THE EFFECT OF AN IMMUNOPOTENTIATOR (LEVAMISOLE) ON COMBINED INACTIVATED RESPIRATORY VIRUS VACCINE WHICH CONTAINING BVD, IBR AND PI-3 VIRUSES (PNEUMO-3) IN CALVES

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ABSTRACT

Evaluation of the immunopotentiator effect of administration of levamisole one dose of 0.7ml/10kg body weight subcutaneously either at 7 days pre-vaccination with double dose of combined inactivated respiratory virus vaccine which containing BVD, IBR and PI-3 (Pneumo-3) intramuscularly, simultaneously or at 7 days post vaccination based on three parameters :

1. *Cell mediated immune response which was expressed as the extent of lymphocyte transfomation measured by MTT.*
2. *Humoral immune response, which was determined by two serological tests (serum neutralization test and ELISA).*
3. *Determination of total and differential leucocytic count.*

The obtained results revealed that improvement of stimulation index of lymphocyte transformation and cattle received levamisole yielded a higher antibody titres more than cattle vaccinated with combined inactivated respiratory virus vaccine (Pneumo-3) alone. Although the four vaccinated groups received whether levamisole and non received developed a detectable SN and ELISA antibody titres that protect the vaccinated animal yet group of cattle that received levamisole at 7 days post vaccination gives a significant higher titres than other groups than those received levamisole at 7 days before vaccination or simultaneously with increase of total and differential leucocytic counts.

INTRODUCTION

Potentiation of normal immune response in calves occurs by activation of the host's immunologic reaction either humoral or cell mediated systems.

Different categories of immunopotentiators are exist and according to their origin, mode of action and method of administration. These agents could be classified into biological modifiers such as corynebacterium or non-microbial modifiers as levamisole hydrochloride.

Levamisole enhances the immune response to viral and bacterial antigens including infectious bovine rhinotracheitis, bovine viral diarrhoea, clostridial vaccine, bovine herpes virus and brucella abortus strain 19 (Irwin et al., 1976; Saperstein et al., 1983; Hogarth Scott et al., 1980; Babiuk and Misra, 1981; Babiuk et al., 1985 and Confer et al., 1985). Immunostimulant effect of levamisole was demonstrated by trebling of antibodies by simultaneous administration of levamisole clostridial vaccines in calves and sheep (Katrinka, 1985). Also, in cattle vaccinated with brucella abortus strain 19 vaccine. The highest mean of antibody titres as determined by all serological tests occurred in steers treated with levamisole at 7 days after vaccination or in those treated at the same time of vaccination and 7 days post vaccination.

This study was aimed to investigate the immunostimulatory effect of levamisole which is highly effective anthelmintic drug on vaccinated cattle with locally combined inactivated respiratory vaccine which containing IBR, BVD and PI-3 viruses (Pneumo-3).

Evaluation of the immunopotential effect of levamisole was done at different intervals through out experimental period by :

1. The extent of lymphocyte blastogenesis assay (cell mediated immune response).
2. Detection of antibody response to inactivated combined respiratory vaccine using serum neutralization test and ELISA (Humeral immune response).
3. Determination of the total and differential leucocytic counts for 2 weeks post treatment.

MATERIAL AND METHODS

1. Levamisole hydrochloride (Citarin) :

Tetramisole hydrochloride 11.79 gm in 100ml obtained from Bayer-Leverkusen, Germany injected with combined inactivated respiratory vaccine (Pneumo-3) as non specific immunostimulant in a therapeutic field dose (0.7ml/10kg body weight subcutaneously) in calves non infested with nematodes (negative faecal sample to nematodes).

