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RESEARCH ARTICLE

The Clinicopathological Effects of the Double Immunization with Formalized Killed Vaccine against *Pasteurella multocida* Challenge in Rabbits

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Abstract

The disease caused by *Pasteurella multocida* is an extremely common and difficult issue of rabbits utilized for biomedical research. The vaccination techniques are dependably the most powerful preventive measures. In the present study, 30 New Zealand rabbits (1.5 kg average body weight and 6–8 weeks old) were divided into 3 groups, control group (I), challenged non-vaccinated group (II), and challenged double vaccinated group (III). 1st dose was administered at the 1st day followed by similar booster dose after 21 days. At the end of the 2nd, 4th, and 6th weeks of the experiment, blood samples were collected from the ear vein for hematological, plasma, and serum examination. At the end of the 6th week, the rabbits were anaesthetized and sacrificed to collect the tissue specimens from liver, kidneys, spleen, lungs and heart for histopathological study. The results showed that double immunization with killed vaccine of *P. multocida* increased the immune response of the animals and the leukocyte phagocytic activity against *P. multocida* and also improved the clinicopathological and histopathological findings. Taken together, our findings proved that double immunization with killed vaccine of *P. multocida* increased the phagocytic activity of the immune cells and the immune status of animals against infection.

Keywords: Pasteurellosis, Phagocytosis, Spleen, Nitric oxide, *P. multocida* formalized killed vaccine.

Introduction

Rabbits are viewed as one of the essential domesticated animals that give high quality protein sustenance. One of the essential diseases that influence rabbits' generation is pasteurellosis [1]. Pasteurellosis affects rabbits of 4– 8 weeks old causing manifestations going from lethal septicemia, serious pleuritis, and pneumonia to less extreme sequelae, for example, various abscesses, perpetual rhinitis, and otitis media. The result of any type of the ailment is extreme financial misfortunes [2]. The utilization of antibiotics has been in part effective in controlling pasteurellosis disease in rabbits, since they don't totally dispose of the bacterium [3]**.** Additionally, *Pasteurella multocida* built up an incredible resistance from the ordinarily utilized antimicrobials [4].

Control of pasteurellosis in rabbits is adept by immunization against *Pasteurella multocida* infection. Defensive resistance can be actuated by a live vaccine or an inactivated entire cell vaccine (bacterin). The live vaccine has advantage over the bacterin, however a genuine inconvenience is that immunization with living vaccines, sometimes, brings about systemic infection. Then again, immunization of rabbits with bacterin frequently brings about insufficient protection in the field when it was used by a single dose [5].

Immunization studies on rabbit's pasteurellosis have been accounted for the utilization of inactivated forms as formalized [6], joined with oil adjuvant [5] or heat treated [7]. Thus, the aim of the present work was to study the effect of formalized killed *P.*

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multocida vaccine injection with double doses against experimental challenge of rabbits with *P. multocida* strain based on selective hematological, biochemical, immunological and histopathological investigations.

Materials and Methods

Animals

A total of 30 white New Zealand apparently healthy rabbits, not vaccinated against pasteurellosis (1.5 kg average body weight and 6–8 weeks old) were obtained from the animal house, Faculty of Veterinary Medicine, Zagazig University. The animals were kept under hygienic conditions, housed in metal cages and fed on balanced ration and water *ad–libitum* and maintained in a 12 h light-dark cycle at a controlled temperature (21–24° C) and humidity (50–60%). They were kept for 15 days without medication for acclimatization before beginning the study. The care and welfare of animals conformed to the guiding principle of the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary.

Vaccine and chemicals

The formalized killed vaccine for *P. multocida* was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. All chemicals and stains were of analytical grade and obtained from Sigma Co., and El Gomhoria Co., Egypt.

Challenging bacteria

The strain of *P. multocida* was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt, as lyophilized ampoules. It was activated by culturing in nutrient broth supplemented with yeast extract followed by I/P inoculation in Swiss mice, and re-isolated from heart blood of mice on nutrient agar plates (Difco., Egypt) supplemented with yeast extract. Bacterial colonies were suspended in sterile saline, and the density was adjusted to contain 5×10^9 bacterial cell/ mL [8]. The suspension was used for S/C injection of rabbits in the challenge test [9].

Experiment design

Thirty New Zealand rabbits were divided into 3 groups (I-III), each group contained 10 rabbits as following:-

Group I: was the negative control group, Group II: was non-vaccinated challenged group and Group III: was the vaccinated challenged group in which rabbits were injected S/C with two doses of 1 mL/kg B W *P. multocida* vaccine, 1st dose at the 1st day of experiment followed by booster dose after 21 days according to Okerman and Spanoghe [6] and Osama [10]. At the end of the 5th week from starting the experiment, rabbits in groups II & III were challenged S/C with broth culture of virulent *P. multocida* strain (0.2 mL/ kg BW) [9].

