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RESEARCH ARTICLE

Effects of Levamisole Application on Immunity System in Anthrax-Vaccinated Cattle

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ABSTRACT

The present study investigated the effect of levamisole administration on the immune system of anthrax-vaccinated cattle. Totally, 40 healthy cattle were employed in the study. Cattle were divided into 4 even groups. In the control group, saline was subcutaneously injected four times as a placebo, every three days, and one dose was administered on the 10th day. In the second group (vaccine group), anthrax vaccine was administered subcutaneously on the 10th day following the administration of physiological saline three times every three days. The 3rd group (vaccine-levamisole) was subcutaneously administered levamisole at a dose of 2.5 mg/kg three times with three-day intervals and on the following 10th day, anthrax vaccine was administered. The 4th group (levamisole) was subcutaneously administered levamisole at a dose of 2.5 mg/kg three times with three-day intervals and physiological saline was subcutaneously injected on the following 10th day. After vaccination neutrophils, lymphocytes, monocytes count, and the serum IgG amount of the vaccine-levamisole group were found to increase significantly ($p<0.05$) compared to the vaccine-only group. It was concluded that subcutaneously administered levamisole at a dose of 2.5 mg/kg three times with three-day intervals before administrating the anthrax vaccine to cattle, had a stimulating effect on the immune system.
Keywords: Anthrax vaccine, cattle, immune system, levamisole.

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Levamizol Uygulanmasının Şarbon Hastalığına Karşı Aşılanmış Sığırlarda Bağışıklık Sistemi Üzerine Etkileri

ÖZ

Bu araştırmada şarbon hastalığına karşı aşılanan sığırlarda levamizol uygulamasının bağışıklık sistemi üzerine olan etkisi araştırıldı. Araştırmada toplam 40 adet sağlıklı sığır kullanıldı. Sığırlar dört eşit gruba ayrıldı. Kontrol grubuna üçer gün arayla ve bir dozu da 10. gün olmak üzere plasebo olarak serum fizyolojik dört defa deri altına enjekte edildi. İkinci gruba (aşı grubu) üçer gün arayla üç kez serum fizyolojik sonrası 10. gün şarbon aşısı deri altı, üçüncü gruba (aşı-levamizol) üçer gün arayla üç kez levamizol çözeltisinden 2,5 mg/kg dozda deri altı ve sonrası 10. gün şarbon aşısı deri altı, dördüncü gruba (levamizol) ise üçer gün arayla üç defa levamizol 2,5 mg/kg dozda deri altı ve sonrası 10. günde serum fizyolojik deri altına enjekte edildi. Aşılama sonrası aşı-levamizol grubunda nötrofil, lenfosit, monosit ve serum İgG miktarının sadece aşı uygulanan gruba göre anlamlı düzeyde ($p<0,05$) arttığı görüldü. Sığırlara şarbon aşısı uygulanmadan önce üçer gün ara ile üç kez 2,5 mg/kg dozda deri altı verilen levamizolün bağışıklık sistemi üzerine uyarıcı etki gösterdiği sonucuna varıldı.
Anahtar kelimeler: Bağışıklık sistemi, levamizol, sığır, şarbon aşısı.

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INTRODUCTION

Anthrax is an infectious disease caused by Bacillus anthracis (Lewerin et al. 2010, Suchitra et al. 2010, Parlak et al. 2015, Gobeli Brawand et al. 2019). The agent responsible for the disease is a gram-positive, spore-forming bacterium (Lewerin et al. 2010). These spores make bacteria resistant to unfavorable environmental conditions (temperature, dry air, changes in pH, chemicals, etc.). Therefore, they maintain their ability to cause disease by maintaining their vitality in the external environment for a long time (Suchitra et al. 2010, Dettwiler et al. 2018, Gobeli Brawand et al. 2019).

Cattle usually ingest the pathogen into the digestive and respiratory tract. Spores that enter the body in this way are captured by macrophages and transported into the lymph nodes. They are transformed from spore-form to vegetative-form in lymph nodes. Bacteria that enter the vegetative-form proliferate quickly. They also synthesize toxins that are responsible for the pathogenesis of the disease. The bacteria enter the bloodstream is resulted in systemic anthrax which is usually fatal (Hanna et al. 1994, Lewerin et al. 2010).

