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The Synergetic effect of propolis in vaccination of rabbits against Pasteurellosis

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ABSTRACT

The present study was designed to evaluate the effect of ethanolic extract of Egyptian propolis in rabbits vaccinated with *Pasteurella multocida*, . A total of twenty clinically healthy male white New Zealand 8 weeks old rabbits were randomly divided into 4 groups each of 5 rabbits. Group (1) was injected S/C with 2mL sterile phosphate buffer saline solution (PBS) and was kept as normal control, group (2) was injected S/C with a single dose of propolis (at dose of 50 mg/kg b.wt.) , group (3) was vaccinated with *Pasteurella multocida* vaccine only(1ml/kg b.wt.), and group (4) was injected S/C with both *Pasteurella multocida* vaccine and propolis. Treatments of propolis and *Pasteurella multocida* vaccine were repeated as a booster dose after three weeks. Blood samples were collected at 2 and 4 weeks post vaccination for evaluating the leukogram, immune response and serum biochemistry in all groups of animals. The result showed that propolis could enhance the antibody titer and improve cellular immune response. No statistically significant differences in serum total protein, albumin, AST, ALT activities, urea and creatinine levels were found between the control and treated groups. In conclusion, ethanolic extract of propolis administrated in combination with inactivated *Pasteurella multocida* vaccine was effective in improving the immune response with no adverse effects on the general health conditions in rabbits.

Introduction

Pasteurella multocida is an important bacterial pathogen which causes a common and widespread respiratory infection leading to great losses among rabbit populations **(Lu et al., 1991).** Prevention is the most likely and potential means to control pasteurellosis through vaccination which is critically important tool in preventing the disease **(Amina el-Bayomy et al., 1997).**

Recently, there has been increasing interest in the possibility of designing new ways for controlling infectious diseases by potential synergistic action of combined vaccine and immunostimulant to enhance the immune response **(Ashry and Ahmad 2012).**

Propolis is a compound formed by honeybees with their mandible gland secretion and various plants resinous. It contains many constituents, such as flavonoid, organic acid, aromatic alcohol, esters, amino acids and enzymes so that it has various biological activities including immune enhancement, antibiosis, antivirus, antioxidation, anticancer, antifatigue and hepatoprotection **(Sforcin, 2007)**. Propolis had been reported to have immunostimulator and immunomodulator activities, in addition to many different biological and pharmacological properties of its different preparations **(Abd El-Aziz et al., 2014)**.

Therefore, the present work was adapted to evaluate the synergetic effect of an ethanolic extract of Egyptian propolis in vaccination of rabbits with *Pasteurella multocida*, with reference to its effects on liver and kidney functions.

Materials and Methods

1. **Experimental Animals**:

A total of twenty clinically healthy male white New Zealand rabbits of 6 weeks old with average weight of 1500 gm were obtained from San El-Hagar Agriculture Company, Egypt. The animals were housed in clean separate metal cages and were fed on well balanced ration. Rabbits were kept at a constant environmental and nutritional condition throughout the period of experiment. The rabbits were not previously vaccinated against pasteurellosis and were left 10 days for acclimatization before the beginning of the experiment.

2. Vaccine:

A formalized killed polyvalent vaccine against *Pasteurella multocida* was obtained from Serum and Vaccine Research Institute, Abassia, Cairo, Egypt.

Dose and rout of vaccination: 1 ml/kg b.wt. S/C injection at age of 60 days, then booster dose after 21 days according to **Osama (1997).**

3- **Extraction of Propolis**:

The propolis adjuvant was prepared as previously described **(Paulino et al. 2002)**. Briefly, the propolis was ground and macerated with absolute ethanol for 10 days, agitation 10 min daily. Then, the solvent was evaporated and the resulting dried matter was dissolved in phosphate buffer solution (PBS, pH 6. 2), in a final concentration of 40 mg /ml. The dose of propolis used in this experiment was 50mg/kg b.wt. **(Turkez et al., 2010).**

4- **Experimental Design**:

The rabbits were assigned into four equal groups each of five rabbits. Treatment of different rabbit groups was as follows: group (1) was injected S/C with 2mL sterile phosphate buffer saline solution (PBS) and was kept as normal control, group (2) was injected S/C with a single dose of propolis(at dose of 50 mg/kg b.wt.), group (3) was vaccinated with *Pasteurella multocida* vaccine only(1ml/kg b.wt.), and group (4) was injected S/C with both the vaccine and propolis. Treatments of propolis and vaccine were repeated as a booster dose after three weeks.