Sampling

Blood samples (10mL) were collected from the marginal ear vein of rabbits in all groups at the end of 2nd, 4th and 6th weeks of the experiment. They were divided into three parts. The 1st part (1mL rabbit blood) was placed at clean Wasserman tubes containing disodium salts of EDTA (El Gomhoria Co., Egypt) for hematological examination. The 2nd part (5mL rabbit blood) was placed in a chemical free test tube without anticoagulant (El Gomhoria Co., Egypt) to separate a clear serum for biochemical analysis [11]. The 3rd part (3 mL rabbit blood) was collected in heparinized tubes (El Gomhoria Co., Egypt) for phagocytosis. Tissue specimens from liver, kidneys, spleen, lungs and heart from all experimental groups were collected at the end of the 6th week of the experiment for histopathological examination.

Hematological studies

Complete blood picture (RBCs, Hb, PCV, MCV, MCHC, PLT, TLC and differential leukocytic count) was carried out using an automated hematology analyzer (Sysmex IV2000, UK).

Clinicobiochemical analysis

All biochemical tests were performed using colorimetric kits (Sigma Co., Egypt) following the instructions described at the enclosed manufacturer's pamphlets. The serum

activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured according to the method of Reitman and Frankel [12]. The serum alkaline phosphatase (ALP) activity was determined according to modified method of Tietz [13]. The serum bilirubin levels (total and direct) were determined according to Jendrassik [14]. Indirect bilirubin was calculated by subtracting the obtained direct bilirubin level from the obtained total bilirubin level. Serum creatinine and urea were determined colorimetrically according to the method of Henry [15] and Fawcett and Scott [16]. Nitric oxide was determined based on Griess reaction [17].

Phagocytic activity

Heat-inactivated *Candida glabrata* (*C. glabrata*) was used as a microbial model to evaluate the phagocytic activity of the rabbit monocytes. In brief, leukocytes were separated from the heparinized blood samples [18,19]**.** One mL of the adjusted viable leukocytes suspension (leukocytes in RPMI 1640 with 5% of pooled rabbit serum) was placed in sterile plastic tube, to which 1 mL of the prepared heat inactivated *Candida glabrata* was added. The tubes were then incubated at 27 ºC for 30 min in a humidified 5% CO2 incubator. Then the tubes were centrifuged at 2500 rpm for 5 min, and the supernatant was removed with Pasteur pipette leaving a small part into which the sediment was re-suspended. Slide smears were prepared from the deposit, air dried and then stained with Leishman's stain (El Gomhoria Co., Egypt) [20]**.** Under a light microscope (Binocular model 500, 3B Scientific, USA) using oil immersion lens, phagocytic cells were counted randomly in about ten microscopic fields. The number of phagocytic cells with engulfed yeast was recorded. The phagocytic activity was evaluated according to the following equation:

Phagocytic percentage **(P %)** =

$$\textbf{P \%} = \frac{\textbf{Number of phagocytes with engulfed yeast cells}}{\textbf{Total number of phagocytes}} \times 100$$

Histopathological examination

Specimens from the liver, kidneys, spleen, lungs and heart from all treated groups were collected at the end of 6th week post-treatment, fixed in 10 % neutral buffered formalin (El Gomhoria Co., Egypt) for 48 hours, then washed overnight under running water. The washed specimens were dehydrated by using up graded concentrations of ethyl alcohol (El Gomhoria Co., Egypt) starting with 75% and ending with absolute alcohol cleaned in xylene (El Gomhoria Co., Egypt) two times, each for 2 h. After that, the specimens were placed in crucible containing soft paraffin (El Gomhoria Co., Egypt) and kept in an oven at 60°C for 12 h. Paraffin sections of 5 microns thickness were prepared and stained with H & E stains (El Gomhoria Co., Egypt) for histopathological examination [21].

Statistical analysis

The obtained data were analyzed using F-test according to Tamhane and Dunlop [22] (SPSS 16.0, 2007 SPSS Inc. TEAM EQX)**.** Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a.

Results

Hematological findings

Regarding to the erythrogram shown in Table 1, comparing with the control non-infected group (I); the challenged group (II) showed non-significant changes at the values of RBCs, Hb, PCV, MCV, MCHC, and platelets at the end of the 2nd and 4th week from starting of the experiment. Meanwhile, at the end of the 6th week, it showed macrocytic hypochromic anemia with thrombocytopenia. On the other hand, the vaccinated rabbits of group III showed normocytic normochromic anemia at the end of the 2nd and 4th weeks with non-significant changes at the counts of platelets, but at the end of the 6th week, they showed non-significant changes at the erythrogram indices with non-significant changes at platelets count also.

