The Lapin Immune States Associated with Intramuscular Injection of Heat Killed *Helicobacter pylori*

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Abstract

Nowadays, *Helicobacter pylori* is considered to be the most common human bacteria in the pathogenesis of chronic gastritis and peptic ulcer diseases. The immunogen administered to an animal may have a profound on the outer come of the immune responses. Four successive doses of heat killed *H.pylori* (HpHK) bacteria was intramuscular injected in rabbits at five days a part manner. HpHK bacteria stimulate specific mucosal and systemic humoral and cellular immune responses determined by raised in the immunoglobulin titers and concentrations, increased the concentrations of C_3 and C_4 which are the complement compartments with significant increase in the lymphoid cells that formed the rosette form , significant migration inhibition index , increased the concentrations of cytokines (IL-4 & IL-8) . HpHk induced positive skin test tuberculin type delayed hypersensitivity, in addition to the histopathological changes at different body organ portions of the animals.

Key words : *Helicobacter pylori* . antigenicity .immune response.

الخلاصة

تعتبر جرثومة اللولبية البوابية من المسببات الشائعة في إحداث أمراض التهاب المعدة المزمن و قرحة المعدة في الإنسان في الوقت الحاضر، ووجد أن تجريع البكترين المقتول بالحرارة والمحضر من هذه الجرثومة للحيوانات المختبرية له تاثير واضح على الاستجابة المناعية ، حيث تم حقن أربع جرع من هذا البكترين عن طريق العضلة في الأرانب المعاملة وعلى فترات زمنية مختلفة ، أذ حفز هذا المستضد استجابة مناعية جهازية وموضعية خلطية وخلوية لوحظت من خلال الارتفاع في معدل تركيز و عيار الأضداد المتخصصة وارتفاع في تركيز مكوني المتمم C_3 و C_4 في المصل مع حدوث زيادة في عدد الخلايا اللمفية المكونة للتشكل الزهري وحدوث تثبيط في هجرة الخلايا البيض في الأوعية الشعرية بوجود الممنع المحفز بالإضافة إلى زيادة تركيز بعض الحركيات الخلوية . كما لوحظ إن هذا البكترين يعمل عمل ارجين يحفز فرط الحساسية المتأخر في الجلد من النوع التيوبركليني بالإضافة إلى حدوث التغيرات المرضية النسجية في الأعضاء المختلفة المأخوذة من أرانب الاختبار.

الكلمات المفتاحية : جرثومة اللولبية البوابية .المستضدية . الأستجابة المناعية .

Introduction

Gastric or duodenal ulcers (commonly referred to as peptic ulcers) are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa. Gastric ulcers mostly occur along the lesser curvature of the stomach, in particular, at the transition from corpus to antrum mucosa **(**Dixon, 2001; Marshall *et al.*, 1985) found that ulcer risk is greater in asymptomatic people infected with *H. pylori*. Shirin *et al.* (2008) found that the importance of regulatory T cells (Treg) and Treg cytokines (transforming growth factor [TGF]-beta and interleukin-10) in the reduction of inflammation and ulceration induced by *H. pylori* .

Intramuscular injection with an immunogen prepared from *H. pylori* resulted in cell – cell cooperation could induced the formation of high endothelial venule from the post-capillary venules which served as portal of entry of circulating lymphocytes to the vessels in the site that join to an immune response . Thus, lymphocytes and other reactive cells can accumulate wherever they needed at muscle. If the response is sufficient , intense and prolonged , the assembled cells may arranged in spatial

manner that resembles a permanent lymphoid organs complete with secondary lymphoid follicle **(**Stites *et al.*, 1994). Lymphocytes (both T cells and B cells), macrophages, neutrophils, mast cells, and dendritic cells (DCs) are usually present (Suzuki *et al*., 2002). This immunogen cause mucosal as well as systemic humoral & cellular immune responses , till now little references regarding the immune status of the lapin animal primed with this bacteria and the time of development of these responses (Kuster *et al.*, 2006 ; Aguemon *et al*., 2004).

