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Characterization and immune effect of the hepatitis B–BCG combined vaccine for using a needle innoculation

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ABSTRACT

Objective: To prepare the hepatitis B–*Mycobacterium bovis* Bacillus Calmette-Guérin combined vaccine (HB–BCG combined vaccine) and resolve a needle problem of the two kinds of hepatitis B vaccine (HB vaccine) and *M. bovis* Bacillus Calmette-Guérin (BCG) for the innoculation.
Methods: The hepatitis B surface antigen (HBsAg) was prepared by the genetic engineering technique, BCG was produced using routine biological technique, and then the finished products of the HB–BCG combined vaccine were processed on the above foundation. The content of HBsAg was measured by Enzyme linked immunosorbent assay (ELISA), the immune effect of BCG was detected by purified protein derivative (PPD) test. Cellular immune response, safety, partial poison and allergy were tested. The stability of HB–BCG combined vaccine was detected by ELISA and viable count method.
Results: The two kinds of antigens (HBsAg and BCG) had good compatibility. The comparison on immune effects of HB–BCG combined vaccine and BCG showed no significant difference. The comparison on immune effects of HB–BCG combined vaccine group (first dose for HB–BCG Combined vaccine, second and third dose for HB vaccine) and HB vaccine group (three dose all for HB vaccine) demonstrated that anti-HBs levels of the HB–BCG combined vaccine group were higher than that of HB vaccine group. No statistical significance was observed between the combined vaccine group and HB vaccine group after three doses immunization schedules. The results of safety in HB–BCG combined vaccine group accorded with that of BCG group, it had been not found the pathological changes of the tuberculosis. The characteristic and process in pathological changes of HB–BCG combined vaccine group and BCG group were similar in the partial poison test. HBsAg did not strengthen the inflammation reaction caused by BCG. Systemic allergy had not been found. The HB–BCG combined vaccine was stable in 2 years.
Conclusion: The immune effects of the HB–BCG combined vaccine were not lower than the two kinds of single dose vaccine, it had good safety.

1. Introduction

China is the high epidemic region of hepatitis B (HB) and tuberculosis (TB). According to statistical date from 2003 to 2005, in the infected number of infectious disease, the first was tuberculosis, the second was HB. So it is necessary to improve prevention work of hepatitis B and tuberculosis. In 1992–1995, the seroepidemiological survey of viral hepatitis in China showed that the carrier rate of HBsAg in general population was 9.75% [1]. In 2006, the seroepidemiological survey of hepatitis B in China showed that the carrier rate of HBsAg in general population was 7.18% [2]. In 1999, the survey on children under 3 years old for 31 provinces in China showed that children under 12 months of age vaccinated with hepatitis B vaccine, the coverage rate was 88.5% in urban area and 62.7% in rural areas, respectively. An average was 70.7%. The protection rate of hepatitis B vaccine immuned for children was 93.0% [3]. The remaining 10 million blood samples of Nationwide Survey of Nutrition and Health in Chinese residents which was conducted in 2002, were detected, the results: (1) among the vaccinated population, the positive rate of HBsAg was 5.28%, and the rate of hepatitis B virus (HBV) infection was 54.44%. For the children from 3 to 12 years old, the rate was 3.63% and 26.88%, respectively. (2) Among the unvaccinated population, the positive rate of HBsAg was 9.51%, and the rate of HBV infection was 64.63%. For the children from 3 to 12 years old, the rate was 11.19% and 47.03%, respectively. (3) Among the vaccinated population, the positive rate of HBsAg was 2.55% in urban area and 7.10% in rural area. For the unvaccinated population, the rate was 7.24% and 10.12%, respectively. Among the vaccinated children from 3 to 12 years old, the positive rate of HBsAg was 1.96%

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in urban area and 6.65% in rural area. For the unvaccinated population, the rate was 2.39% and 10.84%, respectively. The results show that the effect of hepatitis B vaccine immunization is prominent, while vaccination of hepatitis B vaccine in rural areas is the focus of prevention and treatment of hepatitis B in China [4]. Nationwide epidemiological survey of TB in China had been launched for four times in 1979, 1984–1985, 1990 and 2000, respectively. In 2000, the fourth survey estimated that there were 5 million patients with active pulmonary tuberculosis, 1.96 million patients with bacteriological positive pulmonary tuberculosis, 1.5 million patients with smear-positive pulmonary tuberculosis. About 0.13 million people died of tuberculosis each year, the nationwide tuberculosis infection rate was 44. 5%, or 550 million people infected with TB [5]. By the end of 2005, the coverage rate of Chinese TB control strategy (directly observed therapy shortcourse, DOTS) was 100%, discovery rate of TB patients was 79%, recovery rate was 91%. 2.05 million cases of infectious tuberculosis were detected and cured from 2001 to 2005 [6]. Among 2001–2009, 7.37 million active TB patients were detected and cured, including 4.01 million smear-positive tuberculosis patients, the first cure rate for smear-positive tuberculosis patients was more than 90%, and the next cure average rate was 84% [7]. BCG vaccination card scar rate was 77.0–94.0% in cities and towns, but 60.0% in rural area. In 2008, BCG vaccination rate was more than 90% in cities and towns [8]. This showed that China had effectively controlled the rise of tuberculosis. Although prevention and treatment in China has made some achievements, the epidemic of tuberculosis is still very serious in China. As long as we persist in long-term efforts, TB could be cured and controlled.

HB and BCG vaccines are products of Immunization Programme, newborn are obligated to inoculate with both HB and BCG vaccines as national requirements within 24 h after birth. Currently, BCG vaccine is provided in a lyophilized form and intracutaneously inoculated into the deltoid muscle of upper arm, while HB vaccine is provided in a liquid form and intramuscularly inoculated into the deltoid muscle of upper arm, Because the difference between the BCG and HB vaccines in inoculation pathway and form, in addition two univalent vaccines contain some components with antagonism each other, such as aluminium adjuvant and antiseptic. Therefore, two kinds of vaccines could not be mixed and used simply, they must be inoculated respectively. It caused much inconvenience. The study of HB–BCG combined vaccine not only could supply convenience for practice, but also reduce inoculation times, simplify immune procedure, enhance inoculation rate and compliance, save money and reach effect of two univalent vaccine.

2. Materials

Bulk of HBsAg and BCG, recombined HB vaccine (Chinese hamsterovary cell, CHO cell), lyophilized BCG for intracutaneous inoculation, HB–BCG combined vaccine and PPD are provided by Changchun Institute of Biological Products.

Gelatin (Shanghai Chemical agent Corporation), Saccharose and Potassium Chloride (Beijing Chermical Works), Sodium glutamate (ShenyangHongmei monosodium Company).

HBsAg ELISA kit (SINO-AMERICAN Biotechnology Company); HBsAb ELISA kit (Wantai Biotechnology Company); IL-2 and IL-4 ELISA kit (Jingmei Biotechnology Company); CD3-FITCAb, CD4-PEAb, CD8-PE Ab (Shenzhen Dakewei Biotechnology Company).

Test animals: guinea pigs (negative in "PPD skin test", 300–400 g in weight each animal) and BALB/c mice (14–16 g in weight each animal) were obtained from Animal House of Changchun Institute of Biological Products.

Microplate Reader (SUNRISE, TECAN, Switzerland); Multipurpose Scintillation Counter (LS6500, BECKMAN); Flow Cytometer (FACS Calibur, BECTON DICKINSON).