Leukogram and immunological results

At the end of the 2nd week of the experiment compared to the control group as

illustrated in Table 2, the infected non-vaccinated group (II) showed a significant decrease at the counts of total leukocytes including neutrophils, and monocytes with non-significant changes at the lymphocytes, eosinophils and basophils counts. Meanwhile, at the end of the 4th week, the infected group (II) showed non-significant changes at the leukogram variables, but at the end of the 6th week, they showed significant increase at total leukocytes including neutrophils with non-significant changes at lymphocytes, monocytes, eosinophils and basophils. On the other side, group III revealed leukocytosis and lymphocytosis at the end of the 2nd week with neutrophilia at the 4th week, and monocytosis along the experimental periods. The phagocytic percent of group II was non-significantly changed than the control group at all experimental periods (Figure 1; a, b, c, d, e, f) but it was elevated in at group III (Figure1; g, h, i).

Biochemical results

At the 2nd and 4th weeks post-challenge as shown in Table 3, group II showed non-significant changes at serum ALT, AST, ALP, total, direct, and indirect bilirubin, urea, creatinine and nitric oxide. These parameters were increased at the 6th week in the infected group. However, group III showed at the 2nd week non-significant changes at serum ALT, AST, ALP, direct bilirubin, urea, creatinine and nitric oxide with significant increase at the total and indirect bilirubin but at the 4th week, they showed marked increase at the serum activity of ALT, AST, ALP, total and indirect bilirubin with non-significant changes at serum level of direct bilirubin, urea, creatinine and nitric oxide. At the 6th week, double immunization of rabbits (group III) resulted in moderate decline in the elevated parameters that recorded in the infected non-vaccinated rabbits (group II). Such changes does not return to the normal level except for indirect bilirubin, urea, and creatinine.

Table 1: Erythrogram and Platelets count (mean values±SE) in rabbit's groups (I-III) at the end of the 2nd, 4th and 6th weeks of challenging

	RBCs 10^6/µL	Hb g/dL	PCV %	MCV fl	MCHC %	Platelets 10^3/µL
			2nd week			
Control Group(I)	5.63 a ±0.14	11.52 a ±0.31	37.46 a ±1	66.58 a ±1.82	30.77 a ±0.55	483.6 a ±57.53
Non-vaccinated Challenged Group(II)	5.68 a ±0.1	11.38 a ±0.1	35.71 a ±0.9	62.89 a ±1.44	31.94 a ±0.67	456.8 a ±13.58
Vaccinated Challenged Group(III)	4.26 b ±0.1	9.32 b ±0.1	29.56 b ±0.37	69.5 a ±0.67	31.53 a ±0.2	469.6 a ±74.97
			4th week			
Control Group(I)	5.76 a ±0.05	11.42 a ±0.17	36.2 a ±0.66	62.88 a ±1.38	31.56 a ±0.37	440.6 a ±30.85
Non-vaccinated Challenged Group(II)	5,64 a ±0.11	11.1 a ±0.27	34.91 a ±1.26	61.87 a ±1.48	31.86 a ±0.57	438.6 a ±26.09
Vaccinated Challenged Group(III)	5.16 b ±0.11	9.96 b ±0.25	30.92 b ±0.91	67.1 a ±1.36	31.7 a ±0.27	466 a ±33.94
			6th week			
Control Group(I)	5.6 a ±0.1	11.34 a ±0.2	34.3 a ±0.65	61.33 b ±1.78	33.11 a ±0.9	469 a ±48.10
Non-vaccinated Challenged Group(II)	3.34 b ±0.14	8.46 b ±0.16	30.86 b ±1.19	102.43 a ±6.87	25.11 b ±0.96	213.2 b ±46.43
Vaccinated Challenged Group(III)	5.17 a ±0.11	10.9 a ±0.17	33 a ±1.01	63.9 b ±2.24	33.13 a ±0.86	454.6 a ±14.70

Group I: negative control group, Group II: non-vaccinated challenged group and Group III: vaccinated challenged group in which rabbits were injected S/C with two doses of 1 mL/kg B W *P. multocida* vaccine, 1st dose at the 1st day of experiment followed by booster dose after 21 days.
Group II & III: challenged S/C with broth culture of virulent *P. multocida* strain (0.2 mL/ kg BW).

Table 2: Leukogram and phagocytic % (mean values±SE) in rabbit's groups (I-III) at the end of the 2nd, 4th and 6th weeks of challenging

