

Effects of weaning on polymorphonuclear cells phagocytic activity, lymphocyte proliferation and serum IgA-concentration in foals

Anas Sarwar^{1*}, Verena Budde², and Heinrich Enbergs²

¹*Department of Veterinary Anatomy, University of Agriculture, Faisalabad, Pakistan*

²*Institute of Anatomy, Physiology and Hygiene of Domestic Animals, Bonn University, Germany*

SARWAR, A., V. BUDDE, H. ENBERGS: Effects of weaning on PMN cells phagocytic activity, lymphocyte proliferation and serum IgA-concentration in foals. Vet. arhiv 78, 1-11, 2008.

ABSTRACT

Eighty-four foals (46 females; 38 males) of different breeds (Thoroughbreds, Standardbred, German Warmbloods), aged 5-6 months, were used in this study to evaluate the effects of weaning and other influencing factors like gender, breed and serum cortisol content on polymorphonuclear cells (PMNC) phagocytic activity, mitogeninduced lymphocyte proliferation and serum IgA-concentration. PMN phagocytic activity was measured by the luminal-dependent chemiluminescence, lymphocyte proliferation rate by MTT-test and serum IgA-level by radial immunodiffusion. PMNC phagocytic activity ranged between 4500-6000 cpm/ μ L in weanling foals. Despite a slight increase in the beginning, PMNC phagocytic activity clearly declined ($P < 0.01$) the next day to weaning and thereafter, rose again. Lymphocyte proliferation showed a marked rise after first day of the study and then remained unaltered until the end of study except for a minor depression following weaning. Weaning induced a slight but not significant rise in serum IgA-concentrations. Among other variables, breed had a major influence on PMNC phagocytic activity and serum IgA-concentration. This may be a real influence of breed or one of stud farm/management. Interestingly, serum cortisol content did not show a correlation with any of the parameters studied. It is conceivable that weaning has a suppressive effect on the phagocytic activity of PMNC.

Key words: foals, weaning, lymphocyte proliferation, phagocytic activity, serum, IgA

Introduction

Weaning is a strong physiological and psychological stressor for foals (NIEZGODA and TISCHNER, 1994) which is considered to be a potential cofactor in the pathogenesis of infectious diseases (GRIFFIN, 1989; BIONDI and ZANNINO, 1997; MOONS et al., 2005).

*Contact address:

Dr. Anas Sarwar, Chairman, Department of Veterinary Anatomy, University of Agriculture, Faisalabad 38040, Pakistan, Phone: +92 41 9200161 70/3102; Fax: +92 41 9200764; E-mail: anas_sarwar@hotmail.com; anas_sarwar@naf.edu.pak

Such stress affects many aspects of the integrative network between the immune, central nervous and endocrine systems in animals and humans (DANTZER and MORMEDE, 1985; LEONARD, 1995; HICKEY et al., 2003). However, the influences of stressors on immune responses are complex and depend not only on the characteristics of the stressor (i.e., its nature, intensity, duration and frequency), but also on the time at which the stressor acts in relation to immune development and competence (DANTZER and KELLEY, 1989).

The immune system can be divided into two mutually communicating areas: the non-specific and specific immune responses. The non-specific response is mediated largely by neutrophilic granulocytes and monocytes. The specific immune reaction depends upon antigen-sensitized lymphocytes and specific antibodies; however, lymphocytes also participate in a series of non-specific responses. Although foals are commonly weaned at 5-6 months of age (MEYER, 1986), the maternal antibodies in the mare's milk disappear at 3-4 months of age, suggesting that the immune system of foals could have attained considerable maturity by weaning time. Excessive weaning stress may affect appetite, metabolism and immune competence (NEWBERNE and CONNER, 1989).

The present study was undertaken to investigate the effects of weaning and other influencing factors like gender, breed and serum cortisol content on PMN phagocytic activity, lymphocyte proliferation ability and serum IgA-concentration in foals.

