences in concentrations of aminophylline that were used for different studies or in species since most authors evaluated platelet aggregation after treatment with various concentrations of methylxantine *in vitro* [4].

In conclusion, the results of the present investigation showed the inhibitory effect of hydrocortisone on *in vitro* platelet aggregation in horses. We also demonstrated ability of aminophylline to promote the equine platelet response *in vitro*. Since compounds able to regulate platelet activities are essential in veterinary clinical practice, additional investigations using different hydrocortisone and aminophylline concentrations are necessary in order to further evaluate equine platelet aggregation.

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