FULL ARTICLE

ISSN Online 1678-4456

Humoral immunity is not altered in overweight pregnant Crioulo mares

Imunidade humoral não é alterada em éguas Crioulas gestantes com sobrepeso

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ABSTRACT

Both pregnancy and obesity can influence significant changes in the immune system. On this basis, the present study proposes to evaluate the humoral immune response of overweight pregnant mares in response to a commercial vaccine. Thirty pregnant Crioulo mares were separated according to body condition score (BCS) into overweight (BCS≥7/9) or lean-control (BCS= 5-6/9). In each group, the animals were subdivided into vaccinated and controls. The mares were vaccinated against EHV-1 in two doses spaced 21 days apart and had their blood collected monthly, for five months, for antibody evaluation. Both vaccinated groups had an increase in specific neutralizing antibodies after the vaccine. However, after the second dose, there was no increase in antibodies in any of the groups. Vaccinated overweight and lean-control mares did not differ at any time point. Therefore, this study demonstrated that obesity does not influence the humoral immune response in pregnant Crioulo mares.

Keywords: Vaccine. Immune system. EHV-1. Gestation.

RESUMO

Tanto a gestação quanto a obesidade podem influenciar o desenvolvimento de alterações significativas no sistema imune, portanto, o presente estudo teve como objetivo avaliar a resposta imune humoral de éguas gestantes com sobrepeso em resposta a uma vacina comercial. Trinta éguas Crioulas gestantes foram separadas de acordo com o escore de condição corporal (ECC) em éguas com sobrepeso (ECC≥7/9) e éguas controles (ECC=5-6/9) e, ainda, em cada grupo, os animais também foram separados em vacinados e controles. As éguas foram vacinadas contra o EHV-1 em duas doses com intervalo de 21 dias, sendo realizadas coletas de sangue mensalmente durante cinco meses para avaliação de anticorpos neutralizantes. Ambos os grupos vacinados tiveram aumento de anticorpos neutralizantes específicos após a vacina, porém, após a segunda dose, não foi observado aumento de anticorpos em nenhum dos grupos. Nenhuma diferença foi observada entre éguas vacinadas com sobrepeso e as éguas controles em nenhum momento. Assim, este estudo demonstrou que a obesidade não é um fator que influencia a resposta imune humoral de éguas Crioulas gestantes.

Palavras-chave: Vacina. Sistema imune. EHV-1. Gestação.

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Received: April 05, 2023
Approved: July 12, 2023

How to cite: Mousquer MA, Moraes BSS, Santos AC, Souza RP, Lima M, Rodrigues PRC, Curcio BR, Nogueira CEW. Humoral immunity is not altered in overweight pregnant Crioulo mares. Braz J Vet Res Anim Sci. 2023;60:e210215. https://doi.org/10.11606/issn.1678-4456.bjvras.2023.210215.

Introduction

Obesity is a complex problem in equine breeding related to several life-threatening conditions. It is commonly accompanied by metabolic alterations and dysfunctional secretion of adipokines and inflammatory cytokines and may also be associated with developing disturbances in immune responses (Knowles & Grieve, 2020; Salinas et al., 2020). Increased concentrations of inflammatory cytokines in adipose tissue and blood circulation have been described in obese horses (Basinska et al., 2015; Johnson et al., 2010). This inflammatory state is associated with chronic activation of the immune system, in which pro-inflammatory cytokines are constantly recruited and interfere negatively with immune function (Frasca & Blomberg, 2020).

Data from experiments with humans and mice suggest that obese individuals are at greater risk of contracting infections and that the response to vaccination is often impaired in these individuals (Milner & Beck, 2012). Compromised B cell function due to inflammation was suggested as a cause for failure to respond to vaccination (Frasca & Blomberg, 2020). Other immune components of innate and adaptative response are also implicated in the loss of antibody production following vaccination (Frasca & Blomberg, 2020; Ghanemi et al., 2021). In horses with metabolic alterations, a probable decrease in an adequate cellular response to vaccine challenge has been identified without changes in humoral response (Elzinga et al., 2018). Other studies also demonstrated differences in the production of neutrophil reactive oxygen species in non-metabolically-altered obese horses and impaired peripheral blood polymorphonuclear phagocytosis in obese insulin-resistant horses (Salinas et al., 2020, 2022).

