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Serological response against *Mannheimia haemolytica* and its leukotoxin in rabbits supplemented with selenium

Respuesta serológica contra *Mannheimia haemolytica* y su leucotoxina en conejos suplementados con selenio

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ABSTRACT

Selenodeficiency has a negative impact on the immune response of animals. The objective of the work was to evaluate selenium supplementation and its effect on the response against *Mannheimia haemolytica* and its leukotoxin. 21 rabbits were used, these were distributed in three groups (n=7); A: Selenium plus bacterin-toxoid was administered; B: the bacterin-toxoid was administered. C: considered control. Blood selenium content was estimated by atomic absorption spectrophotometry. The response to antigens was evaluated through an ELISA. An analysis of variance and Tukey were performed to determine statistical significance, considering a P value <0.05. In selenium quantification, a difference was observed between A with respect to B and C (P <0.05). In the evaluation of IgG against *M. haemolytica*, there was a difference between A with respect to B and C (P <0.05). For IgG against leukotoxin, no differences were observed between A and B (P > 0.05), but of these with respect to C (P <0.05). In conclusion, the supplemented animals had higher concentrations of selenium. This had positive effects on the response against *M. haemolytica*, however, no differences were found for the response against leukotoxin.

Keywords: *Mannheimia haemolytica*, selenium, rabbits, immunity.

RESUMEN

La selenodeficiencia tiene un impacto negativo en la respuesta inmune de los animales. El objetivo del trabajo fue evaluar la suplementación con selenio y su efecto sobre la respuesta contra *Mannheimia haemolytica* y su leucotoxina. Se utilizaron 21 conejos; estos fueron distribuidos en tres grupos (n = 7); A: se les administró selenio más una bacteria-toxoide; B: se les administró bacteria-toxoide. C: considerado control. Se estimó el contenido de selenio en sangre por espectrofotometría de absorción atómica. Se evaluó la respuesta a los antígenos a través una ELISA. Se realizó un análisis de varianza y de Tukey para determinar la significancia estadística, considerando un valor P<0.05. En la cuantificación de selenio se observó una diferencia entre A con respecto a B y C (P<0.05). En la evaluación de IgG contra *M. haemolytica*, hubo una diferencia entre A con respecto a B y C (P<0.05). Para IgG contra la leucotoxina, no se observaron diferencias entre A y B (P>0.05), pero sí de estos con respecto a C (P<0.05). En conclusión, los animales suplementados tuvieron mayores concentraciones de selenio. Ésta tuvo efectos positivos sobre la respuesta contra *M. haemolytica*, sin embargo, no se encontraron diferencias para la respuesta contra la leucotoxina.

Palabras clave: *Mannheimia haemolytica*, selenio, conejos e inmunidad.

INTRODUCTION

Mannheimia haemolytica is a bacterium that inhabits the upper respiratory tract of ruminants, which can cause pneumonic problems associated with immunosuppression, which can lead to the death of animals (De la Rosa *et al.*, 2012). It was observed that deficiency of some minerals in animals results in a negative effect on the immune response against the presence of antigens (Radwinska y Zarczynska, 2014). That is why there is an interest in the use of supplements that level the mineral needs, to improve the immunological status and the production processes of the animals (Campos, 2015).

Currently, pre-mixes or parenteral solutions are used to avoid the lack of minerals such as selenium, which is an essential micronutrient for mammals, necessary in the process of growth, production and reproduction of animals (Mehdi y Dufrasne, 2016). Through selenoproteins, they have antioxidant functions and, therefore, participate in the proper functioning of organs, such as: heart, liver, kidneys, pancreas, testicles and thyroid (Ghany y Tórtora-Pérez, 2010).

It was observed that the deficiency of this mineral affects the ability of phagocytosis neutrophils and macrophages to destroy antigens; in addition to the fact that the life of these cells is reduced, affecting the phenomena of antigenic presentation and the subsequent production of immunoglobulins in the blood; factor that determines higher prevalence and severity of diseases (Avery y Hoffmann, 2018).

Selenium supplementation can improve the immune response in different animal disorders. The concentration of antibodies increases in animals supplemented with selenium, against the challenge with different antigens; compared to animals that were not supplemented (Gelderman y Clapper, 2013).

