

Changes in leukocyte and antibody response following exercise in horses with booster vaccination against influenza and equine herpes virus 4/1

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SUMMARY

The aim of the study was to investigate the influence of strenuous repeated exercise applied during the effector phase of the immune response (14 days after booster vaccination) upon several parameters of the innate and specific immune responses.

Twelve healthy Hannoverian horses were revaccinated against influenza virus (IV) and equine herpes viruses types 1 and 4 (EHV1/4) and divided into 2 equal groups. In experimental group horses were besides submitted to repeated exercise (jumping for 4 consecutive days) beginning at the 14th day post revaccination, whereas control horses were only revaccinated.

The exercise, combined to revaccination, resulted to significantly increase the counts of band neutrophils on the 1st day after exercise and to induce monocytosis and eosinopenia 2 hours after the end of the exercise.

Neither the specific immune response against influenza virus types A1 and A2 nor the antibody response against EHV-1 and EHV-4 were reduced, evidencing that the applied exercise did not influence the immune response magnitude against vaccinal antigens.

Keywords : horse - booster vaccination - exercise - antibodies - leukocytes.

RÉSUMÉ

Changements dans les réponses leucocytaires et sérologiques induites par un exercice chez des chevaux ayant subi un rappel vaccinal envers le virus grippal et les herpes virus équin de types 1 et 4. Par D. GOUNDASHEVA, I. CHENCHEV, R. KATSAROVA, T. KARADJOV, I. TSACHEV et G. BARZEV.

Le but de cette étude a été d'analyser l'influence d'un exercice intense et répété, réalisé pendant la phase effectrice de la réponse immune (14 jours après un rappel vaccinal) sur plusieurs paramètres caractérisant les réponses immunitaires innées et spécifiques.

Douze chevaux sains de race Hanovre ont été revaccinés contre le virus de la grippe (IV) et les herpes virus équin de types 1 et 4 (EHV 1/4) et répartis en 2 groupes égaux. Les chevaux du groupe essai ont été soumis à un exercice répété (sauts d'obstacles pendant 4 jours consécutifs) commençant le 14^{ème} jour après le rappel vaccinal, alors que les chevaux contrôles ont été seulement revaccinés.

L'exercice combiné au rappel a engendré une augmentation significative du nombre de neutrophiles jeunes le 1^{er} jour suivant l'exercice et a induit une monocytose associée à une éosinopénie 2 heures après la fin de l'exercice.

Ni la réponse spécifique contre les virus grippaux de type A1 et A2, ni la production d'anticorps contre les virus EHV-1 et EHV-4 n'ont été réduites, ce qui montre que la réalisation d'un exercice n'influence pas l'intensité de la réponse immune envers les antigènes vaccinaux.

Mots-clés : cheval - rappel vaccinal - exercice - anticorps - leucocytes.

Introduction

Stress factors, including exercise, could influence the systemic defence mechanisms and be involved in the aetiology of many infectious diseases. Various forms of exercise, applied to human athletes and laboratory animals were accompanied by a dual effect on immune system.

From one part, the continuous moderate exercise stimulates the immune system and protects the individuals from infections [4, 20, 21, 23].

On the other hand, an immune suppression occurs following a continuous intensive exercise or a short-time strenuous training (exhaustive competitions or over-training).

This decrease of immune function is evidenced by the reduction of blood lymphocyte counts, a lower CD4+/CD8+ ratio associated to a leukocytosis, a depressed proliferate response of blood lymphocytes, changes of NK-cell function, lower neutrophile and macrophage activities and altered concentrations of secretory IgA and IgM, plasma cytokines and hormones [3, 9, 12, 14, 20, 23, 24, 29]. Consequently, the susceptibility of the organism towards infections increase especially of the upper respiratory tract.

In vaccinated sport horses the studies about the influence of exercise on the susceptibility to infectious diseases are however relatively few [5, 19]. The aims of the present study were to analyse the variations of several parameters of the

innate and specific immune response in horses, re-vaccinated against *influenza virus* (IV) and *equine herpes virus* 4/1 (EHV 4/1) following repeated exercise during the effector phase of the immune response.