Pregnancy also involves a complex relationship with the immune system in which the balance between the recognition of self and foreign antigens becomes essential for developing a healthy fetus (Antczak, 2020; Morelli et al., 2015). During this period, the mother develops tolerance to the semi-allogeneic fetus through a series of interactions between the maternal immune system and the fetal-placental unit (Antczak, 2020; Morelli et al., 2015). The maintenance of the conceptus is possible through the modulation of an adaptive immune response throughout the gestational period (Fedorka et al., 2020).

In addition, the hypothesis that the maternal immune system would be suppressed throughout the gestation period to prevent the recognition and rejection of the fetus was mistakenly supported for a long time (Billingham et al., 2003; Mor et al., 2017). However, a responsive immune system is essential for the implantation and growth of the fetus (Mor et al., 2017). Thus, different immunological mechanisms, both innate and adaptive, are necessary for fertilization, implantation, and the maintenance of pregnancy (Fedorka et al., 2020; Mor et al., 2017).

Considering that pregnancy and obesity can contribute to the development of significant changes in the immune system, the interaction of both can affect maternal health and the fetus. The close relationship between obesity, pregnancy, and immune dysfunction is well-recognized in humans (Hersoug & Linneberg, 2007; Wilson & Messaoudi, 2015). Therefore, the present study aimed to evaluate the humoral immune response of overweight pregnant mares in the final third of gestation after a vaccination.

Materials and Methods

Animals

All procedures performed in this study were approved by the Animal Ethics and Experimentation Committee of the Federal University of Pelotas (CEEA-UFPel) (approval no. 34960-2019). Thirty pregnant Crioulo mares aged between six and 11 years were included. The mares were to a farm in Santa Vitória do Palmar - RS, Brazil (33°31'08" S and 53°22'05" W), housed exclusively in an extensive management system during the study.

The animals were selected based on pregnancy status and body condition score (BCS), assessed by the same trained observer at the beginning of data collection, based on the nine-point scale described by Henneke et al. (1983). The mares were divided into two groups: overweight (BCS≥7/9, n=20) and lean-control (BCS 5-6/9, n=10). Within these groups, the animals were subdivided between vaccinated (BCS≥7/9, n=13; BCS 5-6/9, n=6) and control (BCS≥7/9, n=7; BCS 5-6/9, n=4).

The mares were weighed on a digital electronic scale for large animals and evaluated for cresty neck score

(CNS). The following morphometric measurements were also performed: neck circumference at 0.25, 0.50, and 0.75 portions along its length using a tape; crest neck height and tail head fat were obtained by ultrasonography (Sonoscape A5v, Domed dominium medical, São Paulo). All the above measurements were obtained as described before in Gentry et al. (2004) and Carter et al. (2009).

Blood samples and vaccine assay

Blood samples were obtained through venipuncture of the external jugular vein in grey-top tubes containing sodium fluoride and red-top tubes with no anticoagulant to assess baseline glucose and insulin, respectively. Both baseline insulin and glucose were evaluated at a single time point (300 days of gestation) as a control to identify whether any animal had increased concentrations. A commercial vaccine (Herpeshorse, Vencofarma®), provided by the manufacturer, was used to evaluate humoral immune response.

At about 300 days of gestation, blood was collected in red top tubes to measure neutralizing antibodies against equine alphaherpesvirus type 1 (EHV-1). Then, the first dose of the vaccine (2 mL) was administered. A booster dose was given at 321 days of gestation, and another blood sample was obtained to evaluate neutralizing antibodies. The control group received 2 mL of PBS+$Al(OH)_3$ at the same time points. After administration of the two doses, blood samples were obtained monthly for the following five months (until D155) to monitor antibody levels (Figure 1).