Materials and methods

Foals. Eighty-four foals of both genders (46 females; 38 males), aged 5 to 6 months, belonging to three breeds i.e., Thoroughbred (n = 45), Standardbred (n = 30) and German Warmbloods (N = 9) were used in this study. The foals belonged to five different stud farms located in Nordrhein Westfalen state of Germany. The management and nutritional conditions were intensive, and comparable to a large extent. After weaning, all foals were kept separate in gender groups during the daytime on the pasture and during the night in the stables. Thoroughbred foals were, however, placed in individual boxes at night after isolation from their mothers. The weanlings received milk from their mothers and pasture before weaning and shifted to a 0.5 kg/per day of a mixture of oats, grains and minerals (1 hand) after weaning. All candidates for weaning were clinically evaluated and found clinically healthy throughout the entire span of study.

Sampling and analyses. Four blood samples were collected from each foal as per schedule: Day - 4: 4 days before weaning; Day - 2: 2 days before weaning; Day 1: one day after weaning; Day 14: 14 days after weaning. Samples of blood were collected between 700 and 900 in the morning. 20 mL blood was collected by jugular venipuncture in heparinized monovette tubes, 2 mL in Fluoride-EDTA labeled tubes and 20 mL in serum gel tubes.

Neutrophilic phagocytic activity and lymphocyte proliferation tests were performed within 6 hours after sampling. The blood was allowed to clot and centrifuged at $1900\times g$ for 10 minutes for serum separation. The serum samples were frozen at $-18\text{ }^{\circ}\text{C}$ until analyzed. Serum levels of cortisol concentrations were measured using enzyme immunoassay testkit 'Serozyme' to evaluate hypothalamo-adrenohypophyseal-axis activity as a traditional measure of stress.

PMNC phagocytic activity. PMNC phagocytic activity was measured by chemiluminescence with the help of 6 canal Biolumat (Biolumate LB 9505 Berthold Wirdbad, Germany) connected with an IBM compatible PC and corresponding software. Briefly, a 100 μL heparinized whole blood was diluted with 400 μL MEM buffer and 40 μL luminol, incubated at $39\text{ }^{\circ}\text{C}$ for 5 minutes and the background value was measured. Thereafter, 40 μL of zymosan was added and chemiluminescence was measured over a period of 25 minutes. The results were recorded in counts per minute/ μL in the form of curves. The evaluation and judgment of results were performed using integral 3 (Integral C-3 = IntegralIPMNC/pi blood = cpmlpi), following NAGAHATA (1988). Enumeration of total leucocyte counts was performed using the Unopette[®] system (Beckton Dickinson, USA).

Lymphocyte proliferation test. Blood samples were layered on the same quantity of Histopaque 1.077 (Sigma Labs., USA) and sodium diatrizote. Samples were centrifuged at $400\times g$ for 30 minutes at room temperature. Lymphocytes were aspirated with the help of a sterilized pipette: Two washing steps were followed in PBS centrifuging at $250\times g$ for 10 minutes. Finally, the pellet was resuspended in a complete medium (RPMI 1640 + 20% autologous serum + 100 U of Penicillin + 100 μg of Streptomycin + 2% Glutamine) and centrifuged again. Cell concentrations were determined using a haemocytometer and were adjusted to 2×10^6 per mL of the complete medium. Viability of cells ($>95\%$).

Isolated lymphocytes (100 in the complete medium were added in triplicate to 96 well flat bottom microtiter plates. 50 μL of phytohaemagglutinin (PHA) as mitogen was added in concentrations of 0% and 1.25% in RPMI 1640.

Cell culture plates were incubated for 72 hours in a 5% humidified incubator at $39\text{ }^{\circ}\text{C}$. Following incubation, 10 μL MTT marking reagent (Tetrazolium) was pipetted in each well, and plates were again incubated for an additional 4 hours. Thereafter, 100 μL solubilisation solution was dispensed in each well and the plates were incubated overnight. Measurements were done with the help of a multiwell spectrophotometer using a wavelength of 550 nm. Data were expressed in terms of stimulation index (SI), which was calculated by subtracting OD with 0% PHA from OD with 1.25 μg PHA.