Material and methods

1) HORSES

Twelve male Hannoverian, 4-9 year old, horses weighing 400-600 kg were housed in the Experimental Equine Base of Trakia University - Stara Zagora in box stalls under ambient light conditions and were fed with a diet containing commercially available pellets and mixed alfalfa-grass hay. Salt and water were given *ad libitum*. Horses were not trained or submitted to strenuous exercise up to day 14 after the revaccination. For a few hours daily, the horses were walked free in paddock. They were divided into two groups : in the control group (n = 6) horses were only re-vaccinated and in the experimental group (n = 6), horses were submitted to exercise and revaccination. One year ago both groups were vaccinated against influenza virus (IV) and EHV 4/1.

The Trakia University Animal Care Committee approved the study protocol and animals were maintained in accordance with the Bulgarian Animal Welfare Regulations.

2) REVACCINATION

At the day of revaccination, a aseptic booster dose (1 ml) of the oily adjuvanted vaccine against *influenza virus* and rhinopneumonitis Fluvac EHV 4/1 Plus (Fort Dodge Laboratories Inc., Fort Dodge, Iowa, USA) was intramuscularly injected in the neck in all horses, previously free of parasites.

During the 14 days following the vaccination, the horses underwent a careful daily clinical examination.

3) EXERCISE PROCEDURE

The exercise procedure consisted in 15 min walk at a speed of 100-200 m/min, then 15 min trot at 250 m/min and 5 min canter at 350 m/min. Afterwards, the horses performed

7 preliminary jumps followed by another 15 jumps in a par-cour with gradually increase of the height from 0.9 to 1.1 m. The height was increased to eliminate the habituation of the hypothalamic-pituitary-adrenal axis resulting in attenuated responses. The horses were submitted to a peak exercise similar to a competition for four consecutive days, between 8 a.m. and 11 a.m. beginning the 14th post revaccination day.

4) BLOOD COLLECTION

Blood samples were taken from *v. jugularis externa* into test tubes without anticoagulant. Blood was allowed no more than one hour to clot at room temperature and was centrifuged at 1200 x g for 10 min. The serum was then collected and used to identify the antibody response one day prior to the revaccination (status) and the 21st and 28th post revaccination days (corresponding to days 4 and 11 after the end of exercise, respectively).

To analyse total and differential leukocyte counts, blood samples were taken in vacuum tubes containing sodium heparin, before exercise (BE), immediately (hour 0) and 2 hours after the last exercise procedure corresponding to the 14th and 17th post revaccination days and on days 1, 2, 4, 11 after the end of exercise (corresponding to 18th, 19th, 21st and 28th post revaccination days (Fig. 1)).

5) BLOOD ANALYSIS

5.1. Blood cell counts

The leukocytes were counted in the Bürker chamber, and the differential leukocyte counts were performed on blood smears stained with May-Grünwald-Giemsa dyes using the Meandre counting method.

5.2. Serological analysis

The EIA combi Test Kit (Svanovir[®], Svanova Biotech, Uppsala, Sweden) allowed to detect and discriminate antibodies specific to EHV-1 and EHV-4 in sera of infected animals. The kit procedure was based on an indirect enzyme immunoassay (indirect EIA). In this procedure, samples (diluted to 1/100 in sample dilution buffer) were then exposed to non-infectious EHV-1 and EHV-4 antigen coated wells and to a well coated with control antigen in microtitre

