Immunomodulatory Effects of Tulathromycin in Rabbits

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Abstract

Tulathromycin is a new injectable macrolid antibiotic used for treating pulmonary disease. Therefore, the present work was aimed to study the effect of tulathromycin administration only or in a combination with vitamin C (immunomodatory agent) on immunomodulating, apoptotic effect and DNA of some immune cells. Moreover, to investigate its effect on serum antioxidant activity. Twenty-five apparently healthy rabbits were divided into 5 equal groups, the 1st group was the control and the 2nd was the vaccinated group with 1 mL/rabbit S.C “*Pasteurella multocida* vaccine”. The 3rd group injected with 17.5 mg/rabbit of vitamin C and 1 mL/rabbit S.C “*Pasteurella multocida* vaccine” while, the 4th group was given 1 mL S.C/rabbit *Pasteurella multocida* vaccine and 2.5 mg/kg BW tulathromycin. The 5th group was treated S.C with 17.5 mg/rabbit vitamin C, 1 mL *Pasteurella multocida* vaccine and 2.5 mg/kg BW tulathromycin. The results showed a significant inhibition of lymphocyte transformation at 3rd day, phagocytic activity and lysozyme activity at 1st, 2nd and 3rd day of vaccination in the 4th group. Moreover, its total globulin level was significantly depressed at the 7th and 14th day with a depression of antibody titre against *Pasteurella* till the 3rd week post vaccination. Comet results revealed a significant increase of DNA damage % on the 3rd and 7th days post vaccination. DNA fragmentation of neutrophil was transiently occurred in the 3rd and 7th days post treatment. It was concluded that, Tulathromycin has a transient immunosuppressive and genotoxic effect, therefore it should be administered in a combination with Vit C to overcome its side effects.

Keywords: Tulathromycin, Vitamin C, Immunity, Genotoxicity, Rabbits

Introduction

Macrolides are a group of antibiotics that have an anti-inflammatory activity, which used in treatment of diffuse pan-bronchiolitis and cystic fibrosis [1]. They have variety of physiological functions, such as anti-viral effects, reduction of sputum production, inhibition of biofilm formation and bacterial virulence factor production [2]. Macrolides have immunosuppressive properties for both cellular and humoral immune responses [3].

Tulathromycin is a semi-synthetic macrolide antimicrobial agent, the chemical formula of the drug is C₄₁H₇₉N₃O₁₂. It is a potent injectable solution and the recommended therapeutic dose is a single dose of 2.5 mg/kg BW S.C [4]. It's administered at a single dose in cattle for controlling and treating bovine respiratory disease caused by *Pasteurella multocida*, *Haemophilus somnus*, *Actinobacillus pleuropneumonia* and recently

Moraxella bovoculi [5]. The present work aimed to study the effect of tulathromycin administration only or in a combination with vitamin C (immunomodatory agent) on immunomodulating, apoptotic effect and DNA of some immune cells. Moreover, to investigate its effect on serum antioxidant activity using rabbit as a model.

Material and Methods

Experimental animals

The present study was conducted on twenty-five apparently healthy rabbits (3 month old) weighing 2±0.25 kg/rabbit, obtained from Laboratory Animal House, Faculty of Veterinary Medicine, Zagazig University. They were divided into 5 equal groups. All groups were vaccinated with *P. multocida* vaccine (1 mL/rabbit, S.C; oil adjuvant polyvalent rabbit *P. multocida* vaccine (4x10⁹/mL CFU of *P. multocida*,

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Vaccine and Sera Institute in Abbasia, Cairo, Egypt) except the first group that was left as a control group (non-vaccinated). The 3rd and 5th groups were administered 17.5mg Vit. C/rabbit S.C (Cevaryl[®], ampules of 5 ml, each contain 1000 mg vitamin C; Memphis Pharmaceutical and Chemical Industries (MPCI), Egypt) for 10 successive days. The therapeutic dose of vitamin C in humans was 250 mg/kg BW/once daily [6] which was converted to the rabbit dose 17.5 mg/kg BW [7]. The 4th and 5th group received a single dose of tulathromycin (Draxxin[®], Pfizer Co., Egypt, 2.5 mg/kg BW S.C). Two Blood samples were collected from each rabbit; the first samples were collected on heparinized tube at the 1st, 3rd, 7th, 14th and 21st days post vaccination, while the second samples were collected without anticoagulant at the 7th, 14th, 21th and 28th days post vaccination.