Glucose and insulin analysis

Baseline glucose was measured by the colorimetric enzymatic method, using commercial kits (Glucose Liquiform, Ref. 133) in an automatic analyzer (Labmax Plenno, Labtest Diagnóstica S.A., Minas Gerais, Brazil). The samples for basal insulin analysis were sent to the Pasin Laboratory - Clinical Analysis (Santa Maria, Rio Grande do Sul, Brazil).

Virus neutralization test

All serum samples collected in the study were tested for antibodies against EHV-1 using the virus neutralization assay described elsewhere (Office International des Épizooties, 2008). Briefly, 25 µL of MEM (minimum essential medium) with 10% FBS (fetal bovine serum) were distributed into 96-well microplates. Then, 25 µL of each sample was added, and dilutions from 1:2 until 1:4096 were used. The viral suspension was prepared at a concentration of 100 DICT50 (50% tissue culture infective doses), and 25 µL was added to each well. The microplates were incubated at 37 °C in an incubator with 5% CO_2 for 1 h. Next, RK-13 cells at an approximate concentration of 3×10^4 were distributed to all wells, and the plates were incubated again. The reading was done between 48-72 h. The titer of each sample was determined at the highest dilution, in which no cytopathic effect was observed, and the cell layer remained intact.

Statistical analysis

Commercial software IBM SPSS Statistics 20 was used. Data normality was tested using the Shapiro-Wilk test. The variables not normally distributed were transformed to log10. Descriptive analysis of all morphometric measurements (weight, neck circumference at the three points, neck crest height, CNS, BCS, and tail head fat), glucose, and insulin are presented as mean ± SEM. A non-paired t-test was used for comparison between groups. Serum neutralization antibodies were analyzed by a repeated-measure ANOVA and are presented as log2. The difference was considered at $p<0.05$.

Results

Weight, neck circumference at all points (0.25, 0.50, 0.75), crest neck height, and CNS were more significant in the group with BCS ≥7 (Table 1). There was no difference in tail head fat between the groups. The group with BCS ≥7 had higher baseline glucose and insulin concentrations (74.81 ± 1 mg/dL and 3.319 µIU/mL) than the group with BCS 5-6 (69.20 ± 1.5 mg/dL and 1.190 µIU/mL), respectively.

All mares showed variable titers of serum-neutralizing antibodies against EHV-1 on the first day (D0). Three animals in the BCS ≥7 group had a baseline serum-neutralizing antibody titer ≥ 1024 and were excluded from the analyses. In the group with BCS ≥7, a difference was observed between vaccinated animals and controls at days 21, 35, and

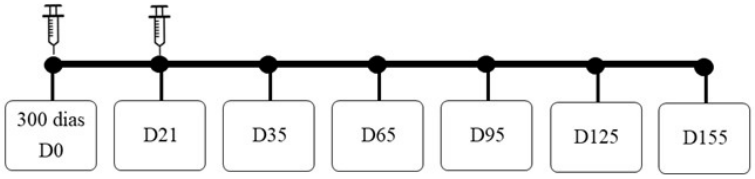

Figure 1 – Timeline showing vaccination time points and blood collection from mares starting at 300 days of gestation (D0).

Table 1 – Morphometric measurements in pregnant lean-control (BCS 5-6/9) and overweight (BCS ≥7/9) mares. Data are presented as Mean ± S.E.M

Morphometric measures	Lean-control mares (BCS 5-6/9)	Overweight mares (BCS≥7/9)
Weight (Kg)	455.44 ± 20.58[a]	583.11 ± 9.16[b]
Neck circunference 0.25 (cm)	71.20 ± 0.82[a]	74.81 ± 0.81[b]
Neck circunference 0.50 (cm)	89.40 ± 1.34[a]	95.26 ± 1.14[b]
Neck circunference 0.75 (cm)	106.40 ± 1.43[a]	119.29 ± 1.42 [b]
Neck crest height (cm)	5.80 ± 0.36[a]	8.48 ± 0.41[b]
Tailhead fat (cm)	1.48 ± 0.15	1.83 ± 0.17
CNS	1.33 ± 0.5[a]	2.57 ± 0.5[b]
BCS	5.67 ± 0.5[a]	7.67 ± 0.48[b]

Different letters represent statistical differences between groups.