The objective of this study was to evaluate, by means of an indirect ELISA, parenteral supplementation with selenium and the antibody response against *Mannheimia haemolytica* serotype A2 and its leukotoxin, using rabbits as a biological model. The hypothesis of this work is that, if rabbits are supplemented with parenteral selenium, they will have a greater serological response to antigens of the serotype A2 of *Mannheimia haemolytica* and its leukotoxin, differently from animals that were not supplemented.

MATERIAL AND METHODS

Characteristics of the experimental units, group distribution and treatment administration.

21 New Zealand rabbits, with an average weight of 3.2 kg and 6 months of age, respectively, were used as biological models. The animals were handled according to the standards of care provided by the National Institute for Forestry, Agricultural and Fisheries Research, at the National Center for Discipline Research in Animal Microbiology, based on the Federal Animal Health Law (third title. Chapter I on animal welfare) and in the official Mexican standard NOM-062-ZOO-1999 (4.2.2; 4.2.2.1; 4.2.2.2; 4.2.2.3) The animals were housed in individual 50 cm x 30 cm stainless steel cages, kept with alfalfa

pellets and water ad libitum; randomly distributed into three groups; group A: (n=7), a selenium solution (10.95 mg of sodium selenite for each mL of solution) was administered, at a dose of 0.25 mg/kg of live weight plus 2 mL of a bacterial toxoid, which contained a bacterial suspension with 1×10^6 colony-forming units per milliliter (CFU/mL), based on *Mannheimia haemolytica* serotype A2 with leukotoxin of the bacterium subcutaneously; group B: (n=7), 2 mL of bacterin toxin were administered subcutaneously and group C: (n=7), 2 mL of 10% physiological saline solution were administered subcutaneously. This group was considered the control group.

Treatments were administered at week 0 and week 2 for all study animals.

Sampling and processing

Samples were collected weekly. Blood was obtained from all experimental groups atrial marginal vein using Vacutainer® system; 1 mL of blood was collected in a neutral tube without anticoagulant and 1 mL in a tube with heparin. The neutral tubes were centrifuged at 4,500 g for 5 min to obtain the serum.

Laboratory tests

Blood and serum tests were performed as follows; to estimate the blood selenium content, the hydride generator atomic absorption spectrophotometry method was used, following the method described by [Ghany-Hefnawy et al., 2007](#). To obtain the antigens for the ELISA tests, sonicated to the serotype A2 from *Mannheimia haemolytica*, to expose its antigens, following the technique described by [Solanet et al., 2011](#). In obtaining leukotoxin from *Mannheimia haemolytica*, the method described by [Morales-Álvarez et al., 1993](#) was followed.

For the evaluation of the response to *Mannheimia haemolytica* and its leukotoxin, an indirect ELISA test was carried out in 96-well flat bottom polyethylene microplates, 100 µL of each of the antigens were added in a 1:20 dilution in each well in triplicate, incubating for 24 hours at 37 °C. After that time, a cycle of three washes with PBS Tween-20 was performed using a microplate washer. Then, a 2% solution of skimmed milk in PBS was added in order to occupy places where there was no adsorption of the antigen, leaving it for 60 min at 37 °C. Subsequently, 3 washes were performed with PBS-Tween 20. Finally, the plates were covered and stored at 4 °C, until use. Subsequently, 100 µL of the test sera were deposited in each well, at a 1:20 dilution in PBS, incubating at 37 °C, in a bacteriological oven for 60 min. Thereafter, three washes were performed with PBS-Tween 20; was added 100 µL of IgG conjugated rabbit anti-sheep to each well at a dilution of 1: 2000 in PBS, incubated 60 min at 37 °C. Thereafter, three washes were performed with PBS-Tween and 20 was added 100 µL of substrate (ABTS from Sigma Chemicals Co).

Finally, the reading was made in a multiple spectrometer, calibrated at 405 nm (EIA multi-well reader of Sigma D).

Statistical analysis

An analysis of variance was performed to determine the statistical significance of each of the variables to be measured; a value of $P < 0.05$ was considered to determine the significance of the data. Subsequently, to identify which groups were no significant differences was performed using the Tukey test statistic. For this, an honestly significant difference or HSD (*Honestly significant difference*) was calculated for each of the variables to be measured. Selenium dose, dose of bacterin toxin and sampling time were considered independent variables. Blood selenium levels and serum absorbances for the evaluated antigens were considered dependent variables. The program *Statgraphics Centurion 16.1.11* was used for the analysis.