Levamisole is a widely used anthelmintic against parasites which settle in the lungs, stomach, and intestines of humans and animals. The drug is very effective against nematodes and eliminates the parasite from the place where it is settled by 90% to 100% (Siwicki and Cossarini-Dunier 1990, Sajid et al. 2006). This drug, belongs to the imidazothiazoles group, has been used since 1970. The drug is indicated to have immunomodulatory and immunostimulating properties as well as potential anthelmintic properties (Siwicki and Cossarini-Dunier 1990, Baydan 1995, Sajid et al., 2006, Kızıltepe 2018). The anthelmintic dose of this drug, widely used in veterinary medicine, is 7.5 mg/kg, and its immune system stimulating dose is 2-2.5 mg/kg. It is suggested that the drug should be used 5-6 times a week or 2-3 successive days to boost the immune system (Baydan 1995). It has been reported to regulate immune function when given to mice at a dose of 150 mg or 2.5 mg/kg body weight for one week (Sajid et al. 2006). A single dose of 2.5mg/kg levamisole administration boosts the immune system for 48 hours, and it is recommended to repeat the drug for a more effective stimulation (Pancarcı et al. 2009). When levamisole is injected into cattle in the non-lactating period decreases the incidence of mastitis. The use of levamisole in an intermittent manner is more effective than continuous use for regulating the immune system (Sajid et al. 2006).

The immunomodulating and stimulating effect of levamisole is still not completely unknown (Kızıltepe 2018). However, some research suggests that by stimulating phagocytes, they become sensitive to mitogens and antigens, and increase the number of T cells. It is reported that levamisole does not have direct effects on B-lymphocytes, but indirectly (by affecting T-lymphocytes and phagocytic cells) stimulates the humoral immune response and causes an increase in antibody level (Van Der Maaten et al. 1983, Baydan 1995, Sajid et al. 2006, Pancarcı et al. 2009). Levamisole demonstrates its effect on the cellular immune response through imidazole and sulphide structures. It is noted that it has a hormone-like effect due to the increase in the amount of intracellular cGMP and serum factors responsible for leukocyte activation in lymphocytes exposed to levamisole (Kızıltepe 2018).

As we know, the immune system is a defense that protects living beings from all kinds of harmful factors. This system is mainly examined under two groups cellular and humoral. While macrophages and T-lymphocytes form cellular immunity, B-lymphocytes are responsible for the humoral system. When a foreign substance (such as bacteria, virus, fungus) enters the organism, they are recognized and processed by macrophages and T-lymphocytes. T-lymphocytes stimulate B-lymphocytes secreting lymphokines (Baydan 1995). Stimulated B lymphocytes convert to plasmocytes and stimulus-specific immunoglobulins (Ig antibodies) are synthesized. These immunoglobulins combine with antigens and impair their ability to cause disease. The main antibodies found in cattle are IgA, IgE, IgM and IgG. Of these antibodies, IgG is the most abundant in serum. This molecule is made of two subunits that are called IgG1 and IgG2 (Arda 1994).

Anthrax progresses quickly in cattle and leads to sudden death. The disease is passed on to people and threatens their health. Therefore, it is very important to fight the disease in order to protect human and animal health and prevent economic losses. As such, healthy animals would need to be vaccinated against the disease. In the literature review, no research was found on the effect of levamisole on the immunity of anthrax-vaccinated cattle. The purpose of this study was to determine the effect of levamisole at the immunomodulatory dose on the immune system of cattle vaccinated against anthrax.

MATERIALS and METHODS

This study was approved by the Committee for Ethics of the University of Kafkas (date of decision 27.11.2020 and number 2020-156) and the Ministry of Agriculture and Forestry (letter of 30.10.2020 and number E-3056045).

