ความปลอดภัยและการตอบสนองของภูมิคุ้มกันชนิด humoral และ cellular ต่อการได้รับวัคซีนป้องกันไวรัสตับอักเสบบีขนาดปกติเทียบกับขนาดสองเท่าในผู้ป่วยเด็กที่ได้รับการปลูกถ่ายตับ

น.ส.พลิตถิยา สินธุเสก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเวชศาสตร์คลินิก ไม่สังกัดภาควิชา/เทียบเท่า
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SAFTY, HUMORAL AND CELLULAR IMMUNOLOGIC RESPONSE OF STANDARD-DOSE COMPARE TO DOUBLE-DOSE HEPATITIS B REVACCINATION IN CHILDREN AFTER LIVER TRANSPLANTATION

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หัวข้อวิทยานิพนธ์	ความปลอดภัยและการตอบสนองของภูมิคุ้มกันชนิด humoral และ cellular ต่อการได้รับวัคซีนป้องกันไวรัสตับอักเสบบีขนาดปกติเทียบกับขนาดสองเท่าในผู้ป่วยเด็กที่ได้รับการปลูกถ่ายตับ
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คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย อนุมัติให้นับวิทยานิพนธ์ฉบับนี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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พลิตถิยา สินธุเสก : ความปลอดภัยและการตอบสนองของภูมิคุ้มกันชนิด humoral และ cellular ต่อการได้รับวัคซีนป้องกันไวรัสตับอักเสบบีขนาดปกติเทียบกับขนาดสองเท่าในผู้ป่วยเด็กที่ได้รับการปลูกถ่ายตับ. (SAFTY, HUMORAL AND CELLULAR IMMUNOLOGIC RESPONSE OF STANDARD-DOSE COMPARE TO DOUBLE-DOSE HEPATITIS B REVACCINATION IN CHILDREN AFTER LIVER TRANSPLANTATION) อ.ที่ปรึกษาหลัก : ศ. นพ.ยง ภู่วรวรรณ, อ.ที่ปรึกษาร่วม : รศ. พญ.วรนุช จงศรีสวัสดิ์

ประวัติความเป็นมา เนื่องจากผู้ป่วยได้รับการปลูกถ่ายตับมีระดับภูมิคุ้มกันต่อไวรัสตับอักเสบบีต่ำกว่าระดับที่สามารถป้องกันโรคได้ภายหลังจากปลูกถ่ายตับและมีรายงานการติดเชื้อไวรัสตับอักเสบบีขึ้น การศึกษานี้จึงมีวัตถุประสงค์เพื่อเปรียบเทียบประสิทธิภาพของวัคซีนป้องกันไวรัสตับอักเสบบีสองขนาดในเด็กที่ได้รับการปลูกถ่ายตับและตรวจพบระดับภูมิคุ้มกันต่อไวรัสตับอักเสบบีมีค่าต่ำ