RESULTS

Quantification of selenium in the blood of experimental groups

The evaluation of quantification of selenium in the blood was conducted in the experimental groups. To determine significant differences, an analysis of variance was performed, considering a value of $P < 0.05$ (Table 1).

Table 1. Analysis of variance of the quantification of selenium in the blood of the experimental groups

Source	Sum of squares	Degrees of freedom	Squares average	F value	P value
Between groups	0.007	2	0.004	4.990	0.026
Within groups	0.009	12	0.001		
Total	0.0158	14			

Table 1 shows the analysis of variance of blood selenium levels in the experimental groups; a value of $P < 0.05$ was obtained; therefore, there is a statistically significant relationship between blood selenium concentrations between experimental groups. These differences can be seen in figure 1.

In figure 1, the standard means and errors for the quantification of selenium in the blood of the experimental groups are observed. Group A has a higher average concentration, unlike groups B and C ($P < 0.05$). During the experiment, the latter with no statistical difference between them ($P > 0.05$). To corroborate, the Tukey test was performed, calculating the HSD, obtaining a value of $0.045 \mu\text{g/g}$. When compared to the value obtained by subtracting the group means, a statistically significant difference was obtained between group A, in relation to group B and group C.

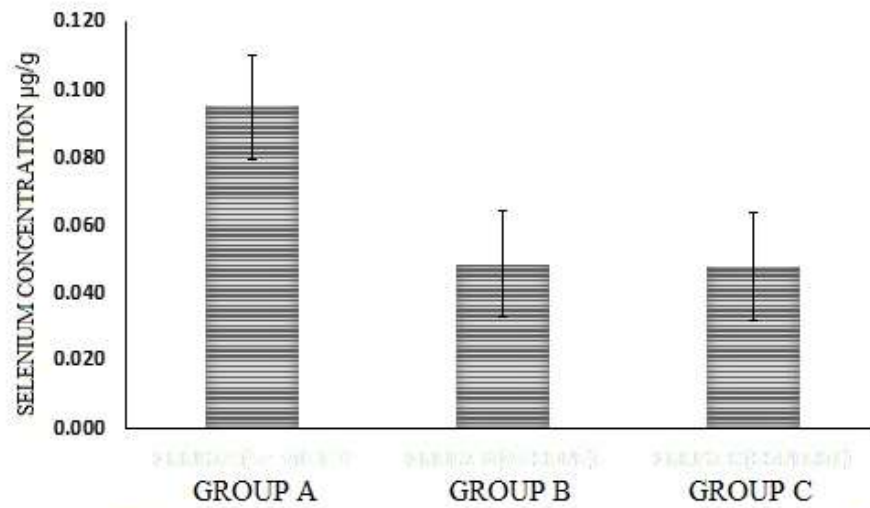


Figure 1. Means and standard errors of selenium quantification in blood of the experimental groups

The weekly blood selenium concentrations for the study groups are shown below (Figure 2). Weekly quantification of selenium in the blood is observed for the experimental groups. It is observed that in weeks 0, 2 and 3, group A has higher concentrations of selenium in the blood, compared to groups B and C ($P < 0.05$); the latter with no significant difference between them ($P > 0.05$), for those weeks. On the other hand, at weeks 1 and 4, the three groups did not show significant differences in blood selenium concentrations ($P > 0.05$).

