*Rocz. Nauk. Zoot., T. 48, z. 1 (2021) 83–91*

# ANALYSIS OF THE EXPRESSION OF IGG2 IN THE BLOOD OF FOALS UNDERGOING IMMUNE STIMULATION RAISED UNDER DIFFERENT FARMING SYSTEMS*

Anna Migdał[1], Łukasz Migdał[1], Adam Okólski[2]

[1]Department of Genetics Animal Breeding and Ethology
[2]University Centre of Veterinary Medicine UJ-UR
University of Agriculture in Krakow, al. Mickiewicza 24/28, 30-059 Kraków, Poland

*Horses express 7 classes of immunoglobulins G, yet the functions of some of these – specifically $IgG_2$ (IGHG2) and $IgG_6$ (IGHG6) – have not been established yet. The aim of the study was concerned with evaluating the concentration of IgG2 in foal during the first 60 days of life as well as assessing the effect of raising foals in different farming systems, and of immunostimulation, on the level of IgG2. The study covered foals from primitive breeds subdivided into the following groups: group C including foals kept with mothers in foaling stalls during the course of the experiment; group E that received two intramuscular injections of 5 ml of Biotropine (Biowet S.A.) at days 35 and 40 of foal age, which stimulates adaptive immune response, and group E2 consisting of free-grazing foals kept in a herd. Blood samples were collected by the following scheme: after birth before first suckling, then at the age of 1, 3, 5, 10, 20, 30, 40, 50 and 60 days. Immunoglobulins were measured using the ELISA (GR106527, Genorise). The pattern of changes in the IgG2 profile was similar in all experimental groups. The highest IgG2 concentration was observed 24 hours after the delivery, in foals from the C group (3.20±1.21 mg/dl). On the subsequent days, the levels of immunoglobulin decreased, reaching the lowest level on day 40, in the E2 group (1.15±0.01 mg/dl). Statistical analysis revealed highly significant differences ($P<0.01$) among all experimental groups in the period from 24 hours to 30 days of age.*

*Key words: foals, immunostimulation, IgG2, raising system*

High morbidity and mortality rate in foal populations in the neonatal period is connected with deficiency in effective immunological mechanisms and the impact of some environmental conditions. Equine placenta prevents transport of large macromolecules, such as immunoglobulins, from the mother's body to the developing foetus. Newborn foals are devoid of adaptive immunity against environmental anti-

*This work was financed by the Ministry of Science and Higher Education of the Republic of Poland (funds for statutory activity, DS 3208 and SUB.201 – D201).

gens and their blood plasma contains low levels of immunoglobulins, which results in increased susceptibility to infections. Foals are highly prone to infections until acquiring passive immunity (Felippe, 2016).

Approximately 20% of foals die before reaching 2 months of age leading to a significant decrease in the number of livestock and financial losses (Kulisa et al., 2009). These negative effects prompt research into discovering new substances stimulating immune response, that is immunostimulators.

Immunoglobulins G can be found in the foal blood plasma as early as on the 180th day of pregnancy in the concentration amounting to approx. 0.005 g/l. Foetus B cells are capable of changing the subclass of the immunoglobulin G at the transcription level. However, in the foetus spleen expression at the level of peptides for genes responsible for immunoglobulin structure (*IGHG1*, *IGHG2*, *IGHG3*, *IGHG4*, *IGHG5*, *IGHG6* and *IGHG7*) is insignificant as compared with the expression in the foal spleen. Before the first suckling the concentration of IgG in the blood plasma is very low. It ranges between 0.02 and 0.17 g/l and contains mainly $IgG_1$ and $IgG_{4/7}$. Reduced immunoglobulin level in the prenatal period is caused most probably by low stimulation of lymphocytes B resulting from developing in a sterile environment (Wagner et al., 2004).

Equine gamma globulins consist of 5 immunoglobulins: IgM, IgA, IgE, IgD and IgG. There are 7 subclasses of immunoglobulin G that differ in the amino acid sequence in the constant region of the heavy chain. Originally in case of horses only 5 different types of immunoglobulin G were described: IgGa, IgGb, IgGc, IgG(T) and occasionally IgG(B). Currently, after sequencing genes coding heavy chain of equine IgG and identifying the locus, we know that horses express 7 genes encoding IgG subclasses (Wagner et al., 2004). Based on molecular analyses immunoglobulin subclasses have been numbered from IgG1 to IgG7, which correspond to those originally described with some genetic redundancy (Wagner, 2006). The function of IgD (*IGHD*), IgG2 (*IGHG2*) and IgG6 (*IGHG6*) has not been determined yet. It is thought that newborn foals are able to generate immune response due to the fact that foetus can produce specific antibodies and shift IgM into IgG as a reaction to an intrauterine vaccine injection administered between 180 and 200 days of pregnancy (Morgan et al., 1975; Hannant et al., 1991).