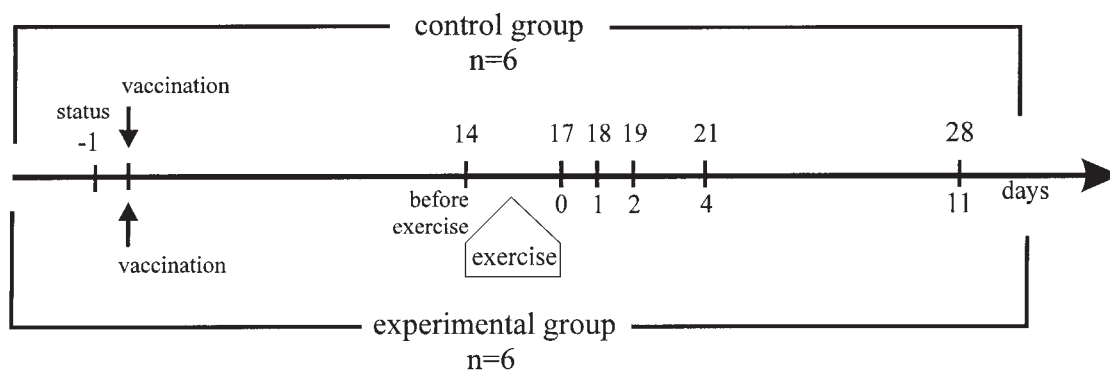


FIGURE 1. — Schedule of the experimental design - booster vaccination and exercise in both experimental and control groups during the whole period of the study.

strips. Whenever antibodies against EHV-1 and EHV-4 were present in the test sample, they bound to virus antigens in the wells. Horseradish peroxidase conjugated with rabbit anti-horse Ig (dilution 1:10 000) bound to all horse antibodies present into wells. When the substrate solution (orto phenylen diamine) was added, a blue colour developed. A positive result was indicated by a strong colour change. The optical density (OD) was measured at 450 nm by a microplate photometer Multiscan 340 (Laboratorial Systems, Helsinki, Finland).

Antibodies against EIV (Equine *Influenza* virus) were detected by the haemagglutination-inhibition test. It was performed in Microsystems according to conventional methods. Sera were treated with 1/90 M KIO₄ (periodate) at room temperature for 60 min. Periodate was then inhibited with 1% glycerine solution in saline (0.15 M NaCl) for 60 min at room temperature, then at 56°C for 30 min and finally adsorbed with chicken erythrocytes. The final test serum dilutions were 1:4, 1:10, etc. of the serum collected in saline solution. The reference - A/equi-1/Prague 56 (H7N7) and A/equi-2/Miami 63 (H3N8) strains were used as antigens in haemagglutinin units. Tests were performed in round-bottomed microtitre plates using volumes of 0.2 ml.

5.3. Cortisol assay

The serum cortisol was determined by a competitive immunoassay using direct chemiluminiscent technology (Chiron Diagnostics ACS:180[®] Automated Chemiluminescence Systems, Chiron Diagnostics Ltd., UK). Cortisol in equine samples competed with acridinium ester-labelled cortisol in the Lite reagent for binding to polyclonal rabbit anti-cortisol antibodies in the Solid Phase. The polyclonal rabbit anti-cortisol antibody was bound to monoclonal mouse anti-rabbit antibody, which was covalently coupled to paramagnetic particles in the Solid Phase. The sensitivity of the method was up to 2069 nmol/l with a detectable minimum concentration of 5.5 nmol/l.

6) STATISTICAL ANALYSIS

The results are given as mean ± SEM and submitted to a statistical analysis of variance (ANOVA). *Post hoc* comparisons of individual group means were carried out by the least significant difference test (LSD). Differences were considered statistically significant at the $p < 0.05$ level.

Results

1) SEROLOGICAL PROFILES

During the experimental period, the antibody response against EHV-1 in control horses (Table I) increased gradually and reached a maximum on the Day 28 after the revaccination ($p < 0.01$). The antibody response against EHV-4 also increased, but earlier (as soon as the day 21, $p < 0.001$) and elevated antibody concentrations persisted up to the day 28 compared to basal values ($p < 0.001$).

The kinetics of antibody responses to EHV-1 and EHV-4 in the experimental group were similar to the control group, and no significant difference was evidenced.

After revaccination against *influenza* viruses types A1 and A2 (Table II), antibody titres were markedly increased on days 21 and 28 compared to initial values in the control and the experimental groups ($p < 0.001$). Against, the vaccinal response was comparable in the 2 groups.