Objectives

The aim of this study is the compare between mucosal and systemic humoral and cellular immune responses in rabbits after intramuscular injection of heat killed *H. pylori* (HpHK) bacteria at different days.

Materials & Methods

1- Antigens:

Heat killed *H.pylori* (HPHK) bacteria was prepared from 24 hour brain heart infusion agar plate culture then we add 6 ml of normal saline to the plats scrape , collect the solution and put it in centrifuge at 4000 rpm for 5 min. , double wash were done with normal saline then compared it with standard opacimeter (WHO) to obtain the concentration equals to 10 IU\ml . The suspension tubes were used after put them in water path at 60 degrees for 30 mints to kill the bacteria and obtained the antigen that was used in immunization the rabbits after done the sterility test . A cell free culture filtrate antigen was also prepared from microaerophilic 72 hour brain heart infusion broth culture of *H. pylori* **(**Svanborg-Eden *et al*., 1985 ; Sachse *et al.*, 2005**)**

2- Animals:

Two groups, each of two rabbits *Oryctolagus cuniculus* were elected , adapted to laboratory conditions and housed under standardized conditions , one served as test & other as control group **(**Schneider *et al.*, 1990**)**

3- Immunization protocol:

Four successive doses of (HpHK) bacteria were injected via intramuscular route into tested rabbits through four weeks , each dose about 2 ml of bacterin that had 10 IU\ml concentration . Control animals received sterile normal saline in same protocol and this protocol was specific for this research.

4- Mucosal samples and immunoglobulines separation:

Gut mucosal samples were obtained from three parts of gut mucosa included esophageous , stomach and duodenum in addition to spleen which is used as lymphoid organ. Then the immunoglobulins were separated from these parts according to **(**Shnawa and Abid, 2005**)**.

5- Blood Samples:

Blood with anticoagulant was processed for E-rosette test and for migration inhibition test. Coagulated blood were collected for the others immunological tests that include: tube agglutination test , measure the concentrations of IgG , IgM ; IL-4 and IL-8 that detected by ELISA test in addition to the concentrations C3 , C4. (Garvey *et al*., 1977**).**

6-Immunolog

Agglutination test was performed by micro titration method, anti *H. pylori* IgG, IgM antibodies were detected by using specialized kits were provided from the (DRG, USA) company . The C_3 and C_4 components were determined by C_3 and C_4 proteins , E-rosette , Capillary Migration Inhibition were performed as in **(**Garvey *et*

al., 1977**)**, IL-4 and IL-8 cytokines were assayed using ELISA kits (provided from RayBio, USA, Company), skin delayed type hypersensitivity was done as in **(**Shnawa and Abid, 2005**).** Histopathology was carried out as in **(**Kuster *et al.*, 2006**).**

Results and Discussion:

The exact correlation between the common mucosal immune compartments and the attributes of the systemic immune system are conflicting subject or it is somewhat unclear. The correlation between local and systemic immune responses affected by several factors includes : immune gene nature , host susceptibility to replicating or non replicating immunogens, nature of immunization protocol, nature of the host immune system and type of experimental design (Brandtizage and Frasted, 1999**)**. *H. pylori* possess several immunogenic subfractions , such immunogens stimulate B cells , T cells as well as the subset Tdh responsible for hypersensitivity **(**Velin *et al*., 2004 ; Choi *et al.*, 2011 ; Michetti, 2011**)** .

We studied the development of immune responses at five days (8 , 12 , 16 , 20 and 27) after immunization with HPHK bacterin via intramuscular route that stimulates *H . pylori* specific humoral systemic and mucosal immunoglobulins titers (Table 1) , also it played an important role in the increased the concentrations of IgG and IgM antibodies in serum and mucosal secretions of immunized rabbits (Table 2). The concentration of C_3 and C_4 which are the complement compartments were significantly increased in the rabbits sera (Table 3).