2. Vaccine :

Local combined inactivated vaccine (Pneumo-3) containing PI-3 (strain 45) 8 log10 TCID50/ml

(Samira 1992) Abou Hammad strain 8 log10 TCID50/ml (El-Sabbagh, 1993) and BVD (Iman strain) 7 log10 TCID50/ml (Ghaly, 1993). The antigens were inactivated by binary ethyleneimine and adsorbed by 30% alhydrogel as adjuvant in 100ml bottle batch No. 1. The vaccine is produced in the Department of Rinderpest Like Diseases, Vet. Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

3 **Reagents :**

a. **Heparin solution** : Heparin ampoules having 5000 I.U /ml were added at a concentration of 200 IU/ml and used at a final concentration of 20-40 IU/ml and was used as anticoagulant for collecting blood for lymphocytic blastogenesis assay.

b. **Phytohaemagglutinin (PHA)** : Sigma, USA was used as a non specific mitogen in the lymphocyte transformation test. It was obtained as powder and reconstituted in 5ml RPMI-1640 medium. The required concentration could be made to 15ug/ml (Peters and Veerkamp, 1982).

c. **Trypan blue dye** : (Merchant et al., 1976). It was used for lymphocytes staining the viable lymphocytes and prepared by dissolving 1.0g of trypan blue powder in 1000ml of phosphate buffer solution filtered through a Whatman filter paper No. 2 and kept in a dark bottle and used as diluent for cell counting and detection of cell viability in the lymphocyte blastogenesis

d. **Giemsa stain** : It was used for staining blood films for differential leucocytic count, according to Schalm et al. (1975).

4. **Media :**

a. **Lymphocyte separating media** : (Mendelsohn et al., 1971). Flow Laboratories Limited Ayrshire KA 128NB. An aqueous solution composed of Ficol 400 (5-7 gm/100ml sodium diatrizoate 9ml/100ml. It was used for the separation of lymphocytes.

b. **RPMI-1640 tissue culture medium** : (Moore et al., 1967). Gibco limited, UK containing L-Glutamine and without sodium bicarbonate. This medium was prepared according to the instruction of manufacture after adding sodium bicarbonate (2g/litre) and antibiotics (penicillin 250IU/ml and streptomycin 100/ml). It was sterilized through Seitz filter and stored at 4oC until used.

c. **MTT** : 3-4,5 dimethyl thiazoyl-2-yl)-2,5 diphenyl tetrazolium bromide.

It was purchased from Sigma Chemical St. Louis, Mo. USA, Cat. no. M-2128.

d. Lauryl sulfate (SDS) : It was obtained from Sigma, Cat. No. L-450.

5. A. Experimental Design :

A total of twelve calves were divided into 4 groups of 3 animals each a follow :

Group I : They were injected subcutaneously (S/C) with levamisole one dose of 0.7ml/10kg body weight at 7 days before vaccination. The same calves were received 5ml intramuscularly 2 times of two weeks interval with combined inactivated respiratory vaccine.

Group II : They were injected subcutaneously (S/C) with one dose of levamisole and combined inactivated respiratory vaccine at the same time and followed with I/M injection of the second dose of vaccine after 2 weeks.

Group III : They were vaccinated I/M with the vaccine followed by S/C injection of 0.7ml / 10 kg body weight levamisole one dose at 7 days post vaccination and take second dose of vaccine I/M after 7 days also.

Group IV : They were va- cinated with combined inactivated vaccine only and not received levamisole with 5ml I/M for 2 times, two weeks apart.

6. Sampling :

Blood samples : They were collected just before administration and at 3, 7, 10, 14 and 21 days post treatment (DPT) with levamisole on anticoagulant for determination of total differential leucocytic counts and lymphocytes transformation according to Schalm et al. (1975) and Lucy (1984) respectively.

Serum samples : They were collected just before vaccination and at 1, 2, 3, 4, 8, 12, 16 and 20 weeks post vaccination (WPV) for screening sera using SNT and ELISA according to Kono (1969) and Voller et al. (1976), respectively.

7. Serological methods :

I. Lymphocyte transformation test :

It was performed according to the method described by Janossy and Greavees (1971), Lucy (1984), Sharma and Woldehiwet (1996).

II. **Serum neutralization test :**

It was carried out following the technique of Kono (1969) against PI-3, BVD and IBR viruses.