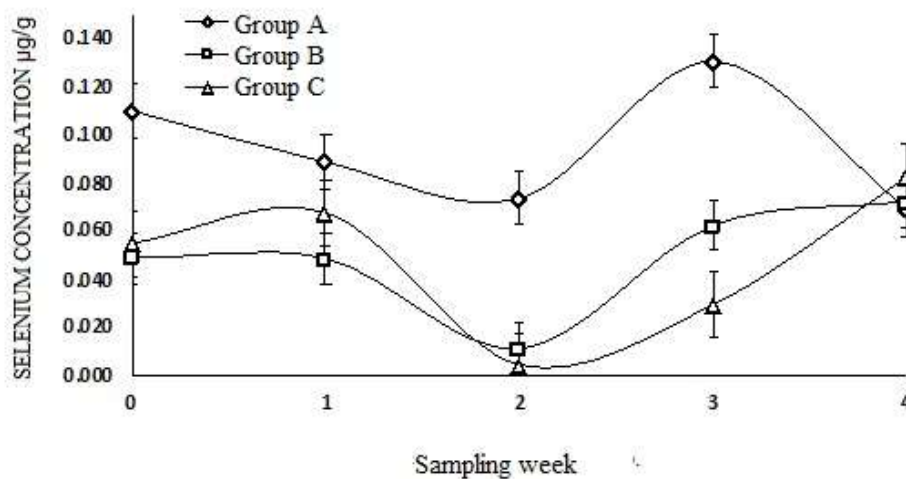


Figure 2. Weekly blood selenium quantification of the experimental groups

Evaluation of the humoral response against *Mannheimia haemolytica* serotype A2 in the serum of the experimental groups

The evaluation of the absorbances at 405 nm for IgG in the sera of the experimental groups was carried out against *Mannhemia haemolytica* antigens. An analysis of variance was performed to determine the statistical significance between the groups, for which a value of $P < 0.05$ was considered (Table 2).

Table 2. Analysis of variance of the absorbances obtained at 405 nm for serum IgG from the experimental groups against the *Mannheimia haemolytica* antigens

Source	Sum of squares	Degrees of freedom	Squares average	F value	P value
Between groups	0.308	2	0.154	4.941	0.027
Within groups	0.375	12	0.031		
Total	0.683	14			

Table 2 shows the analysis of variance for the absorbances obtained at 405 nm for IgG in the sera of the experimental groups, against *Mannhemia haemolytica* antigens. It is observed a P value < 0.05 , then there is a statistically significant correlation between IgG absorbances for the study groups. These differences can be seen in figure 3.

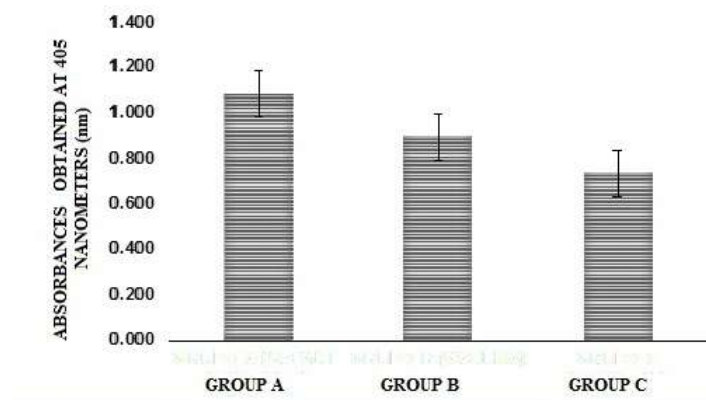


Figure 3. Means and standard errors of the absorbances for serum IgG of the experimental groups against *Mannheimia haemolytica*

Figure 3 shows the means and standard errors of the absorbances obtained at 405 nm for IgG in the sera of the experimental groups, against *Mannhemia haemolytica* antigens. Group A showed higher absorbances, differently from groups B and C ($P < 0.05$). During the experiment, the latter with no statistically significant difference ($P > 0.05$). The Tukey test was performed, the HSD calculated, obtaining a value of 0.272. When compared with the value obtained by subtracting the means of the group, there was a statistically significant difference between group A, in relation to group B and group C.

The weekly absorbances obtained at 405 nm for IgG are shown below, against *Mannheimia haemolytica* for the study groups (Figure 4).