	TLC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Phagocytic %
			2nd week				
Control Group(I)	9.16 b ±0.74	4.11 a ±0.15	3.98 b ±0.47	0.78 a ±0.28	0.06 a ±.01	0.23 a ±0.06	26.2b ±1.39
Non-vaccinated Challenged Group(II)	9.47 b ±0.45	4.21 a ±0.33	4.21 b ±0.28	٠.73 a ±0.07	0.08 a ±0.02	0.24 a ±0.09	25.6 b ±1.50
Vaccinated Challenged Group(III)	11.64 a ±0.15	4.39 a ±0.18	6.81 a ±0.25	0.78 a ±0.03	0.08 a ±0.01	0.21 a ±0.05	36.4 a ±1.12
			4th week				
Control Group(I)	9.08 b ±0.40	4.19 b ±0.06	3.93b ±0.39	0.73 a ±0.06	0.08 a ±0.02	0.15 a ±0.06	26.8 b ±1.24
Non-vaccinated Challenged Group(II)	9.12 b ±0.50	4.01 b ±0.24	3.75 b ±0.29	0.7 a ±0.05	0.06 a ±0.01	0.21 a ±0.08	25.4 b ±1.36
Vaccinated Challenged Group(III)	14.35 a ±0.30	7.6 a ±0.22	5.81 a ±0.14	0.7 a ±0.11	0.06 a ±0.01	0.18 a ±0.07	35.4 a ±1.36
			6th week				
Control Group(I)	9.29 b ±0.41	4.4 c ±0.19	4.03 b ±0.60	0.59 b ±0.04	0.06 a ±0.01	0.21 a ±0.08	25.6 b ±1.50
Non-vaccinated Challenged Group(II)	13.35 a ±0.20	8.01 a ±0.25	4.5 b ±0.15	0.63 b ±0.04	0.05 a ±0.00	0.16 a ±0.05	23.8 b ±1.24
Vaccinated Challenged Group(III)	13.33 a ±0.40	5.47b ±0.12	6.66 a ±0.14	0.96 a ±0.24	0.07 a ±0.03	0.18 a ±0.06	43.6 a ±0.51

Group I: negative control group, Group II: non-vaccinated challenged group and Group III: vaccinated challenged group in which rabbits were injected S/C with two doses of 1 mL/kg B W *P. multocida* vaccine, 1st dose at the 1st day of experiment followed by booster dose after 21 days.
Group II & III: challenged S/C with broth culture of virulent *P. multocida* strain (0.2 mL/ kg BW).

Table 3: Clinicobiochemical results (mean values±SE) in rabbit's groups (I-III) at the end of the 2nd, 4th and 6th weeks of challenging

	ALT U/l	AST U/l	ALP U/l	T. bilirubin mg%	D. bilirubin mg%	In. bilirubin mg%	Urea mg/ dL	Creatinine mg/dL	NO mg/dL
				2nd week					
Control Group(I)	16.18 a ±0.37	15.56 a ±0.41	41.07a ±2.08	1.00 b ±0.04	0.22 a ±0.03	0.77 b ±0.04	33.49 a ±1.19	0.94 a ±0.05	0.66 a ±0.11
Non-vaccinated Challenged Group(II)	16.5 a ±0.39	15.2 a ±0.41	40.12 a ±1.68	0.94 b ±0.06	0.24 a ±0.02	0.71 b ±0.07	34.74 a ±1.05	0.96 a ±0.05	0.7 a ±0.12
Vaccinated ChallengedGroup(III)	18.04 a ±0.45	18.74 a ±0.66	46.16 a ±1.42	1.46 a ±0.08	0.3 a ±0.01	1.15 a ±0.08	31.41 a ±0.76	1 a ±0.04	0.68 a ±.07
				4th week					
Control Group(I)	16.72 b ±0.26	14.9 b ±0.58	41.53 b ±2.21	1.00 b ±0.04	0.25 a ±0.03	0.74 c ±0.03	33.31 a ±1.12	1.01 a ±0.06	0.71 a ±0.09
Non-vaccinated Challenged Group(II)	16.57 b ±0.28	15.28b ±0.43	41.9 b ±1.36	0.95 b ±0.04	0.23 a ±0.03	0.72 c ±0.06	33.91 a ±0.96	0.97 a ±0.07	0.65 a ±0.06
Vaccinated Challenged Group(III)	23.45 a ±0.87	23.92 a ±2.06	53.2 a ±1.41	1.23 a ±0.06	0.29 a ±0.01	0.94 b ±0.07	33.34 a ±1.29	0.95 a ±0.07	0.7 a ±0.12
				6th week					
Control Group(I)	14.86 c ±0.44	13.64 c ±0.49	39.7 c ±2.65	0.95 c ±0.04	0.30 b ±0.02	0.65 c ±0.05	34.14 b ±1.54	0.95 b ±0.07	0.58 c ±0.08
Non-vaccinated Challenged Group(II)	32.03 a ±1.08	27.57 a ±1.19	55.45 a ±2.03	2.05 a ±0.09	0.43 a ±0.03	1.62 a ±0.09	48.65 a ±1.21	1.57 a ±0.05	2.98 a ±0.15
Vaccinated ChallengedGroup(III)	21.35 b ±0.63	21 b ±1.06	44.38 b ±1.86	1.37b ±0.05	0.31 b ±0.01	1.06 b ±0.06	33.21 b ±0.95	1 b ±0.06	1.93 b ±0.25

Group I: negative control group, Group II: non-vaccinated challenged group and Group III: vaccinated challenged group in which rabbits were injected S/C with two doses of 1 mL/kg B W *P. multocida* vaccine, 1st dose at the 1st day of experiment followed by booster dose after 21 days.
Group II & III: challenged S/C with broth culture of virulent *P. multocida* strain (0.2 mL/ kg BW).