Serum IgA - concentration assay. Serum IgA-concentration was determined with the help of a simple radial immunodiffusion test using a commercially available testkit Bind

a RID™ (The Bionding Site, Germany, Serono Diagnostika, GmbH Germany) especially designed for the quantization of the IgA- concentration in the serum of horses. The results were expressed in mg/l. Serum IgA-concentrations were measured on day -4 and day -1 before weaning, and on day 1 and 14 after weaning.

Statistics. Covariance analysis was performed for all parameters to ascertain the effect of different influencing factors including time, breed and serum cortisol level as well as sex/time and breed/time. The effect of time was further ascertained with the help of explorative data analysis. Besides covariance analysis and data explorative analysis, the *t*-test was used for paired samples to compare pre- and post-weaning values. All computations were done with the statistical programme BMDP (Statistical Software, Inc., USA).

Results

The courses of PMNC phagocytic activity, lymphocyte proliferation and serum IgA concentration for the total contingent of foals and for different breeds are presented in Fig 1-3. Despite a slight increase in the beginning, PMNC phagocytic activity clearly declined (15%) on day 1 following weaning but thereafter, rose. Lymphocyte proliferation showed a marked rise on day-1 and then remained unaltered until the end of study except a minor depression following weaning. Weaning-induced stress did not modify lymphocyte proliferation. Serum IgA showed slight but non significant increase following weaning.

Mean \pm SD values of all parameters under investigation for the entire period of study are presented in Table 1. The effects of different factors on PMN phagocytic activity, lymphocyte proliferation and serum IgA-concentrations are summarised in Table 2.

Time and breed as well as breed/time affected PMN phagocytic activity. Above all, the postweaning values of neutrophilic phagocytic activity were found to be lower than preweaning values. Time as well as breed/time affected lymphocyte proliferation. However, the differences between pre- and postweaning values of lymphocyte proliferation were not significant. Serum IgA-concentrations were found slightly greater after weaning as compared with preweaning values. Different breeds showed highly significant differences in IgA-concentrations.

Table 1. Mean \pm SD of PMN phagocytic activity (cpm/ μ L), lymphocyte proliferation test (SI) and serum IgA-concentration (mg/L) for the total contingent of foals (n = 84) throughout the study period.

Parameter	Pre-weaning		Post-weaning	
	Day -4	Day -2	Day 1	Day 14
PMN Phagocytic activity (cpm/ μ L)	5947.85 \pm 1896.81	6175.58 \pm 2667	4833.23 \pm 1749.36	5354.66 \pm 2352.15
Lymphocyte proliferation test (SI)	10.27 \pm 4.67	11.62 \pm 2.97	11.13 \pm 3.15	11.57 \pm 3.67
Serum IgA-concentration (mg/L)	1316.60 \pm 399.53	1323.90 \pm 425.23	1369.80 \pm 430.84	1370.80 \pm 416.63

Table 2. Results of statistical tests of PMN phagocytic activity (cpm/ μ L) lymphocyte proliferation test (SI) and serum IgA-concentration (mg/L) for the total contingent of foals throughout the study period

Test	Variable	PMN Phagocytic activity	Lymphocyte proliferation test	Serum IgA concentration
		P-Value	P-Value	P-Value
Covariance analysis	Time	0.000***	0.001**	0.794 n.s.
	Sex	0.254 n.	0.144n.s	0.056 n.s.
	Breed	0.000***	0.737 n.s	0.000***
	Cortisol	0.026 n.s.	0.682 n.s.	0.824 n.s.
	Sex/Time	0.101 n.s.	0.779 n.s.	0.124 n.s.
	Breed/Time	0.001**	0.000***	0.106 n.s.
Explorative data analysis	Time	0.000***	0.018*	0.033*
T-test for paired samples (comparison of pre- and postweaning means)		0.000***	0.55ln.s	0.007**

P>0.05 = non significant (n.s); + = P<0.05; ** = P<0.01; *** = P<0.001; Time (day 2 and 1 preweaning; day 1 and 14 postweaning), sex (46 females; 38 females); breed: thoroughbred (n = 45), standardbred (n = 30) and German Warmbloods (n = 9), cortisol sampling days (day 2 and 1 preweaning; day 1 and 14 postweaning).