The animal experimentation part of the research consisted of 40 cattle of different breeds and gender, aged from 10 to 15 months on a cattle farm in the Ardahan region. Cows that had not been vaccinated with the anthrax vaccine and were in good health following the exam were used in the study. The cattle were divided into 4 groups with 10 animals in each group. The groups were determined as control, vaccine, vaccine-levamisole, and levamisole groups

respectively. The cattle used in the study were fed ad libitum with grass and tap water obtained from the same resource in the same environment. 0.2 mg/kg of ivermectin (Vilmectin-Vilsan®, Turkey) was given subcutaneously for the antiparasitic purpose to all animals in the groups. There was one month waiting period after the administration. In the control group, 4 mL of saline (Polifleks-Polifarma®, Turkey) was subcutaneously injected four times as a placebo, every three days, and one dose was administered on the 10th day. In the second group (vaccine group), 1 mL of anthrax vaccine (Ant Etvac-Ministry of Agriculture and Forestry®, Turkey) was administered subcutaneously on the 10th day following the administration of physiological saline three times every three days. The 3rd group (vaccine-levamisole) was subcutaneously administered 10% levamisole HCl (Levatek %10-Teknovet®, Turkey) solution at a dose of 2.5 mg/kg three times at three-day intervals and on the following 10th day, 1ml of the anthrax vaccine was administered. The 4th group (levamisole) was subcutaneously administered 10% levamisole HCl solution at a dose of 2.5 mg/kg three times with three-day intervals and 4 mL of physiological saline was subcutaneously injected on the following 10th day. Before the vaccination and the drug administration (day zero) and on the 7th, 14th, 28th, 35th, and 45th days following vaccination, blood samples from all animals in the groups were collected 2 mL tubes with anticoagulant (EDTA) (BD Vacutainer® K2E 5.4 mg, UK) and 10 mL tubes without anticoagulant (BD Vacutainer® CAT, UK) from vena jugularis. The blood samples were brought to laboratory. The blood samples from the no-anticoagulant tubes were centrifuged at 3000 rpm and 20 minutes. The serum obtained was stored at -20 ^{0}C in Eppendorf (ISOLAB®, Germany) tubes prior to analysis. The number of formula leucocytes in the blood sample collected to contain the anticoagulant was determined using the conventional method (Yaman 2016).

Bovine Immunoglobulin G ELISA kit (Bioassay Technology Laboratory, Cat. No: E0010Bo, China) was used in the determination of total IgG amounts in the serum sample. The results of the analysis were recorded using a 450 nm wavelength reading in an ELISA reader (BioTek ELx800, U.S.A) (Aydin 2015).

IBM SPSS 20.0 software was used for statistical evaluation of research findings. The normal distribution curve was checked using the Shapiro-Wilk test. In comparison, of group means, one-way analysis of variance (ANOVA) and multiple comparisons were performed using Tamhane's T2 test. The results were presented as a mean ($\bar{x}$) and standard deviation (SD). In this survey, $P<0.05$ was found to be statistically significant.

RESULTS

In this study, the number of formula leukocyte in the groups is given in Table 1, and the amount of IgG in Table 2. As shown in Table 1 above, there is no difference in the number of neutrophils, eosinophils, monocytes and lymphocytes in the blood sample collected on day zero among the groups. On the 7th days of drug and vaccine administration, an increase in the number of neutrophils and monocytes in the vaccine group-levamisole is statistically significant ($p<0.05$) and increase of the number of lymphocytes is not important statistically ($p>0.05$) compared to the vaccine group. The number of neutrophils, monocytes and lymphocytes in the levamisole-vaccine group increased dramatically ($p<0.05$) compared to the 14th day vaccine group. It is determined that there was a single difference in the number of lymphocytes on the days 28th, 35th and 45th when the blood sample was taken. The number of lymphocytes in the vaccine-levamisole group is higher than the vaccine group during 28th, 35th and 45th days. The amount of serum IgG in the cattle groups is given in Table 2 above. It has been demonstrated that there is no difference in the amount of serum IgG among groups on day zero. It is determined that the amount of serum IgG in the vaccine group is 98.90 ± 22.99 μg/mL, and the mean of vaccine-levamisole group is 232.50 ± 69.49 μg/mL on the 7th days. It appears that the increase in the amount of antibodies is statistically significant ($P<0.05$). The amount of serum IgG in the vaccine-levamisole group increased substantially ($P<0.05$) compared to the vaccine group on the 14th, 28th, 35th, and 45th. There is no difference between the control and levamisole groups in terms of mean antibody. It was observed that the amount of mean antibodies in the vaccine and vaccine-levamisole groups boosted over time.