Histolopathological alterations

In group II, liver of rabbit showed peliosis **,**hepatis (multiple, randomly distributed, blood-filled cavities throughout the liver (Figure 2A). Furthermore, vascular congestion and severe hydropic degeneration in the epithelial lining of the tubular epithelium of kidney were recorded (Figure 2C). Meanwhile, spleen showed lymphoid depletion of the lymphoid follicle associated with hemosiderosis (numerous small golden-brown hemosiderin pigment) (Figure 2 E). Lung also showed bronchiolitis (infiltration of the bronchiolar wall and lumen with inflammatory cells) (Figure 2 G). Heart showed extensive hemorrhages, intermuscular edema, with vacuolation of the cardiomyocytes (Figure 2 I). The challenged –vaccinated group (III) showed sinusoidal dilatation with congestion of the central vein of the liver (Figure 2 B), mild vacuolations in the renal epithelium (Figure 2 D) of the kidney, mild lymphoid depletion in the germinal center of the lymphoid follicle of the spleen (Figure 2 F), mild thickening of the alveolar walls by leukocytes of the lung (Figure 2 H), and numerous vacuolation in some myocardiocytes (Figure 2 J).

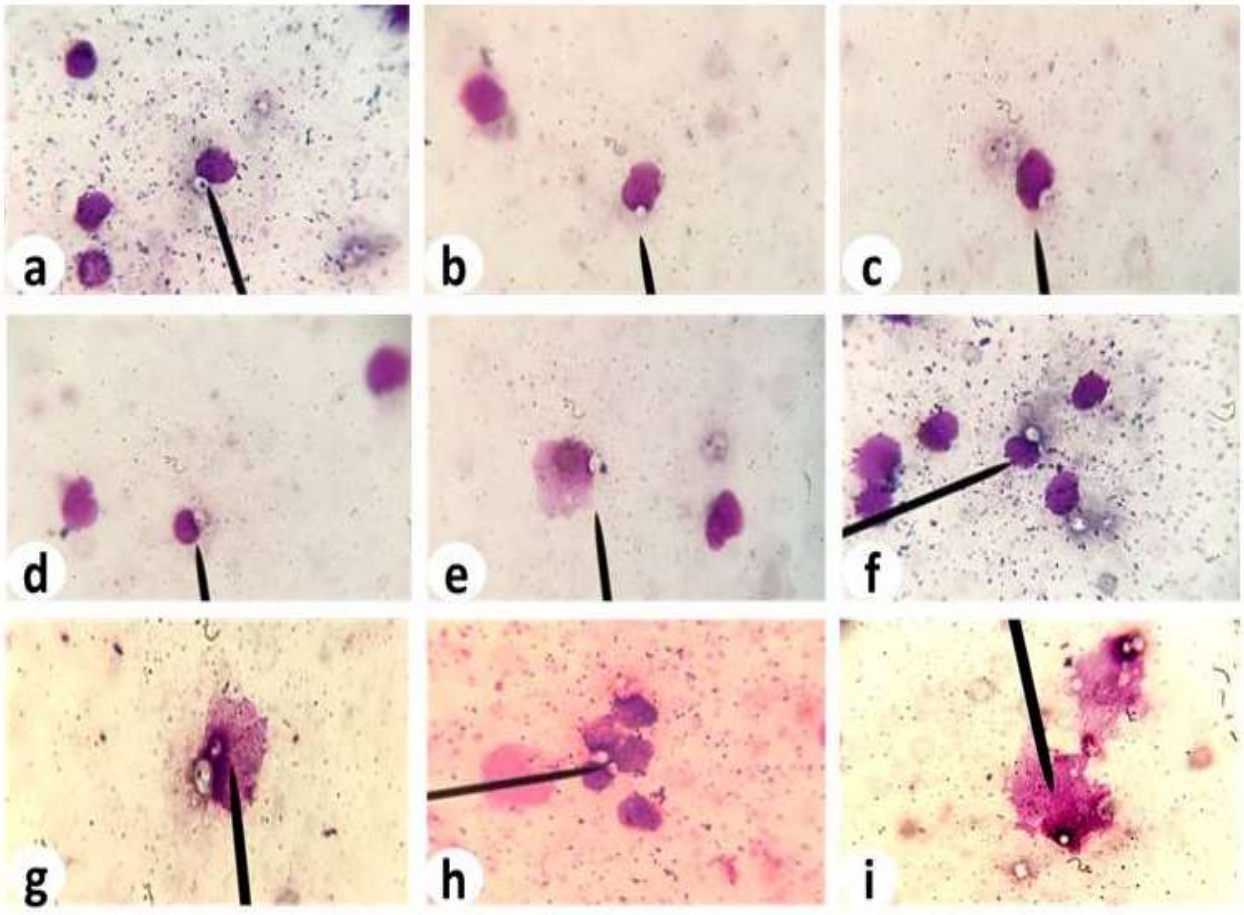

Figure 1: Representative photo of rabbit's phagocytic activity in three groups showing mild phagocytic activity at group I (a, b, c), group II; challenged with *Pasteurella mulocida* (d, e, f) while mild, moderate and high at group III ; vaccinated and challenged(g, h, i) at the 2nd ,4th ,6th week post-challenge, respectively, arrows indicate phagocytes with engulfed yeast cells.