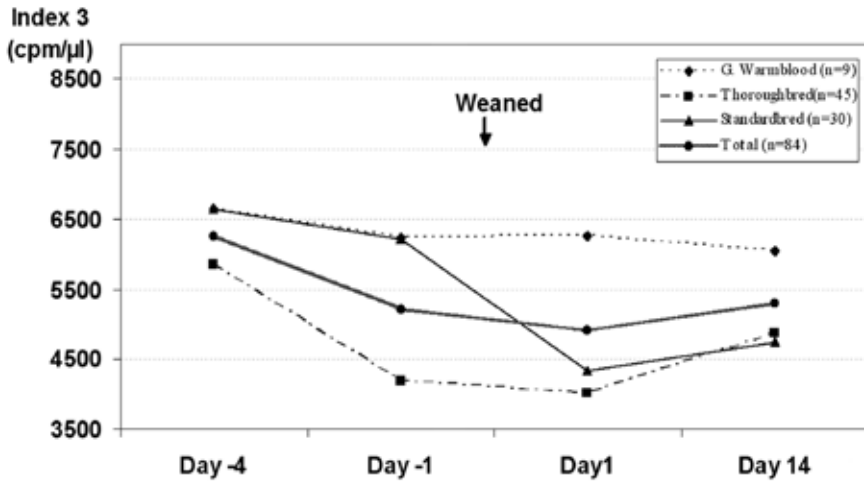


Fig. 1. Courses of PMNC phagocytic activity in different breeds and total contingent of foals throughout study period. $P < 0.05$

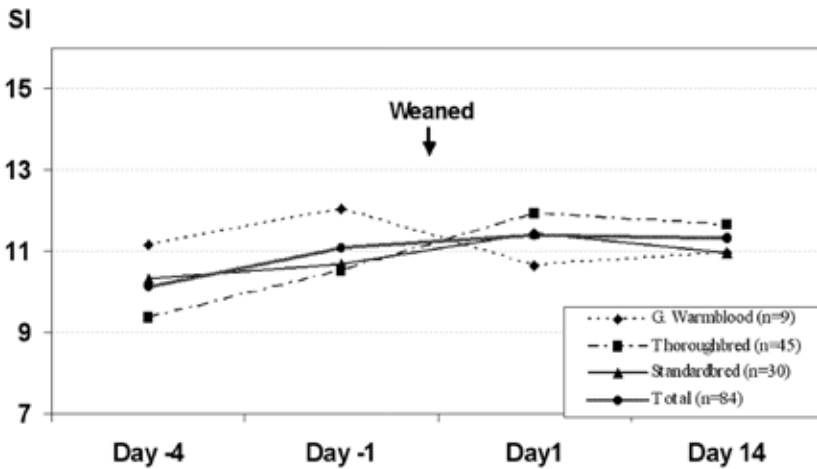


Fig. 2. Courses of lymphocyte proliferation different breeds and total contingent of foals throughout study period. $P < 0.05$

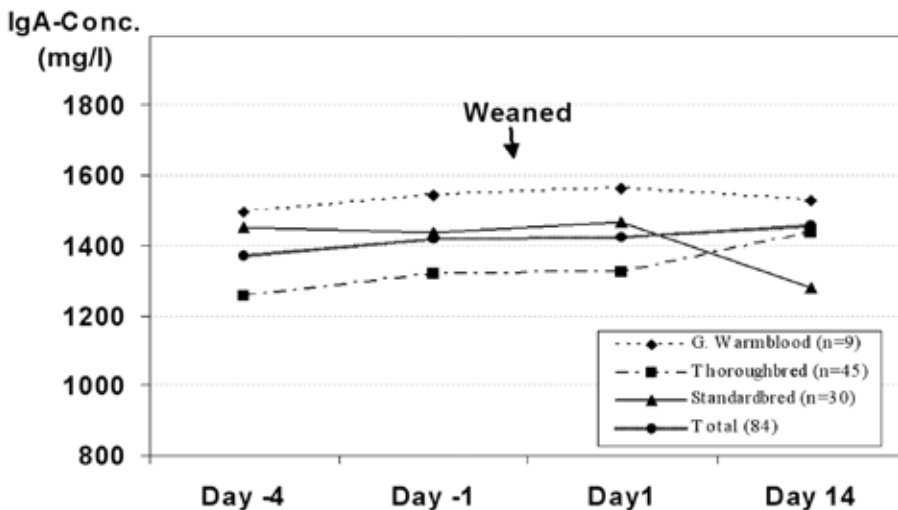


Fig. 3. Courses of serum Ig-A concentration in different breeds and total contingent of foals throughout study period. $P < 0.05$