The cellular systemic and mucosal immune responses were represented by significant increase in the lymphoid cells that formed the rosette form , significant migration inhibition index with more than 30% inhibition as well (Table 4), furthermore, the HPHK bacterin able to inducing tuberculin type delayed hypersensitivity, Hence, their epitopes can be of T dependent type through the activation of Th1 and Th2 (Table 5) **(**Kayaselcuk *et al*., 2002 ; Velin *et al.*, 2004 ; Michetti, 2011**).**

IL-4 is one of anti-inflammatory cytokines and IL-8 is a pro-inflammatory cytokine were be detected in serum and mucosal secretions of rabbits with significant increase in their concentrations (Table 6) . Thus *H. pylori* antigens were B and T cells dependent types **(**Ferrero, 2005; Svennerholm and Lundgren, 2007**).**

The histopathological study was showed the splenic reactive hyperplasia in red pulp in the days 8 and 12 respectively in addition to the presence of large number of lymphocytes that converted to the plasmocytes (Picture 1) , increased the surface area of the pits of mucosal layer of rabbit's stomach, also we have seen the infiltration of neutrophils to the lamina propria of the stomach (Picture 2), with incomplete of epithelial column of the pits of the small intestine (Duodenum) in addition to the infiltration of lymphocytes , polymorphonuclear cells at mucosal layer of the intestine (Picture 3) while the esophageous had been normal in all treated rabbits .

Table (1): Titers of antibodies in rabbits immunized with killed *H. pylori*

Titer						
	Days					Control
Types of immune response	8	12	16	20	27	
Humoral systemic(serum)	40	640	1280	1280	2560	10
Humoral mucosal						
Esophageous	4	16	32	32	64	1
Stomach	32	64	128	256	512	
Duodenum	8	16	32	64	128	

Table (2): Concentrations of anti-IgG and anti-IgM (IU\ml) in rabbits immunized with killed *H. pylori*

Types of immune response	IgG concentration(Iu\ml) M±S.D.					IgM concentration(Iu\ml) M±S.D.				
	Days					Days				
	8	12	16	20	27	8	12	16	20	27
Humoral systemic(serum)	3.931± 0.013	4.136± 0.002	4.341± 0.004	4.551± 0.008	4.551± 0.007	3.494± 0.006	3.573± 0.002	3.813± 0.096	3.973± 0.001	3.973± 0.001
Humeral mucosal										
Esophageous	3.723± 0.003	3.726± 0.007	4.136± 0.002	4.137± 0.002	4.373± 0.209	3.021± 0.014	3.019± 0.000	3.813± 0.124	3.975± 0.012	3.973± 0.083
Stomach	3.928± 0.007	4.136± 0.003	4.136± 0.002	4.343± 0.203	4.346± 0.004	3.177± 0.000	3.336± 0.002	4.131± 0.004	4.342± 0.002	3.813± 0.004
Duodenum	3.721± 0.002	3.722± 0.006	4.140± 0.196	4.434± 0.284	4.766± 0.138	3.020± 0.002	3.335± 0.006	3.813± 0.089	3.814± 0.021	3.636± 0.053
Control	4.076± 0.036					3.638± 0.001				

Table (3) Concentrations of C_3 and C_4 in rabbits sera immunized with killed *H. pylori*

Days	C_3 concentration (mg\dc) M±S.D.	C_4 concentration (mg\dc) M±S.D.
8	151.600±3.252	36.500±0.989
12	151.600±3.252	47.950±1.060
16	200.600±3.677	54.100±1.131
20	216.450±3.747	55.700±1.131
27	255.400±4.101	73.600±2.545
Control	110.500±0.000	37.200±0.000

Table(4): Percentage of E-rosette and LIF in rabbits immunized with killed *H. pylori*

