The Efficacy of Combine Application of Levamisole and Enterotoxemia Vaccine in Sheep

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Summary

The effect of levamisole on potency of enterotoxeima vaccine and also effect of the vaccine on anthelminthic activity of the drug were evaluated in naturally parastie infected sheep. 25 sheep were divided into 5 groups each of 5. The first group vaccinated with enterotoxemia alone, the second and third groups were injected subcutaneously with levamisole combined with vaccine and levamisole alone respectively. The two other groups were selected as control. Parasitology examination, was carried out by floatation technique and McMaster method while epsilon and beta antitoxins were detected in sheep sera by enzyme-linked immunosorbant assay and serum neutralization tests. Faecal egg per gram measurments and post-examination measurments showed no significantly differences between sheep injected with levamisole combined with vaccine and that received levamisole alone. Both groups were cured successfully with 100% efficacy. The results appeared that administration of levamisole in combination with enterotoxemia vaccine can effectively increase the serum epsilon antitoxin.

Key words: levamisole, enterotoxemia vaccine, epsilon and beta antitoxins, sheep

Introduction

Levamisole hydrocholoride, an imidazolthiazole derivative, is the L isomer of DL-tetramisole and is toxic to a broad range of gasterointestinal and systemic nematodes that infected man and animals (Janssen 1976). Numerous reports have shown that, injectable levamisole has a high level of anthelmintic activity against most gasterointestinal and respiratory tract nematodes of sheep (Forsyth & Wynne-Jones 1980). However, levamisole has also been reported to effect both humoral and cellular immune responses in humans and animals. Enhancement of cell-mediated immunity has shown to be more manifest than enhancement humoral immunity (Munson *et al* 1996). Levamisole can act as a vaccine potentiator, particularly in low

responding animals. The efficacy of combination of levamisole and some vaccine including polyvalent closteridial vaccine were evaluated by some investigators (Babiuk & Misra 1982, Bivena *et al* 1986, Burdarov *et al* 1984, Chukwu 1985, Gultekin *et al* 1994, Forysth & Wynne-Jones 1980, Hogarth-Scott *et al* 1980, Jenkins *et al* 1987, Katrinka 1985). In present study, the anthelmintic activity of levamisole-entrotoxemia vaccine combination and the efficacy of combined levamisole-enterotoxemia vaccine on antibody response of sheep were also evaluated.

Materials and Methods

Drug. Choloro-levamisole powder corresponding to 10% W/V levamisole base was purchased from Damloran L.td. company, Iran, dissoleved in distilled water.

Vaccine. Enterotoxemia vaccine was obtained from Razi Vaccine & Serum Research Institute, Iran. This vaccine is effective against *Closteridium perfringens* type B, C and D as well as *Cl.septicum*. The recommended dose for an adult sheep is 3ml subcutanously, repeated 4 weeks later.

Drug-vaccine combination. The drug-vaccine combination was a mixture of choloro-levamisole and entertoxemia vaccine. It was formulated for a single injection (4ml).

Animals. 25 Iranian breed ewes with 25-30 kg body weight that naturally infected with either lung and/or digestive tract nematodes were selected. The Egg per gram (EPG) of most animals was higher than 200.

Experimental design. 25 sheep divided into five groups, each of 5. The animals received the drug, the vaccine or both subcutanously into the neck based on body weight and four weeks apart as follows:

Group 1: 3ml vaccine alone.
Group 2: 10mg/kg levamisole base plus 3ml vaccine in combination form.
Group 3: 10 mg/kg levamisole base alone.
Group 4 (control 1): 10mg/kg levamisole base plus 3ml medium culture of vaccine.
Group 5 (control 2): 1ml levamisole solvent plus 3ml medium culture of vaccine.

Parasitological examination. Faecal examination and egg counts were carried out by floatation technique and McMaster method on all animals on 9 times, 5 before treatment and 4 after treatment. All the animals were killed two weeks after the second injection. The lung and gasterointestinal tract were removed and worms recovered from the organs by washing and passage their content through a 100-mesh sieve. Then the worms were counted and preserved in 10% formalin.

Sera. The animals were bled three times, before the first injection, two weeks after the first injection and two weeks after the second injection. Sera of each sheep was collected and stored in -20C.