Discussion

Neutrophilic phagocytic activity was measured between 4500-6000 cpm/ μ L in weanlings in the present study. WICHEL et al. (1991) studied the killing capacity and phagocytic activity of neutrophils in foals from birth to six months of age. They reported the values of both parameters fluctuating within the range for adult horses. However, at the age of six months, the killing capacity of neutrophils decreased whereas phagocytic activity increased.

No literature was available to compare the effects of weaning on phagocytic activity in foals. The decline in PMN phagocytic activity by weaning might be an effect of stress and related weakness of defence power. A slight recovery occurred over two weeks following weaning indicating that these values could probably have attained the initial level again in an appropriate prolongation of the study period.

Interestingly, the values of phagocytic activity fell among all foals one day before weaning. This was probably due to a change in the usual morning routine during blood-sampling which caused mental perturbation, leading to a stressful state in foals up to sampling. Since the cortisol values measured in this study rose first on day -1 following weaning they could not be considered responsible for modifying phagocytic activity.

This emphasizes that corticosteroids induced mechanisms are not alone responsible for immunosuppression (DANTZER and KELLEY, 1989; KHANSARI et al., 1990).

Among different breeds, Standardbred showed no change in phagocytic activity following weaning. It might be related to the weaning method, as Standardbred were placed in groups of 10 foals on average in playpens following weaning, whereas the majority of thoroughbred foals were placed in individual boxes immediately after isolation from their mothers. McCALL et al. (1987) studied different methods of weaning foals and found considerably higher plasma cortisol values as an indicator of stress in foals after abrupt separation from their dams as compared with those foals which remained in partial contact with their dams following weaning. Comparing foals weaned singly or in pairs, HOFFMAN et al. (1995) also indicated elevated cortisol values and more stressful behaviour in singly weaned foals. Cortisol values were, however, not found to be associated with weaning method in the present study.

Mitogen-induced lymphocyte proliferation is a widely used and the most recognised assay to determine immune status in human beings as well as in animals (LANCKI et al., 1984). Although it is almost impossible to compare the results of this test due to wide variations in test preparations, including composition of medium, serum, mitogen, cell count, antibiotics, glutamin and etc. However, there were no studies available on lymphocyte activity in weaning foals for comparison.

The rise in lymphocyte proliferation on day -2 in this study could be attributed either to the immediate reaction to the first blood sampling and related stress-inducing circumstances for animals such as restraint, unfamiliar people etc. However, these effects did not continue afterwards until the end of study.

In this study, Standardbred, which were placed in groups following weaning, showed a strong decline in lymphocyte proliferation, whereas Thoroughbred foals placed in individual boxes after weaning had raised values. These variations suggest that different weaning methods have a strong effect on lymphocyte activation, as reported earlier (McCALL et al., 1987; HOFFMANN et al., 1995). However, it should be emphasized that Standardbred showed considerably higher values at the beginning of study as compared with Thoroughbreds. Compared to PMN phagocytic activity, lymphocyte proliferation responded more strongly to weaning among different breeds.

BROWN-BORG et al. (1993) described how piglets with high cortisol response to stress showed lower lymphocyte proliferation as compared with their counterparts with low cortisol response. WALLGREN (1994) also reported a reduced lymphocyte proliferation in response to enhanced cortisol concentration in pigs. However, this association between cortisol concentration and lymphocyte activation was not found in the present study. DANTZER and KELLEY (1989) as well as KHANSARI et al. (1990) studied the effect of stress on the immune system. They emphasised the existence of such mechanisms, which could

influence lymphocyte proliferation directly without any involvement of corticosteroids. This might be an explanation for our finding that cortisol values measured in this study were not associated with lymphocyte activity.