Cellular response	Percentage of E-rosette(%) M±S.D.					Percentage of LIF(%) M±S.D.				
	Days					Days				
	8	12	16	20	27	8	12	16	20	27
Systemic	28.000± 1.272	30.000± 0.212	32.250± 0.565	33.000± 0.141	34.600± 0.777	91.150± 0.070	90.700± 0.565	88.550± 0.494	75.900± 1.484	68.500± 1.202
Mucosal										
Esophageous	28.300± 0.282	30.750± 0.070	33.050± 1.060	33.250± 0.212	34.700± 0.141	89.300± 0.141	88.550± 0.212	85.100± 0.282	73.300± 0.494	60.000± 0.494
Stomach	29.650± 0.070	32.950± 0.636	35.150± 0.636	35.100± 0.707	35.200± 0.565	79.300± 1.697	75.400± 1.414	72.250± 0.919	68.300± 0.282	64.750± 0.636
Duodenum	29.500± 0.565	31.95± 0.070	33.750± 1.060	34.450± 0.636	35.400± 0.707	79.400± 1.555	75.350± 0.353	73.900± 0.282	70.950± 0.212	67.400± 1.131
Control	27.500± 0.427					93.500± 0.427				

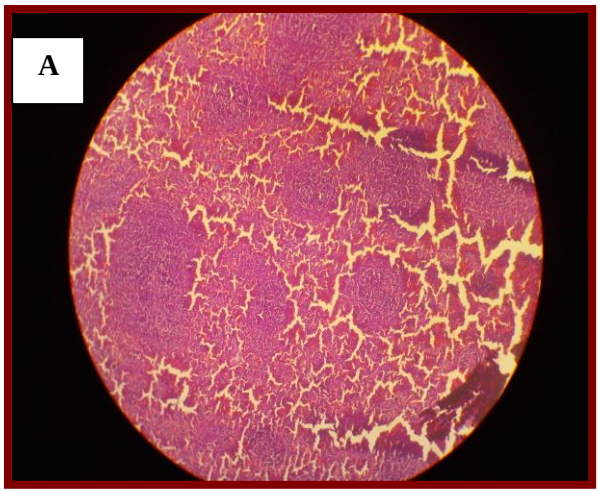
Table (5): Skin test of rabbits immunized with killed *H. pylori*

Date of inoculation CFC \ Days	Skin test \ Hours					Immune reaction
	6	18	24	48	72	
6	-	E	EIN	EIN	EIN	Notes
	-	-	15	15	15	Reaction area (mm)
10	-	E	EI	EIN	EIN	Notes
	-	-	22	22	22	Reaction area (mm)
14	-	E	EI	EIN	EIN	Notes
	-	-	21	21	21	Reaction area (mm)
18	-	E	EI	EIN	EIN	Notes
	-	-	21	21	21	Reaction area (mm)
25	-	E	EI	EIN	EIN	Notes
	-	-	16.5	16.5	16.5	Reaction area (mm)

N- Necrosis, I- Induration, E- Erythema.

Table (6): Concentrations of IL-4 and IL-8 (pg\ml) in rabbits immunized with killed *H. pylori*

