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Research Article

Effect of vitamin C on the immune system in cattle immunized with blackleg vaccine

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ABSTRACT

The purpose of this study is to determine the effect of vitamin C on the immune system in cattle immunized against blackleg. The study employed 28 cattle of different breeds and genders, aged 15-20 months, bred in the Ardahan region. The cattle were divided into four groups, seven in each group. The first group (control group) 2 ml saline, the second group (vitamin group) 5 mg/kg vitamin c, the third group (vaccine group) 2 ml blackleg vaccine, and the fourth group (vaccine-vitamin group) 2 ml Blackleg vaccine and 5 mg/kg vitamin c was administered subcutaneously. Blood samples were collected from all animals in the groups just prior to vaccination and vitamin administration (day zero) and on the 14th and 28th days following administration. The number of formula leukocytes and the amount of serum IgG was measured in the blood samples. Serum IgG was found to increase substantially ($p<0.05$) in the vitamin-vaccine group on days 14 and 28 compared to the vaccine group. It was determined that the number of lymphocytes in days 14 and 28 of the vaccine-vitamin group increased in comparison with the vaccine group. In this study, it was found that vitamin c administered to cattle with the blackleg vaccine has a stimulating effect on the immune system. It is recommended to improve the protective potency of the vaccine using vitamin c along with the blackleg vaccine in cattle.

INTRODUCTION

Blackleg disease is a bacterial disease caused by *Clostridium chauvoei* (*Cl. Chauvoei*). The agent responsible for the disease is gram-positive, anaerobic and spored. These spores protect the bacteria against adverse environmental conditions. Because of spores, bacteria stay alive in the soil for a long time and maintain their ability to cause disease. In animal species, cattle are the most vulnerable to illness. It mainly affects cattle 6 to 30 months old. The disease progresses quickly and causes cattle to die (Abreu et al., 2017; Nicholson et al., 2019). The disease is also reported to be present in sheep and other animal species other than cattle (Abreu et al., 2017).

The disease has quite a complex pathogenesis. Spores ingested orally are carried from the digestive tract to muscle tissue through macrophages. These spores, which are latent in muscle tissue, change to a vegetative form when damaged. Some toxins are synthesized by bacteria that go through the vegetative form (Hansford, 2020). These toxins are responsible for muscle tissue inflammation and death (Abreu et al., 2017; Nicholson et al., 2019). Hyaluronidase, DNase, haemolysin, neuraminidase and leukocyte toxins play an important role in the pathogenesis of the disease. Moreover, it is suggested that the bacterial structural flagella are also effective in pathogenicity (Nicholson et al., 2019).

The disease begins in the muscle tissue. The affected muscular tissue is oedematous. In the future, the gas builds up and the cracking is felt. The infected animals have high fever. Anorexia and stagnation attract interest. The affected muscle tissue has a dark red or black appearance. Furthermore, fibrous areas and bleeding can be observed in the heart. Mortality is high in the illness (Abreu et al., 2017).

Vitamin C, also called ascorbic acid, is a water-soluble vitamin with important biological functions. In cattle, it is synthesized from d-glucose or d-galactose by glucuronic acid in the liver. Vitamin C is a co-factor in certain enzymes. It is required for the synthesis of collagen, catecholamines, peptide hormones, vasopressin, carnitine, cholesterol and tyrosine (Mousavi et al., 2019; Pogge & Hansen, 2013; Ranjan et al., 2012). Vitamin C deficiency leads to a disease known as scurvy (Matsui, 2012). Scurvy is characterized by a weakness in the structure of collagen. It has been reported that wound healing is delayed and susceptibility to life-threatening conditions such as pneumonia increases in patients with scurvy (Carr & Maggini, 2017; Mousavi et al., 2019). Vitamin C is decomposed by ruminal microflora in cattle and is not used orally. It is used parenterally and the dose is one to two grams (Kaya, 1997).

The best way to prevent blackleg disease is to immunize healthy animals. Vaccines are used for disease control around the world (Nicholson et al., 2019; Santos et al., 2021). In this country, cattle over four months old are vaccinated against the disease before they go to pasture. The vaccine is given behind the shoulder and subcutaneously (Mamak et al., 2018).