3. Methods

3.1. Preparation of combined vaccine

The qualified bulk of HBsAg was added to normal saline for injection and cryoprotective agent, diluted to 50 μg/ml, 100 μg/ml and 200 μg/ml, respectively. The examined bulk of BCG was added to cryoprotective agent and diluted to 1 mg/ml. The diluted bulk of BCG and HBsAg (three dosages) were mixed well in a proportion of 1:1 (v/v) to prepare semi-finished products of the HBsAg-BCG combined vaccines. The semi-finished products were distributed, lyophilized and filled with N_2, then sealed. The finished products of the HBsAg-BCG combined vaccine were prepared, the components also contained 0.8%Gelatin, 10%Saccharose, 1%KCl, 1%Sodium glutamate. Three different dosages of combined vaccines were HBsAg 2.5 μg/BCG 5×10^4 CFU (Colony Forming Unit)/mg, HBsAg 5 μg/BCG 5×10^4 CFU/mg and HBsAg 10 μg/BCG 5×10^4 CFU/mg, respectively.

3.2. Quality control of the finished products of combined vaccines

Identity test, water content, pure bacterial test, no toxic Myco test, count plate, hot stability test, BCG efficacy determination and quality control of bulk of HBsAg and semi-finished products were carried out according to the requirement of China Biological product Regulations (2000 edition). The content of HBsAg was measured by ELISA to determine the efficacy of HB vaccine. Quality control of the finished product of combined vaccine was carried out according to the relative requirement of China Biological product Regulations (2000 edition).

3.3. Influence of freeze drying on BCG and HBsAg

3.3.1. Observation by electron microscope

To prepare slide for the bulk of HBsAg, BCG and HB–BCG combined vaccine, then observe the morphous of HBsAg particle and HB–BCG combined vaccine.

3.3.2. Detection of the content of HBsAg

To detect content of HBsAg of the semi-finished products and finished products by ELISA, compare influence of freeze drying on HBsAg.

3.3.3. Detection of the viable count of BCG

To detect the viable count of BCG of the semi-finished products and finished products by count plate method, compare influence of freeze drying on BCG.

3.4. Study of immunity effect

3.4.1. The comparison of immunogenicity between the HB–BCG combined vaccine and univalent HB vaccine

The guinea pigs were negative in "PPD skin test" and divided into five groups, 10 animals in each group. The test groups were inoculated with HB–BCG combined vaccines for 0.1 ml by intradermal injection at 0, 1 and 2 months, respectively (group1: HBsAg 2.5 μg/BCG 5×10^4 CFU/mg for each guinea pig; group2: HBsAg 5 μg/BCG 5×10^4 CFU/mg for each guinea pig; group3: HBsAg 10 μg/BCG 5×10^4 CFU/mg for each guinea pig). In the second and third injections, each guinea pig was injected with 10 μg/1 ml of univalent HB vaccine for intraperitoneal injection. The control group was inoculated with HBsAg vaccine for intraperitoneal injection at 0, 1 and 2 months, respectively, 10 μg/1 ml for each guinea pig. The blank group: there was no vaccine injected. The test animals were breeded in the same environment. Blood samples were taken at 1 month after each immunization and the antibody titers

of serum were measured by ELISA method. Blood samples of blank groups were collected as the negative control. To calculate geometric mean titer (GMT) value and use *t*-test to compare the difference between groups.

3.4.2. The comparison of immunogenicity between the HB–BCG combined vaccine and univalent BCG vaccine

The guinea pigs were negative in "PPD skin test" and divided into four groups, four guinea pigs for each group. The test groups were inoculated with HB–BCG combined vaccines (the dosage for 10 persons) by subcutaneous injection, 1 ml for each guinea pig (group1: HBsAg 25 μg/BCG 5×10^5 CFU/mg for each guinea pig; group2: HBsAg 50 μg/BCG 5×10^5 CFU/mg for each guinea pig; group3: HBsAg 100 μg/BCG 5×10^5 CFU/mg for each guinea pig). The control group was inoculated with BCG (the same lot) by subcutaneous injection, 1 ml (5×10^5 CFU/mg) for each guinea pig. After 5 weeks, PPD for 10 IU/0.2 ml was used to perform skin test. The diameter of scleroma for local reaction was determined after 24 h. To calculate GMT value and use *t*-test to compare the difference between groups.

3.4.3. Cellular immunity effect of the HB–BCG combined vaccine

Immunization: BALB/c mice were divided into five groups, 20 mice for each group. Blank group: there was no vaccine injected; protective agent group was inoculated with protective agent by intradermal injection, that was used in the combined vaccine (the dosage for 2 persons), 0.1 ml for each mouse; HB vaccine group was inoculated with HB vaccine for intraperitoneal injection, 20 μg for each mouse; BCG vaccine group was inoculated with BCG by intradermal injection, 1×10^5 CFU/mg for each mouse; HB–BCG combined vaccine group was inoculated with the HBsAg-BCG combined vaccine by intradermal injection 0.1 ml for each mouse (HBsAg 10 μg/BCG 1×10^5 CFU/mg).

Immunodetection: BALB/c mice of all groups (10 mice for each group) were inoculated for 1 month, spleen cells of mice were isolated by aseptic method, lymphocyte transformation test was detected by the method of ^{3}H-Tdr; IL-2 and IL-4 were detected by ELISA. BALB/c mice of all groups (10 mice for each group) were inoculated for 2 months, spleen cells of mice were isolated by aseptic method, surface molecular markers of T lymphocyte were detected.

3.4.3.1. Lymphocyte transformation test. Prepare cell suspension: The spleen from the BALB/c mouse immunized with vaccine were isolated by aseptic method and grinded, then the culture medium was added, the tissue blocks were filtered by the filter paper or multi-layer gauze to obtain the single cell suspension. Cell suspension were counted under a microscope and diluted to 1×10^6 cells/ml with culture medium. The cell suspension (1×10^6 cells/ml) inoculate 96 wells plate, each well for 0.1 ml, each detected sample was conducted in three test wells and three control wells. Test wells were added 15 μg/ml ConA, incubated under the condition of 37 °C, 5% CO_2 for 48 h, each well was added ^{3}H-Tdr of 0.5 μl Ci, continued to incubate for 8–10 h, collected on nitrocellulose filter. To count by Multi-purpose Scintillation Counter, calculate cmp value.

3.4.3.2. Detection of cytokine. Spleen cells of mice were isolated by aseptic method to prepare cell suspension (1×10^6 cells/ml). To inoculate 96 wells plate, each well for 0.1 ml, incubate under the condition of 37 °C, 5% CO_2 for 48 h, IL-2 and IL-4 of culture supernatant were detected by ELISA kit.

3.4.3.3. Detection of surface molecular markers of T lymphocyte. Surface molecular markers of T lymphocyte were detected by flow cytometry. Spleen cells of mice were isolated by aseptic method to prepare single cell suspension (1×10^6 cells/ml). To take it for 0.1 ml into falcon tube, then add to CD3-FITC Ab, CD4-PE Ab, CD8-PE Ab, the tube was put in the condition of room temperature and dark place for 30 min, washed by PBS for two times, then added 0.5 ml PBS to mix. Positive expression rate of CD3-FITC, CD4-PE, CD8-PE of T lymphocyte were analyzed for two parameter by CELLQuest functional software and calculate ratio of CD4/CD8.