There is lack of information about horse IgG2. Expression of this subclass was not investigated previously, therefore information about its possible influence on horse immune system development is missing. Moreover we decided to analyse possible immune response of IgG2 on bacterial antigen. The aim of the study was to determine the concentration of IgG2 in foals during 60 first days of life as well as to conduct analysis of the impact of raising conditions and immunostimulation on the level of IgG2 in foals.

## Material and methods

The experiment was conducted after permission from Local Ethics Committee in Kraków (no 37, 30 May 2016) was granted.

**Animals and feeding**

The experiments were conducted on 25 foals of the Polish Pony (Konik, Polish Konik) and Hucul breeds; foals were maintained at the Experimental Station of the Department of Reproduction and Animal Anatomy of the University of Agriculture in Krakow and the Hucul Pony foals at the Experimental Station of the National Research Institute of Animal Production in Zabierzów. All animals were clinically healthy during the experimental period. The horses had all been used by university students in the teaching program.

No horses were used for equestrian purposes. Mares were not vaccinated during pregnancy. Inclusion criteria consisted of foals born from healthy mares with no placentitis, a normal gestational period, an uneventful birth, and having normal physical and neurological examination findings. The foals had to successfully stand and nurse within 2 hours of birth and remain clinically healthy during the study period. Foal birth weight was 27–42 kg, weight loss on the first day of life was <1.5%.

The foals were divided into groups:

– the control group C (n=9) – Polish Pony foals that were kept with mothers in foaling stalls throughout the entire experiment (controlled foal raising). Two weeks before delivery, birthing alarms (Abfohlsystem, Jan Wolters) were placed in the labia, and mares were moved to box stalls inside a stable lit with natural light. All foals with mares were kept in the same stable in individual boxes (size 2.15 × 3.50 m) on permanent straw. Mares 5 to 17 years of age and 270 to 340 kg live body weight, were fed *ad libitum* with hay (*Lolium* 40%, *Trifolium* L. 20%) with addition of oat: 1.5 kg/mare per day (according to Institute of Physiology and Animal Nutrition standards, 1997). Foals were fed only with colostrum and mother's milk *ad libitum* without additional supplementation. Water was offered from automatic water bowls (flow ~10 l/min);

– the experimental group E1 (n=7) – the Polish Pony foals, kept under controlled conditions (same as for group C), that were administered an immunostimulating agent.

For immunostimutalion, commercially available immunostimulator was used in the present study, namely the Biotropine (Biowet Drwalew S.A.). It consisted of a mixture of inactivated gram-positive and gram-negative bacteria e.g: *Escherichia coli* (123 mg/ml), *Staphylococcus aureus* (74 mg/ml), *Streptococcus zooepidermicus* (24.6 mg/ml), *Streptococcus equi* (24.6 mg/ml), *Streptococcus equisimilis* (24.6 mg/ml), *Streptococcus agalactiae* (24.6 mg/ml), *Streptococcus dysgalactiae* (24.6 mg/ml), *Pasteurella multocida* (123 mg/ml), and *Erysipelothrix insidiosa* (49 mg/ml) as well as pork spleen extract (10 mg/ml). On day 35 and day 40 after birth, the foals from the experimental group received intramuscular (*m. pectoralis descendens*) injection of 5 ul of Biotropine;

– the experimental group E2 (n=9) – foals representing the Hucul breed raised in a herd kept on a pasture (natural raising). The mares 7 to 19 years of age and 340 to 390 kg live body weight during the whole pregnancy were on the pasture with a herd of horses. Five days before delivery, birthing alarms (Abfohlsystem, Jan Wolters) were placed in the labia, and mares were moved to box stalls inside a stable lit with natural light. All foals with mares were kept in the same stable in individual boxes (size 2.15 × 3.50 m) on permanent straw. Two days after delivery, the mares

with foals returned to the herd to pasture. Mares were fed *ad libitum* fresh forage or hay grass-clover mixture (*Phleum pratense* L. 10%, *Poa pratensis* L. 10%, *Festuca rubra* 15%, *Festuca arundinacea* 15%, *Lolium perenne* 30%, *Lolium westerwoldicum* 10% *Trifolium repens* L. 10%) with addition of oat. Water was offered from a watering trough. Foals were fed only with colostrum and mother's milk *ad libitum* without additional supplementation.