Immunological evaluation. For measuring beta antitoxin (*Cl.perfringens* type C) and epsilon antitoxin (*Cl. Perfringens* type D) in serum of the sheep, two methods were used:

a) Enzyme-linked immunosorbent assay (ELISA). The method was carried out as in Sojka *et al* (1989) with some modification, freeze-dried beta and epsilon protoxin were used as antigens (0.01mg/ml in PBS, pH7.2). The 96 wells microplate were coated with 0.15 ml per well of epsilon and beta solutions and incubated overnight at 4C. Then the microplate was washed five times with PBS 0.01M and Tween 20 (0.05%) pH 7.2. Sheep sera, standard epsilon and beta antitoxin (anaerobic bacterial vaccine production, Razi Ins. Iran and Wellcome) were diluted 50, 100, 200 and 400 folds in PBS, 0.01M and 0.1% gelatine pH 7.2. Then 0.15ml of each dilution was added per well. All dilutions of unknown sera and standard antitoxin were tested in duplicate wells. Blanks were included in each assay and microplates were incubated for 2h at 24C. After washing, 0.1ml of rabbit antisheep IgG peroxidase conjugate (Sigma) diluted at 1:10000 was added to each well and incubated for 20 min at 24C. The microplate then washed and 0.1ml substrate consisting of hydrogen peroxide and *O*-phenylene diamine was added and incubated for 20 min at 4C. The reaction was stopped by using sulfuric acid 2N. The absorbency of the yellow coloured product was read at 492 nm by an Immunoskan ELISA reader. Finally, the amounts of epsilon and beta antitoxins were calculated based on standard antitoxins and unknown sera OD.

b) Serum neutralization test. 20 white albino rabbits were allocated into 2 groups each of 10 animals. The first group treated with 3ml enterotoxemia vaccine and 1ml (250 mg/ml) levamisole and the second group treated with 3ml enterotoxemia vaccine alone. After 4 weeks the animals were bled and pooled sera for each group were collected. 20 mixtures were prepared for each pooled serum. 0.4mg of standard epsilon toxin was put into 10 tube, then into 5 of them 0.1, 0.3, 0.5, 0.7 and 1ml of standard epsilon antitoxin and into the other tubes 0.1, 0.3, 0.5, 0.7 and 1ml of unknown sheep sera were add. Similar procedure was carried out for beta antitoxin into 10 other tubes. Saline was added to the tubes to the final volume of 2ml. The tubes were kept at room temperature, protected from the light, for 30 min. Then, 2 mice were injected intravenously with 0.5ml of each mixture. The mice were

observed for 72h and, based on comparison between the mortality in mice that had received unknown sera mixtures and the mice received standard mixtures, the amount of epsilon and beta antitoxins per ml of sheep sera were calculated (Farzan *et al* 1996).

Statistical analysis. Antibody response was compared among groups by one-way analysis of variance. The least significant differences (LSD) test was applied to determine whether there are significant differences among means.

Results

In faecal examination of experimental groups, all animals that received levamisole (group 2, 3, and 4) were treated successfully. Injection of levamisole alone or mixed with enterotoxemia vaccine were shown to be highly effective against all gasterointestinal and respiratory nematodes. In post-mortem examination of these groups, no nematodes were recovered from their digestive or respiratory tracts. Otherwise in both faecal and post-mortem examination of group 1 and 5, infection with *Ostertagia* spp., *Nematodirus* spp., *Trichostrongylus* spp., *Dictyocalus viviparous* and *Trichuris ovis* were observed (Table 1).

Table 1. *Egg per gram (EPG) of helminths in different groups of sheep before and after first injection of enterotoxemia or/and levamisole*

Groups	Before first injection		After first injection		p*	LSD***
	No. of faecal exam	EPG Mean ± SD	No. of faecal exam	EPG Mean ± SD	All groups	Between groups at (0.05 level)
Vaccine alone	25	194±124.43	20	262.50±164.54	S**	2,3,4
Vaccine plus levamisole	25	440±206.66	20	0	S	1,5
Levamisole alone	25	324±196.63	20	0	S	1,5
Vaccine culture medium plus levamisole (con. 1)	25	284±177.20	20	0	S	1,5
Vaccine culture medium plus drug solvent(con. 2)	25	254±110.79	16	340.00±114.02	S	2,3,4

*One way analysis of variance testing a null hypothesis of no significant differences between group means

** Significant (P<0.05)

** *LSD Least Significant Differences procedure.

The results of titre measurements of serum epsilon and beta antitoxins by ELISA in different groups are shown in tables 2 and 3. In the groups treated with vaccine alone or in combination with levamisole higher serum antibody titre was detected, when

compared with other groups. However the epsilon antitoxin in sheep treated with levamisole-enterotoxemia were statistically higher than that in sheep received vaccine alone.