3.5. Safety study

3.5.1. Safety test of the guinea pigs

The guinea pigs were 300–400 g in weight and divided into three groups, six guinea pigs for each group. The test group was inoculated subcutaneously with the HB–BCG combined vaccine (HBsAg 250 μg/BCG 2.5×10^6 CFU/mg, corresponding to a dosage for 50 persons), while the control group was inoculated with BCG vaccine (BCG 2.5×10^6 CFU/mg, corresponding to a dosage for 50 persons). The blank group: there was no vaccine injected. After observation for 42 days, the guines pigs were dissected. The viscera were observed by eye largely, pathological examination was carried out under the light microscope. The operations of pathological examination and the organs of pathological examination:

Firstly, the thorax, pleura, lung lobes, pericardium, heart, abdominal cavity, peritoneum, omentum, liver, spleen, pancreas, kidney, adrenal gland, cervical lymph nodes, hilar lymph nodes, mesenteric lymph nodes, local inoculated skin, left groin lymph node, right groin lymph nodes, genitals, stomach, intestines serosa and brain were examined by macrography.

Secondly, lung, hilar lymph nodes, liver, spleen, local inoculated skin, left groin lymph node, right groin lymph nodes and mesenteric lymph nodes were taken out, and fixed in 10% neutral formalin solution, conventionally produced pathological section, they were observed with HE staining by light microscopy, and recorded by microphotography.

Thirdly, the organs of pathological examination: lung, liver, spleen, local lymph nodes, local skin.

3.5.2. Local toxicity test of mice

The mice were 14–15 g in weigh and divided into three groups, 10 mice for black control group, 40 mice for control group, 40 mice for test group, the number of male and female mice was same. The test group was inoculated intracutaneously with the HB–BCG combined vaccine (HBsAg 20 μg/BCG 1×10^5 CFU/mg, corresponding to a dosage for 2 persons), while the control group was inoculated intracutaneously with BCG vaccine (BCG 1×10^5 CFU/mg, corresponding to a dosage for 2 persons). The blank group was injected with protective agent (the dosage for 2 persons). The three mice of test group and control group were dissected at 3, 5, 7, 9, 11, 15, 20, 25 and 30 days, respectively. The blank group was dissected at 3 and 5 days. The viscera were observed by eye largely, pathological examination was carried out under the light microscope. The operations of dissection and the organs of pathological examination:

Firstly, mice were killed, the body surface, thorax, pleura, lung lobes, heart, abdominal cavity, omentum, liver, spleen, pancreas, kidney, adrenal gland, cervical lymph nodes, hilar lymph nodes, mesenteric lymph nodes, stomach, intestines, local inoculated skin, groin lymph node and brain were observed.

Secondly, local inoculated skin was taken out, and fixed in 10% neutral formalin solution, conventionally produced pathological section, they were observed with HE staining by light microscopy.

Thirdly, the organs of pathological examination: local inoculated skin.

3.5.3. Allergy test

The guinea pigs were 300–400 g in weight and divided into five groups (six guinea pigs for each group). Positive control group was immunized with 20% bovine calf serum, 0.5 ml for each guinea

Table 1
Detection of finished products in nine lots.

Lot	20031010	20031011	20031012	20031013	20031014	20031015	20031116	20031117	20031118
Specification									
HBsAg	2.5 μg	2.5 μg	2.5 μg	5 μg	5 μg	5 μg	10 μg	10 μg	10 μg
BCG	5×10^4 CFU/mg	5×10^4 CFU/mg	5×10^4 CFU/mg	5×10^4 CFU/mg	5×10^4 CFU/mg	5×10^4 CFU/mg	5×10^4 CFU/mg	5×10^4 CFU/mg	5×10^4 CFU/mg
Identity test	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification
Solution time (s)	60	60	60	70	60	60	50	60	50
Appearance	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification
Vacuity	Filled N_2	Filled N_2	Filled N_2	Filled N_2	Filled N_2	Filled N_2	Filled N_2	Filled N_2	Filled N_2
Water content (%)	1.27	1.12	1.06	1.26	1.34	1.42	2.22	2.08	2.17
Pure bacterial test	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification
No toxic Myco test	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification	Qualification
Viable count ($\times 10^4$ CFU/mg)	460	526	495	608	544	420	566	610	592
Hot stability ($\times 10^4$ CFU/mg)	290	237	211	314	328	230	277	336	343
HBsAg content (μg/dose)	5.97	5.92	5.77	9.84	9.94	11.63	21.96	21.72	19.26
Induration diameter(mm)	8.50	9.50	8.85	7.75	7.15	8.85	8.50	8.75	9.00

Note: the content of finished products for each dose was double specification dosage (0.2 ml), containing package addition content, specification dosage was utilization dosage (0.1 ml).

pig. Negative control group was inoculated with water for injection, 0.5 ml for each guinea pig. The low dosage group of HB–BCG combined vaccine was inoculated with 0.5 ml (HBsAg 5 μg/BCG 5×10^4 CFU/mg) for each guinea pig. The high dosage group of HB–BCG combined vaccine was inoculated with 0.6 ml (HBsAg 30 μg/BCG 3×10^5 CFU/mg) for each guinea pig. BCG vaccine group was inoculated with (3×10^5 CFU/mg/0.6 ml) for each guinea pig. All groups were intraperitoneally injected for four times, interval 1 day. All groups were injected with the same samples (double dosage) last time by intravenous injection at 14 days after the fourth time immunization.

3.6. Stability test

The HB–BCG combined vaccine and BCG vaccine were tested in different temperature 37 °C and 25 °C, the HBsAg content, viable count of BCG and water content were measured at 1, 2, 3 and 6 months respectively. The HB–BCG combined vaccine and BCG vaccine were tested at 4 °C, the HBsAg content, viable count of BCG and water content were measured at 1, 2, 3, 6,12 and 24 months, respectively.

4. Results

4.1. Detection of finished products of the combined vaccine in nine lots

The nine lots finished products of the combined vaccine were prepared for three different dosages, each dosage for three lots. All detective quality controls were qualified (Table 1). The content of HBsAg showed no significant difference ($p > 0.05$) before and after lyophilization. Lyophilization had no influence on HBsAg, HBsAg was not destroyed. The viable count of BCG showed significant degression because of lyophilization, but it was in valid confine (Table 2). It was demonstrated that lyophilization process was stable, feasible and suitable for scale production.

4.2. Observation results by electron microscope

The bulk of HBsAg, BCG and HB–BCG combined vaccine were observed by electron microscope, it was seen that HBsAg presented particle state, particle structure of HBsAg was similar in the bulk of HBsAg and HB–BCG combined vaccine, there was no difference; but it was not seen that particle of HBsAg combined with Bacillus tuberculosis in the HB–BCG combined vaccine, particle of HBsAg presented loose adhesion; morphous of Bacillus tuberculosis was similar in the BCG and HB–BCG combined vaccine, there was no significant difference (Figs. 1–3).

Table 2
HBsAg content and viable count of BCG before and after lyophilization.