Table 1. The number of formula leukocyte in cattle groups (%)
[a,b,c]: Those with different letters in the same row in the range of P<0.05 were found to be statistically significant

Days	Parameters	Control	Vaccine	Vaccine-Levamisole	Levamisole
Day 0	Neutrophils	25.80 ± 2.57	25.80 ± 21.61	26.50 ± 1.43	27.00 ± 1.05
	Eosinophils	6.40 ± 1.57	6.70 ± 1.49	7.60 ± 1.07	7.50 ± 0.84
	Monocytes	2.30 ± 0.94	3.20 ± 1.03	2.60 ± 1.07	3.40 ± 0.96
	Lymphocytes	57.50 ± 1.84	59.60 ± 2.50	58.50 ± 1.71	58.50 ± 2.06
Day 7	Neutrophils	24.90 ± 1.59 [a]	33.80 ± 2.85 [b]	36.60 ± 1.34 [c]	25.70 ± 1.88 [a]
	Eosinophils	5.80 ± 1.68	5.40 ± 1.07	5.50 ± 0.84	6.00 ± 1.33
	Monocytes	2.80 ± 0.91 [a]	3.10 ± 1.10 [a]	5.20 ± 1.03 [b]	4.10 ± 1.44 [a]
	Lymphocytes	57.60 ± 1.71 [a]	67.60 ± 1.83 [b]	70.40 ± 3.20 [b]	58.30 ± 2.31 [a]
Day 14	Neutrophils	25.10 ± 1.52 [a]	26.70 ± 0.94 [a]	32.70 ± 1.94 [b]	25.50 ± 1.08 [a]
	Eosinophils	4.30 ± 0.94	4.80 ± 1.31	4.60 ± 0.96	4.70 ± 0.94
	Monocytes	2.60 ± 1.07 [a]	4.70 ± 0.94 [b]	7.00 ± 1.24 [c]	2.70 ± 0.94 [a]
	Lymphocytes	55.20 ± 1.13 [a]	67.80 ± 3.96 [b]	75.70 ± 1.56 [c]	54.30 ± 1.88 [a]
Day 28	Neutrophils	25.80 ± 2.57	26.20 ± 2.29	26.50 ± 1.43	26.30 ± 1.41
	Eosinophils	6.40 ± 1.57	6.70 ± 1.49	7.60 ± 1.07	7.10 ± 1.37
	Monocytes	2.30 ± 0.94	3.20 ± 1.03	3.20 ± 1.47	2.80 ± 1.03
	Lymphocytes	57.80 ± 1.81 [a]	61.60 ± 2.27 [b]	71.70 ± 1.70 [c]	58.70 ± 2.35 [a]
Day 35	Neutrophils	21.70 ± 3.36	23.90 ± 2.28	23.10 ± 1.91	23.90 ± 2.02
	Eosinophils	5.70 ± 1.15	5.30 ± 1.49	6.30 ± 1.33	6.10 ± 1.44
	Monocytes	2.00 ± 1.33	2.70 ± 1.05	2.80 ± 1.31	2.50 ± 1.35
	Lymphocytes	57.20 ± 2.09 [a]	61.30 ± 2.26 [b]	70.00 ± 2.94 [c]	57.70 ± 2.35 [a]
Day 45	Neutrophils	21.30 ± 2.94	23.10 ± 3.54	22.00 ± 2.21	23.50 ± 2.63
	Eosinophils	5.40 ± 1.07	5.70 ± 1.15	5.60 ± 1.64	5.50 ± 1.84
	Monocytes	2.70 ± 0.94	2.60 ± 0.84	2.00 ± 1.24	2.20 ± 1.31
	Lymphocytes	56.00 ± 3.01 [a]	61.60 ± 1.95 [b]	69.90 ± 0.99 [c]	56.10 ± 2.07 [a]

Table 2. The amount of IgG in serum of the cattle groups (μg/mL)
[a,b,c]: Those with different letters in the same row in the range of P<0.05 were found to be statistically significant