วิธีการ ผู้ป่วยเด็กที่ได้รับการปลูกถ่ายตับที่โรงพยาบาลจุฬาลงกรณ์ทุกคนได้รับคัดเลือกให้เข้าร่วมในการวิจัยโดยพิจารณาเกณฑ์ต่อไปนี้คือมีประวัติเคยได้รับการฉีดวัคซีนตับอักเสบบีมาก่อนแต่ตรวจพบว่าระดับภูมิคุ้มกันต่อไวรัส ≤100 mIU/mL โดยการแบ่งชั้นตามระยะเวลานับที่ได้รับการปลูกถ่ายตับ จากนั้นทำการสุ่มชนิด block of four เป็น 2 กลุ่มคือ อาสาสมัครที่ได้รับวัคซีนขนาดมาตรฐาน (0.5 มล.) 3 ครั้งและขนาดสองเท่าของมาตรฐาน (1 มล.) 3 ครั้งเข้ากล้ามที่เวลา 0-1-6 เดือน โดยผู้ปกครองของอาสาสมัครจะไม่ทราบว่าบุตรหลานได้รับการจัดอยู่ในกลุ่มใด มีการนัดติดตามที่ช่วงระยะเวลา (time point) 0, 1, 6, 7-9 และ 9-12 เดือน แบ่งการตอบสนองต่อวัคซีนเป็น 2 กลุ่ม โดยอาสาสมัครที่มีระดับภูมิคุ้มกันมากกว่า 10 mIU/mL ภายหลังได้รับวัคซีนครบ 3 เข็มอาสาสมัครที่มีระดับภูมิคุ้มกันน้อยกว่า 10 mIU/mL ภายหลังได้รับวัคซีนครบ 3 เข็มเป็น responder และ nonresponder ทั้งนี้ระดับภูมิคุ้มกันต้อง ≤10 mIU/mL ก่อนได้รับวัคซีน สำหรับตัวอย่างเลือดจะนำไปสกัดเม็ดเลือดขาว (periphearal blood mononuclear cells หรือ PBMCs) และทำการตรวจโดยวิธี the enzyme-linked immune absorbent spot (ELISpot) assay และ flow cytometry มีการศึกษาสะกิดผิวหนังด้วยวัคซีนตับอักเสบบีในอาสาสมัครก่อนได้รับวัคซีนด้วยวิธี Mantoux และอ่านผลที่ 48 และ 72 ชั่วโมง โดยรอยนูนที่ผิวหนังที่มขนาดไม่น้อยกว่า 5 มิลลิเมตรอ่านผลเป็นบวก ผลการศึกษาหลักคือสัดส่วนของ responder และ nonresponder ภายหลังการได้รับวัคซีนและระดับภูมิคุ้มกันที่เวลา 7-9 เดือน โดยการศึกษานี้จดทะเบียนใน Thai Clinical Trials Registry with study (TCTR 20180723002) ก่อนเริ่มทำการศึกษา

ผลการวิจัย อาสาสมัครจำนวน 66 คนได้รับการคัดเลือกตามเกณฑ์และสุ่มเป็น 2 กลุ่ม กลุ่มละ 33 คน อาสาสมัครจำนวน 3 และ 4 คนจากกลุ่มที่ได้รับวัคซีนขนาดมาตรฐานและขนาดสองเท่าได้ถูกทำการคัดออกระหว่างการศึกษา จึงมีอาสาสมัครทั้งหมด 30 และ 29 คนเข้ารับการศึกษาจนถึงจุดสิ้นสุดในกลุ่มที่ได้รับวัคซีนขนาดมาตรฐานและขนาดสองเท่า ที่ time point 4 จำนวน seroconversion เท่ากับ 23 (92.0%) จาก 25 (95% CI 73.9-99.0) ในกลุ่มได้รับวัคซีนขนาดมาตรฐานและ 16 (88.9%) จาก 18 (95% CI 65.3-98.6) ในกลุ่มได้รับวัคซีนขนาดสองเท่าโดยไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของระดับภูมิคุ้มกันของทั้งสองกลุ่มในทั้ง 5 time point อย่างไรก็ตาม ระดับภูมิคุ้มกันที่ time point 4 (1372.4 [95% CI 650.2-2896.7] ในกลุ่มได้รับวัคซีนขนาดมาตรฐานและ 730 [95% CI 262.7-2031.6] mIU/mL ในกลุ่มได้รับวัคซีนขนาดสองเท่า) มีค่าสูงกว่าที่ time point 2 (241.3 [95% CI 90.9-641.0] ในกลุ่มได้รับวัคซีนขนาดมาตรฐานและ 181 [95% CI 63.8-516.1] mIU / mL ในกลุ่มได้รับวัคซีนขนาดสองเท่า) ในทั้งสองกลุ่มอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ ในกลุ่มที่ได้รับวัคซีนขนาดสองเท่าที่ time point 5 พบระดับภูมิคุ้มกันมีค่าสูงกว่าที่ time point 2 อย่างมีนัยสำคัญทางสถิติ (969 [95% CI 328.2-2861.4] และ 181.5 [95% CI 63.8-516.1] mIU/mL) ในด้านของความปลอดภัยของการได้รับวัคซีน ไม่พบรายงานอาการไม่พึงประสงค์ที่รุนแรงของวัคซีนตับอักเสบบีทั้งสองกลุ่ม พบ IFN-γ ที่ time point 4 สูงกว่า time point 1 (32 [4, 68] และ 14 [0, 23] spot forming cells/106 PBMCs, P<0.05) อย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตาม ไม่พบความแตกต่างในกลุ่มประชากรย่อยของ Treg, CD4 T cell , CD8 T cell, B cell และ NK cell ในกลุ่ม responder (38 คน) และ nonresponder (4 คน) ปัจจัยที่มีผลต่อการตอบสนองของระดับภูมิคุ้มกันต่อไวรัสตับอักเสบบี ได้แก่ ระยะเวลาภายหลังได้รับการปลูกถ่ายตับ (1.95 [0.66, 4.95] และ 0.58 [0.54, 0.65] ปี)และระดับยากดภูมิคุ้มกัน tacrolimus ที่ time point 1 (3.6 [2.6, 5.7] และ 6.7 [5.8, 7.8] ng/mL) ในกลุ่ม responder และ nonresponder)