**Blood sampling and blood analysis**

Blood samples were collected from umbilical cord and from foals by jugular venipuncture. Blood samples were collected till 60 days after birth according to the following scheme: after birth before first suckling, at 1, 3, 5, 10, 20, 30, 40, 50 and 60 days after birth. 3 ml of blood was collected into tubes with EDTAk2. Collected samples were centrifuged in 5000 rpm 5 min and plasma were stored in –20°C till further analysis.

Immunoglobulins were measured using ELISA assay in Spectramax Plus 384 Microplate Reader (Molecular Devices) using 96-well plates coated with monoclonal antibodies against equine IgG2 (GR106527, Genorise). 100 µl of plasma per well were added. Each well was washed three times with 1x Assay Buffer after each step. 100 µl of working dilution of Detection Antibody were added and incubated 1 hour. After wash 100 µl of working dilution of conjugate were added to each well and incubated 20 min. After wash 100 µl of substrate solution were added to each well and incubated 20 min. After wash 50 µl of stop solution were added to each well. Duplicate wells were used for each sample. Plates were read using microplate reader set to 450 nm. Results were calculated based on standard curve. High standard concentration of 80 ng/ml was prepared, vortexed for 15 sec and allowed to sit for 5 min. A seven-point standard curve was generated using 2-fold serial dilutions in the assay buffer.

**Statistical analysis**

Data are presented as means ± standard error. The results were analysed using SAS 9.4 software (SAS Institute Inc., USA). The Shapiro-Wilk test was considered the best test to check the normality of the distribution of a random variable. Because the data did not have normal distribution, Kruskal-Wallis test was used with immunostimulant and age as effects. The degree of association between the parameters was examined using a nonparametric Spearman's rank correlation coefficient. The value ranges from 0.0 to 0.5, from 0.5 to 1.0, from –0.5 to 0.0 and from –1.0 to –0.5 indicate weak positive, strong positive, weak negative and strong negative correlation, respectively.

## Results

The changes in the concentration of IgG2 observed during the course of the experiment followed the same pattern in all studied groups. The highest IgG2 concentration in foals raised under controlled conditions was reported 24 hours after the delivery (group C; 3.20 ± 1.21 mg/dl). In foals kept freely in a herd (group E2) and those raised

under controlled farming system that were immunostimulated (group E1) the highest concentration of IgG2 was also observed at 24 h after birth (1.51 ± 0.10 mg/dl and 2.97 ± 0.68 mg/dl, respectively). On the subsequent days the level of immunoglobulin decreased and the lowest values were obtained at day 40 in free-range foals (group E2; 1.15 ± 0.01 mg/dl). In the group raised under controlled conditions (group C) and immunostimulated foals (group E1) the lowest concentrations were also observed at day 40 (1.68 ± 0.32 mg/dl and 1.99 ± 0.28 mg/dl, respectively). On the remaining days concentration of IgG2 decreased in all experimental groups (Figure 1).

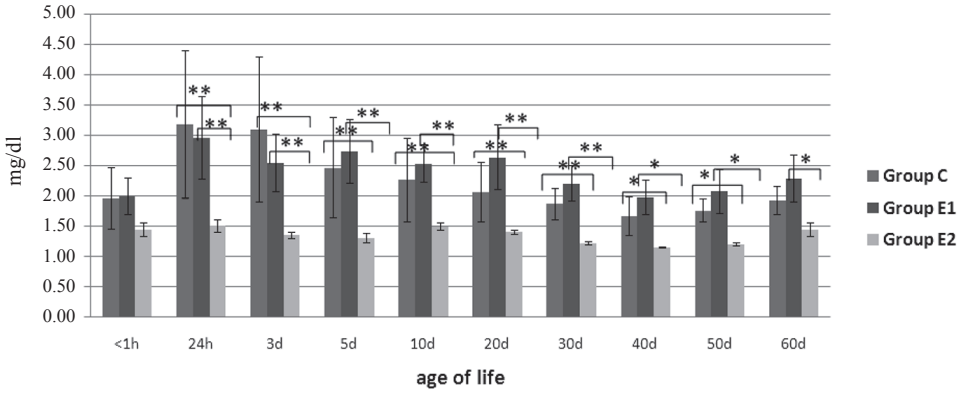


Figure 1. Concentration of IgG2 (mg/dl) in the foal blood in all studied groups on the subsequent days of life (mean ± SE).
Group C – control group.
Group E1 – experimental l group.
Group E2 – experimental 2 group.
* Means in lines differ statistically at P<0.05.
** Means in lines highly differ statistically at P<0.01.