5- Blood sampling:

Three blood samples were collected from each rabbit via ear vein at the end of the 2^{nd} and 4^{th} week post vaccination. Sample (1) was 1 ml of blood collected on EDTA for leukogram studies. Sample (2) was 2 ml of blood collected in a sterile plastic centrifuge tube containing heparin (50 IU/ml) to be used for cellular immune investigation. Sample (3) was 3 ml of blood taken without anticoagulant in a clean and dry centrifuge tube, left to clot at room temperature and then centrifuged at 3000 rpm for 15 minutes. The sera were collected for both biochemical and humoral immunological studies.

6- Leukogram studies:

Leukocytic and differential leukocytic counts were performed using automatic cell counter Sysmex 2000 iv.

7- Determination of Immunoglobulins titer:

Immunoglobulins (G and M) were measured by radial immune-diffusion (RIM) plates according to **Berne (1974).**

8- Cellular immune response:

a) Phagocytic % and index:

Peripheral blood mononuclear cells (PBMC) were isolated and resuspended in RPMI-1640 media according to **Goddeeris et al., (1986).** Phagocytic activity of viable leukocytic using heat inactivated *C.albicans* was determined according to **Wilikinson (1976).** The phagocytic activity is considered as the percentage of phagocytic cells by microscope field. The phagocytic index is the mean number of *C.albicans* ingested by one phagocytic cell.

b) Chemotaxis under agarose:

Chemotaxis and spontaneous migration of polymorphonuclear leukocytes were measured according to **Nelson et al., (1975).**

9- **Biochemical assessments**

Serum total protein was determined according to **Grant et al., (1987).** Serum albumin was determined according to **Doumas et al., (1981).** Serum globulin was calculated by subtraction of the obtained albumin level from total protein level. The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) were determined according to **Reitman and Frankel (1957)**. Serum creatinine and urea levels were determined according to **Henry (1974)** and **Tietz (1995)** respectively.

10- Statistical analysis:

The obtained data were statistically analysed by F-test according to **Tamhane and Dunlop (2000)** using MSTAT-C computer program. Means in the same column followed by different small letter were statistically significant and the highest values will be represented with the letter (a).

Results and Discussion

The successful vaccination depends on their association with potent adjuvant which can increase the immunogenicity of vaccine. A better adjuvant can activate specific effectors of the immune system and strengthen the humoral and /or cellular immune responses against that antigen **(Barr et al. 2006).** On the other hand, suitable adjuvant should have lower toxicity and side effects **(Aguilar and Rodriguez 2007)**. Therefore, it is urgent to develop a new adjuvant with high efficacy, safety and low cost.

Regarding the results of this study, the leukogram revealed leukocytosis in all the experimental groups except those treated with the propolis only (group 2) as shown in table (1). Leukocytosis was associated with heterophilia and lymphocytosis two weeks post vaccination and monocytosis four weeks post vaccination. The highest levels were recorded in group 4 treated with both propolis and *Pasteurella multocida* vaccine.

The vaccine caused increase utilization of heterophils as the *Pasteurella multocida* strain caused inflammatory condition and increased values of lymphocytes than normal due to antigenic stimulation **(Randa Hassan 1996).** Also, the obtained results may indicate an immune-stimulatory effect of propolis when combined with the *Pasteurella multocida* vaccine (**Dimov et al., 1991)**. It has been reported that propolis has a direct regulatory effect on the basic functional properties of immune cells **(Ansorge et al., 2003).**

The humeral immune response of rabbits revealed elevation of serum antibodies (IgG and IgM) in 3rd and 4th group which were more pronounced in 4th goup treated with propolis and *Pasteurella multocida* vaccine (Table 2). This finding agreement with **(Orsilik et al. 2005)** who found that the *Pasteurella multocida* vaccine with propolis increased the potency of the humoral immune response when compared to the *Pasteurella multocida* vaccine without propolis as suggested by **Cox and Coulter (1997)**.

Cellular immune response measured by the phagocytic % and index revealed positive stimulation at the 2nd to the 4th week post vaccination in group 3 and 4. The chemotaxis index results revealed significant increase in all groups with the highest values recorded in group 4 treated with propolis and *Pasteurella multocida* vaccine (Table 2).

Due to the high chemical complexity of propolis, it is extremely difficult to identify which substances are responsible for its biological activities (**Sforcin, et al. 2005**). Artepillin C which is one of propolis components has been described to activate the immune system by increasing phagocytic activity as well as number of lymphocytes (**Kimoto et al., 1998)**. Propolis extract may increase production of the lymphocyte activating factor IL-1 which enhances B- and T-cell proliferation (**Orsolic and Basic 2003**) and has potent effect on different cells of innate immune response **(Orsi et al., 2005).** CAPI which is one of propolis components increase T lymphocyte proliferation as well as secretion of IL-1 and IL- 2 by splenocytes **(Park et al., 2004).** Also, **Chu (2006)** mentioned that propolis could activate antigen presenting cells (e.g., macrophages) to produce cytokines which activate T and B lymphocytes.

