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Levamisole-Phosphate Used In Dairy Cattle on Pre- Parturient and Reproductive Performance during Post-Parturient Period.

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Abstract: *In the milk activity, the problems that affect the reproductive tract of postpartum females such as retention of placenta, metritis and endometrites, cause great losses, such as decrease on milk production, drug expenses, milk discharge and low reproductive performance, are generally related to the animal's systemic immune status. For this reason, several products are indicated as immunostimulators or immunopotentiators in pre-calving cows. In this work, two groups of bovine, Jersey andHolstein females were tested for the immunostimulatory activity of Levamisole, a parasite control drug that has on its properties an increase in the immune response in animals. The work consisted in the application of Levamisole twice in the prepartum and collections of serum and colostrum from the animals during the experiment, measuring the immunoglobulins type G by immunoaffinity with protein A. There were no differences between the races or between the control groups and the groups This is in agreement with several authors that the use of levamisole as immunopotentiator does not show results in bovine females, nor was there any difference in the reproductive performance of the same, regardless of the race studied.*

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I. Introduction

In the Brazilian dairy industry many farmers use animals of the Holstein breed because of their high production and persistence. According to the Brazilian Association of Holstein Livestock Breeders, the Brazilian herd has a milk production around 8,047 kg in adulthood. About 84.0% of farmers are located at the States of São Paulo, Paraná and Minas Gerais. During the past decades the dairy production of the Holstein cows has increased considerably due to programs of genetic improvement and other tools introduced in the management of these herds. However, other indices were affected such as increased metabolic problems, mastitis and postpartum problems that affect reproductive performance[1]. Due to these problems, Holstein cattle have been considered a breed of lower resistance or rusticity which has led some producers to use other breeds such as the Jersey breed, that has a lower milk yield but is considered rustic and more resistant.

In a study carried out in the States of Goias and São Paulo, in five farms with a Holstein herd kept in an intensive system, 2,083 cows were observed, evaluating the main reasons for the cows discarding, where they obtained 296 discards, pointed as in the first place, the reproductive problems (27.7%) and second, problems in the locomotor system (18.5%) [2].

Within clinical pathologies, placental retention is a result of the lack of detachment of the fetal membranes from the maternal caruncles due to low motility or uterine atony. Some cows are able to expel the placenta after delivery in up to three hours, but most cows expel their fetal attachments around 6 hours postpartum and a small number of cows 12 hours postpartum. The placenta is considered retained when the release does not occur after six hours of delivery. Ruckebuschet al[3] adopt eight to 12 hours and other authors[4] consider retained placenta more than 24 hours postpartum.

In a study carried out in a commercial farm located in the city of Araras / SP, 522 bovine primiparous and multiparous Holstein cows were followed in the postpartum period, with an average daily production of 35 kg and average accumulated production at 305 days of 10,747 kg. Retention of placenta and metritis were the most frequent diseases in this herd, with few cases of milk fever and displacement of abomasum[5] .With this, some strategies or management practices have been incorporated over the years in the dairy farms.

Antibodies (Ac), immunoglobulins (Ig) or gammaglobulins are glycoproteins synthesized and excreted by plasma cells derived from B lymphocytes, the plasma cells, present in plasma, tissues and secretions that attack proteins foreign to the body, called antigens, thus performing the defense of organism (humoral immunity). All antibodies are constructed in the same way, from four polypeptide chains, there are five different

classes called IgM, IgD, IgG, IgA, IgE. IgG is the most abundant in blood plasma, being produced at high levels in both primary and secondary immune responses[6].

The discovery of immunomodulation occurred during the global campaign for smallpox eradication through vaccination with an attenuated virus vaccine, discovered by Edward Jenner, in the 18th century. Following mass vaccinations of the population, beneficial and collateral effects began to be observed that this vaccine exerted on individuals. It was therefore appropriate to choose the term immunomodulation for the effects of the smallpox vaccine, as these could be related to both immune stimulation and immunosuppression[6] .

The bovine placenta prevents the passage of antibodies to the fetus during gestation consequently the neonatal calves have an immature immune system, making it essential to transfer immunity through supply of colostrum in the first six hours of life for the neonates.