Table 2. *Beta antitoxin measurement by ELISA in different groups of sheep treated with enterotoxemia vaccine or/and levamisole*

Groups	**Beta serum titer (IU/ml)**		
	Before first injection Mean ±SD	2 wk after first injection Mean ±SD	2 wk after second injection Mean±SD
Vaccine alone	2.54±1.04	2.67±1.25	4.02±1.18
Vaccine plus levamisole	2.65±1.02	3.43±1.12	4.91±0.75
Levamisole alone	2.78±0.31	2.46±0.28	2.02±0.23
Vaccine culture medium plus levamisole (con. 1)	2.76±0.53	1.86±0.60	2.10±0.29
Vaccine culture medium plus drug solvent (con. 2)	2.44±0.25	1.79±0.57	1.91±0.53

Table 3. *Epsilon antitoxin measurement by ELISA in different groups of sheep received enterotoximia or/and levamisole*

Groups	**Epsilon serum titer (IU/ml)**		
	Before first injection Mean±SD	2 wk after first injection Mean±SD	2 wk after second injection Mean±SD
Vaccine alone	1.24±0.44	1.74±0.51	2.00±0.50
Vaccine plus levamisole	1.33±0.60	1.96±0.48	3.35±1.90
Levamisole alone	1.70±0.16	1.45±0.29	1.24±0.32
Vaccineculture medium plus levamisole (con. 1)	2.37±0.78	1.69±0.61	2.01±0.57
Vaccine culture medium plus drug solvent (con. 2)	1.48±0.33	1.21±0.33	1.52±0.13

The titres of antibody against both epsilon and beta toxins measured by SN test in pooled serum samples are shown in table 4. Equally increased antibody titres were observed in both sheep treated with vaccine and vaccine-levamisole combination.

Table 4. *Beta and epsilon antitoxin measurements by SN in different groups of sheep treated with enterotoximia or/and levamisole*

Groups	Beta antitoxin (IU/ml)	Epsilon antitoxin (IU/ml)
Vaccine alone	6	15
Vaccine plus levamisole	6	15

Discussion

Numerous reports have indicated that levamisole in combination with various animal vaccines has kept its anthelmintic activity against gasterointestinal and respiratory tract nematodes in sheep and the mixture does not decrease the already proven anthelmintic efficacy of the drug. The study of Forsyth and Wynne-Jones (1980) suggesting that, levamisole in combination with polyvalent closteridial vaccines gave high efficacy against all of major nematode parasites of Australian and New Zealand sheep. The finding of Gultekin *et al* (1994) showing, 99.1% of the numbers of egg and 97.9% of larvae were decreased after the first injection of sheep with levamisole-enterotoxemia vaccine. In addition 100% of egg and 94.5% larvae were decreased after the first injection of with only levamisole. Our results also indicated that, injection of levamisole alone or in combination with enterotoxemia vaccine were shown to be highly effective against sheep nematodes.

However, levamisole has also been reported to effect both humoral and cellular immune responses in man and animals and acts as a vaccine potentiator (Babiuk & Misra 1982, Bivena *et al* 1986, Burdarov *et al* 1984, Chukwu 1985, Gultekin *et al* 1994, Foorysth & Wynne-Jones 1980, Hogarth-Scott *et al* 1980, Jenkins *et al* 1987, Katrinka 1985). The study of Hograth-Scott *et al* (1980) showing levamisole in combination with polyvalent closteridial vaccine, significantly increases antibody titre of sheep. Gultekin *et al* (1994) also indicated that, the serum antitoxin level of beta and epsilon of both combined enterotoxemia vaccine and vaccine alone were found to be the same. Our finding conducted using ELISA technique shows that the antibody titres against epsilon in sera from groups treated with enterotoxemia-levamisole were statistically higher than in sera from group received vaccine alone.

The drug at higher doses acts as an immunomodulator for cell-mediated immunity (Dipalma & Digregorio 1990). The mode of action in immunomodulation or immunostimulation is probably by action on T.cell. The drug potentiates the stimulation of lymphocytes, granulocystes and macrophages by such stimuli as antigen, mitogen, lymphokine and chemotactic factors. The effect on the T-cell

system has been postulated to result from the induction of a thymic hormone-like factor from liver (Ritter *et al* 1995, Munson *et al* 1996).

More research should be carried out to evaluate the output of the present study concerning field application, drug resistance and socio-economical impacts.

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