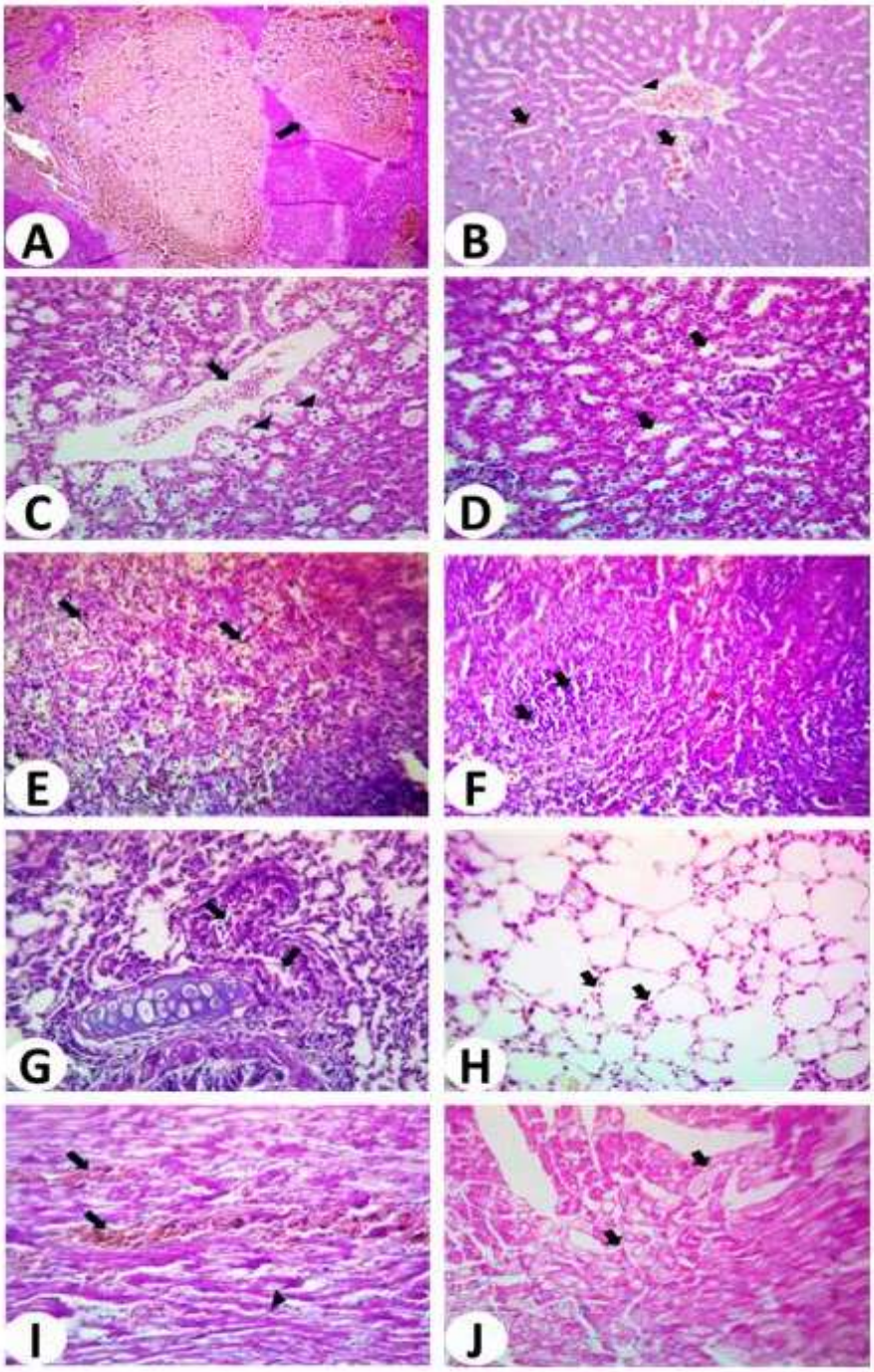

Figure 2: Photomicrograph of H&E-stained tissue sections: liver (A&B) of group (II); challenged with *Pasteurella mulocida*, showing peliosis, hepatitis (arrows), X 100 (A) and sinusoidal dilatation (arrows) with congestion (arrowhead) of the central vein of group III; vaccinated and challenged , X 400 (B). Kidney (C&D) of group II, showing vascular congestion (arrow) and severe hydropic degeneration (arrowheads), X 400 (C) and mild vacuolations (arrows) in the renal epithelium of group III X 400(D). Spleen (E&F) of group II, showing severe lymphoid depletion of the lymphoid follicle associated with hemosiderosis (arrows), X 400 (E) which was mild at group III (F). Lung (G&H) of group II, showing bronchiolitis (arrows), X 400 (G) and mild thickening of the alveolar walls by leukocytes (arrows) at group III, X 400 (H). Heart (I&J) of group II, showing extensive hemorrhages (arrows), intermuscular edema, with vacuolation of the cardiomyocytes (arrowheads), X 400(I) and numerous vacuolations in some myocardiocytes (arrows) at group (III), X 400(J).