ผลสรุป การได้รับวัคซีนป้องกันไวรัสตับอักเสบบีในขนาดมาตรฐานและขนาดสองเท่าจำนวน 3 เข็ม มีประสิทธิภาพสูงและปลอดภัยสำหรับเด็กที่ได้รับการปลูกถ่ายตับ โดยพบว่าระดับภูมิคุ้มกันต่อไวรัสตับอักเสบบีในกลุ่มที่ได้วัคซีนขนาดสองเท่ามีค่าสูงอย่างมีนัยสำคัญในการติดตามระยะสั้น โดยปัจจัยที่ทำให้การตอบสนองต่อวัคซีนไวรัสตับอักเสบบีได้ผลสำเร็จคือ ระยะการให้วัคซีนไวรัสตับอักเสบบีในผู้ป่วยเด็กหลังปลูกถ่ายตับไม่ควรเร็วกว่า 6 เดือนซึ่งเป็นช่วงที่ระดับยากดภูมิคุ้มกันมีค่าสูงอยู่

สาขาวิชา	เวชศาสตร์คลินิก	ลายมือชื่อนิสิต .....
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KEYWORD: Children, Immunity, Liver transplant, Hepatitis B, Vaccine

Palittiya Sintusek : SAFTY, HUMORAL AND CELLULAR IMMUNOLOGIC RESPONSE OF STANDARD-DOSE COMPARE TO DOUBLE-DOSE HEPATITIS B REVACCINATION IN CHILDREN AFTER LIVER TRANSPLANTATION. Advisor: Prof. Yong Poovorawan, M.D. Co-advisor: Assoc. Prof. VORANUSH CHONGSRISAWAT, M.D.

Background: High prevalence of hepatitis B (HB)-antibody loss after liver transplantation (LT) and de novo HB infection were documented. This study aimed to compare the effectiveness of two revaccination regimens in inducing protective immunity in children with liver transplants.

Methods: Children who underwent liver transplantation at King Chulalongkorn Memorial Hospital were recruited. All received primary HB immunization but anti-HBs antibodies after LT if HBs antibodies ≤100 mU/mL. Children were stratified by age at transplantation and then allocated with block of four randomization into two groups; standard (0.5 ml) 3-dose and double (1 ml) 3-dose HB vaccine intramuscularly at 0, 1, and 6 months. The parents were blind with respect to the vaccine regimen. Anti-HBs titers were assessed at 0, 1, 6, 7-9, and 9-12 months. A participant was categorized as a responder if the participant had anti-HBs levels <10 mU/mL before revaccination but had seroconversion (anti-HBs >10 mU/mL) after the 3-dose vaccination regimen. Participants were defined as nonresponders if they had anti-HBs levels <10 mU/mL before revaccination and had no seroconversion after the 3-dose vaccination regimen. Peripheral blood mononuclear cells (PBMCs) were extracted for in vitro cellular immune study by the enzyme-linked immune absorbent spot assay (ELISpot) and flow cytometry. In vivo cellular immune study with delayed-type hypersensitivity (DTH) skin test was performed at beginning with hepatitis B vaccine by Mantoux method. Skin duration was measured by their guardians and the investigators via pictures at 48 hours and 72 hours after the test. The induration size ≥ 5 mm and larger than control was considered positive results. The primary outcome was the percentage of responders and geometric mean titer (GMT) of anti-HBs levels at 7-9 months. The trial was registered in Thai Clinical Trials Registry with study number TCTR 20180723002.