2) HAEMATOLOGICAL CHANGES

In control horses, a significant increase of monocyte counts compared to basal values (Day 14) was observed on the 18th day ($p < 0.05$), and the lymphocyte counts increased on the 17th ($p < 0.05$), 18th ($p < 0.001$) and 28th ($p < 0.01$) days after revaccination, but no significant changes in total and other specific leukocyte counts was noticed (Table III).

By contrast, total leukocyte counts significantly decreased in experimental horses on the 4th and 11th days after the end

	Anti EHV-1			Anti EHV-4		
	Status (Day -1)	Day 21	Day 28	Status (Day -1)	Day 21	Day 28
Control group	0.36 ± 0.06	0.86 ± 0.31	1.28 ± 0.12 ^b	0.35 ± 0.04	2.97 ± 0.27 ^c	3.03 ± 0.16 ^c
Experimental group	0.30 ± 0.07	0.54 ± 0.20	1.25 ± 0.05 ^c	0.32 ± 0.04	2.78 ± 0.17 ^c	3.20 ± 0.13 ^c

^b : $p < 0.01$ and ^c : $p < 0.001$ vs. the status values.

TABLE I. — Antibody response (optical density - OD) to EHV-1 and EHV-4 according to time after revaccination in sera of control (n = 6) and experimental (n = 6) horses. Results are expressed as mean ± SEM.

	Anti EIVA ₁			Anti EIVA ₂		
	Status (Day -1)	Day 21	Day 28	Status (Day -1)	Day 21	Day 28
Control group	0.60 ± 0.08	1.80 ± 0.06 ^c	2.00 ± 0.06 ^c	0.50 ± 0.06	1.90 ± 0.08 ^c	2.15 ± 0.09 ^c
Experimental group	0.55 ± 0.09	1.60 ± 0.08 ^c	1.75 ± 0.07 ^c	0.60 ± 0.08	1.95 ± 0.09 ^c	2.10 ± 0.10 ^c

^c : $p < 0.001$ vs. the status values.

TABLE II. — Antibody responses (lg10) to EIV (Equine *Influenza* Virus) types A₁ and A₂ according to time after revaccination in sera in control (n = 6) and experimental (n = 6) horses. Results are expressed as mean ± SEM.

Parameters	Days after the revaccination							
	14	17	18	19	21	28		
	Time intervals after the end of exercise							
	Before	0 hour	2 hours	1 day	2 days	4 days	11 days	
Control group	WBC	6.48 ± 0.36	5.95 ± 0.36	6.50 ± 0.33	7.75 ± 0.75	6.15 ± 0.62	5.32 ± 0.61	7.00 ± 0.82
	Band neutrophils	0.15 ± 0.04	0.12 ± 0.03	0.11 ± 0.03	0.12 ± 0.02	0.14 ± 0.02	0.12 ± 0.02	0.20 ± 0.06
	Segmented neutrophils	3.59 ± 0.30	2.80 ± 0.24	3.16 ± 0.16	3.97 ± 0.65	2.92 ± 0.31	2.69 ± 0.30	3.49 ± 0.49
	Eosinophils	0.24 ± 0.04	0.18 ± 0.05	0.22 ± 0.02	0.24 ± 0.03	0.17 ± 0.03	0.18 ± 0.05	0.21 ± 0.05
	Monocytes	0.14 ± 0.03	0.10 ± 0.04	0.12 ± 0.02	0.24 ± 0.04 ^a	0.21 ± 0.04	0.11 ± 0.03	0.14 ± 0.02
	Lymphocytes	2.41 ± 0.13	2.72 ± 0.37	2.85 ± 0.26 ^a	3.18 ± 0.22 ^c	2.72 ± 0.32	2.26 ± 0.34	3.01 ± 0.45 ^b
Experimental group	WBC	7.07 ± 0.61	5.90 ± 0.20	6.30 ± 0.50	6.25 ± 0.64	5.80 ± 0.46	5.15 ± 0.49 ^a	5.10 ± 0.73 ^a
	Band neutrophils	0.11 ± 0.03	0.15 ± 0.04	0.11 ± 0.04	0.43 ± 0.05 ^{c3}	0.39 ± 0.04 ^c	0.27 ± 0.05 ^b	0.18 ± 0.04
	Segmented neutrophils	4.15 ± 0.55	2.92 ± 0.18	3.19 ± 0.42	2.85 ± 0.38 ^a	2.17 ± 0.25 ^c	2.33 ± 0.39 ^c	2.54 ± 0.46 ^b
	Eosinophils	0.20 ± 0.06	0.22 ± 0.06	0.04 ± 0.01 ^{a3}	0.24 ± 0.08	0.25 ± 0.05	0.24 ± 0.03	0.21 ± 0.04
	Monocytes	0.19 ± 0.06	0.19 ± 0.05	0.28 ± 0.04 ²	0.13 ± 0.07	0.19 ± 0.03	0.09 ± 0.01	0.09 ± 0.02
	Lymphocytes	2.48 ± 0.19	2.42 ± 0.19	2.69 ± 0.27	2.61 ± 0.49	2.82 ± 0.19	2.21 ± 0.22	2.14 ± 0.29