Measurement of Lysozyme activity, lymphocyte proliferation and phagocytic activity of macrophage

Lysozyme activity was estimated by the diameter of the clear zone ring of lysis that developed in the translucent agarose gel after diffusion of lysozymes through the agarose gel containing a suspension of *Micrococcus lysodeikticus* [8]. The lymphocyte proliferative response was evaluated via lymphocyte separation by centrifugation through a ficoll-hypaque at 400 xg. The number of lymphocyte was concentrated as 1x10⁶cells/mL and cultured in 96 well tissue culture plate with 10% fetal calf serum at 37°C and 5% CO₂ for 72h. Proliferation of lymphocytes in response to phytohemagglutinin mitogen was estimated using MTT reduction assay [9]. Phagocytic activity of macrophage was determined by incubating a mixture of Candida and macrophage at 37°C for 2 h with regular stirring and then centrifugated at 2000 rpm. for 5m at 4°C. The sediment was smeared on glass slide and stained with Leishman stain to examine 100 cells. Consequently, the total number of phagocytic cells, number of phagocyte with ingested Candida cells and number of ingested Candida cells in each phagocyte were determined [10].

Comet assay

The genotoxic effect of Tulathromycin on neutrophil was estimated using comet assay.

And DNA damage was determined according to Singh *et al.* [11]. A volume of 10µL of cell sediment was mixed with 90µL of low melting point agarose (0.7% in PBS) at 37°C and added to a fully frosted microscope slide coated with normal melting point agarose, the slides were placed in lysis buffer (2.5 mol/L NaCl, 100 mmol/L Na₂EDTA, 10 mmol/L Tris, (pH 10)) with freshly added 1% Triton X-100 and 10% DMSO for 2 h at 4°C. Subsequently, unwinding in alkaline buffer (300 mmol/L NaOH, 1 mmol/L Na₂EDTA (pH>13)) for 15 min at 4°C and electrophoresis for 30 min at 25 V and 300 mA. The slides were neutralized (0.4 mol/L Tris (pH 7.5)) and stained (50µL of ethidium bromide (2mg/mL)) then observed using Optika Axioscope fluorescence microscope at 400 magnifications. For each sample 100 cells were photographed and scanned. For each cell, the length of DNA migration (tail length), the percentage of DNA in the tail and the tail moment was estimated.

Fragmentation of DNA (apoptotic effects)

The extraction buffer (500 µL) was mixed with 200 µL of cell suspension in 1.5 mL microfuge tube and then 50 µL of Proteinase-K solution (10 mg/mL) was added and incubated at 37°C overnight. Subsequently, 0.7-0.8 mL phenol: chloroform: iso-amylalcohol, in 25:24:1 was added and vortexed for 2-5 sec, followed by centrifugation at 12.000 rpm for 3-5 min. The aqueous layer (400-500 µL) of each sample was removed to a new tube and 40-50 µL 3M sodium acetate pH 5.3 was added to each tube, ethanol 100% was added till mark 1.5 mL and inverted to mix and DNA was let to set at -20°C overnight, then centrifuged at 12.000 rpm for 20min. The supernatant was then removed and the pellet was dissolved with 50µL of tris EDTA buffer overnight till complete dissolving. Finally, the samples were run on electrophoresis (1.2% agarose and 50 volt) and the gel was stained using ethidium bromide. Samples were analyzed using image analyses software [12].