Results: Sixty-six children were recruited and randomly assigned into two groups with 33 participants in each group. At the end point, three in the standard-dose and four participants in the double-dose group dropped out. Thirty and 29 participants from standard-dose and double-dose regimens, respectively, were included per protocol analysis. At months 7-9, the percentage of seroconversion was 23 (92.0%) of 25 (95% CI: 73.9-99.0) in the standard-dose group and 16 (88.9%) of 18 (95% CI: 65.3-98.6) in the double-dose group. Regarding the GMT of anti-HBs antibodies, there was no significant difference between the two groups at all five time points. However, the GMT of anti-HBs antibodies at time point 4 (1372.4 [95% CI: 650.2-2896.7] in the standard-dose group and 730 [95% CI: 262.7-2031.6] mU/mL in the double-dose group) was significantly higher than at time point 2 (241.3 [95% CI: 90.9-641.0] in the standard-dose group and 181 [95% CI: 63.8-516.1] mU/mL in the double-dose group) in both groups (P< 0.05). No serious adverse reactions to the HB vaccine were reported. After time point 5, the GMT of anti-HBs levels in the double-dose group was significantly higher than after a booster dose (time point 1) (969 [95% CI: 328.2-2861.4] and 181.5 [95% CI: 63.8-516.1] mU/mL). IFN-γ at time point 4 was significantly higher than at time point 1 (32 [4,68] and 14 [0,23] spot-forming cells/106PBMCs, P<0.05). There was no significant difference in the subpopulations of T-reg, CD4 T cells, CD8 T cells, B cells, and NK cells. 57 (96.7%) of participants were performed DTH skin testing with hepatitis B vaccine. Comparing the result of DTH skin test with seroconversion of anti-HBs after first vaccination and third vaccination, the sensitivity, specificity, negative predictive value, positive predictive value and accuracy were 75%, 53%, 85%, 39% and 70% vs 60%, 79%, 97%, 11% and 61%, respectively. In comparing responders (n=38) and nonresponders (n=4), the time of revaccination after LT and the tacrolimus level were the significant factors in seroconversion. The time of revaccination after liver transplantation in responders and nonresponders was 1.95 (0.66, 4.95) and 0.58 (0.54, 0.65) years, respectively. The tacrolimus levels in responders and nonresponders were 3.6 (2.6, 5.7) and 6.7 (5.8, 7.8) ng/mL, respectively.

Conclusion: The 3-standard-dose and 3-double-dose HB regimens were highly effective and safe for children with liver transplants, and the double-dose regimen maintained the high anti-HBs level at short-term follow up. The negative results from DTH skin test could predict slow responder and nonresponder in liver-transplanted children For successful reimmunization with a robust humoral response, anti-HBs antibodies should be monitored post-liver transplant and HB revaccination should be introduced not earlier than 6 months after LT when the immunosuppressant level is still high.

Field of Study: Clinical Sciences
Academic Year: 2020

Student's Signature .....
Advisor's Signature .....
Co-advisor's Signature .....

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สารบัญ

หน้า

Part 1

1.1 Introduction

King Chulalongkorn Memorial Hospital (KCMH) established liver transplantation unit for children in 1988 and 124 children have undergone liver transplantation since then. Of particular interest was a 5-year-old child[1] with biliary cirrhosis whose previous immunization included four doses of hepatitis B virus (HBV) vaccine (anti-HBs antibodies >1,000 mIU/mL) but was diagnosed with *de novo* hepatitis B (DNH) posttransplant. DNH infection may have resulted after transplantation when this child lost protective immunity to HBV despite pre-transplant high levels of anti-HBs antibodies (Figure 1).

Figure 1 A child who was diagnosed DNH after liver transplantation despite complete hepatitis B immunization pretransplant[1]

Revaccination with hepatitis B vaccine after liver transplantation has been recommended, but other vaccine regimens as alternatives to a booster have not yet been assessed. Moreover, there has been no solid evidence for an HBV protective antibody levels though, antibody monitoring after revaccination of children with liver transplant. In addition, there are no data on revaccination for other vaccine-preventable diseases (VPIs) in children after liver transplant. Just as the antibody loss for hepatitis B in the aforementioned case, the impact of other VPIs should be further evaluated to determine whether these children should be revaccinated post-transplant. As study on the burden of VPIs and the impact of incomplete immunization in children with liver transplants could guide clinicians in developing strategies necessary to prevent VPIs post-transplant.