Days	Control	Vaccine	Vaccine-Levamisole	Levamisole
Day 0	79.60 ± 39.38	77.10 ± 12.54	75.40 ± 8.24	81.10 ± 17.52
Day 7	82.40 ± 9.55 [a]	98.90 ± 22.99 [a]	232.50 ± 69.49 [b]	77.90 ± 7.40 [a]
Day 14	69.20 ± 17.99 [a]	141.30 ± 27.77 [b]	235.10 ± 57.64 [c]	65.60 ± 12.73 [a]
Day 28	71.90 ± 30.25 [a]	153.70 ± 38.60 [b]	242.30 ± 36.58 [c]	73.70 ± 28.11 [a]
Day 35	72.30 ± 12.86 [a]	170.00 ± 56.37 [b]	247.50 ± 48.20 [c]	70.00 ± 27.48 [a]
Day 45	74.60 ± 15.43 [a]	174.00 ± 43.38 [b]	251.50 ± 47.84 [c]	72.50 ± 21.24 [a]

DISCUSSION

Levamisole is used to accelerate healing and to protect against some diseases in cattle. It has been suggested that levamisole accelerates healing by increasing serum IgG amount when intramammal (Yarım and Salmanoğlu 2002) and oral used in cattle with subclinical mastitis during 6 days after milking (Ishikawa et al. 1982). It is reported that levamisole adminstration in non-milking cattle decreased incidance of mastitis (Sajid et al. 2006). Levamisole, given intramuscularly at a dose of 2.5 mg/kg starting 5 or 6 weeks before birth and until two weeks before birth, has shown positive effects on reproductive organs by accelerating postpartum uterine involution, providing early follicle wave development (Pancarcı et al. 2009). In addition, it is reported that positive results are obtained with the use of levamisole in the early period of Malignant catarrhal fever in cattle (Van Der Maaten et al. 1983).

For treatment of some diseases such as leprosy, rheumatoid arthritis, systemic lupus erythromatosis, human Immunodeficiency Virus-HIV, colorectal cancers in human is used levamisole due to stimulating effect on the immune system. It is also used to accelerate recovery in malnourished children with disease (Baydan 1995, Yarım and Salmanoğlu 2002, Sajid et al. 2006).

In this study, the usage of levamisole increased the number of neutrophils before administrating anthrax vaccine for cattle. This increase in the number of neutrophils is consistent with the results reported by Mohri et al. (2005) and Bilandžić et al. (2010). In a study, it has been reported that PPR vaccine and levamisole administration to goat did not show any change in neutrophil count (Undiandeye et al. 2014), while in another study, neutrophil count decreased in sheep that received FMD vaccine (Rahman et al. 2002, Abdullah and Başbuğan 2020). In the current study, the anthrax vaccine injected into cattle is a bacterial vaccine. The reason for the increase in the number of neutrophils may be due to both vaccines administered includes bacteria (number of neutrophils increases to bacterial infections) and neutrophils of levamisole sensitivity to antigen. Some studies have shown that levamisole does not affect or reduce neutrophil numbers (Rahman et al. 2002, Undiandeye et al. 2014, Abdullah and Başbuğan 2020). It is believed that the reason for this finding may be that the vaccines used with levamisole are viral and that levamisole is not used in immunostimulating amounts.

In the study, on the 7th and 14th days following the vaccine and drug administration, the number of monocytes in the levamisole-vaccine group increased compared with the vaccine group only. The results of the research are similar to those reported by Stelletta et al. (2004), Mohri et al. (2005), Undiandeye et al. (2014), Das et al. (2016), and Rao et al. (2017). It is thought that the increase in monocytes in the levamisole-vaccine group could be due to the stimulating effect of levamisole on macrophages for antigens. Abdullah and Başbuğan (2020) reported that the FMD vaccine in combination with 5 mg/kg levamisole decreased the number of monocytes. This is thought to be due to the fact that levamisole is not used in immunostimulants and may be due to the viral structure of the vaccine.

