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Full Length Research Paper

Oral immunization with silkworm pupae powder containing recombinant UreB of *Helicobacter pylori* affords prophylactic and therapeutic effects against *Helicobacter pylori* infection in mice

Xiaolin Zhang[1,2], Shitang Ma[1], Meisong Qin[1], Guoliang Zhou[1], Lili Zhou[1], Yanxi Fang[1], Hao Yu[1], and Chengliang Gong[2]*

[1]Anhui Science and Technology University, Fengyang 233100, China.
[2]School of Pre-clinical Medicine and Biological Science, Medical College, Soochow University, Suzhou, 215123 China, People's Republic of China.

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To study whether silkworm pupae powder (SPP) containing recombinant *Helicobacter pylori* urease B subunit (rUreB) could show immune prophylactic and therapeutic effect against *H. pylori* infection using a mouse model. The findings demonstrated that the mice were protected significantly from *H. pylori* infection based on the results of biopsy urease tests, biopsy bacterial culture and polymerase chain reaction (PCR) test with biopsy specimens of mice stomachs and histological grade assessment of *H. pylori* colonization and gastritis with tissue section stains. The colonization of *H. pylori* was obviously decreased and the gastritis was significantly improved after oral vaccination with SPP containing rUreB, which increased the content levels of specific anti-urease serum IgG for both prophylactic and therapeutic vaccination protocols. These results showed that SPP containing rUreB could be used as direct edible antigen in the development of an oral vaccine against *H. pylori* infection.

Key words: *Helicobacter pylori* urease B subunit (rUreB), prophylactic and therapeutic effect, oral vaccine.

INTRODUCTION

Helicobacter pylori are a microaerophilic, spiral and Gram-negative bacillus first isolated from a patient with chronic active gastritis in 1982 (Marshall et al., 1984). *H. pylori* are one of the most widespread human pathogens. It has been recognized as a human-specific gastric pathogen that colonizes the stomaches of over half the world's population, where 90% or more of the population usually acquires the infection early in life in developing countries (Thomas et al., 1999). This pathogen is a major cause of chronic gastritis, gastric and duodenal ulcers, and gastric carcinomas, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma, and is considered as a class I carcinogen by the World Health Organization (Nakamura et al., 2001; Uemura et al., 2001; Casella et al., 2001; Graham, 2000).

Treatment for *H. pylori* infection includes a combination of a proton pump inhibitor and two antibiotics. However, side effects, poor compliance, antibiotic resistance, high cost and recurrence of infection make world-wide eradication through antibiotic drug therapy problematic. To overcome the drawbacks of the antibiotic pharmacological treatment, Vaccine development against this infection appears to be a preferable strategy. Vaccine has been applied successfully as a potential therapeutic strategy for preventing and treating *H. pylori* infection in mice and human (in China) (Xiaolin et al., 2011).

The challenge lies in the establishment of long-term

*Corresponding author. E-mail: zhangxiaolin8@126.com or gongcl@suda.edu.cn. Tel: +86-512-65880183. Fax: +86-512-65880183.

oral immunity to protect of *H. pylori* infection has not yet to be reported. The ability to induce a long-term immunity to *H. pylori* is necessary for the repeated administration of antigens. The potential therapeutic applications in humans would be limited by the high cost using expensive fermentation techniques and stringent purification protocols and the low expression production levels of the available expression technologies such as prokaryotes, cultivated mammalian cells and transgenic animals and plants. Therefore, the recombinant therapeutic protein production becomes a major bottleneck for utilization.

In other hand, the silkworm bioreactor has showed its advantages such as high expression efficiency and low feeding cost, natural activity for its expressed products and safety for both environment and human (Maeda, 1994). Therefore, it is very promising to use the silkworm as vector for industrial large-scale and low cost mass production (Choudary et al., 1995). In our previous reports, we used a practical *Bombyx mori* nuclear polyhedrosis virus (BmNPV) bacmid system to express the urease B subunit (rUreB) protein in silkworm pupae by the recombinant bacmid baculoviruses successfully (Xiaolin et al., 2011). In this paper, we further investigated whether SPP containing rUreB possessed immune prophylactic and therapeutic effect against *H. pylori* infection and used it as an edible vaccine against *H. pylori* infection by mouse animal model.