^a : p<0.05 ; ^b : p<0.01 ; ^c : p<0.001 vs. the status values ; ² : p<0.01 and ³ : p<0.001 vs. the control values obtained at the same time.

TABLE III. — Variations of hematological parameters (total (WBC : White Blood Cells) and differential leukocyte count) ($\times 10^9/L$) according to time after revaccination and exercise in control (n = 6) and experimental (n = 6) horses. Results are expressed as mean \pm SEM.

of exercise (corresponding to days 21 and 28 after revaccination) ($p<0.05$). Numbers of band neutrophils significantly increased from day 1 (day 18 post vaccination) to day 4 after exercise (day 21 post vaccination) ($p<0.001$ on days 1 and 2 vs. basal values and $p<0.001$ vs. control values on day 1 and $p<0.01$ on day 4 vs. basal values). However, the proportions of segmented neutrophils significantly decreased from day 1 to day 11 after exercise, ($p<0.05$ on day 1, $p<0.001$ on days 2 and 4, and $p<0.01$ on day 11 vs. basal values).

Moreover, 2 hours following exercise, significant variations of eosinophil and monocyte counts compared to values obtained in control horses ($p<0.001$ for eosinophils, and $p<0.01$ for monocytes) were evidenced : eosinophil number dramatically decreased and monocytes increased. The number of eosinophils decreased also compared to initial values ($p<0.05$). The lymphocyte counts tended to increase on day 2 after exercise (day 19 after revaccination).

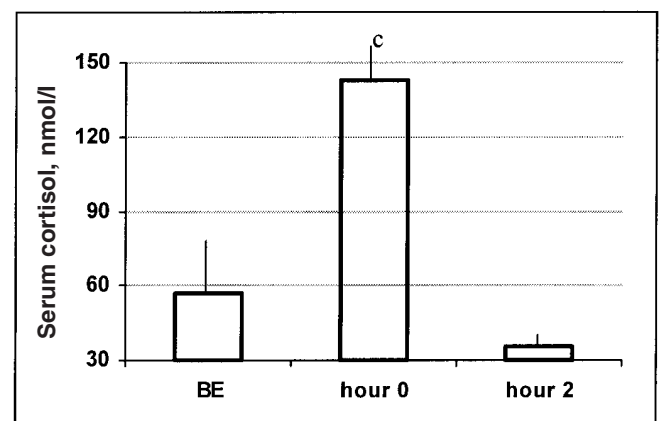
3) CHANGES IN CORTISOL CONCENTRATIONS

The serum cortisol concentrations in control horses did not significantly vary according to time after revaccination. The values were comprised between 58.1 and 53.53 nmol/l and the average serum cortisol concentration was 55.74 ± 1.80 nmol/l.

In experimental horses, cortisol concentrations were markedly enhanced immediately after the training (initial values 57.33 ± 16.47 nmol/l - exercise values : 142.83 ± 12.5 nmol/l ; $p<0.001$) and thereafter (2 hours after the end of exercise), they returned to basal level (Fig. 2).