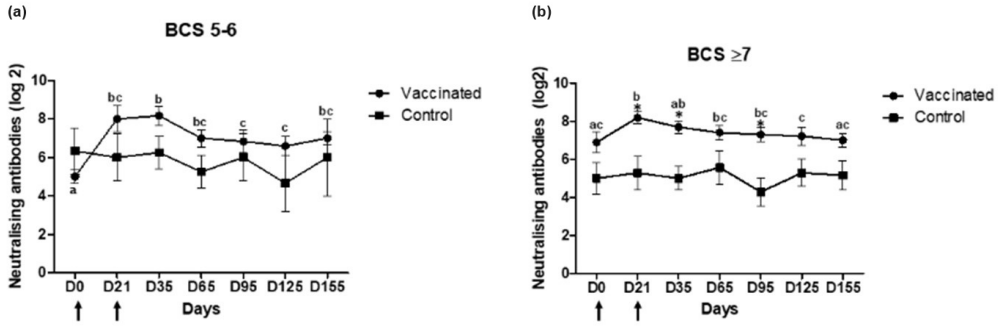

Figure 2 – (a) Serum-neutralizing antibodies in control and vaccinated mares with BCS 5-6 from D0 (baseline) to D155; (b) Serum-neutralizing antibodies in vaccinated and control mares with BCS ≥7. The arrows indicate vaccination (first and second doses). Different letters indicate statistical differences between time points. Asterisk (*) shows the difference between control and vaccinated groups at each time point, $p < 0.05$. The data represents mean ± S.E.M of log2 transformed variables.

95 after administration of the first dose (Figure 2b). When evaluating the titers between days after the first dose of the vaccine, there was an increase in neutralizing antibodies from D0 to D35 in the group with BCS ≥7.

There was no difference between vaccinated animals and controls in the BCS 5-6 group. However, the curve of vaccinated animals increased from D0 to D155 (Figure 2a). Among the vaccinated animals, there was no difference in neutralizing antibody response between the groups with BCS ≥7 and BCS 5-6 at any time point (Figure 3).

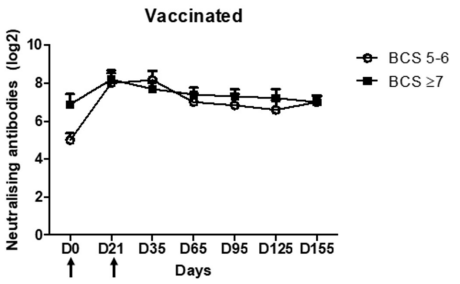

Figure 3 – Serum neutralizing antibodies in vaccinated mares from groups BCS 5-6 and BCS ≥7, from D0 to D155. The arrows indicate vaccination. The data represents mean ± S.E.M of log2 transformed variables.

Discussion

The overweight and lean-control pregnant mares used in this study responded to vaccination by producing neutralizing antibodies against EHV-1. However, the second dose of the vaccine did not provide a boosting effect on antibody production. Despite this, humoral immune responses lasted up to 155 days post-vaccination.

Overweight mares showed higher weight values, neck circumference, crest neck height, and CNS. Among all morphometric measurements evaluated, CNS has been considered more effective than BCS in differentiating animals with a greater predisposition to the development of metabolic alterations (Fitzgerald et al., 2019). Changes in glucose and insulin dynamics have been identified in animals with fat deposition above grade 2 (Carter et al., 2009; Fitzgerald et al., 2019). All overweight mares in this study had a CNS ≥2. However, although baseline glucose and insulin concentrations were higher in this group than in the group with BCS 5-6, they were within the reference range for the species (Frank & Tadros, 2014).

The assessment of baseline insulin and glucose concentrations should be interpreted with care. Although insulin sensitivity can be evaluated using baseline measurements, there are better choices for identifying animals with metabolic alterations than this test (Dunbar et al., 2016; Frank & Tadros,

2014). Dynamic tests are preferred (Dunbar et al., 2016). However, we chose not to perform dynamic tests in this study to not interfere with the work routine at the property. Furthermore, besides the fact that no test was performed to identify insulin resistance secondary to obesity, it is essential to consider that the mares were sampled in the final third of pregnancy, which is physiologically characterized by an increase in insulin resistance that occurs to increase glucose supply for the development of fetal-placental tissues (Fowden et al., 1984; George at al., 2011).