Serum total proteins, albumin and globulin level

The total proteins and albumin levels were estimated [13,14]. The serum globulin level

was obtained by subtraction of the total proteins and albumin.

Determination of antibody titer using ELISA

It was applied to determine the effect of the tested drug on the titer of antibody against *P. multocida* after vaccination [15].

Statistical analysis

Statistical analysis of the results was carried out using student's T-test [16]. All the experimental groups were compared.

Results

Estimation of cellular immune responses

The effect of S.C administration of Tulathromycin 2.5 mg/kg BW given once, vitamin C 17.5 mg/rabbit administered for 10 successive days and their combination on serum lysozyme levels, lymphocyte transformation index and phagocytic percentage in rabbits vaccinated against *P. multocida* vaccine was depicted in Table (1).

Serum lysozyme level

Tulathromycin induced a significant decrease in lysozyme levels $11.47 \pm 0.45 \mu\text{g/mL}$ (9.91%) on the 1st day, 11.47 ± 1.77 (9.05%) the 2nd day and 13.38 ± 1.69 (9.52%) the 3rd day post vaccination when compared with vaccinated group. Co-administration of vitamin C with Tulathromycin elicited a significant increase in lysozyme levels $16.50 \pm 1.03 \mu\text{g/mL}$ (15.82%) on the 1st day, 16.10 ± 2.04 (13.97%) the 2nd day and $13.18.25 \pm 1.85$ (14.53%) the 3rd day post vaccination when compared with Tulathromycin group.

Lymphocyte transformation assay

Tulathromycin induced a significant decrease of lymphocyte transformation 0.811 ± 0.003 (49.15%) on the 3rd day post vaccination when compared with the

vaccinated group. Concurrent administration of vitamin C and Tulathromycin evoked a significant increase in lymphocyte transformation 1.33 ± 0.004 (158.64%) on the 3rd day post vaccination when compared with tulathromycin group.

Phagocytic activity of macrophage

Tulathromycin induced a significant decrease in phagocytic % $5.20 \pm 0.12\%$ (7.72%) on the 1st day, 4.60 ± 0.42 (6.75%) the 2nd day and 4.00 ± 0.31 (5.78%) the 3rd day post vaccination when compared with the vaccinated group, while co-administration of vitamin C with Tulathromycin evoked a significant increase in phagocytic % $6.00 \pm 0.41\%$ (9.65%) on the 1st day, 6.30 ± 0.23 (9.75%) the 2nd day and 6.00 ± 0.45 9.20%) the 3rd day post vaccination when compared with Tulathromycin group.

Estimation of humoral immune responses

The antibody titer against *P. multocida* using ELISA in rabbits as illustrated in Table (2). Tulathromycin induced a non-significant decrease in total globulins levels when compared with vaccinated group, while concurrent administration of vitamin C with Tulathromycin evoked a non-significant increase in total globulins levels when compared with Tulathromycin group. Tulathromycin induced a significant decrease in antibody titer 0.006 ± 0.001 (5.45%) on the 7th day, 0.008 ± 0.001 (7.08%) on the 14th day and 0.008 ± 0.003 (7.02%) on the 21th day post vaccination when compared with vaccinated group, while concurrent administration of vitamin C with Tulathromycin evoked a significant increase in antibody titre 0.006 ± 0.005 (5.77%) on the 7th day, 0.007 ± 0.003 (6.67%) on the 14th day and 0.10 ± 0.006 (9.43%) on the 21th day post vaccination when compared with Tulathromycin group.