Colostrum contains several soluble and cellular immunoprotective elements that are capable of conferring immunity against a variety of pathogenic microorganisms responsible for the high rates of neonatal morbidity and mortality in dairy herds in Brazil and other countries[7].The amount of colostrum immunoglobulins increases according to the number of lactations, and the main immunoglobulins present in bovine colostrum are: IgG (70-80%), IgM (10-15%) and IgA (10-15%). In veterinary medicine there are situations in which it is desirable to potentiate the immune response, such as increased resistance to infections and the treatment of immunosuppressive or infectious diseases of multifactorial origin or better colostrum with higher levels of immunoglobulins[8,9].

Immunomodulators may be specific, non-specific and passive active. Some of these are: interferons and interferon-inducers, interleukins, Calmett-Guérin bacillus (BCG) and their derivatives, *Propionibacterium acnes (Corynebacteriumparvum*), mixed bacterial vaccine, PIND-ORF, Phosprenyl, *Quillajasaponis, Bordetella pertussis*, wall components of *Staphylococcus aureus*, avridine and levamisole.According to Spinosa[10] immunostimulators have specific actions, such as interferons and interferon inducers that inhibit or stimulate the production of proteins, induce receptors for G-immunoglobulins (IgG) and Complement Fixers (FC) in monocytes and macrophages besides stimulating macrophages and cells (NK).

Levamisol is a broad spectrum drug anthelmintic used in the treatment of intestinal parasites. Concomitantly with the anthelmintic action, this drug acts in the immune system in a manner similar to the hormone thymopoietin, produced in the thymus that influences the maturation of the T lymphocytes. It stimulates the T-cell action and the response to the antigens, potentiates the production of interferons and increases phagocytic activity of macrophages and neutrophils, stimulates cell-mediated cytotoxicity, lymphocyte production [9].Its use as an immunopotentiator was discovered in 1974 , authors [11] worked on mice infected with Brucella. Since there is ample evidence that host immunity may be affected by different non-specific mechanisms, the use of levamisole was widespread as an immunoprotector or immunopotentiator, as well as its functions in the control of parasites[12]. Other authors also question the action of levamisole as an immunomodulator[13,14] using *Plasmodium berghei* and *Trypanosomacruzi* as parasites in the experiments.

There were already reported [15] the advantages of using levamisole in comparison todoramectin in the control of parasites in cattle, and could bring other benefits to the herd, which could or should not be attributed to the use of drugs to control parasites, such as gain of herd weight. Despite the divergence of the action of levamisole as an immunopotentiator, this drug has advantages in the restoration of defective neutrophils and in phagocytosis[12].

In the 2009the beneficial effect of levamisole in the group of 20 cows treated with the drug in the dosage of 2.5 mg kg -1 of PV was reported and the control group (n=13) received the same dosage but of saline solution. Cows received the first dose of levamisole between 5 and 6 weeks and the second dose 2 weeks before the expected calving. The animals treated with levamisole presented a better uterine involution and the onset of ovarian activity earlier in the postpartum period in relation to the animals in the control group[16].

Treatment with levamisoleimmunomodulator in milk cows with placental retention or purulent uterine discharge resulted in a stronger immune reaction leading to a significant increase in the circulation of neutrophils and eosinophils in the postpartum period[16] .

Levamisole applications during the prepartum period around six weeks before delivery significantly reduced the incidence of mastitis, fetal deaths and metritis due to its immunostimulatory activity in cows[18].The efficacy of levamisole in the prophylactic treatment[19] of aphthous stomatitis, expecting an increase in the immunological capacity of the levamisole group as a stimulatory drug, however the results were not significant, compared to the control group, who received a placebo. Although levamisole is indicated for restoration of the cellular immune response, several studies have been reviewed and there are discussions about the actual effect of levamisole in the experiments.

Other immunostimulants used are: *Propionibacterium acnes* - is a Gram-positive bacterium, natural resident of the sebaceous gland of the hair follicle of human skin, live in anaerobic environment, growing more effectively in this, however, some strains are aerotolerant. *P. acnes* was successfully used in the therapy of endometriosis, osteomyelitis in horses and in the treatment of wounds[20].

BCG is an attenuated vaccine strain of Mycobacterium bovis is one of the most potent potentiators of cytokine synthesis due to the activation of macrophages, but also exerts a generalized potentiation of phagocytosis, B cell and T cell mediated responses[9].