MATERIALS AND METHODS

Antigen preparation for immunization

Preparation of SPP containing rUreB of *H. pylori* which was expressed according to our previous report (Xiaolin et al., 2011). The pupa expressed UreB 96 h post-inoculation with recombinant virus (rBacmid/ BmNPV/*UreB*) were collected and dried with a vacuum dryer under low temperature of -50°C. The dried pupae were homogenized to powder and stored at -20°C up to use.

Animal and breeding conditions

Specific-pathogen (*H. pylori*)-free (SPF) female BALB/c mice were purchased from animal experiment center of Medical College, Soochow University, China and used between the ages of 8 and 12 weeks. The animals were housed in polycarbonate cages and fed on a commercial pellet diet with water ad libitum. All procedures were conducted in accordance with the P. R. China legislation under No. 8910M047 on the use and care of laboratory animals and with the guidelines established by the Institute for Experimental Animals of Soochow University and were approved by the university committee for animal experiments.

Growth of *H. pylori* for infection of mice

Two clinical strains of *H. pylori* was isolated from two patients with gastric ulcer and gastric cancer, which were obtained from the Jiangsu University (Zhengjiang, China), were cultured on brain heart infusion (BHI) agar plates supplemented with 7% horse blood, trimethoprim (5 µg/ml), polymixin B (5 µg/ml) and vancomycin (10 µg/ml) under microaerophilic conditions at 37°C for 3 to 4 days. The bacteria were collected in 0.2 M sodium bicarbonate (SB) buffer (pH9.0) and diluted to 1×10^8CFUs/ml before inoculation.

Prophylactic vaccination

The mice were randomly divided into three groups. The control group (n = 10) received 0.5 ml 0.2 M SB, the SPP + CTB group (n = 10) received 0.5 ml 0.2 M SB + 200 mg SPP without containing rUreB + 10 µg cholera toxin B-subunit (CTB) which could be used as a mucosal immune adjuvant; the immune protection (SPP + rUreB + CTB) group (n = 10) administrated through feeding with 0.5 ml of 0.2 M SB + 200 mg SPP containing 1 mg rUreB +10 µg CTB. Mice were administered by gavage, using a ball-end feeding needle. Each mouse of groups was immunised orally with an above-mentioned dose on day 0 and booster doses on days 10 and 20. In 10th day after the final immunisation, each mouse was challenged with three doses of 0.3 ml (10^8 CFU/ml) of *H. pylori* diluent at 2-day intervals. Mice were sacrificed at 6 weeks after receiving the last immune protection.

Therapeutic vaccination

Mice were challenged with *H. pylori* as it was earlier. Four weeks after completion of challenge, mice were immunised four times at a week intervals. The control group (n = 10) received 0.5 ml 0.2 M SB; the SPP + CTB group (n = 10) received 0.5 ml 0.2 M SB + 200 mg SPP without containing rUreB + 10 µg CTB; the immune treatment (SPP + rUreB + CTB) group (n = 10) received 0.5 ml 0.2 M SB + 200 mg SPP containing 1 mg rUreB + 10 µg CTB. Mice were sacrificed at 4 weeks after receiving the last immune treatment.

Sampling and evaluation of *H. pylori* infection

At each experiment, mice were euthanized with a suitable dose of pentobarbital sodium solution. Blood was taken by heart puncture and gastric tissue was taken for further testing. Each stomach was dissected into three equal longitudinal sections which containing body and antrum. The prophylactic group biopsy specimens were collected for urease tests, bacterial culture and PCR test. The basic urease assay was used as the method had been developed by Hazell et al. (1987) which are commonly used by many laboratories worldwide. Urease positivity determined by an increase in pH, indicated by a colour change from orange to red. Bacterial culture was conducted as described previously (Handt et al., 1997, 1995) and plated on solid GAB-Camp medium and incubated for 48 h at 37°C under microaerophilic conditions. *H. pylori* were identified based on their characteristic morphology by Gram staining under microscope, and positive for urease and oxidase activities. PCR amplification identification of *H. pylori* was performed as described previously (Monteiro et al., 1997). The forward primer contained the sequence 5' (AAGCTTTTAGGGGTGTTAGGGGTTT)-3' and the reverse primer the sequence 5'(AAGCTTACTTTCTAACACT ACGC)3', derived from the *H. pylori ureC* gene sequence (accession numbers, EMBL X57132 and GenBank M60398), which amplified that a 249-bp DNA fragment (Monteiro et al., 1997) PCR was done under the following conditions: preheating at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis.