Table 1: The effect of S.C administration of tulathromycin 2.5 mg/kg BW given once, vitamin C 17.5 mg/ rabbit administered for 10 successive days and their combination on serum lysozyme levels, lymphocyte transformation (in OD) and phagocytic % in rabbits vaccinated with pneumobac vaccine

Group	Serum lysozyme levels ($\mu\text{g/ml}$)			Lymphocyte transformation index		Phagocytic %		
				Time post vaccination (day)				
	1 st	2 nd	3 rd	3 rd	7 th	1 st	2 nd	3 rd
1) Control	89.67 $\pm$ 4.73	87.30 $\pm$ 6.70	89.67 $\pm$ 4.33	1.23 $\pm$ 0.004	1.35 $\pm$ 0.006	61.40 $\pm$ 0.24	61.20 $\pm$ 0.37	61.20 $\pm$ 0.37
2) Vaccinated	115.78 ^{**} $\pm$ 6.7	126.4 ^{***} $\pm$ 5	140.5 ^{***} $\pm$ 4.43	1.65 [*] $\pm$ 0.006	1.56 $\pm$ 0.005	67.4 ⁺⁺ $\pm$ 0.51	68.2 ^{***} $\pm$ 0.55	69.2 ^{***} $\pm$ 0.37
3) Vaccinated + vit. C	123.77 ⁺ $\pm$ 7	135.2 ⁺ $\pm$ 6.7	150.73 ⁺ $\pm$ 5.07	2.38 ⁺⁺⁺ $\pm$ 0.005	1.65 $\pm$ 0.007	73 ⁺⁺ $\pm$ 0.31	74 ⁺⁺ $\pm$ 0.44	75.80 ⁺⁺ $\pm$ 0.37
4) Vaccinated + tulathromycin	104.31 ⁺ $\pm$ 7	115.3 ⁺ $\pm$ 5.7	127.15 ⁺⁺ $\pm$ 6	0.839 ⁺⁺⁺ $\pm$ 0.003	1.42 $\pm$ 0.006	62.2 ⁺⁺ $\pm$ 0.37	63.6 ⁺⁺ $\pm$ 0.51	65.2 ⁺ $\pm$ 0.37
5) Vaccinated+vit.C+tulathromycin	120.8 ⁰⁰ $\pm$ 6	131.4 ⁰⁰ $\pm$ 7.7	145.4 ⁰⁰ $\pm$ 4.33	2.17 ⁰⁰⁰ $\pm$ 0.007	1.52 $\pm$ 0.005	68.2 ⁰⁰ $\pm$ 0.37	69.8 ⁰⁰ $\pm$ 0.37	71.2 ⁰⁰ $\pm$ 0.37

* P < 0.05

** P < 0.01

*** P < 0.001

+ P < 0.05

++ P < 0.01

+++ P < 0.001

0 P < 0.05

00 P < 0.01

000 P < 0.001

* Compared with control group

+ Compared with vaccinated.

0 Compared with vaccinated + tulathromycin

Table 2: The effect of S.C administration of tulathromycin 2.5 mg/kg BW given once, vitamin C 17.5 mg/rabbit administered for 10 successive days and their combination on serum total globulins levels and antibody titer levels using ELISA test in rabbits vaccinated with pneumobac vaccine

Group	Days	Serum total globulins levels (g/dl)				Antibody titer			
		Time (post vaccination)				Time (post vaccination)			
		Day				Day			
		7 th	14 th	21 th	28 th	7 th	14 th	21 th	28 th
1) Control		2.68 $\pm$ 0.16	3.02 $\pm$ 0.15	2.79 $\pm$ 0.14	2.97 $\pm$ 0.15	0.092 $\pm$ 0.002	0.089 $\pm$ 0.003	0.095 $\pm$ 0.003	0.091 $\pm$ 0.006
2) Vaccinated		3.74 ^{**} $\pm$ 0.13	3.78 [*] $\pm$ 0.11	3.49 $\pm$ 0.12	2.81 $\pm$ 0.14	0.110 ^{***} $\pm$ 0.001	0.113 ^{**} $\pm$ 0.001	0.114 ^{***} $\pm$ 0.005	0.114 $\pm$ 0.009
3) Vaccinated + vit. C		4.04 $\pm$ 0.12	4.05 $\pm$ 0.15	3.72 $\pm$ 0.13	2.86 $\pm$ 0.14	0.112 $\pm$ 0.002	0.115 $\pm$ 0.002	0.118 $\pm$ 0.001	0.116 $\pm$ 0.008
4) Vaccinated + tulathromycin		3.32 $\pm$ 0.15	3.59 $\pm$ 0.13	3.41 $\pm$ 0.14	2.73 $\pm$ 0.05	0.104 ⁺ $\pm$ 0.002	0.105 ⁺ $\pm$ 0.001	0.106 ⁺ $\pm$ 0.002	0.113 $\pm$ 0.012
5) Vaccinated+vit.C+tulathromycin		3.82 $\pm$ 0.15	3.91 $\pm$ 0.12	3.74 $\pm$ 0.14	2.86 $\pm$ 0.11	0.110 ⁰ $\pm$ 0.008	0.112 ⁰ $\pm$ 0.002	0.116 ⁰ $\pm$ 0.005	0.112 $\pm$ 0.003