III. **Enzyme linked immunosorbent assay (ELISA) :** Local strains of IBR, PI-3 and BVD viruses were propagated in MDBK cells, after complete infection of MDBK cells, the whole culture were frozen and thawed 3 times, centrifuged at 3000 r.p.m. for 30 minutes and the supernatant fluid were collected. These fluids were dialysed against polyethylene glycol 6000 (PEG) and kept over night at +4oC to be concentrated 10X and used for coating ELISA plates at a final dilution 1:50 in carbonate buffer pH 9.0 according to Peter and Lori (1990).

RESULTS AND DISCUSSION

The immunopotentiatory effect of levamisole which is widely used as anthelmintic drug was studied as follows :

1. **Cell mediated immune response :**

Regarding the results of cell mediated immune response for calves received levamisole and vaccinated with (Pneumo-3), was measured using the lymphocyte blastogenesis assay by MTT and recorded in table (1) and fig. (1).

The results obtained showed an increase in the lymphocyte transformation for mitogen in calves (Group I). In addition, group II showed that vaccination at the 3rd day post administration of levamisole with a figure of (0.423, 0.576, 0 423, 0.600), respectively for PHA mitogen and continued at higher levels till 10 days post administration with a reading of (0.578, 0.682, 0 85). The stimulation index declined by 14th day post administration being (0.402, 0.525, 0.650), respectively on within 21 days the stimulation index declined to (0.228, 0.379, 0 390) post administration of levamisole.

The highest stimulation was obtained in calves received levamisole at 7 days post vaccination (Group III) than other groups. Several investigators (Janossy and Greaves, 1971; Lucy, 1977, 1984, Mendez et al., 1981), Parigrahy et al. (1979); Mayer et al. (1986) and Archambault et al. (1989) have demonstrated enhancement of blastogenesis of peripheral blood lymphocytes to (PHA) mitogen after first three days of administration of levamisole in calves.

Similarly, also Afify (1987) reported that levamisole potentiates the cell mediated immunity against Newcastle disease (ND) virus and Flesh et al. (1982) studied the immunopotentiating effect of levamisole in the prevention of bovine mastitis, foetal death and endometritis. In the con-

trol group (IV) cattle (vaccinated only), the stimulation of lymphocytes increased at first 3 days post vaccination for mitogen as in figure (1) with a reading of 0.377 then decreased by the 10th day post vaccination with a reading 0.309. The stimulation response continued to be detectable up even at a low level to 21 days post vaccination. These results were previously reported by Eman (1995) who mentioned that there is a weak cell mediated immune response with inactivated vaccines.

2. Humoral immune response :

Administration of levamisole in a dose mentioned before significantly improved serum neutralizing antibody response of calves to combined inactivated respiratory vaccine (Pneumo-3) in the different groups as measured by SNT and ELISA (table 2). The results in sera of cattle received levamisole at 7 days pre-vaccination increased from (0.55 to 1.80 log10) with SNT and from (0.70 - 2.25) with ELISA against IBR virus. Moreover PI-3 was from (0.45 to 1.95 log10) with SNT and from (0.90 to 2.40 log10) with ELISA while, against BVD virus the titre increased from (0.35 to 1.75) with SNT and from (0.65 to 2.15 log10) with ELISA from the 2nd week to the 4th week post vaccination. However, the levamisole non-received cattle (Group IV) SNT and ELISA titres from 2-4 week post vaccination were increased from (0.50 to 1.75), (0.45 to 1.80) and (0.35 to 1.65), while with SNT from (0.75 to 2.00), (0.90 to 2.10) and (0.60 to 1.95) with ELISA against IBR, PI-3 and BVD viruses, respectively. The maximum antibody responses for each group reached on day 28 as measured by SNT and ELISA tests.

The obtained results revealed that cattle received levamisole yielded a higher antibody titre more than that vaccinated with combined inactivated respiratory vaccine alone. Although the two groups received levamisole and non-received one developed a detectable SNT and ELISA antibody titres that protect the vaccinated animal while the group of cattle that received levamisole at 7 days post vaccination gives a significant higher titres than the other group that received levamisole before vaccination and at the same time of vaccination.