It is noticed that the number of lymphocytes in the vaccine-levamisole group insignificantly increased compared to vaccine-only group on the 7th days ($P>0.05$), and significantly increased on the 14th, 28th, 35th, and 45th days ($P<0.05$). It is reported by Rahman et al. (2002), Gürbulak and Kılıçarslan (2004), Mojžišová et al. (2004), Undiandeye et al. (2014), Das et al. (2016), Rao et al. (2017), and Abdullah and Başbuğan (2020) that the administration of levamisole increased the number of lymphocytes. The results obtained form this study are consistent with the results of the research given above. The reason for the increased number of lymphocytes may be due to the stimulant effect of levamisole on T- lymphocytes by imidazole and its sulphur structure. After vaccination, antigen-specific antibodies are synthesized thanks to B lymphocytes that transform into plasmocytes. This increase in the number of lymphocytes during the post-vaccination period is due to the stimulation of the cellular and humoral immune response. It has been reported that the number of lymphocytes decreased 35 days after the 5 mg/kg dose of levamisole injected into sheep with enterotoxemia vaccine (Rashid and Yüksek 2019). Many factors (such as toxoid vaccine, animal type, levamisole dose used, uptake time and number of replicates) are believed to play a role among the reasons for this decline in lymphocytes.

In current study, the administration of levamisol dramatically increased the amount of serum IgG. Similar finding with the outcome of this research have reported by some researcher in cattle with the Hemorrhagic septicemia-Pasteurella multocida (Sharma et al. 1990), Brucella S-19 (Sajid et al. 2006), foot and mouth vaccine (Rao et al. 2017), bovine viral diarrhea-BVD (Sayed-Ahmed et al. 2015), FMD vaccine in buffaloes (Qureshi et al. 2000), Enterotoxemia vaccine in sheep (Rashid and Yüksek 2019), FMD vaccine in sheep (Abdullah and Başbuğan 2020), blue tongue (Stelletta et al. 2004), sheep pox (Rao Dabbir and Nanjundaiah 2020), plague in goat-peste des petits ruminants-PPR (Undiandeye et al. 2014, Das et al. 2016), parvovirus in dogs (Mojžišová et al. 2004), and inactivated influenza vaccine in chickens (Ismail et al. 2018). The results of the present study are consistent with the results of the research described above. The level of serum IgG in the levamisole-vaccine group increased significantly compared to the vaccine group alone. This may be due to the fact that levamisole indirectly stimulates the humoral response by sensitizing phagocytes to antigens and increasing the number of T lymphocytes. As evidenced by the above studies, levamisole is used to support the immune system in vaccines and infections in humans and animals. Levamisole shows

immunomodulatory and immunostimulant effects when used repeatedly in low doses (usually one third of the treatment dose-2.5 mg/kg) before, during or after vaccination in different animal species (Baydan 1995, Sajid et al. 2006, Rao Dabbir and Nanjundaiah 2020). In one study, higher doses of anthelmintic and repeated levamisole did not increase antibody levels in bovine and ovine animals infected with the leukaemia virus. When levamisole is used to reinforce the immune system, it should be used in immunostimulating and repeated doses (Van Der Maaten et al. 1983). In this study, administration of levamisole at 2.5 mg/kg three times every three days prior to anthrax vaccination in cattle led to an increase in serum IgG. Based on this information, levamisole used at a dose of 2.5 mg/kg boosted the immune system in cattle vaccinated against anthrax.

CONCLUSION

In conclusion, levamisole, which was administered to cattle at a dose of 2.5 mg/kg three times with three-day intervals, increased the number of neutrophils, monocytes and lymphocytes on the 7th and 14th days after anthrax vaccine. The lymphocyte count increased on the 28th, 35th, and 45th days. In addition, while the mean serum IgG amount of the 7th day vaccine group was 98.90 ± 22.99 μg/mL, and the mean of the vaccine-levamisole group was 232.50 ± 69.49 μg/mL. The mean amount of antibodies in the levamisole-vaccine group on the 14th, 28th, 35th, and 45th days showed a significant increase ($P<0.05$) in comparison with the vaccine group. There was no difference in the mean antibody quantity between the control and levamisole groups. As we know, anthrax threatens both human and cattle health. Given that the disease is zoonotic, meat obtained from dead animals is not eaten. Therefore, effective disease control is important from a health and economic point of view. It is recommended to boost the immune system by using levamisole prior to anthrax vaccination in cattle. It is believed that this practice will be used to strengthen immunity against anthrax and to protect human and animal health.

Ethics Committee Information: This study was approved by the Committee for Ethics of the University of Kafkas (date of decision 27.11.2020 and number 2020-156) and the Ministry of Agriculture and Forestry (letter of 30.10.2020 and number E-3056045).

Conflict of Interest: The author declares that there is no conflict of interest.

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