In the therapeutic group, one tissue strip from each stomach was fixed by formalin and embedded in paraffin after cutting from the stomach. Then, the tissues were cut and stained by the Giemsa

Table 1. *H. pylori* urease test, culture and PCR test from mouse gastric biopsies.

Oral immunised with	Animal number	Urease test (positive) (%)	Culture (positive) (%)	PCR test (positive) (%)
SB(control) (prophylactic, therapeutic vaccination)	10	10 (100)	10 (100)	10 (100)
SSP + CTB (prophylactic, therapeutic vaccination)	10	10 (100)	10 (100)	10 (100)
SSP + rUreB + CTB (prophylactic vaccination)	10	1 (10)	2 (20)	2 (20)
SSP + rUreB + CTB (therapeutic vaccination)	10	7 (70)	7(70)	7 (70)

techniques for assessment of *H. pylori* colonization. The degree of colonisation in *H. pylori* infected mice was assessed by semi-quantitative analysis of blinded slides, whereby bacterial colonisation was graded from 0 to 4 where 0 = no bacteria, 1 = 1 to 2 bacteria/crypt, 2 = 3 to 10 bacteria/crypt, 3 = 11 to 20 bacteria/ crypt, 4 = >20 bacteria/crypt (Nolan et al., 2002; Genta et al., 1994). The reminder of the stomachs were cut and stained with hematoxylin and eosin (HE) and analyzed for *H. pylori* associated inflammation. For evaluation of gastritis, HE-stained sections were scored based on the degree of infiltrating lymphocytes, plasma cells, and neutrophils (Guy et al., 1998). The scoring grades were defined as follows: 0, none; 1, a few leukocytes scattered in the deep mucosa; 2, moderate numbers of leukocytes in the deep to mid mucosa and occasional neutrophils in the gastric glands (microabscesses); 3, dense infiltrates in the deep to mid mucosa, a few microabscesses; and 4, dense, diffuse infiltrates throughout the lamina propria and into the submucosa, frequent microabscesses. The other two biopsy specimens were collected for urease tests, bacterial culture and PCR test as above.

Antigen-specific antibody assays

Serum samples, collected from the heart of all mice, were used to titrate humoral responses against *H. pylori* UreB. Serial dilutions of individual sera were tested in enzyme-linked immunosorbent assays (ELISAs). The determination was performed as our previous report (Xiaolin et al., 2011).

Statistical analysis

The primary analysis was a comparison between immunised groups and mock controls. All data were presented as mean ± SEM. With all statistical analyses by t-test, differences between groups were considered statistically significant for $P < 0.05$.

RESULTS

Prophylactic and therapeutic vaccination protects mice from *H. pyl*ori infection

Based on the results of the urease test, bacteria culture and PCR test, mice immunised with SSP + rUreB + CTB were protected against *H. pylori* infection, compared with the control and SPP + CTB group (Table1). Bacteria culture identification: after 48 h culture of tissue homogenates in GAB-Camp medium, individual, pinpoint, clear colonies were observed. Colonies were confirmed as *H. pylori* if they were urease, catalase and oxidase positive and if the cells were Gram negative and had the characteristic curved-rod appearance during microscopical examination. By bacteria culture identification, two out of 10 (20%) of the 10 BALB/c mice orally immunised with SSP + rUreB + CTB, while all 10 mice (100%) of the feeding with SPP + CTB group were positive for *H. pylori*. However, by urease test assay,10 mice (100%) were positive for the body and the antrum from all animals of the control and SPP + CTB group, but only one animal (10%) of the SSP + rUreB + CTB group had evidence of the presence of *H. pylori*, as determined by this test. The PCR assay successfully amplified a DNA fragment of the expected 249 bp with the *ure*C primers. When genomic DNAs prepared from the gastric tissue specimens were used as templates. In the immunised with SSP + rUreB + CTB group, 8 animals were found to be free of the pathogen, 20% animals were infected. While in the control and SPP + CTB group PCR test showed that all the animals (100%) were positive. However, in immune therapeutic group, only 30% healing rates were obtained from *H. pylori*-infected mice after treatment with SPP + rUreB + CTB (Figure 1 and Table 1).