Lot	HBsAg content (μg/dose)		Viable count ($\times 10^4$ CFU/mg)	
	Before lyophilization	After lyophilization	Before lyophilization	After lyophilization
20031010	5.41	5.97	2400	460
20031011	4.75	5.92	2350	526
20031012	4.72	5.77	2540	495
20031013	10.02	9.84	2700	608
20031014	9.34	9.94	2330	544
20031015	10.54	11.63	2330	420
20031116	19.86	21.96	1940	566
20031117	21.23	21.72	2170	610
20031118	20.15	19.26	2660	592

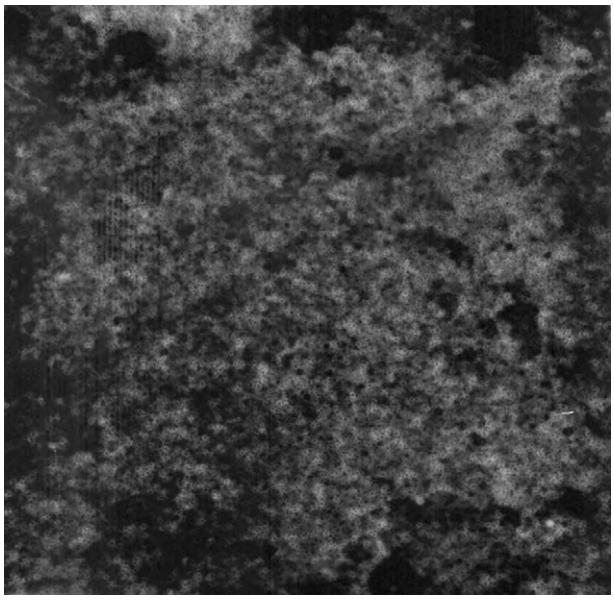

Fig. 1. Bulk of HBsAg.

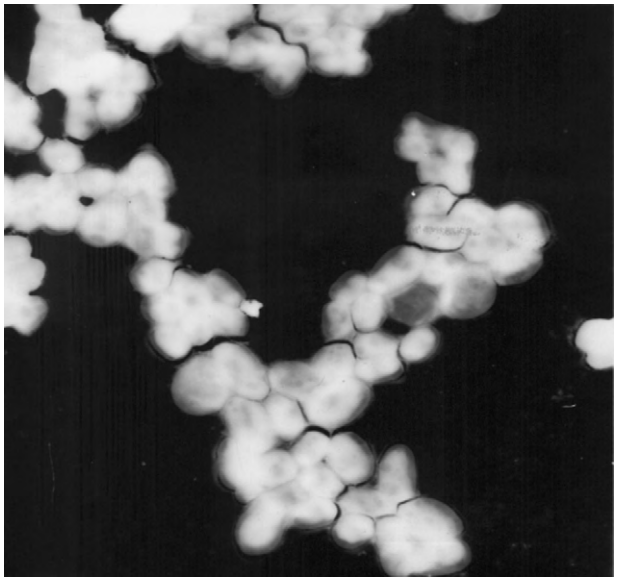

Fig. 2. Bulk of BCG.

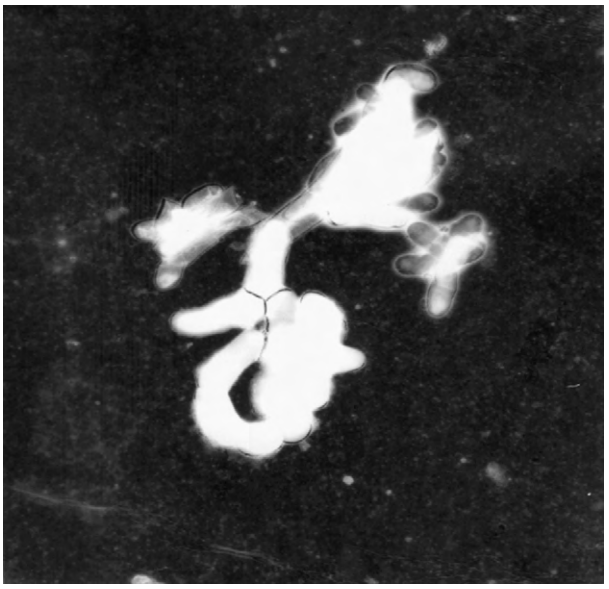

Fig. 3. Combined vaccine.

Table 3
HBsAb titers induced by HB–BCG combined vaccine of three dosages (mIU GMT ± s).

Group	First injection	Second injection	Third injection
Test 1	276 ± 31.16	24656 ± 2052	63244 ± 8068
Test 2	296 ± 123.0	31424 ± 2788	62540 ± 7724
Test 3	380 ± 102.60	35184 ± 2328	75168 ± 8052
Control	604 ± 20.23	22384 ± 2736	45084 ± 7500
	$p > 0.05$	$p > 0.05$	$p > 0.05$

4.3. The comparison of immunogenicity between the HB–BCG combined vaccine and univalent HB vaccine

After the first injection, antibody titers of HB–BCG combined vaccine group for three dosage (test groups 1, 2 and 3) were lower than that of control group, but the statistical analysis of results showed no significant difference ($p > 0.05$); antibody titers of three test groups were higher than that of control group at 1 month after the second and third injection, there was no significant difference ($p > 0.05$). Blood serum antibody titers of three test groups were similar after the primary immunization, there was no significant difference ($p > 0.05$); blood serum antibody titers of three test groups after the second and third injection, showed no significant difference ($p > 0.05$) (Table 3).

Table 4
Result of PPD test induced by HB–BCG combined vaccine of three dosages.

Group	Lot	Diameter of scleroma (mm)				$\bar{x} \pm s$	p
Test 1	20021201	8	12	14	8.5	10.62 ± 2.86	
Test 1	20021202	11	9	13	8.5	10.31 ± 2.05	$p > 0.05$
Test 1	20021203	10	9.5	14	10.5	11.00 ± 2.04	
Control	20021225	9	9.5	10	10.5	9.74 ± 0.64	
Test 2	20021204	11.5	10	12	9	10.62 ± 1.37	
Test 2	20021205	14	8	12	8.5	10.60 ± 2.86	$p > 0.05$
Test 2	20021206	12	12	10.5	8	11.32 ± 2.13	
Control	20021226	–	10.5	8.5	9	10.00 ± 1.58	
Test 3	20021207	9	8.5	7	10	8.62 ± 1.25	
Test3	20021208	6.5	8	12	9	8.83 ± 2.32	$p > 0.05$
Test 3	20021209	7.5	8.5	11	9.5	9.15 ± 1.49	
Control	20021227	8.5	9.5	10	11.5	9.82 ± 1.25	

Table 5
Result of lymphocyte transformation test.

Group	Lymphocyte transformation stimulating index ($\bar{x} \pm s$)	cpm
Control group		
Blank	4.35 ± 0.59	7439
Protective agent	3.50 ± 0.46	6613
Test group		
HB	6.56 ± 0.34	11,709
BCG	6.89 ± 0.24	7309
HB–BCG	8.05 ± 0.48	11,988

Note: test groups compared with control groups, $p < 0.001$.
HB–BCG group compared with HB group, BCG group, $p < 0.001$.

Table 6
Determination of cytokine content.

Group	IL-2 (pg/ml)	IL-4 (pg/ml)
Control group		
Blank	240 ± 48.77	179 ± 43.63
Protective agent	280 ± 43.07	180 ± 40.71
Test group		
HB	358 ± 58.16	213 ± 43.71
BCG	695 ± 116.40	187 ± 39.20
HB–BCG	972 ± 88.54	204 ± 39.56

Note: IL-2 content of the test groups compared with control groups, $p < 0.001$.
IL-2 content of HB–BCG group compared with HB group, BCG group, $p < 0.001$.
IL-4 content showed no significant difference between groups, $p > 0.05$.