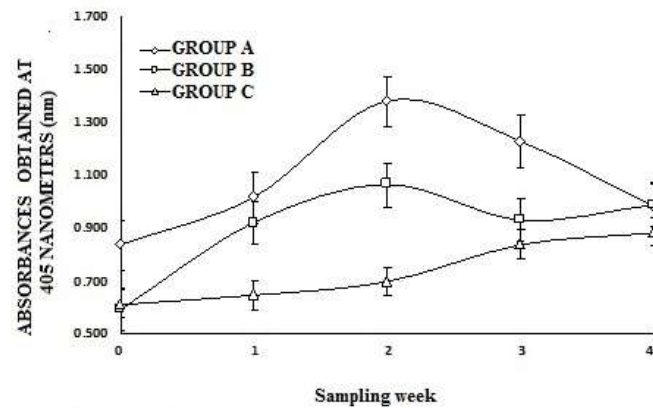


Figure 4. Weekly absorptions for IgG from the sera of the experimental groups, against *Mannheimia haemolytica*

Figure 4 shows the weekly absorbances obtained at 405 nm for IgG, against *Mannheimia haemolytica* in the study groups. At weeks 0, 2 and 3, it is observed that group A has higher absorbances, compared to groups B and C ($P < 0.05$). At weeks 1 and 4, no differences were observed between A and B and between A, B and C, respectively ($P > 0.05$).

Evaluation of the humoral response to *Mannheimia haemolytica* leukotoxin in the serum of the experimental groups

The absorbances obtained at 405 nm were evaluated for the serum IgG of the experimental groups, against *Mannheimia haemolytica* leukotoxin. An analysis of variance was performed to determine significant differences, considering a value of $P < 0.05$ (Table 3).

Table 3. Analysis of variance of the absorbances obtained at 405 nm for *Mannheimia haemolytica* leukotoxin from the sera of the experimental groups

Source	Sum of squares	Degrees of freedom	Squares average	F value	P value
Between groups	0.827	2	0.414	4.026	0.046
Within groups	1.233	12	0.103		
Total	2.060	14			

Table 3 shows the analysis of variance for the absorbances obtained at 405 nm for serum IgG from the experimental groups, against the leukotoxin of *Mannheimia haemolytica*. A value of $P < 0.05$ is observed, therefore, there is a statistically significant relationship for absorbances for IgG between the study groups. These differences can be seen in figure 5.

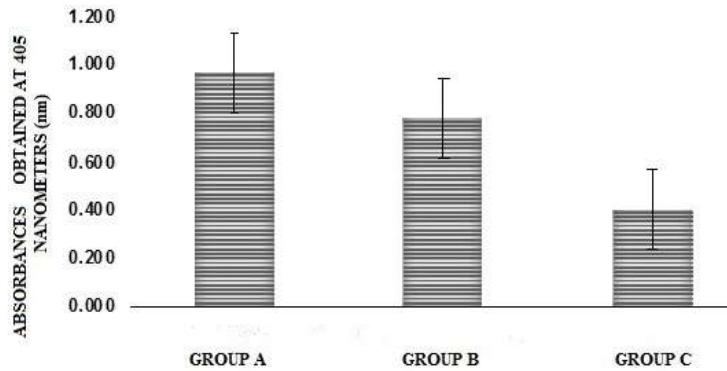


Figure 5. Serum absorbance averages for IgG of the experimental groups of serum *Mannheimia haemolytica* leukotoxin serotype A2

Figure 5 shows the means and standard errors of the absorbances obtained at 405 nm for serum IgG of the experimental groups, against the leukotoxin of *Mannheimia haemolytica*. Groups A and B do not show significant differences between them ($P > 0.05$); however, they show higher absorbances than group C ($P < 0.05$) during the experiment. Tukey's test was performed, HSD calculated, obtaining a value of 0.540. When comparing it with the value obtained by subtracting the means of the group, there is a statistically significant difference in group A in relation to group C; that has not been challenged with leukotoxin.

The weekly absorbances obtained at 405 nm for IgG against leukotoxin for the study groups are shown below (Figure 6).