Our findings were similar to that obtained by Confer et al. (1985) who reported that serum antibody titres to brucella abortus were the highest in steers treated with levamisole at the same time of vaccination and in steers treated at 7 days post vaccination and they suggested this increase in antibody formation was due to the stimulator effects of levamisole on macrophages which reduces suppressor T cell function because brucella abortus tends to be an intracellular pathogen and macrophages are thought to be important in host defense in bovine brucellosis. Therefore, enhanced phagocytosis and killing of B. abortus strain 19 along with enhanced helper T lymphocyte function or reduced suppressor cell function allow for more efficient antigen processing and an enhanced response.

Similar results were obtained by Babiuk et al. (1985) who studied the effect of levamisole on the immune response to herpes virus in steers vaccinated with attenuated infectious bovine rhinotracheitis (IBR) virus. Levamisole given at 6mg/kg body weight on the day of vaccination showed the greatest immune response in animals.

Also, our results were in agreement with that obtained by Burdarov et al. (1985) who found that simultaneous injection of levamisole and inactivated vaccine against salmonella abortus ovis, enteritis and typhimurium enhanced the formation of agglutinins. The same results obtained by Katrinka (1985) who recorded that immunostimulant effect of levamisole was demonstrated by a trebling of antibodies by simultaneous administration of levamisole and clostridial vaccines in calves and sheep.

3. Determination of total and differential leucocytic counts :

From tables (3), it is clear that total leucocytic counts increased in the group (received levamisole) at 7 days pre-vaccination increased from (7.300 - 9.500 X 103 mm3) and lymphocytes percentage increased from (63% to 69%) and neutrophils increased from (22 - 25) while basophils % decreased from (4 - 2) and monocytes with in the normal limit.

The total leucocytic counts increased in the group (II) received levamisole at the same time of vaccination from (7.500 - 9.800 X 103 mm3) while lymphocyte % increased from (58 - 69) and neutrophils % increased from (28-39) but eosinophils % decreased from (5-2) while basophils was not present.

The total leucocytic count were increased in cattle received levamisole at 7 days post vaccination (Group III) from (7.400 - 10.800 X 103 mm3). While lymphocyte % was increased from (60-70%) and neutrophils % increased from (33-40) but eosinophil % decreased from (5% - 2%). The total leucocytic counts were in cattle non-received levamisole (Group IV) increased from (6.800 - 8.500 X 103 mm3) lymphocyte % increased from (59% - 62%) eosinophils increased also from (2-3%) and neutrophils increased from (32 - 38)

Our results were similar to that obtained by Saperstein et al. (1983) who found that injection of levamisole to calves experimentally infected with BVD virus resulting in high increase in white blood cell counts in treated group than that in the control group. The differential leucocytic count revealed highly significant increase in the percentage of lymphocytes from (55.5 - 59.0%) which may be due to the potentiating effect of levamisole and in addition to the stimulation of the vaccine on the immune system. Benjamin (1978) suggested that the increase in leucocytic count (leucocytosis) may be observed as a result of foreign protein reaction as he found lympho-

cytosis after vaccination.

Kollar (1982) and Jayoppa and Loken (1982) concluded that levamisole had a broad range of effects on the immune system. It stimulates phagocytosis, lysosomal enzyme release and intracellular killing and the differentiation of precursor T-cells. It augments chemotaxis for neutrophils and monocytes, migration inhibition factor, lymphocyte mediated toxicity.

While our results were disagreed with that obtained by Abdel Wahab (1978) who found that nematodes infested sheep which treated with levamisole showed a highly significant increase in total leucocytic counts from (8.66 - 10.22 X 103 mm3, at 21 days post vaccination with Rift Valley Fever (RVF). He also found that lymphocytosis from (55.0 - 59.0%) and decrease in the eosinophil % from (9.40 - 6.80% at 21 days post vaccination and the percentage of neutrophils, basophils and monocytes was non signifficantly changed after vaccination.