4.4. The comparison of immunogenicity between the HB–BCG combined vaccine and univalent BCG vaccine

The test groups and the corresponding control groups were prepared from the same lot of BCG bulk, immunogenicity of all lot vaccines was showed by $\bar{x} \pm s$ for diameter of scleroma. The test and control groups used *t*-test to compare the difference between groups ($t = 0.298$–0.546). The statistical analysis of the results showed no significant difference between the HB–BCG combined vaccine and the univalent BCG vaccine ($p > 0.05$). It was investigated that HBsAg had no influence on immunogenicity of BCG (Table 4).

4.5. Lymphocyte transformation test

The HB–BCG combined vaccines surpassed BCG and HB vaccines on promote lymphocyte blastogenesis, it had significant cellular immune effect. Lymphocyte transformation stimulating index of the test groups (HB–BCG combined vaccine, HB vaccine and BCG vaccine) and control groups (blank and protective agent) showed significant difference ($p < 0.001$). Lymphocyte transformation stimulating index of the HB–BCG combined vaccine group and HB vaccine, BCG groups showed significant difference ($p < 0.001$) (Table 5).

4.6. Detection of cytokine

The HB–BCG combined vaccine could stimulate effectively organism to produce IL-2.IL-2 content of the test groups (HB–BCG combined vaccine, HB vaccine and BCG vaccine) and control groups (blank and protective agent) showed significant difference ($p < 0.001$). IL-2 content of the HB–BCG combined vaccine group and HB vaccine group, BCG group showed significant difference ($p < 0.001$). The HB–BCG combined vaccine had no significant influence on production of IL-4, IL-4 content showed no significant difference between groups ($p > 0.05$) (Table 6).

4.7. Detection of surface molecular markers of T lymphocyte

The results of all groups were showed by $\bar{x} \pm s$, used *t*-test to compare the difference between groups. $CD3^+$ and $CD4^+$ cell subpopulation of all groups showed no significant difference; $CD8^+$ cell subpopulation in the BCG and protective agent groups were obviously lower than that in the HB vaccine group, but $CD8^+$ cell subpopulation in the BCG group, protective agent group and HB–BCG combined vaccine groups showed no significant difference (Figs. 4 and 5). The proportionality of $CD4^+/CD8^+$ in the HB–BCG combined vaccine group and BCG group was higher than that in the HB vaccine group, but the proportionality of $CD4^+/CD8^+$ in the HB–BCG combined vaccine group, BCG group and protective

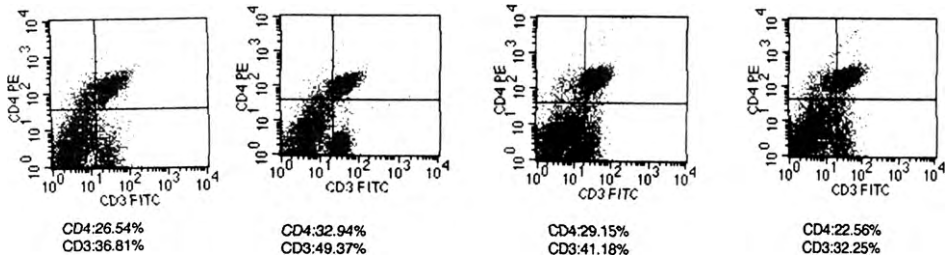

Fig. 4. The map of detection for $CD4^+$ T lymphocytes.

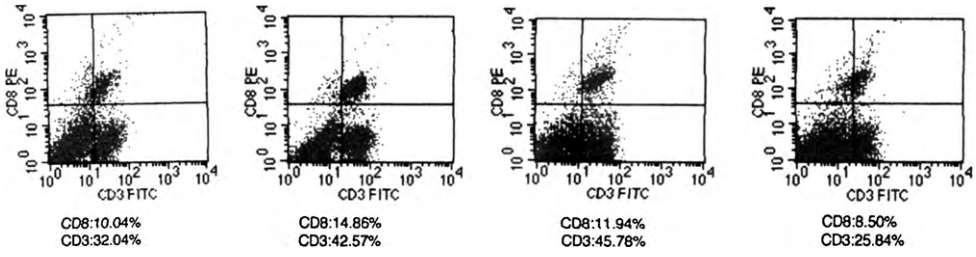

Fig. 5. The map of detection for $CD8^+$ T lymphocytes.

Table 7
Detection of surface marker of lymphocyte ($\bar{x} \pm s$).

Group	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
(1) Protective agent	36.12 ± 4.03	25.20 ± 5.67	9.82 ± 1.18	2.55 ± 0.48
(2) HB	41.41 ± 10.03	29.70 ± 4.33	14.98 ± 2.72	2.07 ± 0.66
(3) BCG	39.77 ± 3.42	31.51 ± 8.45	11.76 ± 5.29	2.87 ± 0.60
(4) HB + BCG	36.35 ± 5.44	27.83 ± 4.26	9.43 ± 0.69	2.93 ± 0.36
p	(1) (2) (3) (4) $p > 0.05$	(1) (2) (3) (4) $p > 0.05$	(1) and (2) $p < 0.001$ (2) and (4) $p < 0.001$ (1) and (3) $p > 0.05$ (1) and (4) $p > 0.05$ (2) and (3) $p > 0.05$ (3) and (4) $p > 0.05$	(2) and (3) $p < 0.01$ (2) and (4) $p < 0.05$ (1) and (2) $p > 0.05$ (1) and (3) $p > 0.05$ (1) and (4) $p > 0.05$ (3) and (4) $p > 0.05$

agent group showed no significant difference (Table 7). The proportionality of $CD4^+/CD8^+$ demonstrated that the activation level of immunological cell in the HB–BCG combined vaccine group was higher than that in the univalent hepatitis B vaccine group, HB–BCG combined vaccine could induce cellular immunity effectively.

4.8. Safety test of the guinea pigs

The observed roughly results demonstrated that the organs of blank group, control group and combined vaccine group had been not seen the pathological changes of the tuberculosis. Inflammatory reaction was observed by the light microscope in the inoculated local skin, lymphatic glands and lung of BCG group and combined vaccine group, but the pathological changes in HB–BCG combined vaccine group and BCG group were similar, pertaining to primary pathologic changes because the sensitive animals were inoculated with BCG (Figs. 6–9). It was not found that HBsAg boosted poison of BCG, It was also not seen that nonmalignant immune response caused by BCG developed malignant phymatiosis focus of infection.

4.9. Local toxicity test of mice

The observed roughly results demonstrated that the organs of mice in all groups had been not seen the pathological changes of tuberculosis. In the initial stage, inflammatory reactions were observed by the light microscope in the inoculated local skin of test group and control group, they began to absorb increasingly after 7 days, focus of infection trended to localization, inflammatory cell infiltrate in the granuloma regressed obviously on the 30th day, the granuloma diminished obviously. The characteristic and process in pathological changes of HB–BCG combined vaccine group and BCG group were similar in the local toxicity test, pertaining to primary focus caused by BCG, HBsAg did not strengthen the inflammation reaction caused by BCG (Figs. 10 and 11).

