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Plasma levels of β-endorphin and in vitro lymphocyte proliferation as indicators of welfare in horses in normal or restrained conditions

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Summary

Plasma β-endorphin levels and lymhocyte proliferation were measured in 14 geldings to evaluate the animals' response to different challenges (application of a lip twitch for five minutes and restraining the animals blindfolded for two hours). The basal β-endorphin levels showed a great variability among the horses, but were consistent for the basal levels measured on different days for each animal. A significant effect of age on β-endorphin concentration was found (p=0.005). The β-endorphin levels showed an increase after the use of the lip twitch (p=0.0001) and returned to the pre treatment values within 30 minutes after the release of the animal. Plasma β-endorphin levels at the end of the blindfold treatment were lower than basal levels (p=0.025) and there was a decrease in the proliferation of lymphocytes (p=0.00001). A negative correlation was found between plasma β-endorphin levels and lymphocyte proliferation for individual horses in samples taken before (ρ=–0.62; p=0.039) and two hours after the application of the hood (ρ=–0.59; p=0.049).

Keywords: horses, β-endorphin, immune response, welfare, stress

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Die β-Endorphinkonzentration im Plasma und die Lymphozytenproliferation in vitro als Indikatoren für das Wohlbefinden von Pferden unter normalen und unter Zwangsbedingungen

Die Studie diente dem Zweck, die Reaktionen von Pferden auf unterschiedliche Bedingungen hin zu erfassen. Als Indikatoren für das Wohlbefinden der Tiere wurde der Plasmaspiegel des β-Endorphins gemessen und eine in-vitro-Lymphozytenproliferation verwendet. Endogene Opioide wie das β-Endorphin gelten schon seit einigen Jahren als Indikatoren für das Wohlbefinden eines Pferdes. In Verbindung mit stressigen Situationen werden vom Körper vermehrt Endorphine freigesetzt. Die genaue Reaktion des Pferdes auf bestimmte Stimuli bzw. Anforderungen ist bisher jedoch wenig erforscht.

In diesem Experiment wurden die Korrelationen zwischen Alter und Geschlecht der Pferde und ihrem Basis-Endorphinspiegel ermittelt und Veränderungen der endogenen Opioidausschüttung nach Zwangsmaßnahmen gemessen. Um die Immunokompetenz der Pferde zu überprüfen, wurde die Lymphozytenproliferation in vitro untersucht. Die Versuchspferde wurden bestimmten Bedingungen ausgesetzt. So wurde z.B. für 5 Minuten eine Nasenbremse angelegt oder den Pferden 2 Stunden lang in der Box die Augen verbunden. Vor und nach jedem Experiment wurden in bestimmten Zeitintervallen Blutproben entnommen.

Die β-Endorphinkonzentration im Plasma wurde mit einem Radioimmunoassay ermittelt, nachdem eine Reverse-Phase-Chromatographie das Material konzentrierte. Die peripheren Lymphozyten der Probe wurden separiert, aufbereitet und radioaktiv markierte Zellkulturen angelegt, welche szintigraphisch ausgewertet wurden.

Der basale Endorphinspiegel der einzelnen Pferde fiel sehr unterschiedlich aus. Er blieb jedoch für jedes Pferd konstant, wie Messungen an verschiedenen Tagen belegten. Es zeigte sich eine deutliche Altersabhängigkeit der Endorphinkonzentration, wobei ältere Pferde höhere endogene Opioidkonzentrationen im Blut besaßen. Es konnte keine Geschlechtsspezifität des β-Endorphinspiegels ermittelt werden.

Nach Anlegen der Nasenbremse nahm der β-Endorphingehalt merklich zu und kehrte innerhalb von 30 Minuten nach Beendigung des Experiments auf seinen Basalwert zurück. Der Versuch, die Pferde mit verbundenen Augen 2 Stunden lang in einer Box festzuhalten, erbrachte zum Schluß Endorphinkonzentrationen, die unter dem Basallevel lagen. Auch die Lymphozytenproliferation war reduziert. Insgesamt ergab sich eine negative Korrelation zwischen dem β-Endorphinspiegel und dem Umfang der Lymphozytenproliferation bei Messungen vor und nach dem Verbinden der Augen.