* P < 0.05

** P < 0.01

*** P < 0.001

+ P < 0.05

++ P < 0.01

+++ P < 0.001

0 P < 0.05

00 P < 0.01

000 P < 0.001

* Compared with control group

+ Compared with vaccinated.

0 Compared with vaccinated + tulathromycin

Table 3: The effect of S.C administration of tulathromycin 2.5 mg/kg BW given once, vitamin C 17.5 mg/rabbit administered for 10 successive days and their combination on tail % DNA and tail moment length in rabbits vaccinated with pneumobac vaccine

Group	Days		Comet assay Time (post vaccination) Day					
	3 rd		7 th		14 th		21 th	
	Tail % DNA	Tail moment	Tail % DNA	Tail moment	Tail % DNA	Tail moment	Tail % DNA	Tail moment
1) Control	12.43±0.23	1.27±0.005	12.3±0.17	1.18±0.008	13.7±0.14	1.06±0.008	12.3±0.17	0.964±0.001
2) Vaccinated	11.5±0.15	1.38± 0.005	13.21±0.14	1.25±0.004	13.5±0.29	1.09±0.007	3.2±0.11	0.984±0.006
3) Vaccinated + vit. C	9.8±0.17	1.02±0.008	11.2±0.15	1.03±0.001	12.1±0.18	0.981±0.001	12.2±0.15	0.967±0.001
4)Vaccinated+ tulathromycin	20.6 ⁺⁺ ±0.16	3.54 ⁺⁺ ±0.015	21.40 ⁺⁺ ±0.23	3.15±0.013	14.8±0.15	1.26±0.004	14.5±0.26	1.22±0.006
5)Vaccinated+ vitC+ tulathromycin	21.5±0.18	3.22±0.018	19.80±0.26	3.28±0.011	14.4±0.22	1.12±0.002	13.9±0.44	0.996±0.006

* P < 0.05

** P < 0.01

*** P < 0.001

+ P < 0.05

++ P < 0.01

+++ P < 0.001

θ P < 0.05

θθ P < 0.01

θθθ P < 0.001

* Compared with control group

+ Compared with vaccinated.

θ Compared with vaccinated + tulathromycin

Genotoxicity and Comet assay for neutrophil

The genotoxic effect expressed by “DNA fragmentation assay” and comet assay in Table (3) and Figures (1 and 2). Tulathromycin induced a significant increase in DNA% in tail $9.10 \pm 0.14\%$ (79.13%) on the 3rd day, 8.19 ± 0.13 (61.98%) on the 7th day and non-significant changes on the 14th and 21th day post vaccination when compared with the vaccinated group. Tulathromycin induced a significant increase in tail moment length

2.16 ± 0.004 (156.52%) on the 3rd day, 1.90 ± 0.002 (152.00%) on the 7th day and non-significant differences on the 14th and 21th days post vaccination when compared with the vaccinated group.