Discussion

The data for controls provided evidence for an active involvement of humoral immune response mechanisms characterised by increases of serum antibodies against vaccinal viruses (EHV-1/4 and EIV A₁/A₂). The virus-dependent increase of the antibody response following immunization with inactivated *influenza virus* was reported by MOLITOR *et al.* [17] and BRUN *et al.* [2]. Others [1, 18, 26, 28] demonstrated elevated antibody titres against EIV A₁, EIV A₂ and EHV-1 after booster vaccination in horses. The immune stimulation after the revaccination was probably related to the proliferation of blood lymphocytes stimulated



^c : p<0.001 vs. values obtained before exercise.

FIGURE 2. — Variations of serum cortisol concentrations in experimental horses (n = 6) according to time after exercise (BE : Before exercise, hour 0 : immediately after exercise and 2 hours after the end of exercise). Results are expressed as mean \pm SEM.

by viral proteins that are known to act as mitogens or superantigens on some lymphocyte subpopulations [31].

The lack of considerable changes in leukocyte counts in these groups suggested that during the period of the study, the specific defense mechanisms prevailed over the natural ones.

The antibody titres against equine *influenza* virus types A₁ and A₂ and against EHV-1 and EHV-4, by days 4 and 11 following exercise (corresponding to the 21st and 28th revaccination days) increased also in experimental horses. The absence of significant differences of antibody titres between the experimental and the control groups demonstrated that training did not modify the immune response against vaccinal antigens. Other studies [11, 15, 32] also illustrated that the vaccinal responses against EIV A₁, EIV A₂ and EHV-1 were adequate after an extensive physical activity.

The physical training was accompanied by a moderate leukopenia related to considerable decrease in segmented neutrophils. This fact is contrary to previous observations [6, 25, 27]. The authors reported that a leukocytosis due to increase of segmented neutrophils occurred after physical training with a various intensity and duration. However, GROSS *et al.* [7] have shown that exercise combined with *influenza* virus infection induced decreases of leukocytes counts. The virus-dependent decrease of segmented neutrophils was also documented following a herpes-virus 1 (EHV-1) infection [28] and influenza epidemics [13, 33].

The increase of band neutrophile percentages, observed between days 1 and 4 after exercise in experimental horses has been also observed by other authors [10, 30]. Furthermore, they have demonstrated that the modifications of band neutrophile percentages were accompanied by release of biologically active substances as plasma growth hormone, adrenaline, IL-6 and IL-1. Most probably, these biologically active substances are responsible for neutrophile mobilisation.

Our study showed the development of monocytosis, and of eosinopenia 2 hours after exercise. Similar increases of monocytes and of IL-1 and IL-6 production from activated monocytes after physical training were also reported by HAAHR [8] and PEDERSEN [22]. But, until now, it is not well established how monocytes become activated in the early hours following exercise.

A decrease in eosinophile percentages in the early hours following exercise was already noted [6, 22], and could probably result from the increase of cortisol concentrations, observed immediately after the exercise. Indeed, it is well known that exogenously administered cortisol [16] induces eosinopenia and consequently eosinopenia could be considered as a manifestation of stress reaction.

The tendency for lymphocyte counts to increase in experimental horses, 2 days after training most probably reflected the systemic immuno-biological adaptation after the revaccination. Such a variation is also evidenced in control horses.

Conclusions

The application of repeated exercise during the effector

phase of the immune response in horses with booster vaccination by the multivalent Fluvac EHV 4/1 Plus vaccine against *influenza* and rhinopneumonitis was accompanied by a significant increase of the counts of band neutrophils on day 1, and monocytosis and eosinopenia, 2 hours after exercise. The magnitude of the immune response against equine influenza viruses types A₁ and A₂ and the antibody response against EHV-1 and EHV-4 were not altered. The observed dominance of specific over the non-specific mechanisms of defence could be useful for equine practice, because after the 14th day of revaccination, the horses could be included in training and competitive programmes without notable alteration of their immune status.

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