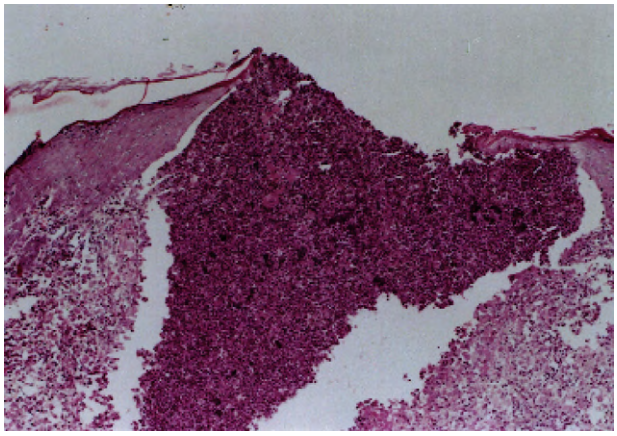

Fig. 7. BCG group inoculated skin on 42 days (HE × 325).

4.10. Allergy test

The guinea pigs in the positive control group were injected after about 1 min and showed typical symptom of allergy, restlessness, scratching nose, then sternutation, cough, anhelation, jumping, spasm and rotation. Six guinea pigs all died finally. The guinea pigs in the negative control group showed no above paradoxical reaction; the experiment was established. The high and low dosage groups of combined vaccine and BCG group had no allergy reaction. It was not found that the combination of HBsAg and BCG occurred systemic allergy reaction.

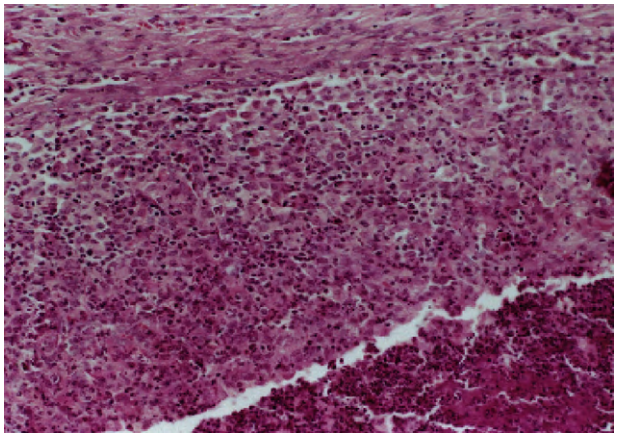

Fig. 6. HB–BCG group inoculated skin on 42 days (HE × 650).

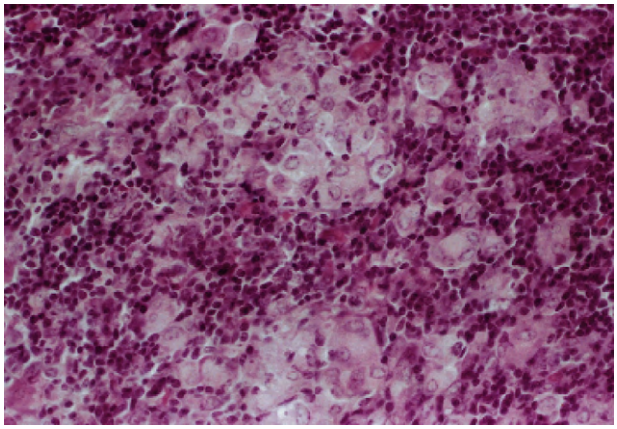

Fig. 8. Lymphatic glands of HB–BCG group on 42 days (HE × 1300).

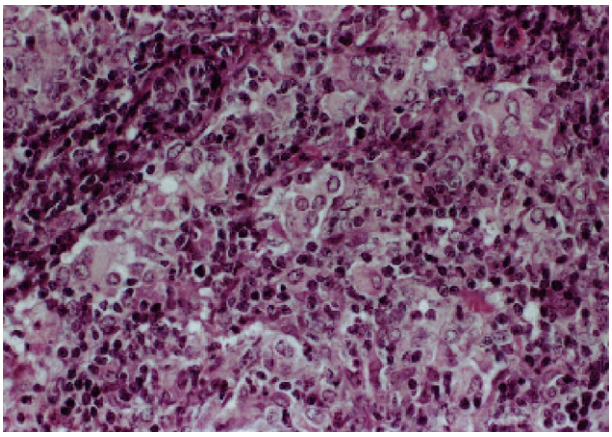

Fig. 9. Lymphatic glands of BCG group on 42 days (HE × 1300).

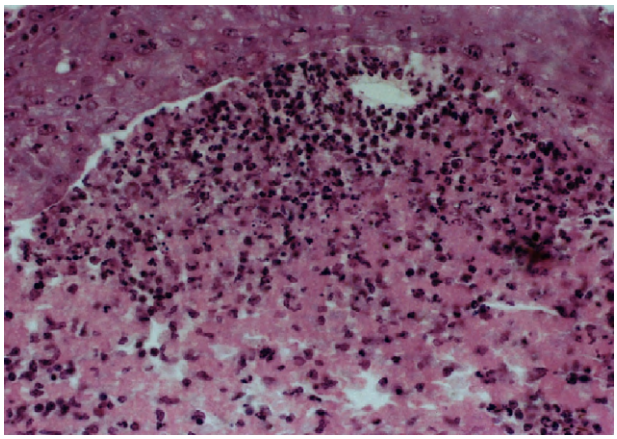

Fig. 10. Pathological slice of BCG group on the fifth day (HE × 1300).

4.11. Stability test

4.11.1. Stability of HBsAg in HB–BCG combined vaccine

HBsAg content in HB–BCG combined vaccine was detected by ELISA, the results of three continuous lot HB–BCG combined vaccines indicated that in the condition of 25 °C for 6 months, 37 °C for 6 months and 4 °C for 24 months, HBsAg content had no obvious change (Table 8). To compare immune effects in the HB–BCG combined vaccine group (first injection for HB–BCG combined vaccine, second and third injection for HB vaccine) in the condition of 4 °C for 24 months and the same lot vaccine in the starting stage (0 month). The stability of HBsAg in HB–BCG combined vaccine was good and no obvious change was observed on immune effect in the condition of 4 °C for 24 months ($p > 0.05$) (Fig. 12).

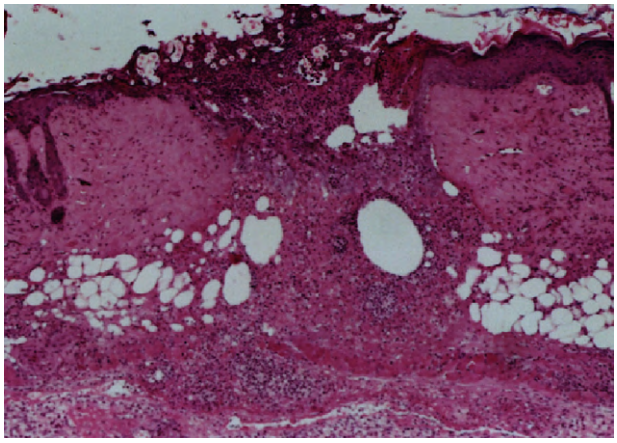

Fig. 11. Pathological slice of HB–BCG group on the fifth day (HE × 325).

Table 8
Detection of HBsAg content in HB–BCG combined vaccine in various temperature for 24 months (μg/dose).