Also, similar results were obtained by Abdel Aoi (1991) who found that a significant increase in total leucocytic counts, lymphocytosis, neutrophilia, monocytosis and eosinophilia. And also by El-Molla et al. (1993) who studied the effect of levamisole administration on the humoral response of calves vaccinated with haemorrhagic septicaemia vaccine (HS).

In conclusion, all cattle received levamisole yielded a higher antibody titres more than cattle vaccinated with combined inactivated respiratory vaccine (Pneumo-3) specially group of cattle that received levamisole at 7 days post vaccination, which gave a significant higher titres than other groups at 4th week post vaccination and continued up to 4 months post vaccination in SNT and ELISA results respectively. The highest immune response was due to the immunopotentiatory effect of levamisole on the humoral immunity of cattle receiving levamisole. From the previous results, it is recommended to inject cattle levamisole at 7 days post vaccination with combined inactivated respiratory vaccine (Pneumo-3) to obtain a higher antibody titres to maintain animals of high level of antibodies for a high protection to against any expected outbreak.

Table 1 : Effect of levamisole treatment on lymphocyte transformation in calves measured by MTT assay (Mean titre using ELISA reader).

Time in days post treatment	Lymphocyte transformation measured by MTT			
	(Group I) 7 days pre-vaccination	(Group II) At the same time	(Group III) Levamisole + Vaccine 7 days post vaccination	(Group IV) Calves non received levamisole & received vaccine
Zero	0.255	0.240	0.259	0.250
3 DPT	0.423	0.567	0.600	0.377
7 DPT	0.528	0.632	0.740	0.440
10 DPT	0.578	0.682	0.859	0.309
14 DPT	0.402	0.525	0.650	0.265
21 DPT	0.228	0.379	0.390	0.250

DPT : Days Post Treatment.
Lev : Levamizole.
Vacc : Vaccine (Pneumo-3).

Table 2 : SN and ELISA titres in sera of calves non-infected with ruminantic viral administered levamisole 7 days before vaccination with combined inactivated respiratory viruses (Pneumo-3) (Group I), at the same time (Group II) and 7 days later and after 4 months post vaccination (Group III) and levamisole administration (Group IV).