Die gewonnenen Daten aus dem Experiment mit der Nasenbremse standen im Einklang mit füheren Messungen. Der streßbedingte Anstieg des β-Endorphinspiegels im Blut war charakteristisch. Die Autoren vermuteten, daß die Messungen während des 2.Experiments mit verbundenen Augen nicht oft genug durchgeführt wurden und so möglicherweise ein Peak in der Endorphinkurve nicht erfaßt wurde. Der Abfall der Opioidkonzentration nach Abnehmen der Augenbinde könnte aufgrund eines negativen Feedback-Mechanismus entstanden sein.

Die negative Korrelation zwischen dem gemessenen Endorphingehalt und der Lymphozytenproliferation wies auf einen immunodepressiven Effekt des β-Endorphins hin. Der Versuch zeigte, daß die Reaktion der Pferde von der Art und Dauer einer Stressor-Einwirkung abhing und daß das Alter der Tiere eine wichtige Rolle spielt.

Schlüsselwörter: Pferde , β-Endorphin, Immunantwort, Tierschutz, Streß

Introduction

There is some evidence that endogenous opioids can be used as indicators of welfare in horses (*Lagerweij* et al., 1984; *McCarthy* et al., 1993; *Gillham* et al., 1994; *McGreevy* and *Nicol*, 1995). The processes underlying the release of β-endorphin in association with stressful stimuli are well understood but there is an apparent lack of in depth studies addressing basic questions such as the consistency of β-endorphin release when an individual horse is subjected to different challenges. When studying β-endorphin levels in association with behavioural problems (*Gillham* et al., 1994) or in response to transport (*McCarthy* et al., 1993) the possible influence of age and gender on plasma β-endorphin levels was not been considered. The in vitro proliferation of lymphocytes is useful as an indicator of immunocompetence in several species, which may be impaired by stress (*Blecha*, 1988; *Sacerdote* et al., 1994). Moreover there is evidence that neuropeptides play a role in the modulation of the immune system (*Blalock*, 1989; *Manfredi* et al., 1993). In the present study the role of gender and age on basal plasma β-endorphin levels was studied. In addition, plasma β-endorphin levels were monitored when horses were subjected to either five minutes restraint using a lip twitch or two hours tied in a box while keeping the animals blindfolded. The possible influences of physiological responses on measures of immunocompetence were investigated in the blood samples collected before and after the blindfolding experiment.

Materials and methods

Animals

Fourteen geldings and 7 mares were studied, ranging in age from 6 to 17 years old. The horses were kept in individual boxes in a riding centre close to Milan, Italy. Horses were fed twice a day with hay and concentrate and were ridden on average one hour/day.

Collection of blood

Blood was collected from the jugular vein in Vacutainer® tubes with EDTA (1 mg/ml blood). This procedure took a few seconds for each horse. No physical restraining was needed, since the operator was a veterinarian who was familiar to the animals. The horses were not visibly stressed by the withdrawal of blood. Samples were collected on three different days, the basal β-endorphin levels being determined for each animal on each day.

1. Day 0 → no manipulation: basal level determined for 21 horses (geldings and mares);
2. Day 7 → short term challenge (restraining the animals using a lip twitch for 5 minutes): blood samples were collected from 14 horses (geldings only) before (basal level) and 5, 15 and 30 minutes after the application of the lip twitch;
3. Day 14 → long term challenge (covering the animals' head with a hood while the horses were tied in the box for two hours): blood samples were collected from 14 horses (geldings only) before application of the hood (basal level) and immediately after its removal.

Blood processing: plasma β-endorphin levels

Aprotinin (Bayer, 500 KIU/ml) was added to the samples immediately after collection. Samples were kept in ice until they were centrifuged at 3000 g for 15 minutes at 4°C within two hours from collection. Plasma samples were acidified by the addition of acetic acid (Merck) to a final concentration of 0.1 M and stored at –20°C until further processing. β-endorphin levels were measured using a commercial radioimmunoassay kit (Peninsula laboratories) in plasma samples which were previously concentrated by reverse phase chromatography using C_{18} Sep-Pak cartridges (*Zanella* et al., in press). β-endorphin values are reported in pg/ml of plasma.