Discussion

The disease caused by *Pasteurella multocida* is an extremely common and difficult issue of rabbits utilized for biomedical research. *P. multocida* causes suppurative rhinitis (snuffles), otitis media, enzootic pneumonia, conjuctivitis, pyometra, orchitis, subcutaneous abscesses, and septicemia in rabbits [23]. Chemotherapeutic treatment of *Pasteurella* is costly, protracted, and insufficient because of the expanding antibiotics resistance of the bacterium in addition to its harmfulness to human buyers. However, biosecurity measures assumed a part in lessening the spread of the pathogen, the vaccination techniques were dependably the most powerful preventive measures [24].

Different methods have been endeavored to control and eliminate pasteurellosis. One of the additionally encouraging methodologies is the improvement of vaccination techniques which over long stretches will ensure animals presented to *P. multocida*. Immunization investigations of rabbits with executed *P. multocida* have indicated variable outcomes [25-27]. By evaluation of erythrogram in the present study, vaccinated challenged rabbits of group III showed a significant decrease in RBCs count, hemoglobin concentration and PCV, associated with normal MCV and MCHC leading to normocytic normochromic anemia at the end of the 2nd and 4th week of challenging. This anemia may be due to adverse effect of formalized killed *P. multocida* vaccine on erythropoiesis process through its effect on erythrocyte precursor cells in the bone marrow [28]. These findings agreed with Jasprica *et al*. [29] and Nassar *et al.* [30].

After infection of rabbits with virulent *P. multocida,* a highly significant decline in RBCs count, Hb concentration, PCV and MCHC with highly significant rise in MCV was noted. This macrocytic hypochromic anemia may be attributed to hemorrhage induced by *P. multocida* [31-33].

Regarding to platelets count, vaccinated challenged rabbits in group III did not show any significant changes in platelets count during all experimental periods when compared with the control group; that suggests the positive effect of killed *P. multocida* vaccine on the function and number of platelets even after bacterial challenging of rabbits with virulent *P. multocida*. While, there was a significant reduction in platelets count in challenged rabbits in group II. These results may demonstrate the reason of hemorrhage induced by *P. multocida* [31-33]. Similar results were previously obtained by Schimmel and Schimmel [34].

Our results were confirmed by the histopathological findings particularly in different organs of non-vaccinated challenged rabbits where liver and spleen of such group showed multiple, randomly distributed, blood-filled cavities. Lungs showed extensive hemorrhage with presence of large numbers of extravasated RBCs in the alveolar lumens.

Regarding to leukogram, group II rabbits after challenging with virulent *P. multocida* showed a highly significant neutrophilia at the end of 6th week; probably due to the severe inflammatory response induced by *P. multocida* as that was resulted by Wu et al. [35], who showed that *P. multocida* induced proinflammatory cytokines expression that caused severe lung inflammation. Also, Galdiero *et al*. [36] said that the porins, major polypeptide of the outer membrane of *P. multocida*, affect different biological functions of cells involved in the immune response as well as in inflammation especially neutrophils where they concluded that pretreatment of bovine neutrophils with various concentrations of porin always caused a concentration-dependent increase in examined biological activities after an *in vitro* study. On the other hand, rabbits received double dose of the killed vaccine (group III) showed increase in lymphocytes along the experimental periods and monocytes at the end of the 6th week. These results could be interpreted as that the killed vaccine enhances immune response which could be augmented by receiving double vaccine dose [37].

Our results were confirmed by the histopathological findings of different organs from rabbits in group II, where lungs, liver, kidneys, spleen and heart showed severe

inflammatory reactions with multiple mononuclear inflammatory cells infiltration. In contrast, vaccinated challenged rabbits (group III) showed very few mononuclear inflammatory cells infiltration in their internal organs.

In concern with phagocytic activity, rabbits administered formalized killed *P. multocida* vaccine (group III) showed a significant increase in the phagocytic activity compared with groups I and II. This result may be attributed to the effect of killed vaccine on induction of immune response and enhancing the phagocytic capacity of the immune cells [37,38].

Our results were emphasized by histopathological changes of the spleen of rabbits, where the most severe lesions seen in non-vaccinated challenged rabbits (group II) which showed severe lymphoid depletion of the lymphoid follicle associated with hemosiderosis. While, group III were enhanced as represented by very mild lymphoid depletion.