Total protein values were non-significantly changed especially in the group treated with the *Pasteurella multocida* vaccine and propolis S/C, (group 4). The elevation was associated with significant increase of globulin values (table 3). Results pointed out to a non-specific immunostimulant effect of propolis as adjuvant to the *Pasteurella multocida* vaccine (**Onlen et al., 2007)** and a specific immune response induced by *Pasteurella multocida* vaccine (**Borkowska et al., 1997).**

Regarding serum enzyme activities, AST and ALT showed non-significant changes in all groups along the period of experiment in comparison with control group. No apparent change in serum AST activity due to single dose of propolis (100 mg) in rats **(Ali 1995)** and rabbits treated with crude propolis extract **(Alves et al., 2008)**.

Results of serum creatinine and urea revealed non-significant changes in all groups compared to control (table 3). Similar results were recorded by **Sforcin (2007)**, he found that propolis did not induce kidney damage in rats as demonstrated by normal levels of urea and creatinine. The demonstrated result in the present study revealed that administration of propolis had no toxic effect on rabbit.

In conclusion, the ethanolic extract of Egyptian propolis, when administrated in combination with formalized inactivated *Pasteurella multocida* vaccine in rabbits' enhanced specific and nonspecific immune response. The present experimental trial can encourage the use of propolis as an immunostimulant with human and animal vaccines.

Table (1) : Leukogram (mean ± S.E) in different experimental groups of rabbits received propolis and vaccine treatments. (n = 5 rabbits)

Group / Parameters	Two weeks post vaccination				Four weeks post vaccination			
	Group 1 control	Group 2 Propolis	Group 3 Vaccine	Group 4 Propolis + vaccine	Group 1 Control	Group 2 Propolis	Group 3 vaccine	Group 4 Propolis + vaccine
TLC 10^3/UL	$9.62^{C} \pm 0.58$	$11.18^{bc} \pm 0.45$	$11.98^{b} \pm 0.58$	$13.86^{a} \pm 0.54$	$9.76^{C} \pm 0.50$	$11.42^{b} \pm 0.43$	$12.32^{b} \pm 0.56$	$14.78^{a} \pm 046$
Heterophils 10^3/UL	$3.24^{b} \pm 0.19$	$3.58^{ab} \pm 0.14$	$3.86^{a} \pm 0.17$	$3.94^{a} \pm 0.26$	$3.29^{C} \pm 0.19$	$3.59^{bc} \pm 0.16$	$3.81^{b} \pm 0.12$	$4.42^{a} \pm 0.17$
Lymphocyte 10^3/UL	$5.42^{C} \pm 0.34$	$6.56^{bc} \pm 0.27$	$7.1^{b} \pm 0.43$	$8.85^{a} \pm 0.55$	$5.52^{C} \pm 0.35$	$6.84^{b} \pm 0.23$	$7.9^{ab} \pm 0.45$	$9.2^{a} \pm 0.54$
Monocytes 10^3/UL	$0.65^{a} \pm 0.03$	$0.69^{a} \pm 0.02$	$0.67^{a} \pm 0.04$	$0.69^{a} \pm 0.03$	$0.64^{b} \pm 0.036$	$0.71^{b} \pm 0.018$	$0.78^{ab} \pm 0.06$	$0.82^{a} \pm 0.035$
Eosinophil 10^3/UL	$0.20^{a} \pm 0.02$	$0.22^{a} \pm 0.01$	$0.23^{a} \pm 0.013$	$0.24^{a} \pm 0.014$	$0.20^{a} \pm 0.019$	$0.18^{a} \pm 0.01$	$0.16^{a} \pm 0.01$	$0.19^{a} \pm 0.014$
Basophil 10^3/UL	$0.11^{a} \pm 0.01$	$0.14^{a} \pm 0.01$	$0.12^{a} \pm 0.013$	$0.14^{a} \pm 0.01$	$0.11^{a} \pm 0.013$	$0.10^{a} \pm 0.011$	$0.12^{a} \pm 0.01$	$0.15^{a} \pm 0.01$

Means followed by different superscripts (a, b, c, d, e) within the same row are significantly different at $P < 0.05$.