Types of immune response	IL-4 concentration(pg\ml) M±S.D.					IL-8 concentration(pg\ml) M±S.D.				
	Days					Days				
	8	12	16	20	27	8	12	16	20	27
Humoral systemic(serum)	8.908± 1.151	8.908± 0.018	14.020± 0.021	14.020± 0.159	44.904± 0.183	18.532± 0.186	18.582± 0.632	20.092± 0.188	20.916± 1.134	21.188± 0.031
Humoral mucosal										
Esophageous	4.032± 0.035	6.292± 0.132	8.200± 0.118	9.176± 0.053	7.732± 0.046	17.884± 3.618	19.888± 0.957	23.864± 2.132	26.568± 0.345	28.200± 0.162
Stomach	6.542± 0.603	7.160± 0.062	8.212± 0.154	9.176± 0.031	7.272± 0.066	18.252± 0.667	21.564± 0.323	26.568± 0.137	29.628± 0.963	43.116± 0.758
Duodenum	5.316± 0.178	6.932± 0.097	7.092± 0.123	7.156± 0.038	7.092± 0.097	21.564± 0.473	21.720± 2.015	24.900± 0.035	24.912± 0.489	28.188± 0.261
Control	7.004± 0.012					19.814± 0.050				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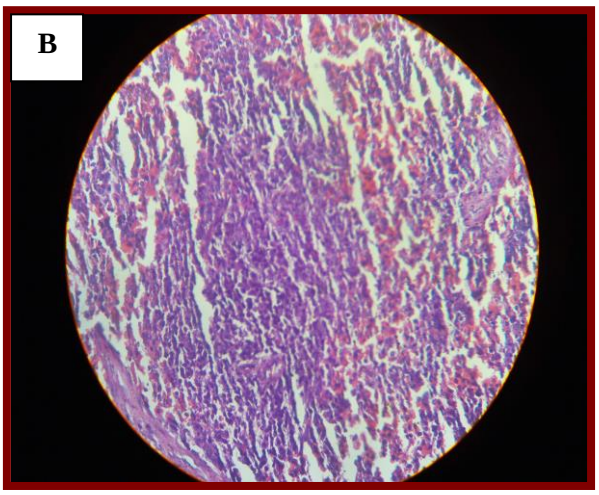
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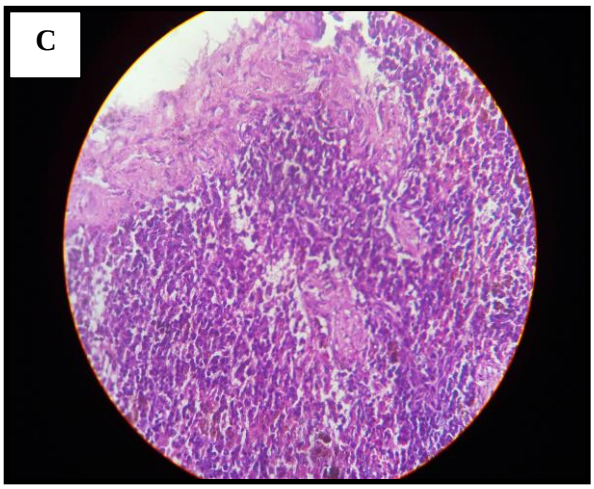