Serum IgA-concentrations ranged 520 and 2780 mg/L which were comparable with the values measured for foals and adult animals (KOHN, 1989; HALLIWELL and GORMAN, 1989; TIZARD, 1982; HEREMANS, 1973). The slight but significant increase in serum IgA-concentration following weaning may be attributed to psychological stress. These findings extend those of previous reports showing that psychological stress significantly increased IgA concentration (KIECOLT-GLASER et al., 1986; MAES et al., 1997).

References

- BIONDI, M., L. ZANNINO (1997): Psychological stress, neuroimmunomodulation and susceptibility to infectious diseases in animal and man: a review. *Psychother. Psychosom.* 66, 3-26.
- BROWN-BORG, H. M., H. G. KLEMCKE, F. BLECHA (1993): Lymphocyte proliferation response in neonatal pigs with high or low plasma cortisol concentration after stress induced by restraint. *Am. J. Vet. Res.* 54, 2015-2020.
- DANTZER, R., K. W. KELLEY (1989): Stress and immunity: an integrated view, of relationships between the brain and the immune system. *Life Sci.* 44, 1995-2008.
- DANTZER, R., P. MORMEDE (1985): Stress in domestic animals: Apsychoneuroendocrine approach. In: *Animal Stress* (Moberg, G. P., Ed.). Am. Phys. Soc., Bethesda, MD.
- GRIFFIN, J. F. T. (1989): Stress and immunity: a unifying concept. *Vet. Immunol. Immunopathol.* 20, 263-312.
- HALLIWELL, R. E. W., A. N. T. GORMAN (1989): *Veterinary Clinical Immunology*. W. B. Saunders Company, Philadelphia, New York, Tokyo, pp. 19-54.
- HEREMANS, J. F. (1973): *Antigens*. Academic Press, USA. pp. 366.
- HICKEY, M. C., M. DRENNAN, B. EARLEY (2003): The effect of abrupt weaning of suckler calves on the plasma concentrations of cortisol, catecholamines, leukocytes, acute phase proteins and in vitro interferon-gamma production. *J. Anim. Sci.* 81, 2847-2855.
- HOFFMAN, R. M., D. S. KRONFELD, J. L. HOLLAND, K. M. GRIEWE-CRANDELL (1995): Pre-weaning diet and stall weaning method influences on stress response in foals. *J. Anim. Sci.* 73, 2922-2930.
- KHANSARI, D. N., J. MURGO, R. E. FAITH (1990): Effects of stress on the immune system. *Immunol. Today* 11, 170-175.
- KIECOLT-GLASER, J. K., R. GLASER, E. C. STOUT, K. L. TARR, J. E. HOLIDA, C. E. SPEICHER (1986): Modulation of cellular immunity in medical students. *Psychosoma. Med.* 46, 7-14.