Estimation of DNA fragmentation in neutrophil

Rabbits given Tulathromycin showed double fragmentation in DNA of neutrophil on the 3rd and 7th day post vaccination when compared with vaccinated group.

Figure 1: DNA fragmentation in neutrophils in rabbits given once tulathromycin 2.5 mg/kg BW, vitamin C 10 mg/kg BW for 10 successive days and their combination. DNA revealed double fragmentation and tail on the 3rd day and 7th post vaccination. M: Genomic DNA Marker, Lane 1: control group, Lane 2: vaccinated group, Lane 3: vaccinated + tulathromycin group, Lane 4: vaccinated + tulathromycin + vitamin C group and Lane 5: vaccinated + vitamin C group.

Discussion

In the current study, the serum lysozyme levels, lymphocyte transformation assay and phagocytic percent was significantly decreased in Tulathromycin treated rabbits that vaccinated with *P. multocida* vaccine. Likewise, Kohyama *et al.* [17] observed that treatment with 14-member of macrolides inhibit IL-8 release by eosinophils and may prevent the autocrine cycle necessary for the recruitment of these cells into the airways. Macrolides inhibit the production of many pro-inflammatory cytokines such as interleukin IL-1, IL-6, IL-8 and TNF- α , possibly by suppressing the transcription factor nuclear- κ B

or activator protein-1 [18]. Macrolides suppress neutrophil migration through interfering with (i) Production of IL-8 and TNF- α by macrophages and structural cells, (ii) Decreased expression of adhesion molecules on vascular endothelium and neutrophils and (iii) Decrease production and release enzymes by neutrophils [19].

In a similar view, [20] reported that macrolides could potentially reduce the number of lymphocytes in the lungs of patients with chronic lower respiratory tract disease. Macrolides decline the proliferation of T-lymphocytes by interfering with (i) expression of nuclear factor Kappa-light-chain-enhancer of activated B cells (NF κ B), (ii) cellular (JNK)

c-Jun N-terminal kinases and (ERK) extracellular-signal-regulated kinases, and (iii) IFN- γ levels [21]. These findings were in agreement with Kikuchi *et al.* [22] who found that macrolides suppress macrophages by suppression of cytokine production (IL-1B, IL-6, IL-8, TNF- α), interfering with intracellular signaling mechanisms and transcription factor activation, resulting in suppression of gene

expression and other mechanism through decreasing NO production by decreased the expression of gene encoding iNOS. Shortening of neutrophil survival was mediated indirectly through inhibition of GM-CSF (granulocyte monocyte-colony stimulating factor) released from epithelial cells or IL-8 production in activated neutrophils [23].

Figure 2: DNA of lymphocytes, macrophages and neutrophils in rabbits administered Tulathromycin depicting nuclei from damaged leukocytes consisted of a head with DNA migrating into tail region as a result of strand breakage on the 3rd and 7th day post vaccination.

In the current study, it was evinced that subcutaneously administration of Tulathromycin provoked a significant decrease in serum total proteins levels and antibody titer compared with pneumobac vaccine. In accordance, Luo *et al.* [24] found that in vitro Rapamycin had direct suppressive effect on B cells, so that Rapamycin suppressed IgG production by pure B cells stimulated with IL2, while measuring IgG production and cell proliferation revealed that Rapamycin acted at the activation stage of T and B cells. Therefore, Rapamycin had a strong suppressive effect on antibody titer. In the current study, we found that Tulathromycin evoked apoptosis in neutrophils. Likewise, Jun *et al.* [25] demonstrated that macrolides shorten neutrophil survival by accelerating neutrophil apoptosis. Moreover, Luo *et al.* [24] reported that Roxithormycin induced apoptosis by enhancing FaS-FaS ligand and caspase-3

but not caspase-8. Tulathromycin with superior clinical efficacy promotes neutrophil apoptosis, which in turn leads to inhibition of the NF- κ B pathway and downstream production of pro-inflammatory mediators. Caspases cleavage (NF- κ B nuclear factor kappa B cells) proteins during apoptosis prevented the transcription of many pro-inflammatory and prosurvival genes [26].