Table (2) : immunoglobulins and cellular immunity (mean ± S.E) in different experimental groups of rabbits received propolis and vaccine treatments. (n = 5 rabbits)

Group / Parameters	Two weeks post vaccination				Four weeks post vaccination			
	Group 1 control	Group 2 Propolis	Group 3 vaccine	Group 4 Propolis + vaccine	Group 1 control	Group 2 Propolis	Group 3 vaccine	Group 4 Propolis + vaccine
IgG Mg/dl	2396 ^{C} ± 195.5	2443 ^{C} ± 189.6	3063 ^{b} ± 170.9	3683 ^{a} ± 160.5	2372 ^{C} ± 190.5	2450 ^{C} ± 182.4	3363 ^{b} ± 134.2	3861 ^{a} ± 162
IgM Mg/dl	273 ^{C} ± 15.5	281 ^{C} ± 6.9	328 ^{b} ± 14.8	388 ^{a} ± 13.6	264 ^{C} ± 16.4	284 ^{C} ± 14.2	348 ^{b} ± 14.6	402 ^{a} ± 16
Phagocytic %	71.2 ^{C} ± 2.7	76.0 ^{bc} ± 1.6	80.0 ^{b} ± 1.2	86.2 ^{a} ± 1.6	70.9 ^{d} ± 1.4	77.4 ^{C} ± 1.7	83.0 ^{b} ± 1.6	88.2 ^{a} ± 1.3
Phagocytic index	3.8 ^{b} ± 0.6	4.1 ^{b} ± 0.4	5.3 $^{a\,b}$ ± 0.5A	5.7 ^{a} ± 0.4	3.6 ^{b} ± 0.3	4.1 ^{b} ± 0.3	5.4 ^{a} ± 0.4	6.1 ^{a} ± 0.5
Chemotaxis index	1.126 ^{C} ± 0.034	1.28 ^{b} ± 0.033	1.3_{5} ^{b} ± 0.035	1.53 ^{a} ± 0.034	1.078 ^{d} ± 0.0338	1.291 ^{C} ± 0.038	1.41 ^{b} ± 0.034	1.68 ^{a} ± 0.05

Means followed by different superscripts (a, b, c, d, e) within the same row are significantly different at $P < 0.05$

Table (3) : Some biochemical parameters (mean ± S.E) in different experimental groups of rabbits received propolis and vaccine treatments. (n = 5 rabbits)

Group / Parameters	Two weeks post vaccination				Four weeks post vaccination			
	Group 1 control	Group 2 Propolis	Group 3 Vaccine	Group 4 Propolis + vaccine	Group 1 Control	Group 2 Propolis	Group 3 vaccine	Group 4 Propolis + vaccine
Total protein gm/dl	6.62 ^{a} ± 0.13	6.68 ^{a} ± 0.12	6.74 ^{a} ± 0.15	6.78 ^{a} ± 0.08	6.7 ^{a} ± 0.14	6.72 ^{a} ± 0.11	6.81 ^{a} ± 0.16	6.86 ^{a} ± 0.13
Albumin gm/dl	3.34 ^{a} ± 0.06	3.31 ^{a} ± 0.08	3.33 ^{a} ± 0.05	3.32 ^{a} ± 0.07	3.36 ^{a} ± 0.05	3.34 ^{a} ± 0.06	3.31 ^{a} ± 0.04	3.32 ^{a} ± 0.05
Globulin gm/dl	3.28 ^{b} ± 0.05	3.37 $^{a\,b}$ ± 0.08	3.41 $^{a\,b}$ ± 0.40	3.46 ^{a} ± 0.05	3.34 ^{C} ± 0.06	3.38 ^{bc} ± 0.08	3.5 $^{a\,b}$ ± 0.03	3.54 ^{a} ± 0.04
AST U/L	41.2 ^{a} ± 1.38	40.6 ^{a} ± 1.52	44.2 ^{a} ± 1.12	42.4 ^{a} ± 1.4	42.4 ^{a} ± 1.12	40.8 ^{a} ± 1.46	44.4 ^{a} ± 1.36	42.8 ^{a} ± 1.2
ALT U/L	44.6 ^{a} ± 1.4	43.8 ^{a} ± 1.5	47.2 ^{a} ± 1.6	44.2 ^{a} ± 1.08	45.2 ^{a} ± 1.2	43.2 ^{a} ± 1.6	47.4 ^{a} ± 1.8	44.8 ^{a} ± 1.14
Urea mg/dl	17.3 ^{a} ± 0.8	17.26 ^{a} ± 0.7	18.6 ^{a} ± 0.9	18.1 ^{a} ± 0.6	17.36 ^{a} ± 0.9	17.33 ^{a} ± 0.8	18.92 ^{a} ± 0.96	18.14 ^{a} ± 0.82
Creatinine Mg/dl	1.26 ^{a} ± 0.11	1.25 ^{a} ± 0.09	1.3 ^{a} ± 0.08	1.28 ^{a} ± 0.07	1.3 ^{a} ± 0.12	1.29 ^{a} ± 0.08	1.33 ^{a} ± 0.09	0.31 ^{a} ± 0.08

Means followed by different superscripts (a, b, c, d, e) within the same row are significantly different at $P < 0.05$

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