- A. Sarwar et al.: Effects of weaning on polymorphonuclear cells phagocytic activity, lymphocyte proliferation and serum IgA-concentration in foals
- KOHN, C. W. (1989): Colostral and serum IgG, and IgA and IgM concentrations in Warmblood mares and their foals at parturition. *J. Am. Vet. Med. Ass.* 195, 64-68.
- LANCKI, D. W., D. I. MA, W. L. H. HAVRAN, F. W. FITCH (1984): Cell surface structure involved in T cell activation. *Immunol. Rev.* 81, 65-94.
- LEONARD, B. E. (1995): Stress and the immune system: immunologic aspects of depressive illness. In: *Stress, the Immune System and Psychiatry.* (Leonard, B. E., K. Miller, Eds.). pp. 133-148.
- MAES, M., D. HANDRIKS, A. V. GASTEL, P. DEMEDTS, A. WAUTERS, H. NEELS, A. JANKA, S. SCHARPE (1997): Effects of psychological stress on serum immunoglobulin, complement and acute phase protein concentrations in normal volunteers. *Psychoneuroendocrino.* 22, 397-409.
- McCALL, C. A., G. D. POTTER, J. L. KREIDER, W. L. JENKINS (1987): Physiological responses in foals weaned by abrupt or gradual methods. *J. Equine Vet. Sci.* 7, 368-374.
- MEYER, A. (1986): Fortschritte bei der Immunisierung und Parammunisierung von Nutztieren. *Prakt. Tierarzt* 10, 865-870.
- MOONS, C. P. H., K. LAUGHLIN, A. J. ZANELLA (2005): Effect of short term maternal separation on weaning stress in foals. *Appl. Anim. Behav. Sci.* 91, 321-335.
- NAGAHATA, H. (1988): The use of lumol- dependent chemiluminescence assay for monitoring the phagocytic and opsonic activity in bovine white blood. *Br. Vet. J.* 144, 158-165.
- NEWBERNE, P. M., M. W. CONNER (1989): The Vitamins. In: *Clinical Biochemistry of Domestic Animals.* 4th ed., (Kaneko, J. J., Ed.), Academic Press, Inc., San Diego, CA. pp. 576-611.
- NIEZGODA, J., M. TISCHNER (1994): Intensity of stress reactions during short-term isolation from mothers from foals. In: *The Proceedings of 6th International Symposium on Equine Reproduction,* Caxambu, Minas Gerais, Brazil 7-13 August, pp. 25-26.
- TIZARD, I. (1982): *An Introduction to Veterinary Immunology.* 2nd ed. W. B. Saunders. p. 48.
- WALLGREN, P. (1994): Influence of experimentally induced endogenous production of cortisol on the immune capacity in swine. *Vet. Immunol. Immunopathol.* 42, 301-316.
- WICHEL, M. G., K. L. LANDERSON, T. V. JOHNSON, U. NATHON, L. SMITH (1991): Influence of age on neutrophil function in foals. *Equine Vet. J.* 23, 466-469.

Received: 18 August 2006
Accepted: 28 December 2007

SARWAR, A., V. BUDDE, H. ENBERGS: Učinci odbića na fagocitnu aktivnost polimorfonuklearnih stanica, limfocitnu proliferaciju i koncentraciju serumskih IgA u ždrebadi. Vet. arhiv 78, 1-11, 2008.

SAŽETAK

Istraživanje je bilo provedeno na 84 ždrebeta (ženskih 46 i muških 38) različitih pasmina (punokrvnjak, standardna pasmina, njemački toplokrvnjak) u dobi od pet do šest mjeseci radi procjene učinka odbića i drugih čimbenika kao što su spol, pasmina i sadržaj serumskoga kortizola na fagocitnu aktivnost polimorfonuklearnih stanica (PMNS), proliferaciju limfocita potaknutu mitogenima te na serumsku koncentraciju IgA. PMN fagocitna aktivnost bila je mjerena kemoluminescencijom, stupanj limfocitne proliferacije MTT-testom, a razina IgA radijalnom imunodifuzijom. Aktivnost PMNS kretala se od 4500 do 6000 cpm/ μ L u odbite ždrebadi. Usprkos blagom povećanju na početku, fagocitna aktivnost PMNS znatno se smanjila ($P < 0,01$) sljedećeg dana po odbiću, a zatim se ponovo povećala. Limfocitna proliferacija znatno se pojačala nakon prvoga dana, a potom je ostala nepromijenjena do kraja istraživanja, s iznimkom neznatnoga smanjenja odmah po odbiću. Odbiće je potaklo blago, ali ne i značajno povećanje koncentracije serumskih IgA. Od drugih promatranih pokazatelja, pasmina je imala veliki utjecaj na fagocitnu aktivnost PMNS i koncentraciju IgA. To može biti stvarni učinak pasmine ili pak rezultat načina upravljanja ergelom. Zanimljivo je da sadržaj serumskog kortizola nije bio u korelaciji s bilo kojim drugim istraživanim pokazateljem. Odbiće je imalo supresijski učinak na aktivnost PMNS.

Cljučne riječi: ždrebad, odbiće, limfocitna proliferacija, fagocitna aktivnost, serum, IgA