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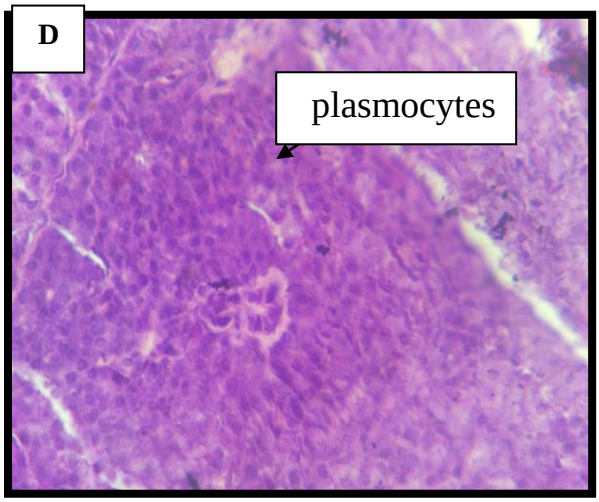
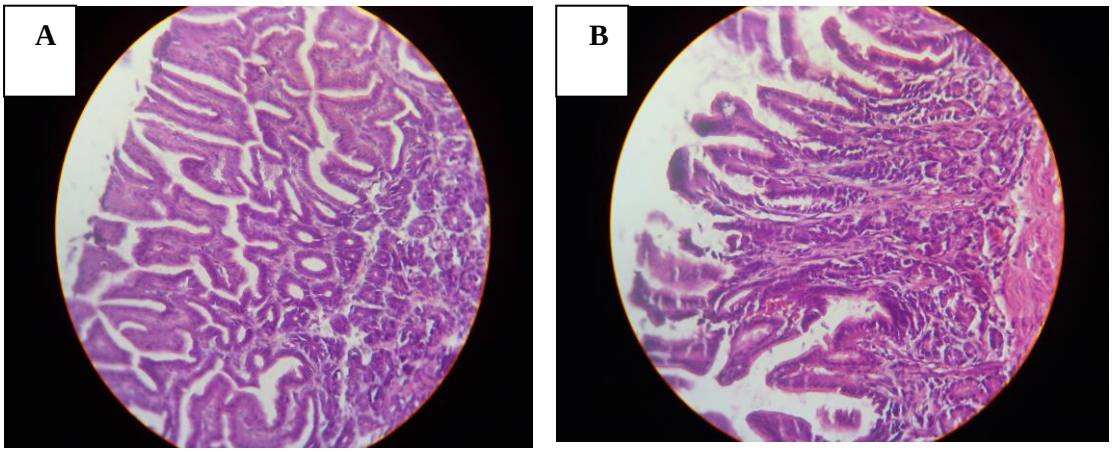
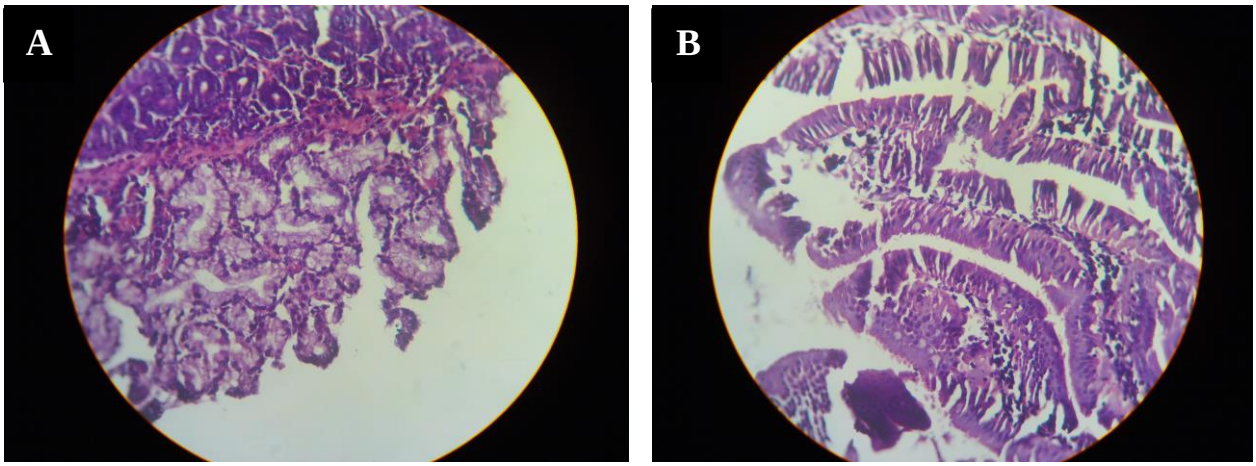

D
plasmocytes

Picture (1): Section in spleen of rabbits immunized with killed *H. pylori*
A- control grope , B- & C- test grope, D- plasmocyte(100X, 400X, 1000X)



Picture (2): Section in Stomach of rabbits immunized with killed *H. pylori*
A- Test group , B- Control group (400X)



Picture (3) Section in Duodenum of rabbits immunized with killed *H. pylori*
A- Test group , B- Control group (400X)

Conclusions

According to the results of this study we can conclude that serum & mucosal anti *H. pylori* antibodies elevated, serum C_3 & C_4 levels , lymphocytes & their secretion of cytokines were correlated to the status of *H. pylori* immunity & protection against recurrent infections.

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