The numbers of *H. pylori* from tissue Giema stain and *H. pylori* histological colonisation assessment were significantly decreased and in mice treated with SPP + rUreB + CTB compared with *H. pylori*-infected control and treated with SPP+CTB animals ($p < 0.01$). There was no significant difference between the control animals and treated with SPP+CTB animals. The infected control and treated with SSP+CTB mice showed severe inflammation in the stomach when compared with normal control mice 6 weeks after inoculation ($p<0.01$). Treated with SSP + rUreB + CTB mice showed a significant decrease in inflammation score compared with infected control ($p < 0.01$) and oral immunised with SSP + CTB animals ($p<0.05$), but still had a significantly higher inflammation score than non-*H. Pylori*- inoculated normal control animal ($p<0.05$).The *H. pylori* histological colonisation assessment and histopathological analysis of these different groups of mice is illustrated in Figures 2 and 3.

H. pylori urease-specific serum IgG response

Serum IgG anti-H. pylori urease-specific antibodies were detected at 6 weeks post the last inoculation with *H. pylori* in the prophylactic treatment route and at 4 weeks

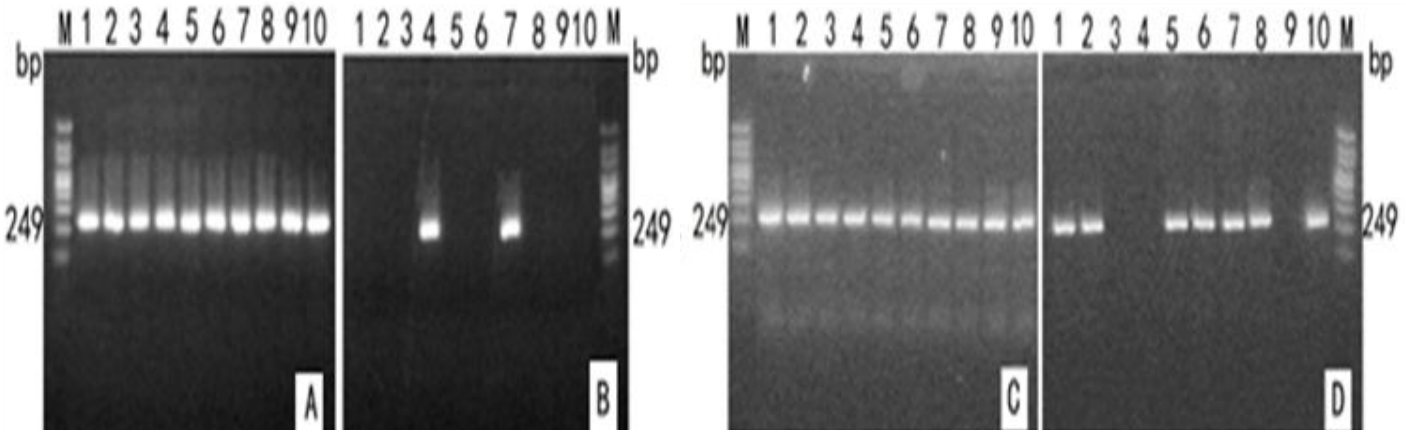

Figure 1. PCR detection of *H. pylori* UreC fragment in the body and antrums of the stomach of mice. Immune prophylactic group: (a) immunised with SSP + CTB, (b) immunised with SSP + rUreB + CTB; immune therapeutic group: (c) immunised with BS, (d) immunised with SSP + rUreB + CTB.

post the last vaccination with SSP + rUreB + CTB, SSP + CTB, SB (control mice) in the therapeutic route. In the two protocols, there were significantly higher levels of specific antibodies IgG, in the group treated with SSP + rUreB + CTB than in the mice vaccinated with SSP+ CTB and control mice ($p<0.01$), but no significant differences between the control mice and immunised with SSP + CTB mice (Figure 4). These results suggested that vaccination with SSP + rUreB + CTB enhanced the immune response and *H. pylori* urease-specific anti bodies might have contributed to the protection of SSP containing rUreB vaccinated mice. Interestingly, there were higher levels of specific UreB antibodies IgG in the group treated with SSP + CTB in mice received *H. pylori* challenge ($p<0.05$). The results might be that mouse stomach *H. pylori* induced immune responses and CTB adjuvant enhanced this effect (Figure 4).