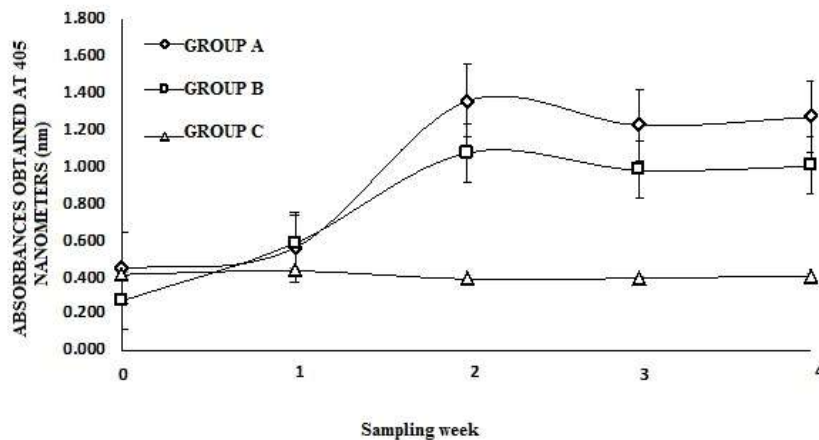


Figure 6. Weekly average of the absorbances for IgG in the sera of the experimental groups, against *Mannheimia haemolytica* leukotoxin

Figure 6 shows the weekly absorbances obtained at 405 nm for IgG in the sera of the study groups, against *Mannheimia haemolytica* leukotoxin. There were no statistically significant differences in the mean absorbances of group A and group B during the study ($P > 0.05$); it is from week 3 that differences between A and B are observed in relation to group C ($P < 0.05$).

DISCUSSION

In this study, rabbits were used as a biological model to observe the effect of selenium supplementation and to evaluate part of the humoral immune response, against *Mannheimia haemolytica* serotype A2 and its leukotoxin; which affects the respiratory tract of ruminants, causing pneumonia and death.

The selenium requirement is low for rabbits, with about 0.05 mg/kg of food, to observe beneficial effects on the productivity of these animals (NRC, 1977; Papadomichelakis *et al.*, 2017); however, doses of 0.2 mg/kg of food have been found to improve productivity in this species (Syvyk *et al.*, 2018).

To supplement the animals parenterally, a dose of 0.025 mg/kg of live weight was used, recommended by Ramírez-Bribiesca *et al.*, 2004 for ruminants, extrapolating this dose to the metabolic weight of rabbits, so that this was the suitable for the species; as well as to avoid poisonings and deaths.

Adequate levels of selenium in the blood of rabbits have been reported to range from 0.074 to 1,000 ppm (Puls, 1988), this depends on the diet and the geographical area in which they are found. After supplementation, it was observed that group A had an average of 0.972 µg/g of selenium in the blood throughout the study; while groups B and C had an average of 0.595 µg/g of selenium in the blood throughout the experiment ($P < 0.05$).

There were no selenium deficiencies in the blood; all three groups maintained adequate levels throughout the experiment. It was observed that mineral supplementation increases blood selenium levels in dairy cows (Khalili, *et al.*, 2020), pigs (Cao *et al.*, 2014), in chickens (Doaa *et al.*, 2019), sheep (Ademi *et al.*, 2017) and goats (Ziaei, 2015), with beneficial effects on animal health and production; however, an excess of mineral in the diet can have negative effects on productivity with the consequent poisoning of animals and death (Żarczyńska *et al.*, 2013).

Variations in selenium concentrations were observed throughout the experiment for all groups. Within weeks, they show a decrease in blood mineral concentrations, particularly in group A, at weeks 2 and 4. This has also been seen in pigs supplemented with selenium and challenged with different antigens (Falka *et al.*, 2018), that it may be due to the biodistribution of the mineral in the body, to maintain the oxidative balance against infections or challenges with antigens, by means of selenoproteins; since they participate in the regulation of oxidative stress, optimizing cellular processes for proper functioning;

including those involved in innate and adaptive immune responses (Dalgaard *et al.*, 2018). However, this synthesis is regulated by the availability of the mineral in the body; a selenium deficiency will have an impact on the correct functioning of cells and, therefore, on immune responses (Howard *et al.*, 2013; Seyedali *et al.*, 2014). Monogastrics, like rabbits, do not have selenium deficiency as marked as ruminants; which are very susceptible to the deficiency of this mineral, mainly in sheep and goats. In these species, the digestibility and absorption of this mineral through the diet is very low (11-18%); compared to monogastric (70-80%), which affects their health and productivity (Ghany y Tórtora-Pérez, 2010). This greater susceptibility of ruminants is attributed to the reticulo-ruminal environment, since part of the ingested selenium is absorbed by the microbiota, which uses it for protein synthesis; or the rumen environment itself reduces the mineral to non-soluble forms (selenides), which cannot be absorbed by the animal (Carbajal *et al.*, 2013).