Month	Lot	Starting	37 °C	25 °C	4 °C
1	20021204	11.34	11.12	11.95	11.25
	20021205	9.95	10.20	10.12	9.86
	20021206	10.33	10.15	9.86	10.40
2	20021204	11.34	10.22	10.44	11.25
	20021205	9.95	11.44	11.28	9.86
	20021206	10.33	9.56	11.33	10.44
3	20021204	11.34	9.23	10.11	10.55
	20021205	9.95	10.88	10.23	10.78
	20021206	10.33	10.24	10.18	9.46
6	20021204	11.34	10.82	10.55	10.55
	20021205	9.95	11.53	11.22	10.67
	20021206	10.33	10.23	11.33	9.46
9	20021204	11.34	–	–	9.86
	20021205	9.95	–	–	11.05
	20021206	10.33	–	–	11.89
12	20021204	11.34	–	–	9.86
	20021205	9.95	–	–	11.25
	20021206	10.33	–	–	11.37
24	20021204	11.34	–	–	9.57
	20021205	9.95	–	–	10.85
	20021206	10.33	–	–	9.62

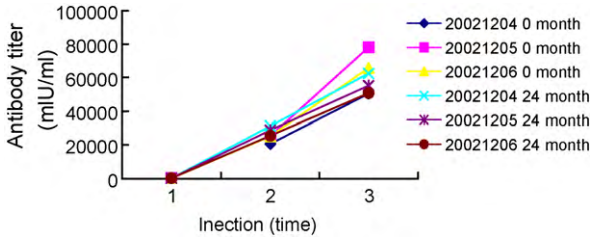

Fig. 12. Immune effect of HBsAg after three injection.

4.11.2. Stability of BCG in the HB–BCG combined vaccine

The results indicated that viable count decreased significantly in the condition of 37 °C and 25 °C, viable count were less than 20% of original viable count at 3 months. Viable count showed no significant difference and were similar to that of the same lot BCG vaccine in the condition of 4 °C for 24 months (Table 9). The thermostability was quantified in the condition of 4 °C after 24 months and similar to the result of same lot BCG vaccine (Table 10).

4.11.3. Stability of moisture in the HB–BCG combined vaccine

Moisture determination results of HB–BCG combined vaccine were all quantified in the condition of 37 °C and 25 °C for 6 months and 4 °C for 24 months, no obvious change was observed (Table 11).

5. Discussion

Currently, the combined vaccine is one development direction of vaccine research worldwide. The combined vaccines are developing rapidly on the basis of hepatitis B vaccine, there are several reasons contributing to the fact: first, people pay more attention to hepatitis B, as the infectious disease worldwide; sec-

Table 9
Viable count of BCG in HB–BCG combined vaccine ($\times 10^4$ CFU/mg).

Month	Lot	37 °C	25 °C	4 °C
1	HB–BCG 20021204	237	356	420
	HB–BCG 20021205	284	367	607
	HB–BCG 20021206	267	276	544
	BCG 20021226	213	289	485
2	HB–BCG 20021204	138	225	389
	HB–BCG 20021205	164	194	579
	HB–BCG 20021206	134	213	384
	BCG 20021226	110	168	415
3	HB–BCG 20021204	57	115	388
	HB–BCG20021205	67	94	479
	HB–BCG 20021206	32	88	526
	BCG 20021226	21	79	402
9	HB–BCG 20021204	–	–	338
	HB–BCG 20021205	–	–	418
	HB–BCG 20021206	–	–	496
	BCG 20021226	–	–	425
12	HB–BCG 20021204	–	–	328
	HB–BCG 20021205	–	–	358
	HB–BCG 20021206	–	–	456
	BCG 20021226	–	–	397
18	HB–BCG 20021204	–	–	365
	HB–BCG 20021205	–	–	448
	HB–BCG 20021206	–	–	428
	BCG 20021226	–	–	383
24	HB–BCG 20021204	–	–	304
	HB–BCG 20021205	–	–	335
	HB–BCG 20021206	–	–	412
	BCG 20021226	–	–	356

Table 10
Thermostability test of BCG in HB–BCG combined vaccine ($\times 10^4$ CFU/mg).

Lot	Starting	12 months	18 months	24 months
HB–BCG 20021204	237	185	150	114
HB–BCG 20021205	284	177	200	152
HB–BCG 20021206	267	201	186	180
BCG	213	185	166	146

ond, HB vaccine is not a vaccine for life immunity, people have three injections for the whole immunity in the first time, one injection for a booster at 3–5 years, it is inoculated for many times; third, more and more new vaccines are going into children basic immunization, some of them have the same time with HB vaccine for inoculation; finally, HBsAg is a recombinant protein, which has the good compatibility with other antigen. Recently, the various combined vaccines were developed on the basis of HB vaccine at abroad: combined hepatitis A and B vaccines (HepA–HepB) [9–12]; combined diphtheria, tetanus toxoid, pertussis and HB vaccines (DTP–HepB) and combined diphtheria, tetanus toxoid, acellular pertussis and HB vaccines (DTaP–HepB) [13]; combined hemophilies influenzae type b and HB vaccines (Hib–HepB) [14]; combined diphtheria, tetanus toxoid, acellular pertussis, hemophilies influenzae type b and HB vaccines (DTaP–Hib–HepB) [15]; combined diphtheria, tetanus toxoid, pertussis, hemophilies influenzae type b and HB vaccines (DTP–Hib–HepB) [16]; combined diphtheria, tetanus toxoid, pertussis, hepatitis B and poliomyelitis vaccines (DTP–Hib–HepB–IPV) [17]; combined diphtheria, tetanus toxoid, pertussis, hepatitis B, poliomyelitis, hemophilies influenzae type b vaccines (DTP–HepB–IPV–Hib) [18]. Some of these combined vaccines have been approved as safe and effective, applied widely, some are still in the clinical observation, they need further verification. In the current literature retrieval, research of HB–BCG combined vaccine has been not reported at home and abroad, the study has obtained national invention patent (a Intradermal injection Combined Vaccine Included HeVac and BCG and its Preparation Method, patent number: ZL03127065.4). Study of HB–BCG combined vaccine has significance of combined vaccine itself, what is more, it consistents with urgent requirement of China. HB vaccine and BCG vaccine play important role, as biological products that China needs in the long term, their required quantity is very large. Recently, hepatitis B and tuberculosis still occupy the first of pathogenetic population in Chinese infectious diseases. The fact comes up with austere challenge for strengthening preservation work. Development of HB–BCG combined vaccine is a positive research to aim directly at emphases of preservation work in China.

Hepatitis B vaccine and BCG vaccine, as planed immunization products that newborn must inoculate, have been applied together for more than 10 years. Clinical effect of HB vaccine and BCG vaccine immunized together manifestated that there was no interference with each other and harmful effects [19,20]. The combination of HB vaccine and BCG vaccine could not only make the best of advantage of combined vaccine, but also enforce effectively planed immunization. Available HB vaccine and BCG vaccine can not be mixed and inoculated simply, the reasons as following up: first, Sodium Mercurothiolate and formaldehyde in available HB vaccine have antagonism on BCG; second, BCG vaccine cannot be adsorbed with $Al(OH)_3$ adjuvant; third, vaccination dosage and immunity pathway in available two vaccines are different. Thus it can be found that available two vaccines make against combination of HBsAg and BCG, but HBsAg and BCG could combine. The two kinds of antigens (HBsAg and BCG) have good compatibility, immunization time of two vaccines is identical, therefore, combination of HBsAg and BCG has theoretical and practical foundation [20–22]. The study was considered from compatibility and safety of HBsAg and BCG, the combined vaccine removed $Al(OH)_3$ adjuvant, Sodium Mercurothiolate and formaldehyde in original HB vaccine, that avoided potential toxicity of Sodium Mercurothiolate and boosted safety of HB–BCG combined vaccine. FDA of America in 1999 considered toxicity of Sodium Mercurothiolate in HB vaccine, it suggested that newborns were inoculated with HB vaccine without Sodium Mercurothiolate, HB vaccine with Sodium Mercurothiolate was inoculated later for newborns after 2 months [17,23]. FDA and Europe Drug Product Inspection Centre have definite regulation on the problem of Sodium Mercurothiolate, vaccine companies in America and Europe should stop using Sodium Mercurothiolate, quality criteria of current new type vaccines pursued do not contain Sodium Mercurothiolate in the vaccines. The combined vaccine reasonably removals $Al(OH)_3$ adjuvant, Sodium Mercurothiolate and formaldehyde in the HB vaccine, which not only makes compatibility of HBsAg and BCG better, but also decreases toxicity side reaction of vaccines.