DISCUSSION

Although an effective oral vaccine (UreB + LTB) of protection H. pylori infection has been fully developed and approved for production in China, its anti-infection protective time is a year and protective rate is 72% (Xiaolin et al., 2011). It is most important to achieve a long-term effective and low cost vaccine so that man can be immunised widely and eliminate *H. pylori* from the world at last. This study aims to investigate whether silkworm expressing recombination urease B subunit of *H. pylori* can possess prophylactic and therapeutic effects against *H. pylori* infection in BALB/c mice model. Many studies have demonstrated that *H. pylori* in infected mouse stomach can be cleared by oral immunization with *H. pylori* urease (Guy et al., 1998; Kleanthous et al., 1998; Ermak et al., 1998; Corthesy-Theulaz et al., 1995). The present findings show that silkworm pupae powder (SPP) containing rUreB prevented *H. pylori* from infection and its protective rate was 80% in immune prophylactic group. Our results show that SSP containing rUreB decreased the gastric colonization of *H. pylori* and eased the gastritis, which had 30% healing rates in the mouse model (Figures 3 and 1).

The culture is considered “Gold standard” of *H. pylori* diagnosis (Attallah et al., 2004), but this method has certain limitations which can contribute to a decrease in its sensitivity, such as demanding conditions for transportation and culture. The urease test is convenient and fast; however, it can fail in *H. pylori* detection, especially if there are few bacteria. PCR is recognized as a promising method for detection of *H. pylori* in gastric biopsy specimens (Thoreson et al., 1995; Clayton et al., 1992; Hammar et al., 1992), its sensitivity reaches examination of the smallest number of 8 to 10 *H. pylori* in gastric biopsy specimens. After amplification, polymerase chain reaction (PCR) products are usually detected by gel electrophoresis (Figure 1). In this study, three methods of diagnosis are conducted to determine the result of *H. pylori* infection (Table 1). Through comparison of histological assessment of *H. pylori* colonization and gastritis grades, we feel it is likely that the low number of bacteria remaining after a therapeutic effect will be of little consequence to the host. This is supported by the finding that the grade of *H. pylori* colonisation in humans was found to be closely related to the grade of gastritis and neutrophil infiltration (Figures 2 and 3) (Bayerdorffer et al., 1992).

In conclusion, these findings demonstrate that administration of SSP containing rUreB results in effect of immune prophylactic and therapeutic against H.pylori infection in mouse model. The results also suggest that the rUreB expressed in silkworm might have potential in medicine application. However, the results show that although the vaccine is immunogenic, it can not prevent