Statistical analysis revealed highly significant differences (P<0.01) among groups C, E1 and E2 from 24 hours to 30 days of age. Statistically significant differences among these groups (P<0.05) were shown from day 40 to 60.

## Discussion

Equine immonoglobulins G include 7 subclasses that are characterised by different amino acid sequences in constant region of the heavy chain. Immunoglobulins such as IgG1 (previously IgGa), IgG3/5 (IgGT) and IgG4/7 (IgGb) are present in horses in large concentrations, while the concentration of IgG2 and IgG6 (IgGc) is lower. Specific functions that are fulfilled by IgG2 are still unknown (Felippe, 2016) and only few authors mention equine IgG2 in their papers. So far there are no references about the alterations in the concentration of this immunoglobulin in foals during the first weeks after delivery. Determining IgG2 function in foals is seriously prevented by the fact that there is no possibility to compare it with other

mammal species as they express different number of IgG subclasses. For instance, cattle express only two IgG subclasses: IgG1, IgG2 (Akita et al., 1998), pigs five: IgG1, IgG2a, IgG2b, IgG3 and IgG4 (Butler and Brown, 1994) and humans four: IgG1, IgG2, IgG3, IgG4 (Roux et al., 1997). Different IgG1 and IgG2 antibody production as a result of stimulation with cytokines was described in mice and cattle. The roles and functions of IgG2 are also unclear in dogs. Many reports suggest that IgG2 plays a role in development of symptomatic infections, therefore assuming that IgG2 plays a stronger immunopathogenic role than IgG1 (Bourdoiseau et al., 1997; Carneiro, 2016; Iniesta et al., 2007; Solano-Gallego, 2001). On the other hand, Deplazes et al. (1995), Lima et al. (2017) and Cardoso et al. (2007) suggested the existence of a dichotomy in IgG1 and IgG2 responses in symptomatic and asymptomatic dogs – in the sense that IgG2 was associated with asymptomatic and IgG1 with symptomatic infections. In mice, during differentiation towards Th1 or Th2 response, increased IgG2 level correlated with high level of IFN-γ (Th1 cytokine), while increased IgG1 level correlated with increased IL-4 secretion (Th2 cytokine) (Toellner et al., 1998). Similar observations were made in cattle, where secretion of IgG1/IgG2 increased after specific activation with cytokine. These results suggest that activation with IFN-γ can induce the production of bovine IgG2 by B cells, while stimulation with IL-4 triggers IgG1 production (Brown et al., 1998). In case of pigs information on the effect of intradermal immunostimulator injection is still scarce. However, some sources indicate that production of IgG1 and IgG2 antibodies by B cells in pigs may be regulated by specific cytokine production (Crawley et al., 2003; Oreskovic et al., 2017). What is more, Ma et al. (2012) revealed that after an intramuscular PRV vaccine injection the level of IgG1 and IgG2 in pigs can change depending on the animal age – older animals produced only low amount of IgG2 combined with increased Th2 cytokine level (IL-4 and IL-5) and decreased Th1 cytokine level (IFNγ and IL-12) (Ma et al., 2012). In our own studies we observed very low level of IgG2 that was very close to the lower level of detection. No significant differences in the concentration of IgG2 between foals raised under natural and controlled conditions were found. Moreover, in that experimental group the level of examined immunoglobulin also decreased between 24 and 30 days of age. Our own studies did not show any effect of immunostimulation with Biotropine on the IgG2 concentration. Similar results were delivered by Lewis et al. (2008) who analysed the influence of stimulation with protein A from *Staphylococcus aureus* or protein G from *Streptococcus equi* on the concentration of different subclasses of immunoglobulin G and found very weak IgG2 reaction. The authors suggest that maximum vaccine efficacy can be obtained provided that it triggers IgG1, IgG3, IgG4 and IgG7 response. Vaccinating infants is a paradox as it is aimed at ensuring rapid protection at an early stage of life and obtaining long-term immune memory despite limitations of the developing immune system and does not take into account interference of maternal antibodies. It is highly recommended that flu vaccines should be delayed until sixth or twelfth month of age (Wilson et al., 2001). Allowing for 30-day half-life period of IgG, it is probable that antibodies from the colostrum will reach low level approximately in the second or third month. Moreover, the transfer of cell-mediated immunity through colostrum is not efficient, which makes foals especially susceptible to infections in the period between degradation

of colostral immunoglobulins and acquiring vaccine-induced immunity. That is why further research aimed at improving immunization strategies in foals is necessary.