According to Van Kampen[20] BCG has been used successfully in the treatment of diarrhea caused by *Escherichia coli* in calves. The treated animals present improvement in the clinical symptoms, in addition to a decrease in the mortality rate.

In studies conducted[8], the effectiveness of the Baypamun® immunomodulator was verified, limiting the spread of HBV-1 (Bovine Herpesvirus type-1). Bovine animals experimentally infected with HBV-1 were divided into treated and non-immunomodulator treated groups. Infected animals receiving Baypamun ® showed clinical signs of the disease. In the same experiment healthy animals cohabited with an infected animal with only half of the healthy animals receiving Baypamun®; As a result, untreated animals developed symptoms of classic and severe disease, whereas animals that received immunomodulators were only partially affected [8].

Vaccination of pregnant females using dead Salmonella sp. adsorbed on different adjuvants, have been shown to produce antibodies[21]. Two doses of subcutaneous SD in the last months of gestation of females and one dose in calves between 15 and 30 days of life were sufficient to reduce the mortality rate.

In Brazil, Ávila et al [22] found that these vaccines induce the production of antibodies in the vaccinated cows and that these are transferred to the calves through the colostrum.

Protein A is a polypeptide found on the cell wall of *Staphylococcus aureus* approximately 98% of S. aureus contains this protein. The mechanism of interaction between antibody and protein A occurs when the protein binds to the 4 potential binding sites of the antibodies but only two of them can be used at the same time[23].

Protein A columns are one of the most versatile methods used for antibody purification. The columns are easy to prepare because the molecules of the antibodies are intended to array the gel through the Fc domain. Antibodies bind directly to protein A because the Fc domain of the antibody has high affinity for protein A.

II. Material And Methods

Animals: The experiment was carried out using two dairy herds in good sanitary and nutritional conditions, belonging to the CAPTA / Milk of the Institute of Animal Science(Nova Odessa) and at the APTA / RibeirãoPreto Experimental Station. The animals were chosen at the end of gestation. The total of 33 females, 17 Holstein breed (Nova Odessa) and 16 Jersey breed (RibeirãoPreto).

Application of Immunostimulant: The immunostimulant chosen was Levamisole Phosphate (Ripercol L-150 Manufacturer Fort Dodge Animal Health). The test was outlined as follows:

1st group (15 pods): 1st dose of 3.75mg Kg -1 levamisole PV 6 weeks before delivery with a booster dose after 21 days after the 1st dose.

2nd group (18 cows): Control group only blood was collected to obtain serum.

Blood samples were collected from all cows on the 1st dose of the immunostimulant and at the 21st daywhen cows received the 2nd dose. Blood samples were also harvested one week before delivery. Soon after delivery the blood samples were also collected. The samples were processed to obtain serum to quantify and qualify IgG immunoglobulin levels. Colostrum was collected shortly after delivery in plastic bottles and stored at -80 ° C.

IgG were quantified using the Sepharose protein Aimmunoaffinity column. Blood sera obtained from each animal during the experimental period, were eluted in a sepharose - protein A (HiTrap Protein A agarose HP 1mL, GE Healthcare) column according to the procedure[23]: the column prepared with 2 cm of agarose gel was washed with 20 ml of PBS buffer, pulsed by peristaltic pump (Model Pump-P-1, Manufacturer Pharmacia Biotech, Flow 6 ml per hour). The serum was prepared, to be eluted on the protein A column, added to each 1.5mL of serum; 4mL of PBS solution and 0.22μm Millipore membrane filtrate.

Then 5.5 mL of the serum-containing solution was passed into the column in a closed system for 6 hours using a peristaltic pump (10 mL per hour flow). After this period, the column was washed with 20 mL of PBS buffer to remove impurities contained in the serum, with only IgG-type immunoglobulins bound in the protein A of the column being retained in the column.

The third step was to wash the column with Glycine Buffer - NaHCL pH3 so that the Immunoglobulins were detached from protein A. 2 ml aliquots were serially collected in labeled tubes from 1 to 9 containing 50 μL of the Tris-HCL Buffer, pH 9.0. The control tube received 50 μlTris-HCL buffer plus 2mL Glycine buffer. After elution, the column was again washed for one hour with PBS solution and 0.1% thymerosal to be conditioned until the next day.