Both vaccinated groups had an increase in neutralizing antibodies after the first vaccination. Nonetheless, no seroconversion was observed after the second dose in either group. Bannai et al. (2019) found a similar effect after vaccinating non-pregnant animals with pre-existing antibodies, suggesting that animals that have already had contact with EHV do not have a boosting effect after a second dose. Another study that evaluated the impact of sequential vaccination in mares from an EHV-free area also did not observe an increase in antibodies after repeated vaccinations (Wagner et al., 2015). Likewise, antibody titers declined but were still present after the second dose until D155 in both vaccinated groups, which is described to occur after the use of inactivated vaccines against EHV-1 (Bannai et al., 2019; Kydd et al., 2006). The animals tested in this study had no previous vaccination history. However, both control and vaccinated animals showed variable titers of neutralizing antibodies at all time points. These observations may suggest the last or recurrent contact of the animals with EHV-1, which usually happens in equine populations in field situations.

Pregnancy poses a constant challenge to the maternal immune system, and species that exhibit more invasive types of placentation, such as humans and rodents, had to develop several tolerance mechanisms to maintain the semi-allogeneic fetus (Antczak, 2020). The equine placenta is epitheliochorial diffuse, considered the least invasive among placental species (Meeusen et al., 2001). However, between days 25 and 36 of gestation, trophoblast cells assume an invasive profile and form endometrial cups (Antczak et al., 2013; Meeusen et al., 2001). After their formation, invasive endometrial cells express class I MHC of maternal and paternal origin, generating an immune response in which high titers of paternal anti-MHC I antibodies develop (Antczak et al., 2013; Antczak, 2020; Chen et al., 2012). This ability to produce antibodies suggests that the humoral immune response is maintained and functions typically during pregnancy (Antczak, 2020), which was observed in this study. All pregnant mares responded to vaccine administration with increased antibodies after the first dose, regardless of the group.

There was no difference in humoral response between the vaccinated overweight and lean-control groups. Similarly, Elzinga et al. (2018) evaluated the vaccine response in horses presenting with equine metabolic syndrome (EMS) and found no effect of metabolic status on antibody response after vaccination. On the other hand, the metabolic rate in obese horses interfered with peripheral blood PMN phagocytosis (Salinas et al., 2022). Obesity was an essential factor in humoral response and neutralizing capacity in a study conducted on mice (Kim et al., 2012). In that same study, obese individuals vaccinated against influenza and challenged with the H1N1 virus exhibited compromised immune response, increased inflammatory response, and increased mortality rate (Kim et al., 2012). In horses, obesity with no endocrine disorders was shown to alter neutrophil reactive oxygen species production (Salinas et al., 2020).

One limitation of the present study was using mares with prior antibodies against EHV-1. Although this is usually observed in field situations in equine populations on farms, it may have interfered with the assessment of immune response in both groups. In this respect, the use of seronegative animals would be more appropriate. Another factor to consider is that we could not evaluate cellular immunity, which could be impaired in both gestations and overweight animals. Further research should address cellular immunity in overweight pregnant mares.

Conclusion

Pregnant Crioulo mares with BCS 5-6 and BCS≥7 vaccinated against EHV-1 responded to vaccination by producing specific neutralizing antibodies. However, a second dose of the vaccine did not induce a boosting effect, and humoral immune responses lasted at least up to 155 days post-vaccination. Overweight pregnant mares showed no difference in humoral immune response to vaccination compared to lean-control pregnant mares. This study demonstrated that obesity is not a factor influencing the humoral immune response in pregnant mares.

Conflict of Interest

The authors declare there are no conflicts of interest.

Ethics Statement

The study was approved by the Ethical Committee for Animal Experimentation of the Universidade Federal de Pelotas (CEEA-UFPel), under protocol number 34960-2019.

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Financial Support: The authors thank the Associação Brasileira de Criadores de Cavalos Crioulos (ABCCC) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for funding this project.