Effects of hydrocortisone on platelet aggregation in jumper horses

Giuseppe Piccione¹*, Stefania Casella¹, Claudia Giannetto¹, Vanessa Messina¹, Pietro P. Niutta², and Elisabetta Giudice²

¹ Dipartimento di Scienze Sperimentali e Biotecnologie Applicate, Facoltà di Medicina Veterinaria Università degli Studi di Messina, Polo Universitario dell’Annunziata, Messina, Italy
² Dipartimento di Sanità Pubblica Veterinaria, Sezione Clinica Medica, Facoltà di Medicina Veterinaria Università degli Studi di Messina, Polo Universitario dell’Annunziata, Messina, Italy


ABSTRACT
Research conducted in human sports medicine showed the variable effect of exercise on platelet aggregation parameters, which consist, for some authors, in a significant increase of aggregation, while for others, in its decline as result of physical activity. The purpose of our study was to test, in athletic horses, the effect of hydrocortisone as an anti-inflammatory steroid drug as an inhibitor of platelet aggregation in vitro. In our research a total of 12 jumper horses, 4 females and 8 geldings (Sella Italiana breed), clinically healthy, specifically trained and in good nutritional condition, were divided into two groups (A and B). From all the blood samples, collected in tubes containing sodium citrate (1 part sodium citrate to 9 parts blood), platelet aggregation was evaluated adding adenosine diphosphate (ADP) as a platelet-activating agent, and also after incubation for 20 minutes with hydrocortisone (IDR) as an inhibitor of platelet aggregation. Using an aggregometer platelet aggregation curves were defined and from these the aggregation rate and the slope of aggregation were evaluated. Multivariate ANOVA showed the significant effect of time (P<0.05) on platelet aggregation and on the average speed of aggregation (P<0.05). The effect of hydrocortisone was observed only on the aggregation slope (P<0.01). Further research should be conducted to assess the effective sensitivity of the slope, and certainly, given the absent effect of hydrocortisone on the platelet aggregation, another kind of anti-aggregation substance should be used to compare our data.

Key words: athletic horse, platelet aggregation, hydrocortisone, adenosine diphosphate, jumper horse

Introduction
Platelets, upon activation, stimulate thrombus formation and recruit additional platelets. Their activation is essential for several physiological and pathological reactions and depends upon their adhesion to the vessel wall and attachment to each other in the

*Corresponding author:
Prof. Giuseppe Piccione, Dipartimento di Scienze Sperimentali e Biotecnologie Applicate, Facoltà di Medicina Veterinaria, Università degli Studi di Messina, Polo Universitario dell’Annunziata, Messina, Italy, Phone: +39 90350 3584; Fax: +39 90350 3975; E-mail: giuseppe.piccione@unime.it

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aggregation process (SAWICKI et al., 1997). Plasma constituents and blood cells other than platelets affect aggregation and secretion in an agonist-dependent manner (THOMAS, 1996). Adenosin diphosphate (ADP) is used as a promoter of aggregation because it stimulates intracellular calcium flux and thereby establishes platelet activation, which leads to the formation of the “haemostatic plug” (JOHNSTONE, 1983). The aggregation response is then enhanced by the secretion of products of arachidonic acid metabolism and by further ADP release (JOHNSTONE, 1983; CATTANEO and GACHET, 1999).

Research conducted in human sports medicine has showed the variable effect of exercise on aggregation parameters, which consist, for some authors, in a significant increase of aggregation, while for others, in its decline as result of the physical activity (HENDRA et al., 1988; PRISCO et al., 1993; HURLEN et al., 2000; PETIDIS et al., 2008; HONG et al., 2009).

The effects of exercise on platelet aggregation and activation have been extensively studied, but the results are still highly variable. Platelet activation and aggregation seem to be directly related to the intensity of physical effort, and the duration and degree of training of the subject (EL SAYED, 2004). Certainly strenuous exercise causes an increase in the number of circulating platelets through the release of platelets from the spleen, and increased concentration of the von Willerbrand factor, but the effect on coagulation is also expressed through other mechanisms not yet well clarified (LIPPI and MAFFULLI, 2009; LIN et al., 1999). The increased fibrinolytic activity appears to counterbalance the exercise-induced increase in coagulability (PICCIONE et al., 2004a and b; PICCIONE et al., 2005). There is evidence that the reduction in bleeding time after exercise is due to the involvement of the platelets (PRISCO et al., 1993). However, long term physical endurance training seems to suppress platelet adhesiveness and aggregation (LEE and LIP, 2003).