One mL of the filtrate was taken from each test tube and the spectrophotometer (Genesys Model 10 uv, Manufacturer Thermo Scientifitic) was read at 280 nm. The correlation absorbance: mg of IgG .mL $^{-1}$ was calculated as 1.3: 1 from the absorbance read.

Statistical analysis

Tukey´s test was used to ascertain the significance of differences between mean values from control group and immune stimulated group. The level $P < 0.05$ was considered as the cutoff value or significance.

III. Result

The herds of Jersey cows had IgG levels between 0.2 and 1.0 mg mL $^{-1}$ and were below the reference levels [24], where serum IgG concentration varies between 8 and 16 mg mL -1 in several species. This result corroborated with other authors [25,26] who observed a decrease in immunoglobulin concentration in the last five weeks before delivery. Some authors believe that the transfer of immunoglobulins from the bloodstream to the mammary gland to form colostrum causes marked drop in blood immunoglobulin levels between two to three weeks before delivery [26].

Figure 1 above shows that the control group took on average 82 days and the treatment group around 90 days after delivery for the first Artificial Insemination. Most cows present the first estrus around 60 days postpartum, and it is recommended that the conception must occur no later than 85 days after calving, so that the cow has a birth interval of 365 days at conception[27].

For some authors the period of service is between the time of delivery and the new conception; however, the period of service can not exceed 90 days, in order to obtain a 12-month delivery interval, one child per year is correlated with factors of the sanitary management and the post - partum uterine involution time[28].

The number of AI services for the control group 1.5 services per conception and for group treatment was 1.3 per design outcome is within the mean 1.8 to 2.2 services per conception however the treatment group achieved a better outcome , and the target for an efficient herd is <1.5 service / design[27]. The number of services per conception is the number of doses used to impregnate a cow. To calculate this index, the total number of doses was divided by the number of pregnant cows.

Another parameter observed was the occurrence of placenta retention, and in the control group there were no cases. However, in the treatment group there was a case of placenta retention. Milk cattle may suffer a higher incidence of this problem than beef cattle in herds with good sanitary and nutritional management and the index varies from 3% to 7% on average [29].

Table 1- Pregnancy rate from Holstein and jersey cows, from the treatment with levamisole and from control cows.

	Pregnancy rate
Holstein	
treated	50%
control	33%
Jersey	
treated	57%
control	55%

The 16 cows from the herd of RibeirãoPreto were inseminated conventionally at the first natural estrus observed the pregnancy rate two groups was around 56% (9/16) (Table 1). The control group with 9 cows in the diagnosis of pregnancy performed 60 days after AI presented 5 pregnant cows with a pregnancy rate of 55%. Already the treatment group with 7 animals in the diagnosis of pregnancy were diagnosed 4 pregnant cows at the pregnancy rate 57%. The percentage of pregnancy relates to the number of pregnant cows and the total number of cows (pregnant and empty). A Pregnancy Rate around 70 and 75% may indicate a good reproductive efficiency of the herd [28] .

The animals from Holstein Herd CAPTA Milk - FazendaPalmeiras - Instituto de Zootecniapresented immunoglobulin levels of IgG levels between 0.150 and 0.400 mg mL -1 lower than the animals of RibeirãoPreto and below the reference levels according to Harlow and Lane[24], the concentration of IgG in the blood serum ranges from 8 to 16 mg mL -1 in several species. With this result in this experiment the serum immunoglobulin levels of the animals of both herds are below those of reference that are composed of different races showing that race variable does not interfere in this variable (Figure 1).

The 17 animals from the Nova Odessa herd (Holstein) were subjected to protocols of fixed time artificial insemination with sexed semen. This technology has been increasingly used due to the difficulties in conventional insemination with the observation of estrus being a bottleneck. Researchers are now developing protocols that synchronize ovulation by the application of hormones and enable the use of Fixed Time Artificial Insemination (IATF) , regardless of the manifestation of estrus behavior. The advantages of using the IATF are based on:

The design rate of the whole group for the 1st TAI protocol was 47% (8/17) pregnant cows. The treatment group composed of 8 cows obtained a design rate in the 1st TAI protocol of 50% (4/8) and a control

group composed of 9 cows had a pregnancy rate of 33% (3/9) (Table 2). In an experiment carried out in 2003 with 108 Holstein cows, the design rate of inseminated animals with pre - fixed time (41.4%) was not different (P> 0.05) from those inseminated in induced (53.1%) or natural (49.1%) [27]. Other authors found results similar to the 40% to 50% obtained in Holstein cows [30,31].