In a human study, vitamin C is reported to accumulate in neutrophils and monocytes (Carr & Maggini, 2017). There is also evidence that vitamin C increases serum antibody levels in humans (Mousavi et al., 2019). Based on this information, we can affirm that there is an important link between vitamin C and the immune system. This study was conducted to

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determine the effect of vitamin C given to cattle with the blackleg vaccine on the immune system.

MATERIAL and METHODS

This study has been approved by the Ethics Committee of Kafkas University (decision date 26.04.2022 and numbered 2022-085) and Ministry of Agriculture and Forestry of Turkey (letter dated 05.04.2022 and numbered E-5125179).

This research was carried out on 28 cattle of different breeds and genders, aged 15-20 months, in the Ardahan region. Cattle kept in the same environment were fed tap water from the same source and grassy grass. A dose of 0.2 mg/kg ivermectin (Vilmectin-Vilsan®) was administered subcutaneously to all cattle in the study of anti-parasitic purposes. There was one month of waiting period after the administration. Following this, the cattle were divided into four groups, seven in each group. 2 ml of saline (Polifleks®-Polifarma) to the first group (control group), 5 mg/kg of vitamin C (Maxivit-C®-baVET) to the second group (vitamin group), 2 ml of blackleg vaccine (VBR CHAUVOEI®-Ata-Fen) to the third group (vaccine group), and 5 mg/kg of vitamin C and 2 ml of blackleg vaccine to the fourth group (vaccine-vitamin group) were administered subcutaneously from different regions. Just before drug and vaccine administration (day zero) and on day 14 and 28 following administration, 2 ml of blood from all animals in the group into tubes with anticoagulant (EDTA) (BD Vacutainer® K2E 5.4 mg) and 10 ml of blood into tubes without anticoagulant (BD Vacutainer® CAT) samples were collected. The blood samples were taken to the laboratory in cold temperatures. Non-anticoagulant tubes were maintained at room temperature for 2 hours. Serums were then obtained by centrifuging at 3000 rpm for 20 minutes. The resultant serum samples were placed into an Eppendorf tube (İSOLAB®) and stored at -20^0 C until analysis. Blood smears were performed from blood samples in tubing containing anticoagulant. The leukocyte formula was determined using the conventional method (Yaman, 2016).

The ELISA kit (Bioassay Technology Laboratory, Cat. No: E0010Bo) was used to determine the quantity of G immunoglobulin (IgG) in serum samples. The analysis was carried out using the methodology described by the manufacturer. After the test, the optical density of the standard and serum samples was measured at 450nm in an ELISA reader (BioTek ELx800, U.S.A). Serum IgG was calculated by comparing the optical density of the samples to the optical density of the standards. This calculation was done by computer and using Microsoft Excel (Aydin, 2015).

The Microsoft Windows, SPSS 20.0 software program was used to statistically calculate the data obtained in the study. Normal distribution conditions were verified using the Shapiro-Wilk test. Group means were compared with one-way analysis of variance (ANOVA), multiple comparisons among groups were made using the Tukey HSD test. The results are presented on average and as a standard deviation. P<0.05 was found to be statistically significant in this study.

RESULTS

The serum IgG measured over 0, 14 and 28 days in cattle groups is presented in Table 1 below.

Table 1. Levels of serum IgG in the cattle groups (µg/ml)

Days	Control	Vitamin	Vaccine	Vaccine-vitamin
Day 0	54.60 ± 6.68	58.17 ± 17.89	57.14 ± 26.23	58.90 ± 29.68
Day 14	50.00 ± 22.40 ^{a}	49.54 ± 11.06 ^{a}	91.04 ± 13.13 ^{b}	116.58 ± 14.64 ^{c}
Day 28	48.55± 9.58 ^{a}	45.98± 17.85 ^{a}	97.31± 16.43 ^{b}	125.19± 14.07 ^{c}

^{a,b,c}: Those that have different letters in the same line in the P < 0.05 range were statistically significant.