Some studies have been conducted in horses to test the effectiveness of various non steroidal anti-inflammatory drugs (JOHNSTONE, 1983), but no studies are available in the current literature which compare platelet aggregation after exercise and the use of a selective inhibitor of platelet aggregation in athletic horses. The purpose of our study was to test the effect of hydrocortisone as an inhibitor of platelet aggregation in vitro in athletic horses.

**Materials and methods**

In our study, a total of 12 jumper horses were used, 4 females and 8 geldings (Sella Italiana breed), clinically healthy, specifically trained and in good nutritional state. All the subjects were divided into two groups (A and B) and examined to exclude general phenomena of dehydration. No medication was administered for one month before the study. Group A was composed of 6 horses, 5 years old and with an average weight of 450 ± 10 kg. Group B was composed of 6 horses aged between 7 and 9 years and with an
average weight of 490 ± 40 kg. Both sets of exercises consisted of a standardized obstacle
course length of 400 meters preceded by warming-up for 15 minutes. The warming-up
consisted of walk, trot, canter and three obstacles of increasing height, 0.6-1.00 meters
for Group A and 0.8 - 1.25 meters for the Group B. The obstacle course length of 400
m height of 10 obstacles 1 meter respectively for Group A and 125 for group B, was
divided into 4 vertical, 5 wide, and a combination of vertical and wide. From all subjects
blood samples were taken by jugular venipuncture using Vacutainers tubes (Terumo
Corporation, Japan) containing K3-EDTA and sodium citrate (1 part sodium citrate to
9 parts blood). Blood samples collected in K3-EDTA were analyzed using an automatic
multiparametric analyzer for haematology (HeCoVet C, SEAC, Italy) in order to obtain
a complete cell blood count. Blood samples collected in sodium citrate were treated to
prepare platelet-rich plasma (PRP) and platelet-poor plasma (PPP). To prepare the PRP,
the samples were centrifuged at 300 g × 20′, then the PRP removed and transferred into
Eppendorf. To prepare the PPP the blood samples were again centrifuged at 3000 g ×
10′ and also the PPP obtained was transferred into Eppendorf. Subsequently, all serum
samples obtained were divided into two aliquots. After the addition of 10 μM adenosine
diphosphate (ADP) as the platelet-activating agent, the sera were analyzed. One of the
aliquots was incubated for 20 minutes with 10 μL of hydrocortisone (Flebocortid Richter,
500 mg/5 mL) as the inhibitor of platelet aggregation (IDR). Using an aggregometer (Clot
2S, Radim-SEAC, Florence, Italy) the curves of platelet aggregation were defined and
the aggregation rate (percentage of maximum amount of clotted platelets) and the slope
of aggregation (percentage of platelets that aggregate in 1 minute) were evaluated from
these curves.

Statistical analysis. Statistical analysis of data was performed from the values of
platelet aggregation and the speed of aggregation obtained in different experimental
conditions. Multivariate ANOVA was applied to assess the statistically significant effect
of time in different experimental conditions (at rest, after exercise, after 30 min and
after 60 min from the end of exercise) and statistical differences due to the addition of
hydrocortisone. Bonferroni’s test was applied as a post-hoc comparison. A P value <0.05
was considered statistically significant. Statistica software version 7.0 (Stat Soft Inc.) was
used to perform the analysis. All results were expressed as mean ± standard deviation
of the mean (SD).

Results
No significance differences emerged in the hemochrome-cytometric parameters. In
particular the platelet count no showed significant variations during the experimental
conditions studied. Table 1 shows mean values and standard deviations of platelet
aggregation and the slope of aggregation, expressed in their conventional units of
measurement, observed during our research.
Table 1. Average values of the parameters considered, expressed in their conventional units of measurement with the related standard error, observed in different experimental condition in 12 jumper horses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
<th>Rest</th>
<th>After exercise</th>
<th>After 30</th>
<th>After 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Aggregation (10 μM ADP)</td>
<td>A</td>
<td>43.00 ± 7.04</td>
<td>26.55 ± 3.50</td>
<td>31.16 ± 8.58</td>
<td>22.23 ± 6.26</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>35.53 ± 9.63</td>
<td>30.93 ± 9.71</td>
<td>43.22 ± 9.87</td>
<td>25.73 ± 4.85</td>
</tr>
<tr>
<td>% Aggregation IDR</td>
<td>A</td>
<td>31.57 ± 10.58</td>
<td>20.01 ± 4.91</td>
<td>34.66 ± 10.93</td>
<td>41.79 ± 5.59</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>41.86 ± 6.78</td>
<td>24.75 ± 4.16</td>
<td>39.5 ± 6.97</td>
<td>33.16 ± 5.49</td>
</tr>
<tr>
<td>Slope (%/min)</td>
<td>A</td>
<td>3.05 ± 1.02</td>
<td>2.91 ± 1.06</td>
<td>3.91 ± 0.86</td>
<td>1.50 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.95 ± 1.01</td>
<td>3.63 ± 0.74</td>
<td>3.93 ± 0.78</td>
<td>3.33 ± 0.81</td>
</tr>
<tr>
<td>Slope % IDR (%/min)</td>
<td>A</td>
<td>3.86 ± 0.87</td>
<td>2.40 ± 0.45</td>
<td>3.00 ± 0.89</td>
<td>3.60 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3.13 ± 1.12</td>
<td>2.99 ± 0.20</td>
<td>5.05 ± 0.91</td>
<td>5.86 ± 0.61</td>
</tr>
</tbody>
</table>