Table 11
Moisture determination of HB–BCG combined vaccine.

Lot	37 °C		25 °C		4 °C			
	2 months	6 months	2 months	6 months	6 months	12 months	18 months	24 months
20021204	1.27	1.22	1.21	1.43	1.31	1.33	1.45	1.54
20021205	1.25	1.43	1.34	1.33	1.45	1.30	1.23	1.48
20021206	1.56	1.32	1.62	1.43	1.52	1.44	1.55	1.68

Acute toxicity test, abnormal toxicity test, allergy test, safety test of the guinea pigs and local toxicity test of mice were performed from safety of combined vaccine. The results of allergy test showed that the guinea pigs were immunized with HB–BCG combined vaccine (HBsAg 30 μg/BCG 3×10^5 CFU/mg, a dosage for 6 persons), allergy reaction was not caused. Safety test of the guinea pigs and local toxicity test demonstrated that inflammatory reaction caused by HB–BCG combined vaccine group was similar to pathologic changes caused by BCG control group, pertaining to normal vaccination reaction caused by BCG vaccine. After the combination of HBsAg and BCG, it was not found that HBsAg boosted toxicity of BCG, other abnormal toxicity was also not observed.

Usually, evaluation of immune effect of vaccine is to determine antibody, because more used vaccines induce mainly humoral immunity. Recent study discovered, infectious diseases of intracellular infection such as tuberculosis and hepatitis B, not only antibody plays important role on anti-infection, but also cellular immunity is more important. Therefore, evaluation of immune effect of HB–BCG combined vaccine should be carried out from humoral immunity and cellular immunity.

Experiment of immunogenicity between the HB–BCG combined vaccine and univalent HB vaccine demonstrated that HBsAb geometric mean titer of combined vaccine group (the first injection with combined vaccine) was lower than that of control group, it is not a satisfied result, at first, it was thought that it was experiment error, but results of repeated experiments still so. Further experiments showed antibody titer of HB–BCG combined vaccine group went up rapidly and were higher than that of univalent HB vaccine group after the second and third injection. So, HB–BCG combined vaccine group (the first injection with HB–BCG combined vaccine, the second and third injection with HB vaccine) was compared with univalent HB vaccine group (three injection with HB vaccine), antibody titer of HB–BCG combined vaccine group was higher than that of univalent HB vaccine group from the whole immune effect of HBsAg. Because BCG vaccine regulates mainly cellular immunity, strengthens immunologic memory. HB–BCG combined vaccine group was compared with univalent BCG vaccine group, the diameter of scleroma for local reaction in the PPD test were similar, efficacy showed no significant difference. Cellular immunity effect of the HB–BCG combined vaccine manifested all index of cellular immunity of HB–BCG combined vaccine were enhanced [24–26], it is connected with immunologic mechanism of BCG vaccine, immunologic enhancement of BCG is major in cellular immunity, it promotes T cell activation, proliferation, differentiation to be effector Th cells (Th1 cells are main) [27,28], lymphocyte transformation and IL-2 detection results of combined vaccine were higher than that of univalent BCG vaccine, it is demonstrated that HBsAg and BCG have promotion each other on cellular immunity. Adjuvant effect of BCG has been generally acknowledged. Combined vaccine, which removals $Al(OH)_3$ adjuvant and utilizes adjuvant effect of BCG, could induce humoral immunity and cellular immunity, it is not existed in the univalent hepatitis B vaccine. Acknowledged adjuvant effect of BCG is showed mainly by inducing strong cellular immunity, which makes up a deficiency that HB vaccine simply induces humoral immunity, it assigns new significance for effective prevention of hepatitis B.

Immune effects of combined vaccine and two kinds of univalent vaccine (hepatitis B vaccine and BCG vaccine) were compared by contrast study, it is demonstrated that immune effects of combined vaccine and two kinds of univalent vaccines showed no significant difference, the two kinds of antigens (HBsAg and BCG) have good compatibility. Hepatitis B–BCG combined vaccine could replace univalent vaccines.

Because of specificity of BCG, HB–BCG combined vaccine will adopt freeze drying form and intracutaneous inoculation, that not only could protect HBsAg protein, but also improve the stability of HBsAg. Meanwhile, intracutaneous inoculation could most effectually stimulate immunological reaction, so it is expected to reduce inoculated dosage of HBsAg and improve immune effect simultaneously. It is found that BCG has favourable potentiation on immunological memory from antigen compatibility test. Intracutaneous inoculation pathway adopted by combined vaccine is the most effective pathway of stimulating immunological reaction, intracutaneous inoculation with low dosage can reach, even exceed inoculation effect with high dosage by other pathway [29–33]. The study by repeated experiments demonstrated immune effect of HB–BCG combined vaccine group (HBsAg 5 μg) surpasses obviously that of univalent HB vaccine (HBsAg10 μg).

Univalent HB vaccine is a liquid form, BCG vaccine is a freeze drying form, HB–BCG combined vaccine adopts freeze drying form. Stability test in the study showed the stability of HBsAg in the lyophilized HB–BCG combined vaccine were more better than that in the aqueous vaccine, there was no obvious change of efficacy at 37 °C for 6 months, but efficacy of univalent HB vaccine (CHO cell) in a liquid form had decreased by about 50% at 37 °C for 2–4 weeks. Stability of BCG in the HB–BCG combined vaccine showed no significant difference and was similar to that of the univalent BCG [34].

In a word, the development of HB–BCG combined vaccine can make newborn inoculated with one injection instead of two injection, simplify innoculation procedure and reduce side reaction. After combination of HBsAg and BCG, immune interference was not caused, side reaction was not aggravated, immune effect was not reduced. The advantages of HB–BCG combined vaccine as following up: first, combined vaccine generates effective immunogenicity; second, freeze dried vaccine could improve stability of HBsAg; third, The HB–BCG combined vaccine does not contained $Al(OH)_3$ adjuvant, Sodium Mercurothiolate and formaldehyde, safety is improved; fourth, BCG, as a strong adjuvant, could enhance cellular immunity and humoral immunity, it makes immune effect of combined vaccine surpass univalent HB vaccine; fifth, intracutaneous inoculation with low dosage can reach, even exceed inoculation effect with high dosage by other pathway. In conclusion, HB–BCG combined vaccine accords with Basic Requirements of vaccine in the aspect of safety, efficacy and practicality, it has good development and application value.

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