1.2 Background and statement of the problem

The burden of VPIs and the impact of incomplete immunization in children with liver transplants

Infection after liver transplant is a serious concern because of the potential morbidity and mortality[2-5]. In addition to more complicated and severe illnesses than those experienced in immunocompetent patients, such infections could give rise to graft rejection, thus affecting short- or long-term graft survival[5]. Therefore, strategies to decrease overall post-transplant infection are warranted. Immunization is considered an effective, less invasive, and affordable way to reduce VPIs[6] such as measles, varicella, influenza, and viral hepatitis A and B. The Infectious Diseases Society of America (IDSA)[7] and the American Society of Transplantation (AST) Infectious Disease Community of Practice[8] encourage using the accelerated vaccine schedule, especially with live vaccines for immunocompromised children with solid organ transplants.

Feldman *et al*[5, 9] studied the impact of VPIs in children after liver transplant and solid organ transplant with respect to morbidity, mortality, and costs. They found a significant rate of VPIs in these children in comparison with the general pediatric population. However, published data on VPIs in children after liver transplant particularly in Thailand are scarce and the impact of VPIs in complete and incomplete vaccination has been not reported elsewhere. Strategies to avoid VPIs should be initiated to improve the quality of life of children with liver transplants by minimizing the serious infectious complications after liver transplant worldwide and particularly in Thailand.

The burden of VPIs at KCMH was investigated previously in the context of hepatitis B vaccination to prevent DNH and the prevalence of loss of humoral immunity in children with liver transplants[1]. The impact of other VPIs post-transplant will be evaluated to prioritize the revaccination of children with liver transplants in Thailand and it is hoped the findings can be generalized to apply in other countries.

Hepatitis B immunologic loss in children after liver transplantation

In 2014, Leung *et al*[10] studied the prevalence of HBV immunity in 160 children after liver transplantation and found that 67% of previously immunized children lost

immunity (anti-HBs <10 mIU/mL) after liver transplantation at a mean time of 5.6 ± 4.6 years. However, no children had DNH. In 2018, Sintusek *et al*[1] assessed the seroprevalence of hepatitis B virus immunity among previously vaccinated children (at least three HBV injections) who underwent liver transplantation and presented a case report on DNH after liver transplantation. Even with full HBV immunization with/without one booster dose before liver transplantation, there was a high incidence of hepatitis B immunity loss (46% at 1 year, 57% at 2 years and 82% at >3 years following liver transplantation). Sintusek *et al* reported a patient who had a very high level of anti-HBs antibodies (>1000 mIu/mL) prior to transplantation but the child lost immunity after 3 years and 10 months posttransplant and was diagnosed as having DNH.

In healthy persons who received HBV vaccine but have lost antibodies (anti-HBs <10 mU/mL), the rapid anamnestic response to increase the anti-HBs level occurs at 5-8 days after HB vaccination. However, there are few data about this cellular immunity and the response to HBV exposure in children with liver transplants. Information on the anamnestic response to booster doses is needed to adequately address immunity in children after liver transplantation.

Humoral response to HBV vaccine in children after liver transplantation

As mentioned previously, disappearance of HBs antibodies might indicate loss of protection in an immunocompromised host. Presence of HBs antibodies has been shown to be a correlate of immunity and offers the simplest way to demonstrate durable protection.

In 2000, Duca *et al*[11] prospectively studied the immunogenicity of the HBV vaccine in unimmunized children (N=47) before liver transplantation with three full courses of recombinant HBV vaccine after liver transplantation (mean time after liver transplantation, 3.56±2.19 years). Among the participants, 70% had anti-HBs antibody levels higher than 10 mU/mL after the HBV series, and 50% (7/14) of the hyporesponders had anti-HBs levels >10 mU/mL after a booster dose. Their findings

indicate a good immunologic response to HBV vaccine in children after liver transplantation.