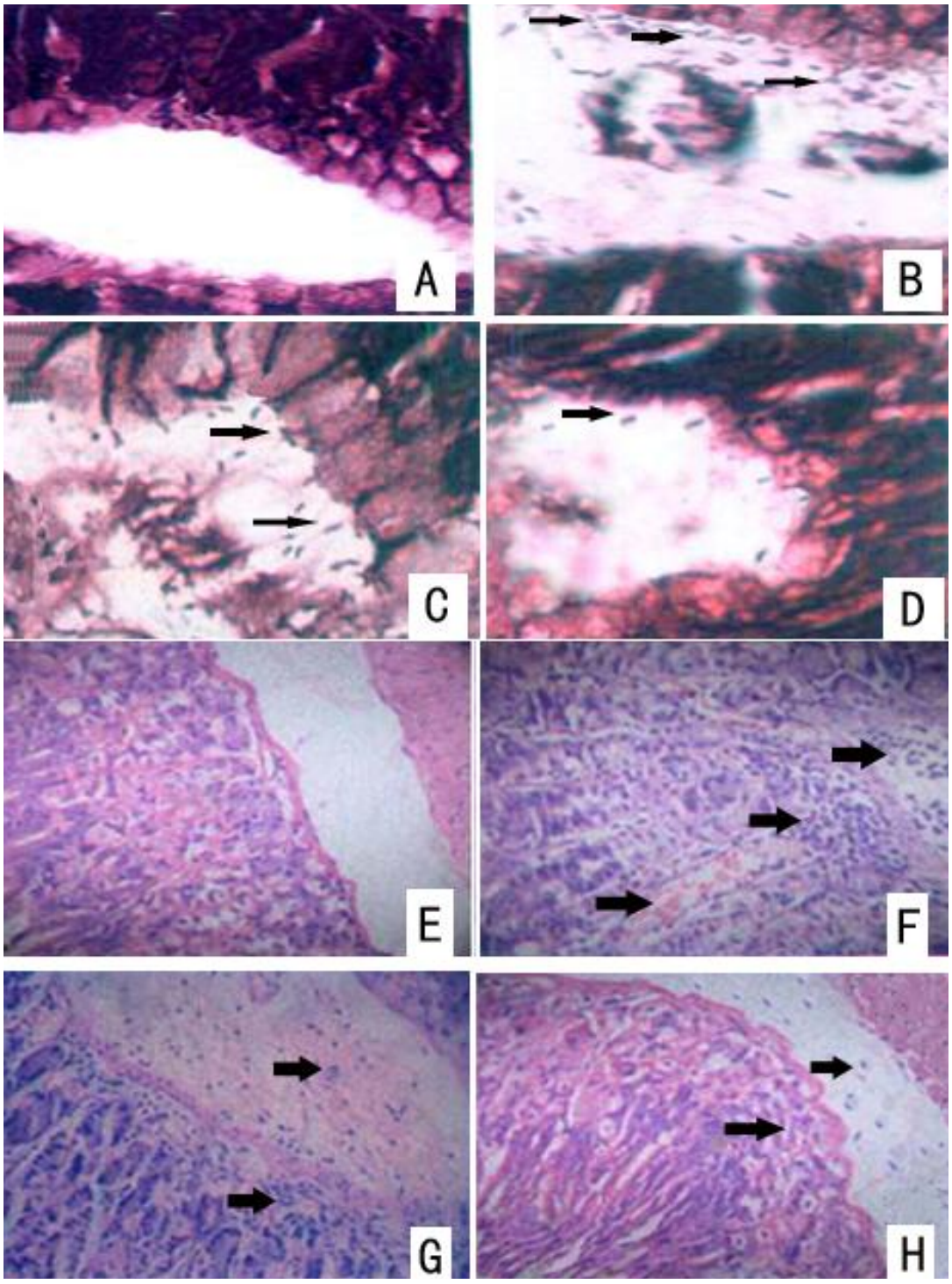

Figure 2. Histological assessment of bacterial colonisation and histopathological analysis of mouse stomach (Giemsa stain,1000 × and HE stain, 200 ×) (a) Normal control animal: almost no bacteria, score 0; (b) non-immunised animal: more than 11 bacteria per crypt, score ≥ 3; (c) immunised with SSP+ CTB animal: more than 3 bacteria per crypt ,score ≥ 2; (d) immunised with SSP + rUreB + CTB animal: 1 to 10 bacteria per crypt, score ≤ 2; (e) Normal control animal: only a few lymphocytes, inflammation score 0.2 or so; (f) H. pylori-infected control animal: heavy infiltrate of granulocytes, lymphocytes and plasma cells close to muscular mucosa (arrows) and also many inflammation cells between crypts, inflammation score 3.0 or so; (g) H. pylori-infected mice treated with SSP + CTB : many cells around the lamina muscularis mucosa and scattered inflammatory cells in-between glands, inflammation, score 2.5 or so;(h) *H. pylori*-infected mice treated with SSP + rUreB + CTB: Very few cells around the lamina muscular is mucosa and scattered inflammatory cells in-between glands, score 1.2 or so.