Multivariate ANOVA showed the significant effect of time ($F_{(3.80)} = 26.41, P<0.0001$) on platelet aggregation. No effect of hydrocortisone was observed on this parameter. In Group A, a significant decrease of platelet aggregation was observed after exercise and after 60 min vs. rest, whereas a significant decrease was observed after 60 min vs. after 30 min. The value recorded 30 min after the end of exercise, however, showed an increase vs. after exercise. The same significant pattern was observed in Group B. On samples treated with IDR from Group A, a significant decrease after exercise vs. rest and a significant increase after 30 and 60 min vs. after exercise was observed. In samples treated with IDR from Group B, a significant decrease after exercise vs. rest and a significant increase 30 min after exercise vs. rest and after exercise was observed. The value recorded after 60 min showed a significant increase vs. after 30 min. The application of multivariate ANOVA on the average speed of aggregation, showed the significant effect of time ($F_{(3.80)} = 4.97, P<0.003$) and hydrocortisone ($F_{(1.80)} = 8.41, P<0.004$). In Group A the value recorded after 60 min showed a statistically significant decrease vs. rest, after exercise and 30 min from the end of exercise. In Group B, ANOVA showed a significant decrease after exercise, after 30 min and after 60 min from the end of exercise vs. rest. On samples treated with IDR from Group A, a statistically significant decrease was observed after exercise vs. reg at rest and an increase and after 30 min vs. rest, whereas a statistical increase after 60 min vs. after exercise was shown. On samples treated with IDR from Group B a statistically significant increase after 30 min and 60 min vs. rest and after exercise was observed. The comparison of samples with and without IDR showed a significant increase after 60 min with the addition of IDR both in Group A and in Group B.
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Fig. 1. Graphical representation of average mean values of platelet aggregation during different experimental conditions. a. Platelet aggregation of groups A and B; b. Platelet aggregation of groups A and B with addition to samples of 10 μL of hydrocortisone (Flebocortid Richter, 500 mg/5 mL).

Significances: *Vs Rest (P<0.05); ■ Vs After exercise (P<0.005); *Vs After 30 min (P<0.001); *Vs After 60 min (P<0.0001).

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Fig. 2. Graphical representation of average mean values of slope of aggregation during different experimental conditions. a. Slope of aggregation of groups A and B; b. Slope of aggregation of groups A and B with addition to samples of 10 μL of hydrocortisone (Flebocortid Richter, 500 mg/5 mL).

Significances: *Vs Rest (P<0.05); ■ Vs After exercise (P<0.005); ● Vs After 30 min (P<0.001); *Vs After 60 min (P<0.0001).

Discussion

Figures 1-2 represent the average values of platelet aggregation and the slope of aggregation during different experimental conditions both with and without the addition
of 10 μL hydrocortisone (Flebocortid Richter, 500 mg/5 mL). Our results showed no statistically significant changes in platelet count, as previously observed by other authors (KINGSTONE et al., 1999; WEISS et al., 1998; LEPHERD, 1977). Authors showed that moderate, rather than intense exercise in humans causes the increased release of catecholamines from the adrenergic system, as well as the increased release of nitric oxide by the cells’ vascular epithelium (WANG, 2006). This phenomenon causes the inhibition of thrombus formation, even under the action of an intense “shear flow”, and a decrease in platelet aggregation in response to exercise, even with the addition of ADP as an aggregation agonist (WANG, 2006; JOHNSTONE et al., 1991). Moreover, results from our previous studies show that training enhances fibrinolitic activity. Some studies in humans have shown that the effect of physical conditioning in the long term, such as from continuous and aerobic exercise, also involves platelet reactivity in general as well as other blood components, and results in a decrease in aggregation following exercise (PETIDIS et al., 2008). This should be considered in the light of research conducted in humans, which demonstrate that, in trained subjects, in contrast to sedentary ones, exercise causes a change in the ability of platelets to aggregate (DAVIS et al., 1990). Usually jumper horses undergo an intensive program of training before the start of their careers. In this study a decrease in aggregation occurred in both Group A and Group B. This means that the workloads used do not cause significant changes in the behaviour of aggregation. In fact, the result of both types of exercise was a decrease in platelet aggregation. This platelet aggregation behaviour is attributable to the increased release of catecholamines during exercise and to the activation of the hypothalamic-pituitary axis (CAYADO et al., 2006; HENDRA et al., 1988; HURLEN et al., 2000; SAKITA et al., 1997).