Blood processing: lymphocyte proliferation

Lymphocyte proliferation was determined only in the long term challenge (n=14). Peripheral lymphocytes were separated by gradient centrifugation on Ficoll-Paque, and micro cultures of 2×10^6 lymphocytes were set up in RPMI 1640, 10% FCS ± Concanavalin A (10 μg/ml, 5 μg/ml and 2.5 μg/ml). After 48 hrs incubation at 37°C, 1 μC of ^{3}H Thymidine (specific activity 2 Ci/mmol, Amersham, UK) was added to all cultures. Eighteen hours later, cells were harvested by an automatic cell harvester (Skatron) and radioactivity was measured in a liquid scintillation counter (Packard, Downers Grove, IL, USA). Background values, i.e. thymidine incorporation of unstimulated cells, were subtracted from mitogen-induced proliferations (*Sacerdote* et al., 1994). Values were expressed as "counts per minute" (CPM).

Statistical analysis

Spearman rank correlations were calculated to investigate the relationships between the three β-endorphin basal levels, between β-endorphin levels before and after the lip twitch and between β-endorphin levels before and after the hood. In addition, the correlations between β-endorphin levels and lymphocyte proliferation both before and after the hood treatment were calculated. For the correlation analysis of lymphocyte proliferation the values obtained at 5 μg/ml Concanavalin A were used, as these were considered the most informative. Non parametric analysis of variance (ANOVA) was used to test the difference for β-endorphin levels between genders. Regression analysis was used to evaluate the relationship between age and β-endorphin levels. *Friedman's* two-ways analysis of variance was used to asses the horses' response to the lip twitch and *Wilcoxon* matched pair t-test was used to compare the β-endorphin basal levels with each of subsequent sampling. Matched-pairs t-test was also used to analyse the effect of hood placement on β-endorphin levels. Lymphocyte proliferation before and after the hood was compared by two-way analysis of variance.

Tab. 1: Spearman's rank correlation matrix for β-endorphin levels before and after the application of the lip twitch (n=14).

	Basal	After 5 min.	After 15 min.	After 30 min.
Basal	1	0.767 p=0.0014	0.763 p=0.0015	0.675 p=0.0081
After 5 min.	–	1	0.877 p=0.0001	0.811 p=0.0004
After 15 min.	–	–	1	0.930 p=0.0001

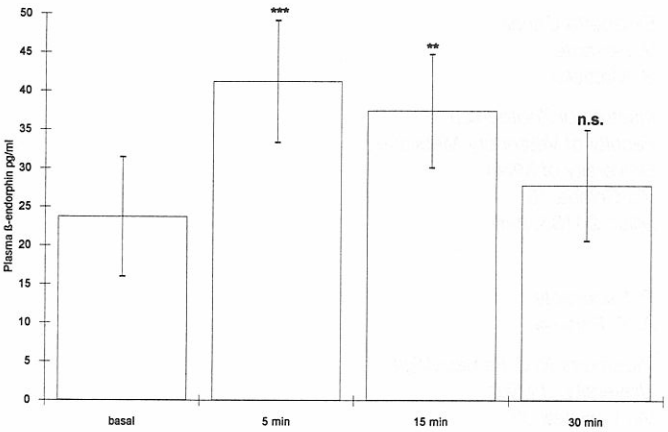

Fig. 1: Differences in plasma β-endorphin levels (means ± SEM) between basal levels and each subsequent sampling after restraining with a lip twitch.
** P<0.01 *** P<0.001 (matched pairs t-test)