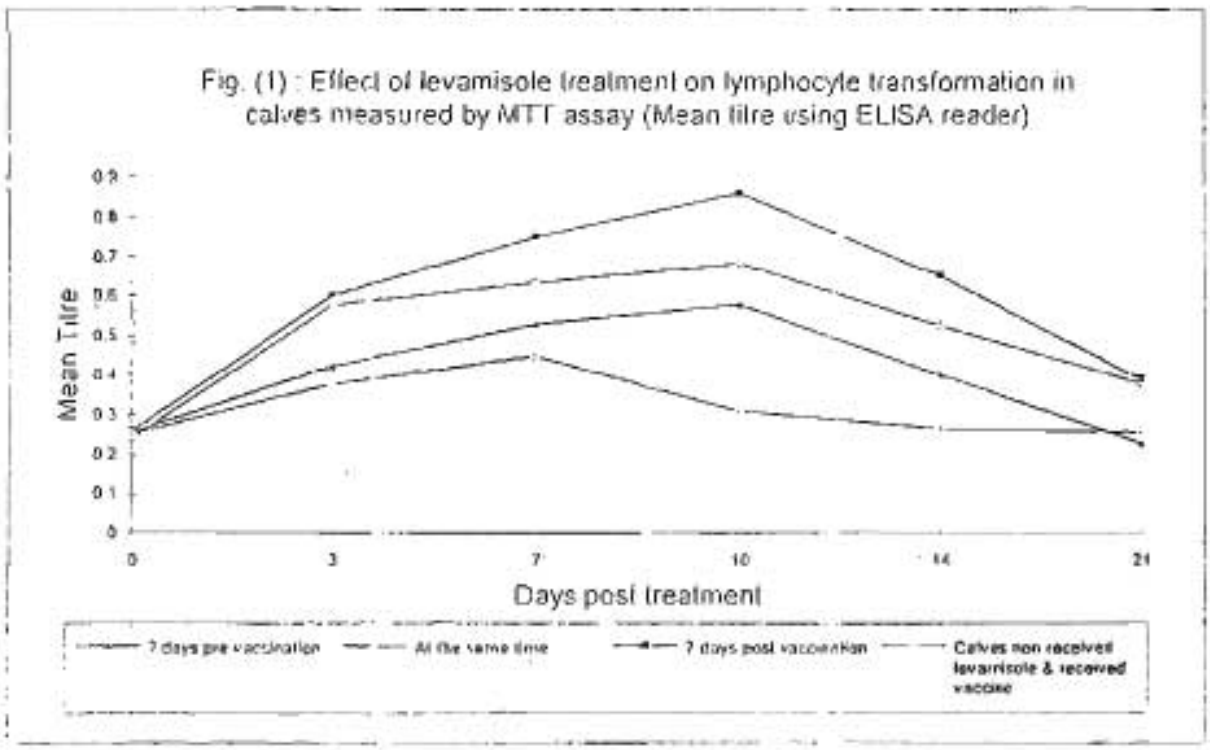
Time	Group (I) Calves received levamisole 7 Days before vaccination						Group (II) At the same time						Group (I) Calves received levamisole 7 Days before vaccination						Group (I) Calves received levamisole 7 Days before vaccination					
	SNT			ELISA			SNT			ELISA			SNT			ELISA			SNT			ELISA		
	IBR	PI-3	BVD	IBR	PI-3	BVD	IBR	PI-3	BVD	IBR	PI-3	BVD	IBR	PI-3	BVD	IBR	PI-3	BVD	IBR	PI-3	BVD	IBR	PI-3	BVD
Prevacc.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 WPV	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 WPV	0.56	0.45	0.35	0.70	0.90	0.85	0.56	0.65	0.45	0.90	1.00	0.80	0.60	0.80	0.55	1.00	1.10	1.00	0.60	0.45	0.35	0.70	0.90	0.80
3 WPV	1.35	1.45	1.25	1.60	1.85	1.80	1.50	1.85	1.40	1.95	2.20	1.90	1.60	1.75	1.50	2.10	2.30	2.00	1.30	1.35	1.15	1.50	1.65	1.55
4 WPV	1.80	1.95	1.75	2.25	2.40	2.15	2.00	2.10	1.95	2.40	2.50	2.30	2.25	2.25	2.10	2.50	2.60	2.35	1.75	1.90	1.65	2.00	2.10	1.95
8 WPV	1.75	1.85	1.70	2.15	2.30	2.10	2.00	2.00	1.80	2.30	2.45	2.20	2.20	2.20	2.00	2.40	2.50	2.25	1.75	1.80	1.60	2.00	2.05	1.90
12 WPV	1.85	1.75	1.60	2.00	2.15	1.90	1.80	1.85	1.70	2.00	2.10	1.95	1.90	1.95	1.80	2.25	2.40	2.10	1.65	1.70	1.50	1.80	1.90	1.75
16 WPV	1.50	1.65	1.40	1.80	1.95	1.75	1.70	1.80	1.55	1.95	2.00	1.80	1.80	1.80	1.70	2.00	2.10	1.85	1.50	1.40	1.25	1.65	1.80	1.65
20 WPV	1.40	1.50	1.25	1.70	1.85	1.60	1.60	1.60	1.45	1.75	1.80	1.70	1.75	1.65	1.50	1.90	1.95	1.80	1.40	1.30	1.00	1.50	1.70	1.45

SN titre : Serum neutralizing titre as $\log_{10} TCID_{50}$
ELISA titre : An ELISA titre is expressed as the $\log_{10}$ of the highest serum dilution giving deparaffinization or colour change to the of the controls

Table 1 : The total and differential leucocytic counts in calves non-infested with nematodes and administered levamisole 7 days before vaccination, at the same time of vaccination and at 7 days later, at 14 days post treatment