Table 1 above shows that there is no difference in the amount of serum IgG on day zero among the groups. Serum IgG in the vaccination group at day 14 was found to have increased significantly (P<0.05) compared to the control and vitamin groups. In addition, the amount of IgG in the vaccination-vitamin group increased compared to the vaccinated group. It was observed that this significant increase in the amount of antibodies in the vaccine-vitamin group compared with the vaccine group continued on day 28.

Table 2 above demonstrates that there is no difference among the groups in terms of the percentage of leukocytes on day zero. The number of lymphocytes in the vaccine and vitamin-vaccine groups was found to increase significantly (P < 0.05) on day 14 compared to the control and vitamin groups. Lymphocytes in the vitamin-vaccine group were observed to increase on days 14 and 28 compared to the vaccine group. However, it was not statistically significant (P > 0.05).

DISCUSSION

Blackleg is a rapidly developing bacterial disease that kills cattle. It causes significant economic losses in the cattle breeding. Vaccines are used to fight the disease in this country and in countries around the world (Mamak et al., 2018; Nicholson et al., 2019; Santos et al., 2021). The synthesis of antibodies in a short period of time and at a protective level with vaccination is of great importance in the control of the disease. It is known that the development of immunity is possible with the stimulation of cells of the immune system. Vitamin C has been reported to have a significant relationship with the immune system. There is some evidence that plasma levels of vitamin C are declining in infectious diseases such as fatty liver disease, liver disease, heat stress, mastitis in cattle (Matsui, 2012; Ranjan et al., 2012).

There has been some suggestion that vitamin C is effective

Table 2. Leukocyte formula of the cattle groups by days (number/100)

Days	Parameters	Control	Vitamin	Vaccine	Vaccine- vitamin
Day 0	Neutrophil	27.71 ± 4.88	27.28 ± 4.30	27.85 ± 4.87	28.57 ± 2.82
	Eosinophil	12.71 ± 2.87	11.28 ± 2.87	10.42 ± 2.63	11.42 ± 2.99
	Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Monocyte	1.28 ± 1.11	1.14 ± 1.21	1.14 ± 1.06	1.00 ± 1.15
	Lymphocyte	55.14 ± 6.71	54.28 ± 5.85	54.71 ± 4.82	57.28 ± 5.25
Day 14	Neutrophil	26.28 ± 5.05	26.57 ± 5.15	28.57 ± 5.34	29.71 ± 1.79
	Eosinophil	11.28 ± 2.92	10.85 ± 2.03	10.57 ± 2.37	10.71 ± 2.49
	Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Monocyte	1.14 ± 1.21	1.00 ± 1.15	1.28 ± 1.38	1.42 ± 1.61
	Lymphocyte	54.71 ± 7.49 [a]	55.71 ± 4.85 [a]	62.42 ± 3.86 [b]	65.57 ± 3.95 [b]
Day 28	Neutrophil	27.28 ± 3.81	25.42 ± 4.42	26.71 ± 4.46	28.28 ± 2.87
	Eosinophil	11.85 ± 2.11	11.28 ± 2.13	10.57 ± 2.82	11.57 ± 2.37
	Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Monocyte	1.00 ± 1.15	1.14 ± 1.21	0.71 ± 0.75	1.14 ± 1.21
	Lymphocyte	53.85 ± 4.37 [a]	54.57 ± 3.50 [a]	60.14 ± 4.94 [b]	64.57 ± 4.72 [b]

[a,b]: Those that have different letters in the same line in the $P < 0.05$ range were statistically significant.

in increasing disease resistance in cattle. Vitamin C use in calves is reported to reduce diarrhea (Cummins & Brunner, 1989; Sahinduran & Albay, 2004; Seifi et al., 1996). It has been observed that when applied to cattle prior to transport, vitamin C prevents the reduction in plasma ascorbate due to transport and increases fattening performance (Deters & Hansen, 2020).