**Acknowledgements**

This work was financed by the Ministry of Science and Higher Education of the Republic of Poland (funds for statutory activity, DS 3208).

**Declaration of interest**

The authors declare that they have no competing interests.

**Ethics committee**

The authors have declared no competing interest. Ethical Animal Research The experiment was conducted upon receiving the permission granted from the Local Ethics Committee in Kraków (no 37, 30 May 2016). Veterinary care and samples collection were performed by veterinary professor Adam Okólski. All animals used in this study were held in experimental station of the University of Agriculture in Krakow.

**References**

Akita E.M., Li-Chan E.C. (1998). Isolation of bovine immunoglobulin G subclasses from milk, colostrum, and whey using immobilized egg yolk antibodies. J. Dairy Sci., 81: 54–63.

Bourdoiseau G., Bonnefont C., Hoareau E., Boehringer C., Stolle T., Chabanne L. (1997). Specific IgGl and IgG2 antibody and lymphocyte subset levels in naturally *Leishmania infantum*-infected treated and untreated dogs. Vet. Immunol. Immunopathol., 59: 21–30.

Brown W., Rice-Ficht A.C., Estes D.M. (1998). Bovine type 1 and type 2 responses. Vet. Immunol. Immunopathol., 63: 45–55.

Butler J.E., Brown W.R. (1994). The immunoglobulins and immunoglobulin genes of swine. Vet. Immunol. Immunopathol., 43: 5–12

Cardoso L., Schallig H.D.F.H., Cordeiro-da-Silva A., Cabral M., Alunda J.M., Rodrigues M. (2007). Anti-*Leishmania* humoral and cellular immune responses in naturally infected symptomatic and asymptomatic dogs. Vet. Immunol. Immunopathol., 117: 35–41.

Carneiro L.A. (2016). Estudo prospectivo sobre a dinâmica da evolução clínica e imunológica da infecção canina por *Leishmania* (*Leishmania*) *infantum* chagasi em área endêmica de leishmaniose visceral no estado do Pará. Tese de Doutorado em Patologia experimental e comparada – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brasil, pp. 1–96.

Crawley A., Raymond C., Wilkie B.N. (2003). Control of immunoglobulin isotype production by porcine B-cells cultured with cytokines. Vet. Immunol. Immunopathol., 91:141–154.

Deplazes P., Smith N.C., Arnold P., Lutz H., Eckert J. (1995). Specific IgG1 and IgG2 antibody responses of dogs to *Leishmania infantum* and other parasites. Parasite Immunology, 17: 451–458.

Felippe M.B. (2016). Equine Clinical Immunology, Wiley-Blackwell.

Hannant D., Rossdale P.D., McGladdery A.J. (1991). Immune responses of the equine foetus to protein antigens. In: Proceedings of the 6th Int. Conf. Equine Infect. Dis., 86 (Abstr).

Iniesta L., Gállego M., Portus M. (2007). Idiotype expression of IgG1 and IgG2 in dogs naturally infected with *Leishmania infantum*. Vet. Immunol. Immunopathol., 119: 189–197.

Kulisa M., Makieła K., Długosz B., Gaj M. (2009). Thoroughbred foals' mortality causes during first six months of life. Part II. Diseases and injuries. Rocz. Nauk. PTZ, 5: 79–84.

Lewis M.J., Wagner B., Woof J.M. (2008). The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. Mol. Immunol., 45: 818–827.

Lima L.V.D.R., Carneiro L.A., Campos M.B., Vasconcelos Dos Santos T., Ramos P.K., Laurenti M.D., Teixeira C.E.C., Silveira F.T. (2017). Further evidence associating IgG1, but not IgG2, with susceptibility to canine visceral leishmaniasis caused by *Leishmania (L.) infantum chagasi*-infection. Parasite, 24: 37.