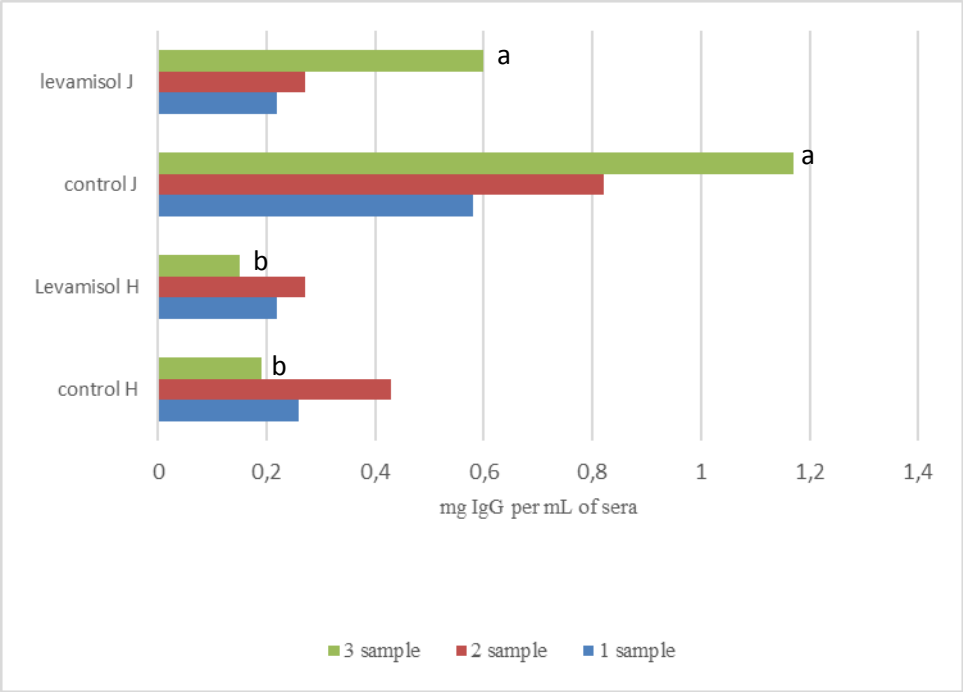

Figure 1 – Immunoglobulins IgG (mg per mL of serum) from every sampling, measured from Holstein herd (control H, levamisole H) and from Jersey herd (control J and Levamisol J). Same letters means there were no significate difference ((P> 0.05) between control and levamisole treatment in both herds.

The control group consisted of 9 cows, 6 multiparous cows and only 3 heifers had a lower pregnancy rate than the treatment group (Table 1). Some researchers recommend that IATF protocols using sexed semen should be performed only on heifers because they present favorable results for this category of animal, however, the three heifers in the control group did not engage, however in the treatment group, the heifers had a better result.

As reported[32],in a combination of several experiments, that conception rates of Holstein heifers inseminated with sexed semen varied from 40% to 68% and with conventional semen from 67% to 82%. One strategy that can be used is the use of sexed semen in heifers at the first insemination and the following inseminations with conventional semen.

One of the possible reasons for the decrease in fertility rates after the use of sexed semen is the shorter time of viability associated with different patterns of sperm motility[33]. Some authors have reported that sexed semen requires less time for training because of the flow cytometry separation process[34]. In theHolstein herd, the control and treatment groups presented a case of retention of placenta per group, showing that this herd has good nutritional and sanitary management. In vitro tests showed the use oflevamisole increasing the fagocitosys of parasites, acting as immunomodulatory [35].

Tukey test (test t) show no statistical difference from control animals and immune stimulated ones, showing the injections done do not increase strength in the immune system.

IV. Conclusion

In this experiment the IgG levels for two groups (control and immune stimulated) and herds were below the reference levels. Decreased serum immunoglobulin concentration in the last six weeks before delivery may have been influenced by the transfer of immunoglobulins from the bloodstream to the mammary gland to form colostrum.

The levamisole-treated groups showed no improvement in postpartum performance. Therefore, the recommendation of treatment with levamisole in cows in the pre-partum period for the improvement of postpartum performance should be reassessed.

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