When evaluating the absorbances for serum IgG, against *Mannheimia haemolytica* serotype A2, it was found that group A had an average absorbance of 1,087 nm, significantly higher than groups B and C, with an average of 0.817 nm in the study ($P < 0.05$)

In other studies, ambiguous results have been observed in relation to selenium supplementation and its effect on the immune response to challenges with different antigens. On the one hand, selenium supplementation has been shown to have a positive effect in chickens in inducing specific antibodies to the vaccine against the infectious bursal disease virus (Shekaro *et al.*, 2012). Likewise, a higher antibody-mediated immune response against *Pasteurella multocida* was observed in sheep supplemented with 0.3 ppm of selenium in the diet (Kumar *et al.*, 2009). On the other hand, when the influence of selenium on the immunity of broilers was studied by supplementation through feed in various concentrations (0, 100, 200, 300 or 400 µg/kg of diet), no effect was found in the production of specific antibodies to the vaccine against Newcastle disease virus (Rao *et al.*, 2013). On the other hand, a study in goats in which the immune response against *Mannheimia haemolytica* was evaluated, when assessing serum IgG, no significant differences were observed in the study groups in the first 28 days of the experiment; however, the supplemented groups showed a significantly higher concentration of IgG since the 28th day, after and until the end of the experiment (Díaz-Sánchez *et al.*, 2017). Finally, despite limited studies in rabbits, California rabbits, supplemented with selenium and challenged against sheep red blood cells (SRBC), have been reported to have higher antibody titers compared to the control group (Ebeid *et al.*, 2013), as observed in this study for the case of *Mannheimia haemolytica*.

There are likely to be more interactions related to selenium supplementation and the immune response to antigens; such as the nutritional status of the animals, age, the

availability of selenium in the body and the type of infectious agent or antigen that entered the animal body; therefore, differences between animals in relation to selenium supplementation and the immune response can be observed (Hoffmann y Berry, 2008). When there is an infection, the production of reactive oxygen species (ROS) participates in the activation and signaling of several endogenous systems (Vladimirov *et al.*, 2009). Phagocytic cells depend on the production of ROS for their bactericidal activities during inflammation, but if this process is not controlled by antioxidants, such as selenoproteins, reactive oxygen products can induce damage to the host, such as cell lipoperoxidation (Lubos *et al.*, 2011). Finally, it was found that the effects of selenium supplementation would not necessarily affect the concentration of antibodies in the same way. Different effects can be expected on targeted antibody responses against T dependent antigens versus independent T antigens (Dalgaard *et al.*, 2018).

Regarding the evaluation of absorbances for IgG, against *Mannheimia haemolytica* leukotoxin, no differences were found between groups A and B ($P > 0.05$); however, both groups had higher absorbances than group C ($P > 0.05$), which did not respond to the antigen, as expected in the study. Leukotoxin is known to induce negative biological effects on ruminant leukocytes in a species-specific manner. The rabbit is not susceptible to this antigen; however, it could have similar effects on its immune system. In ruminants, it induces the secretion and release of vasoactive chemotactic peptides; as well as the number of leukocytes available at the inflammation site, where fibrous deposits occur. This process causes acute fibrinopurulent pneumonia. Any opportunity for a secondary immune response is interrupted by leukotoxin activity, which prevents lymphocyte blastogenesis and destruction of the leukocytes themselves (Jaramillo *et al.*, 2009). Finally, Jaramillo *en el* 2000, achieved the purification of an adhesin, capable of specifically binding to rabbit erythrocytes, concluding that *Mannhemia haemolytica* adhesins play an important role in infection. This may suggest why there was a stronger IgG response to *Mannhemia haemolytica* serotype A2 than to leukotoxin in this study.

CONCLUSION

The animals that were supplemented had a higher concentration of selenium in the blood. When using the rabbit as a biological model to evaluate the antigenic response, it was found that supplementation with selenium had positive effects on the response to antigens of serotype A2 of *Mannheimia haemolytica*, obtaining greater absorbance in the supplemented rabbits, compared to those that were not supplemented. Regarding the antigen response to *Mannheimia haemolytica* leukotoxin, no differences were found between the groups studied.

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