Many studies have demonstrated the association between vitamin C and antibody levels in cattle. It has been reported that when the calves received colostrum are used orally 3 grams a day in the first week, 2 grams in the second week, and 1 gram in the third week, the amount of serum gamma globulin is increased significantly on the 14th day but not significantly on the 28th day (Lotfollahzadeh et al., 2005). In a study, vitamin C administered to calves was found to not affect the amount of total plasma IgG (Hidiroglou et al., 1995). In another study, calves were given 1 g of vitamin C per day from 2 weeks to 8 weeks of age, and the same calves were vaccinated with inactivated *Histophilus somni* vaccine when they reached 4 and 8 weeks of age. It is reported that 12 weeks after the second vaccination, vitamin C greatly increased the amount of antibodies synthesized against *Histophilus somni* (Otomaru et al., 2021). When vitamin C is administered to cattle on the 3rd day of foot-and-mouth vaccination, it leads to an increase in serum gamma globules (Kızıl & Gül, 2010). Another study suggests that vitamin C accelerates treatment and increases the amount of milk immunoglobulin in dairy cattle suffering from mastitis (Chaiyotwittayakun et al., 2002).

The effect of vitamin C on the immune system has been studied in some animal species besides cattle. It was suggested that 200 mg/kg of vitamin C administered intramuscularly to sheep prior to transplant is effective in preventing stress from transport (Yanar, 2020). When the goats were 21 days old, they

were separated from their mothers and exposed to stress and given 100 mg of vitamin C orally per day. The same goats were vaccinated against *Brucella melitensis* at 14 weeks old. It was reported that there was no significant increase in serum antibody levels in the vitamin C group at three weeks post-vaccination (İmik et al., 2000).

The effect of vitamin C on the immune system has also been studied in poultry. It is claimed that ascorbic acid added to the diets of chickens exposed to stress by applying heat for 35 days increases the growth performance and the amount of antibodies against Newcastle virus (Gouda et al., 2020). In another study, chickens raised in an overcrowded environment were supplemented with vitamin C in their diet and immunized against the Newcastle virus. It is reported that the quantity of antibodies synthesized against the vaccine does not increase significantly 7 and 14 days after vaccination (Mirfendereski & Jahanian, 2015). In another study, chickens were vaccinated against Newcastle and infectious bronchitis after being molted with zinc oxide added to their feed. After vaccination, the level of antibodies in the group to which vitamin C was added increased insignificant relative to the control group (Khan et al., 2014). In another study, it was reported that daily supplementation of 500 mg/kg ascorbic acid to broilers exposed to heat stress from 22 days to 42 days increased the amount of antibodies against Newcastle disease and reduced the negative effects of heat (Toplu et al., 2014). In another study, the amount of antibodies against Infectious Bursal Disease was found to be higher in chickens supplemented with ascorbic acid in their diets than in the group that did not receive ascorbic acid (Wu et al., 2000). It has been reported that vitamin C added to the feed laying hens has no effect on the antibody titer against Newcastle disease (Coşkun et al., 1998). It is suggested that vitamin C injected at a dose of 3 mg on the 15th day increases the hatchability rate, the amount of IgG and IgM (Zhu et al., 2019).

Human studies have suggested that there is a positive relationship between vitamin C and antibody levels (IgA, IgG, IgM) (Carr & Maggini, 2017). Enhanced antibody production (IgG and IgM) was observed in human lymphocytes exposed to vitamin C in vitro (Tanaka et al., 1994). It has been reported that intra-peritoneal administration of vitamin C in guinea pigs increases mitotic activity and levels of post-vaccination antibodies in lymphocytes (Carr & Maggini, 2017). The FMD inactivated intramuscular vaccine was administered 7 and 14 days after 1 mg and 10 mg intra-peritoneal injection of vitamin C to the rats divided into groups. The high dose group for vitamin C was reported to show a significant increase in serum IgG (Wu et al., 2018).