In the current work, The Tulathromycin treated group displayed genotoxicity (DNA fragmentation) through a significant increase in DNA percent in the comet tail and tail moment in groups challenged with pneumobac vaccine. These findings coincide with Maletić *et al.* [27] who noted that Tulathromycin induces genotoxic effects. Neutrophils lose some of their secretory properties and undergo distinct of chromatin, and DNA fragmentation [28]. An early biochemical change during apoptosis is the loss of membrane

phospholipid asymmetry, as phosphatidyl serine translocates to the outer leaflet of the plasma membrane [29].

Conclusion

It was concluded that, Tulathromycin has transient immunosuppressive and genotoxic side effects which can be avoided using immunostimulant and antioxidant such as Vit C to overcome these side effects.

Conflict of interest

None of the authors have any conflict of interest

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الملخص العربي

التغيرات المناعية للتولاثرومايسين في الارانب

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التولاثرومايسين نوع جديد من الماكرولايد ويستخدم فى علاج الامراض التنفسية أجريت هذه الدراسة لإلقاء الضوء على الآثار الجانبية الضارة للتولاثرومايسين على التأثير الفارماكولوجى المناعى الذى يتمثل فى المناعة الخلوية والعضدية فى الارانب بالإضافة الى التأثير السمي الجينى على كرات الدم البيضاء مع استكشاف تأثير فيتامين سى على مقاومة هذه الآثار الضارة ومحاولة التغلب عليها . تم استخدام خمسة وعشرون ٢٥ من ذكور الارانب النيوزيلندى حيث قسمت الى ٥ مجموعات متساوية وكل مجموعة منهم تحتوى على ٥ أرانب كالتالى: المجموعة الأولى "مجموعة ضابطة": أرانب لم تعالج بأى من الأدوية أو التحصينات. المجموعة الثانية: تم تحصينها باللقاح الزيتى الميت للباستريلا بجرعة مقدارها ١ مللى تحت جلد الرقبة . المجموعة الثالثة: محصنة باللقاح الزيتى الميت للباستريلا بجرعه مقدارها ١ مللى تحت جلد الرقبة وتم علاجها ب١٧,٥ مللى جرام من فيتامين سى لكل أرنب تحت الجلد. المجموعة الرابعة: محصنة باللقاح الزيتى الميت للباستريلا بجرعه مقدارها ١ مللى تحت جلد الرقبة وتم علاجها ب١٧,٥ مللى جرام من فيتامين سى لكل أرنب تحت الجلد. المجموعة الخامسة: محصنة بـ ٢,٥ مللى جرام/كجم من وزن الجسم عن طريق الحقن تحت الجلد جرعة واحدة وتم علاجها أيضاً بـ ١٧,٥ مللى جرام من فيتامين سى حقن تحت الجلد. اعطاء التولاثرومايسين للأرانب المحصنة كان سبب نقصاً معنوياً فى معدل تحول كرات الدم البيضاء فى اليوم الثالث للتحصين وكذلك نقصاً معنوياً فى نشاط الليزوزيم والخلايا الالتهامية فى اليوم الاول والثانى والثالث للتحصين بالمجموعة الرابعة وكذلك انخفاض فى مستوى الجلوبيولين فى الاسبوع الاول والثانى وكذلك انخفاض فى مستوى الاجسام المناعية فى الاسبوع الاول والثانى والثالث للتحصين. اعطاء التولاثرومايسين للأرانب المحصنة ادى الى تكسير وتحطم الحمض النووى فى اليوم الثالث والسابع لاعطاء التحصين لذلك التولاثرومايسين له تأثير مثبط للمناعة وتأثير سمي جينى لذلك ينصح باعطاء فيتامين سى مع التولاثرومايسين.