Hepatocytes injury was detected by measuring the serum activities of enzymes that have leaked from hepatocytes. Necrosis in a tissue can produce high serum enzyme activity [39]. The liver ALT activity is lower in rabbits than in other species and there is less organ specificity [40]. AST is found in the liver, heart, skeletal muscle, kidney and pancreas of rabbits with the highest activity in the liver and skeletal muscle. Raised AST level can be found in association with liver disease [41].

Regarding to the biochemical analysis for evaluation of liver functions, vaccinated rabbits (group III) showed a significant increase in ALT and AST activities at the end of the 2nd week. This could be attributed to mild hepatic irritating effect induced by formalized killed *P. multocida* vaccine injection. The same results previously obtained by Nassar *et al*. [9]. However, these enzymes activities showed moderate decline at the end of the 6th week especially when compared with non-vaccinated challenged rabbits (group II) that showed a highly significant rise in the hepatic enzymes activities when compared with the control; this suggests that the protective effect of double immunization with killed vaccine against *P. multocida* had damaging hepatic effect

Our results were confirmed by the histopathological findings of liver from rabbits in group II, where it showed multiple focal areas of coagulative necrosis and multiple, randomly distributed, blood-filled cavities. While, the liver of vaccinated challenged rabbits (group III) had less severe lesion represented by mild sinusoidal dilatation and congestion of central vein. These results were in consistent with Mathy *et a*l. [42] and Nassar *et a*l. [30].

At 6th week of the experiment, serum activity of ALP was significantly rise in non-vaccinated challenged rabbits (group II) which could be discussed due to hyperplastic and irritating effect of *Paseurella multocida* on the bile duct epithelium resulted in release of ALP into serum, this result agreed with Thurston *et al.* [43]. This result was proved by histopathological findings of liver and bile duct of rabbits in this group which revealed hyperplasia of the bile duct epithelium.

Serum urea and creatinine levels in non-vaccinated bacterial challenged rabbits (group II) revealed a highly significant rise. In contrast, vaccinated challenged rabbits (group III) showed no significant changes in serum urea and creatinine levels, in comparison with the control (group I).These results proved the renal damaging effect of virulent *P. multocida* which can be prevented by administration of double killed vaccine.

Our results confirmed by the histopathological findings of kidneys from rabbits in group (II), where they showed vascular congestion, severe hydropic degeneration in the epithelial lining of the tubular epithelium and hyaline casts in the lumens of the renal tubules, while the kidneys of vaccinated challenged rabbits (group III) had slight vacuolation in renal epithelium.

Conclusion

As evident from the current study, it can be concluded that the use of formalized killed *P. multocida* vaccine by double doses, in spite of its mild hepatic alteration effect, it

is very effective in increasing immune protection of rabbits against pasteurellosis.

Conflict of interest

None of the authors have any conflict of interest to declare

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الملخص العربى

التاثيرات االباثولوجية-الاكلينيكية لجرعتى التحصين باستخدام لقاح الباستيريلا الفورمالينى الميت ضد التحدي ببكتريا الباستيريلا ملتوسيدا في الارانب

محمد عبد العظيم هاشم ،عصام عبده محمود و محمد فرج محمد فرج
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يعد المرض الناجم عن الباستيريلا ملتوسيدا من الامراض الشائعة والخطيرة للغاية في الارانب المستخدمة في البحث الطبي الحيوى. ويعتبر التحصين ضد الباستيريلا هو أكثر الإجراءات الوقائية فاعلية. في هذه الدراسة تم تقسيم ثلاثين أرنب نيوزيلاندى (تتراوح اعمارهم من ٦ الى ٨ اسابيع ومتوسط الاوزان حوالى كيلو ونصف) الى ثلاث مجموعات : المجموعة الأولى كانت مجموعة ضابطة للتجربة والمجموعة الثانية لم يتم تحصينها والمجموعة الثالثة تم تحصينها بجرعتين من لقاح الباستيريلا حيث كانت الجرعة الاولى فى اليوم الاول من التجربة يليها جرعة تعزيزية مماثلة بعد ٢١ يوماً. وفى نهاية الاسبوع الثانى والرابع والسادس تم تجميع عينات دم من وريد الاذن لفحص الدم والبلازما والسيرم. فى نهاية الاسبوع السادس تم تخدير الارانب وذبحها لتجميع عينات الانسجة من الكبد والكلى والطحال والرئتين والقلب للدراسة النسيجية. واوضحت النتايج بان التحصين المزودوج بلقاح الباستريلا مالتوسيدا زاد من الاستجابة المناعية للحيوانات ونشاط البلعمة لكرات الدم البيضاء ضد الباستريلا مالتوسيدا كما انه عمل على تحسين النتائج النسيجية الاكلينيكية. ونستخلص من ذلك انه قد أثبتت النتائج التي توصلنا إليها أن التحصين المزدوج زاد من النشاط البلاعمية للخلايا المناعية والحالة المناعية للحيوانات ضد العدوى بالباستريلا.