The trend shown by platelet aggregation can be explained by the substance used as aggregating agent. These observations were made by some authors who demonstrated that in equine platelets aggregation is only significantly decreased in response to ADP (KINGSTONE et al., 1999). Other research previously showed that different types of agonists or the ADP itself provide different results at higher amounts (PETIDIS et al., 2008; JOHNSTONE, 1983).

Several authors wanted to test on platelets the effect of the addition of substances to samples that antagonize the adhesion of platelets in vitro (JOHNSTONE, 1983; BAYLY et al., 1983; JARVIS and EVANS, 1996). The substances mostly used for this purpose are inhibitors of cyclooxygenase, i.e. non steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. These substances, by blocking the conversion of arachidonic acid into prostaglandins, prostacyclin and thromboxanes, act by preventing the platelet activation mediated by them. However, the action of hydrocortisone did not show any influence on platelet aggregation. Hydrocortisone has been used in some research as an anti-aggregation drug, with various results, especially dose-dependent. Hydrocortisone has a variety of immunological and haematological effects. Against platelets are known effects
of a decrease in aggregate dose-dependent, although are also known opposed effects. In particular in the case of ADP, a higher dose of hydrocortisone is needed to inhibit platelet aggregation than the dose required to inhibit aggregation mediated by other antagonists, such as collagen and prostaglandins (GLASS et al., 1981; SCHUERHOLZ et al., 2007).

The slope of the aggregation curve showed a significant decrease related to exercise and the anti-aggregation drug. This should be read in the light of the work of other authors, where it is proposed that the aggregation parameter alone may be measured as a function of the slope of the aggregation curve and for this reason it may be seen as a more sensitive parameter in detecting and recording changes in physical conditions such as temperature, pH, sample preparation and the presence of electrolytes (SEE et al., 1992; DE PAULA et al., 2009). We can assume, that in fact the presence of hydrocortisone, which did not significantly affect the platelet aggregation, caused a decrease in the values of the slope.

Further research should be conducted to assess the effective sensitivity of the slope, and certainly, given the absent effect of hydrocortisone on the platelet aggregation, another kind of anti-aggregation substance should be used to compare our data. Moreover, to clarify the results obtained, it would be interesting to test different workloads, to see how far the exercise interferes with the function of platelets in the equine species.

References


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SAŽETAK

Istraživanje poduzeta u humanoj športskoj medicini pokazala su da postoji različit učinak tjelesnog vježbanja na nakupljanje krvnih pločica pa tako neki autori smatraju da tjelesno vježbanje značajno povećava njihovo nakupljanje dok drugi smatraju da ona smanjuje nakupljanje krvnih pločica. Svraća je ovog rada u športskim konjima istražiti učinak protuupalnoga steroidnoga lijeka hidrokortizona kao inhibitora nakupljanja krvnih pločica in vitro. U istraživanje je bilo uzeto ukupno 12 preponskih konja, četiri kobile i osam kastrata pasmine Sella Italiana. Svi su bili klinički zdravi, uvježbani i dobrog gojbenog stanja, a bili su podijeljeni u dvije skupine (A i B). Uzorci krvi bili su uzet u epruvete s natrijevim citratom (jedan dio natrijevog citrata na devet dijelova krvi). Nakupljanje pločica bilo je određivano dodavanjem adenozin-difosfata (ADP) kao activatora nakupljanja i, nakon inkubacije od 20 minuta, dodavanjem hidrokortizona kao inhibitora nakupljanja pločica. Krivulje nakupljanja pločica bile su određene uporabom agregometra te je tako vrednovan stupanj nakupljanja. ANOVA analizom pokazan je značajan učinak vremena (P<0.05) na nakupljanje i na prosječnu brzinu nakupljanja (P<0.05). Učinak hidrokortizona ustanovljen je samo na krivulji nakupljanja (P<0.01).

Ključne riječi: športski konj, nakupljanje krvnih pločica, hidrokortizon, adenozin-difosfat, preponski konji