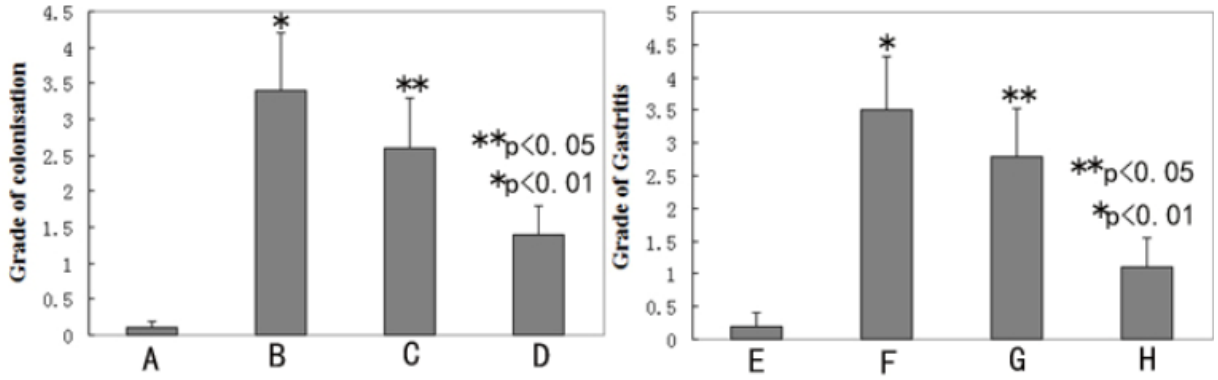

Figure 3. Histological grade of bacterial colonisation and histopathological grade of gastritis of mice stomachs (a, e) Normal control animals: almost no bacteria and inflammatory cells; (b, f) H. pylori- infected control animals: there was a large number of *H.pylori* colonisation and inflammatory cells. (c, g) *H. pylori*-infected mice treated with SSP + CTB: the number of *H. pylori* colonisation and inflammatory cells reduced to some extent; (d, h) H. pylori-infected mice treated with SSP + rUreB + CTB: the number of *H. pylori* colonisation and inflammatory cells reduced significantly. *p<0.01, **p<0.05, n = 10.

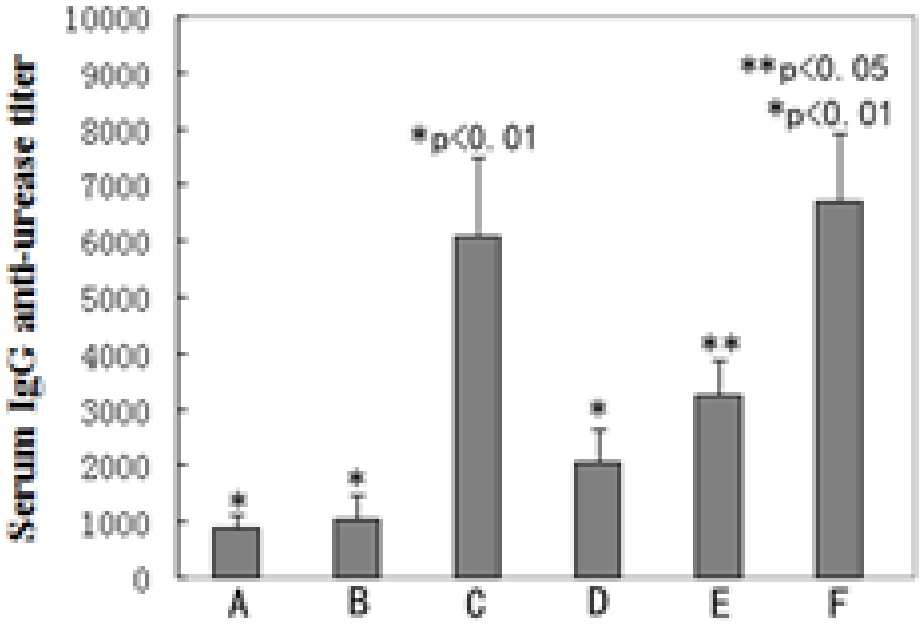

Figure 4. Titers of serum IgG specific anti-*H pylori* UreB in the immune protection group and the immune treatment group mice. Immune protection animals: (a) control mice; (b) oral immunised with SSP + CTB; (c) oral immunised with SSP + rUreB + CTB *p<0.01, n = 10. Immune treatment animals: (d) control mice; (e) oral immunised with SSP + CTB; (f) oral immunised with SSP + rUreB + CTB. *p<0.01, **p<0.05, n = 10

infection completely. An effective vaccine to prevent *H. pylori* infection will probably require more different or more additional antigens, as well as improvements in stimulation of the host immune response. A longer duration of therapy possibly yields better results. To solve the antigen mass production problem, the present studies further administrate that the method proposed in our research has many advantages such as high expression efficiency and low cost without demanding rigorous purification, natural activity for its expressed products and safety for both environment and human .

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