Results

The basal β-endorphin levels showed a great variability among the horses, nevertheless the three basal samples for the 14 geldings (at day 0, day 7 and day 14) were significantly correlated (day 0–day 7:ρ=0.70, p=0.005; day 0–day 14: ρ=0.86, p=0.0001; day 7–day 14: ρ=0.68, p=0.007).
There was no significant difference between gender on basal β-endorphin concentrations (females: n=7, mean 69.75 ± 16.93; geldings: n=14, mean 45.81 ± 15.25).
There was a significant effect of age on β-endorphin concentration, where older horses had higher levels of plasma β-endorphin (n=21, r=0.59, p=0.005).
Table 1 shows the correlation matrix from the different β-endorphin measures (basal, +5 min, +15 min and +30 min after the twitch). β-endorphin levels showed a significant increase after the use of the lip twitch (chi-Squared=23.4, p=0.0001). β-endorphin levels had returned to the pre treatment values 30 minutes after the release of the animal (fig. 1).
The plasma β-endorphin levels before and after covering the horses head were positively correlated (n=14, ρ=0.89, p=0.002). The plasma β-endorphin levels 2 hours after the hood were significantly lower than the basal levels (mean difference before and after the hood =–3.47; p=0.02).

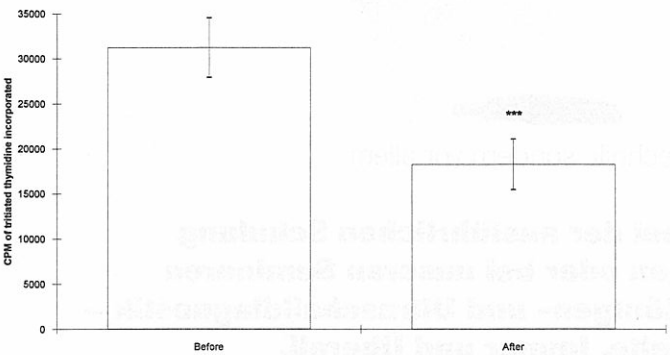

Fig. 2: In vitro lymphocite proliferation (means±SEM) before and after the application of the hood, in response to 5 μg Concanavalin A.
*** P<0.001 (two-ways analysis of variance)

There was a significant decrease in the proliferation of lymphocytes in samples taken after the removal of the hood (F=31.36; p=0.00001; fig. 2).
There was a negative correlation between plasma β-endorphin levels and lymphocyte proliferation for individual horses in samples taken before (ρ=–0.622, p=0.039) and two hours after the application of the hood (ρ=-0.59, p=0.05).

Discussion and conclusion

As older horses had higher levels of plasma β-endorphin, the great variability in basal levels among horses may be due to different ages of the animals. It would seem advisable that this variability be taken in account for future studies and the experimental design carefully planned to avoid the confounding effect of age. Gender (considering geldings and female only), on the other hand, seems to have no effect on β-endorphin levels in these data. In spite of the great variability among individuals, the significant positive correlation between β-endorphin levels in the three basal samples within the same horse seems to indicate that this opioid has a relatively constant basal concentration within each individual.
The present study is in agreement with previous works (*Lagerweij* et al., 1984; *McCarthy* et al., 1993) demonstrating that β-endorphin levels immediately increase after the application of a stressor as in the case of the restraint with the lip twitch. It also showed the rapid decrease of this endogenous opioid after the removal of the stressor. In the case of the two hours restraint and blindfolding the lack of an increase of β-endorphin levels may be due to the sampling schedule. It is possible that a sudden peak could have been missed as blood was taken two hours after the placement of the hood. A more frequent sampling could possibly have shown a trend similar to the one observed with the lip twitch. The reduction in β-endorphin levels detected after the removal of the hood might be explained by a lasting negative feed-back effect. The negative correlation found between β-endorphin levels and lymphocyte proliferation is consistent with the view that opioids may produce an immunodepressive effect, as already reported by *Manfredi* et al. (1993).
It is surprising that an apparently mild challenge, represented by blindfolding and restraint for two hours, had such a marked effect on lymphocyte proliferation. Given the fact that this practice, for shorter periods, is used during certain management procedures, further studies are recommended in order to clarify its effects on the welfare of horses. These findings suggest that the animals' responses to challenging situations are influenced by the type of stressor, its duration and also individual factors, such as the age of the horse, may play an important role. A combination of physiological, immunological and behavioural indicators can provide complementary information for assessment of animal welfare.

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