	Group (I) Calves treated with levamisole 7 Days before treatment					Group (II) Simultaneously at the same time					Group (III) 7 days post vaccination					Group (IV) Untreated levamisole			
	Zero	3DPT	7DPT	10DPT	14DPT	Zero	3DPT	7DPT	10DPT	14DPT	Zero	3DPT	7DPT	10DPT	14DPT	Zero	3DPT	7DPT	10DPT
Total leucocytic count ($X 10^3$ / mm^3)	7,300	7,600	8,700	8,800	9,000	7,500	8,700	9,500	9,800	9,300	7,400	8,500	7,500	10,800	10,000	6,500	7,000	6,200	6,100
Lymphocytes (%)	63	65	66	68	69	58	59	61	69	71	60	62	66	70	65	59	61	62	60
Neutrophils (%)	32	28	26	28	28	28	37	39	35	32	33	39	40	38	36	32	35	36	33
Basophils (%)	4	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Eosinophils (%)	3	1	3	3	1	5	5	2	3	2	5	2	3	2	1	2	3	1	1
Monocytes (%)	2	3	2	2	3	1	1	0	0	0	1	1	1	1	1	1	1	1	1

Results : It is clear that calves received levamisole showing total and differential leucocytic counts higher than calves non-received and also proved immunostimulatory effect of levamisole



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الملخص العربى

دراسة تأثير بعض منشطات الجهاز المناعى مثل ليفاميزول على المناعة المكتسبة للقاح التنفسى الجماعى الميت (نيمو - ٣) فى العجول

المشتركون فى البحث

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دراسة تأثير بعض منشطات الجهاز المناعى مثل ليفاميزول على المناعة المكتسبة من اللقاح التنفسى الجماعى الميت (نيمو - ٣) والذى يحتوى على كل من فيروس *الميكوزا والتهاب القصبة الهوائية والبارا أنفلونزا*-٣ حيث تم إختبار عدد ١٢ من العجول *الغير* مصابة بالطفيليات الخارجية والداخلية وقسمت إلى أربعة مجموعات. المجموعة الأولى تم حقنها بجرعة واحدة من الليفاميزول وهى ٧ر. ملى/ ١٠ كجم من الوزن تحت الجلد قبل التحصين باللقاح التنفسى (نيمو ٣) بسبعة أيام والمجموعة الثانية تم حقنها بالليفاميزول تحت الجلد واللقاح التنفسى (نيمو-٣) فى العضل سوياً فى نفس الوقت والمجموعة الثالثة تم حقنها بالليفاميزول تحت الجلد بعد التحصين بسبعة أيام والمجموعة الرابعة تم حقنها باللقاح التنفسى الميت (نيمو-٣) فقط فى العضل. تم إختبار القدرة المناعية وفاعلية الليفاميزول باختبار أمال العجول بالنسبة لوجود أجسام مناعية تخصصية ضد جميع الفيروسات المحتوى عليها اللقاح وذلك باستخدام التجارب السيرولوجية مثل إختبار السيرم المتعادل واختبار الاليزا وتم تقييم المناعة الخلوية عن طريق إختبار تحور الخلايا الليمفاوية كذلك عدد خلايا الدم البيضاء المختلفة وبينت الاختبارات المختلفة زيادة القوى التحكوينية للأجسام المعادلة المضادة وتحسن المناعة الخلوية فى المجموعات التى تم حقنها بالليفاميزول حيث وجد تأثير إيجابياً على الخلايا نوع T cell بواسطة مادة الفيتوهيماجلوتنين (PHA) ووجد أن تأثير اللقاح الميت على المناعة الخلوية ضعيفاً. كما وجد زيادة فى عدد كرات الدم البيضاء. كما تلاحظ أيضاً أن درجة الاستجابة المناعية كانت أعلى بالنسبة لمجموعة العجول التى تم تحصينها باللقاح (نيمو-٣) ثم حقنها بالليفاميزول بعد التحصين بسبعة أيام على الرغم من أن كل المجموعات التى حقنت بالليفاميزول مع اللقاح والتى حقنت باللقاح فقط أعطت مناعة قادرة على التصدى للمرض وعلى حماية الحيوانات.