Ma M., Wang L., Yang J., Cai H., Shi J., Zhang S. (2012). Age-related impaired Th1 responses to PRV vaccine *in vivo* in aged pigs. Mol. Immunol. 52: 217–223.

Morgan D.O., Bryans J.T., Mock R.E. (1975). Immunoglobulins produced by the antigenized equine fetus. J. Reprod. Fertil. Suppl. 23: 735–738.

Oreskovic Z., Kudlackova H., Krejci J., Nechvatalova K., Faldyna M. (2017). Oil-based adjuvants delivered intradermally induce high primary IgG2 immune response in swine. Res. Vet. Sci., 114: 41–43.

Roux K.H., Strelets L., Michaelsen T.E. (1997). Flexibility of human IgG subclasses. J. Immunol., 159: 3372–3382.

Solano-Gallego L., Riera C., Roura X., Iniesta L., Gallego M., Valladares J.E., Fisa R., Castilejo S., Alberola J., Ferrer L., Arboix M., Portus M. (2001). *Leishmania infantum*-specific IgG, IgG1 and IgG2 antibody responses in healthy and ill dogs from endemic areas. Evolution in the course of infection and after the treatment. Vet. Parasitol., 96: 265–276.

Toellner K.M., Luther S.A., Sze D.M., Choy R.K., Taylor D.R., MacLennan I.C. (1998). T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. J. Exp. Med., 187: 1193–1204.

Wagner B. (2006). Immunoglobulins and immunoglobulin genes of the horse. Dev. Comp. Immunol., 30: 155–164.

Wagner B., Miller D.C., Lear T.L., Antczak D.F. (2004). The complete map of the Ig heavy chain constant gene region reveals evidence for seven IgG isotypes and for IgD in the horse. J. Immunol., 173: 3230–3242.

Wilson W.D., Mihalyi J.E., Hussey S., Lunn D.P. (2001). Passive transfer of maternal immunoglobulin isotype antibodies against tetanus and influenza and their effect on the response of foals to vaccination. Equine Vet. J., 33: 644–650.

Accepted for printing: 27 IV 2021

ANNA MIGDAŁ, ŁUKASZ MIGDAŁ, ADAM OKÓLSKI

**Analiza ekspresji IgG2 we krwi źrebiąt poddanych immunologicznej stymulacji utrzymywanych w różnych warunkach odchowu**

STRESZCZENIE

U koni występuje 7 klas immunoglobulin G, nie zidentyfikowano jednak jeszcze funkcji immunoglobulin IgD (IGHD), IgG2 (IGHG2) oraz IgG6 (IGHG6). Celem przeprowadzonego badania była ocena stężenia immunoglobuliny IgG2 u źrebiąt w pierwszych 60 dniach życia oraz ocena wpływu warunków odchowu i immunostymulacji na stężenie IgG2 u źrebiąt. Badania przeprowadzono na źrebiętach ras prymitywnych podzielonych na grupy: grupa N (n=9) – źrebięta odchowywane na pastwisku w tabunie koni; grupa K (n=9) – źrebięta, które przez cały okres badań pozostawały z matką w boksach porodowych; grupa KI (n=7) – źrebięta, którym dwukrotnie w 35. i 40. dniu życia podano w iniekcji domięśniowej 5 ml zawiesiny stymulującej swoistą odpowiedź immunologiczną Biotropina (Biowet Drwalew S.A.). Próbki krwi pobierano do 60. dnia życia zgodnie ze schematem: po urodzeniu przed pierwszym ssaniem, w 1., 3., 5., 10., 20., 30., 40., 50. i 60. dniu życia. 3 ml krwi pobrano do probówek z EDTAk2. Oznaczanie immunoglobulin przeprowadzono metodą ELISA (GR106527, Genorise). We wszystkich grupach doświadczalnych obserwowano zbliżony profil zmian stężenia immunoglobulin IgG2. Najwyższe stężenie IgG2 obserwowano w 24. godzinie życia źrebiąt z grupy K 3,20±1,21 mg/dl. W kolejnych dniach

obserwowano spadek poziomu immunoglobuliny, najniższe stężenie wykazano 40. dnia w grupie N 1, 15±0,01 mg/dl. Analiza statystyczna wykazała wysoko istotne różnice (P<0,01) między grupami źrebiąt w czasie od 24. godziny do 30. dnia życia.

Słowa kluczowe: źrebięta, immunostymulacja, IgG2, system utrzymania