In the present study, it was found that there was no difference between serum IgG in the control groups and vitamins in days 0, 14 and 28. The amount of IgG measured on days 14 and 28 in the vaccine-vitamin group was found to have increased significantly ($P<0.05$) compared to the vaccine group. It has been reported that vitamin C increases the amount of serum antibody in newborn calves (Lotfollahzadeh et al., 2005), calves vaccinated against *Histophilus somni* (Otomaru et al., 2021), in beef cattle vaccinated against Foot and Mouth disease (Kızıl & Gül, 2010), in dairy cattle with mastitis (Chaiyotwittayakun et al., 2002), in goats vaccinated with *Brucella melitensis* (İmik et al., 2000), chickens vaccinated against Newcastle virus (Gouda et al., 2020; Khan et al., 2014; Mirfendereski & Jahanian, 2015; Toplu et al., 2014), in chickens vaccinated against Infectious Bronchitis (Khan et al., 2014), in chickens vaccinated against Infectious Bursal Disease (Wu et al., 2000), when injected into embryonated eggs (Zhu et al., 2019), in human lymphocytes in vitro (Tanaka et al., 1994), in mice vaccinated with Foot and Mount Disease (Wu et al., 2018). The results obtained in this study are consistent with the research results presented above. Serum IgG levels in the vaccine-vitamin group showed a significant increase in comparison to the vaccine group. This increase may be because of vitamin C stimulating the mitotic activity of lymphocytes. It may be interpreted that lymphocytes with increased mitotic activity differ into antibody synthesis plasma cells and cause increased antibody production.

Contrary to the findings of this study, it is reported that vitamin C does not affect the level of antibody in calves (Hidiroglou et al., 1995), and in chicken vaccinated with Newcastle vaccine (Coşkun et al., 1998). In some studies, it has been suggested that vitamin C supplementation decreases the incidence of diarrhea in calves (Cummins & Brunner, 1989; Sahinduran & Albay, 2004; Seifi et al., 1996), improves the fattening performance of cattle (Deters & Hansen, 2020), and sheep (Yanar, 2020). It is thought that the stress factors that animals are exposed to may be effective among the reasons for obtaining different results between vitamin C administration and the amount of antibodies.

In this study, it was found that there was no difference in the number of leukocytes in the control and vitamin groups during the 0, 14 and 28 days. It is observed that the lymphocyte counts of the vaccine and vaccine-vitamin group increased significantly ($P < 0.05$) on the 14th and 28th days compared to the control and vitamin groups. It was observed that the lymphocyte counts of the vaccine-vitamin group increased on the 14th and 28th days compared to the vaccine group. However, the increase was statistically insignificant ($P > 0.05$). No differences were found between the neutrophil, eosinophil, basophil and monocyte counts in the 0th, 14th and 28th day groups. One study indicates that lymphocytes have 10 to 100 times more vitamin C than plasma (Mousavi et al., 2019). It has been suggested that intra-peritoneal administration of vitamin C to guinea pigs increases the mitotic activity of lymphocytes (Carr & Maggini, 2017). In another study, it was reported that vitamin C administered at different doses (25mg, 50mg and 75mg/kg/day for 30 days) to sheep exposed to heat stress increased the lymphocyte count (Babe, 2011). The results of this research are similar to those of Babe (2011), Carr and Maggini (2017), and Mousavi et al. (2019). The reason for this increase in the number of lymphocytes may be due to the accumulation of vitamin C in lymphocytes and stimulating lymphocytes in the direction of maturation, differentiation and proliferation.

CONCLUSION

Blackleg is a rapidly developing bacterial disease that kills cattle. It causes significant economic losses in the cattle

breeding. The best way to prevent blackleg disease is to immunize healthy animals. In this study, it was observed that vitamin C, injected with 5mg/kg in different regions together with the Blackleg vaccine, stimulated the immune system by increasing the amount of serum IgG and lymphocyte count. Because of this immune-boosting effect, it is recommended to use vitamin C along with the blackleg vaccine in cattle. Thus, the protective effect of the vaccine may be increased against acute and fatal blackleg disease, which does not have a cure.

DECLARATIONS

Ethics Approval

This study has been approved by the Ethics Committee of Kafkas University (decision date 26.04.2022 and numbered 2022-085) and Ministry of Agriculture and Forestry of Turkey (letter dated 05.04.2022 and numbered E-5125179).

Conflict of Interest

The author declares that there is no conflict of interest.

Author contributions

All applications were performed by the author.

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Data availability

The data used to prepare this manuscript are available from the corresponding author when requested.

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Not applicable.

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