In 2007, Lin *et al*[12] prospectively studied children after liver transplantation (N=60) by monitoring anti-HBs antibodies every 1-3 months. Children had received the HBV booster if the anti-HBs antibody level was less than 1,000 mU/mL. The median follow-up periods were 51 and 57 months for children with liver donors negative and positive for anti-HBc antibodies, respectively. There were two children diagnosed with DNH in this study.

In 2009, Su *et al*[13] retrospectively studied the incidence of and risk factors for DNH in children after liver transplantation (N=51). They found nine cases of DNH (one from a negative anti-HBc liver donor). The major risk factor for DNH was low anti-HBs titers (less than 200 mU/mL) before liver transplantation. This study also indicated that an anti-HBs antibody titer >200 mU/mL before liver transplantation might be sufficient to prevent DNH in HBsAg-negative recipients.

In 2008, Ni *et al*[14] demonstrated that the incidence of HBV immunologic loss in pediatric liver transplant candidates was high. However, 63% (N=7) and 100% (N=4) had anti-HBs antibody levels more than 10 mU/mL after the first and second booster, respectively.

Cellular response to HBV vaccine in children after liver transplantation

After HBV vaccination, the immune mechanism to protect against HBV infection depends on HBsAg-specific B and T cell-mediated immune memory. HBsAg-specific immune memory can have an anamnestic response to as booster dose of hepatitis B vaccine, normally at 5-8 days after the re-exposure to the HBsAg and peaks after about 14 days.[15, 16] Consequently, there is no need for healthy persons who had undetectable levels of anti-HBs antibodies after complete HBV immunization. However, there is little information on HBsAg-specific immune memory and the response after HBsAg exposure especially in liver-transplanted recipients.

***In vitro* cellular immune response to vaccination**

In 2007, Bauer *et al*[16] conducted a pilot analysis of cellular immune response determining HBsAg T and B cells in 16 adults after liver transplantation, including 6 healthy immunized persons and 21 healthy unvaccinated persons. The study demonstrated that the specific induction of T-reg cells secreting IL-10 corresponded with the poor response of liver transplant recipients to revaccination, while healthy immunized and nonimmunized persons had a strong Th1-type immune response, with HBsAg T cells secreting IL-2, interferon gamma, and tumor necrosis factor alpha. This study highlighted the role of a strong inhibitory effect of T-reg cells in the immunologic response after hepatitis B revaccination.

In 2008, Ni *et al*[14] studied both humoral and cellular immunity after booster hepatitis B vaccines in children with liver transplants by measuring the anti-HBs antibody level and HBsAg-specific cytokine production using the enzyme-linked immune absorbent spot assay (ELISpot). They found that 2 months after a booster, 7 of 11 children with a liver transplant developed protective anti-HBs antibodies (>10 mU/mL). Moreover, the cellular immunity result was compatible with that of the anti-HBs antibodies because each patient had more than one spot-forming cell to HBsAg-specific interferon gamma and IL-5, which reflects the good response of Th1-cells and Th2-cells.

In vivo cellular immune response to vaccination

Delayed-type hypersensitivity (DTH) skin testing is a cost-effective *in vivo* test to evaluate T cell-mediated immunity. Siripassorn *et al*[17] found three suitable antigens for DTH skin testing in Thailand including purified protein derivative (PPD), tetanus toxoid (TT) and *Candida albicans;* the positive response in healthy adults was 92.6%, 83.2%, and 82.1%, respectively. However, only 5.3% of subjects in this study had a positive response to HBsAg, which might be explained by suboptimal exposure to this antigen, the long period of vaccination, or no previous hepatitis B vaccination before the DTH testing in adults. Because hepatitis B vaccine is part of the expanded program immunization (EPI) vaccines that our children receive at birth and 2, 4, and 6 months of age, the DTH skin test with HBsAg might be the appropriate *in vivo* tool to evaluate

both T-cell response and the HBsAg-specific T-cell response in children after liver transplantation.

Gaps in knowledge

- There is no study comparing the prevalence of VPIs and the impact of incomplete vaccination before liver transplantation in children.

- To date, there has been no consensus guideline or solid information on HBV protective antibody levels, antibody monitoring, and appropriate revaccination regimen for children with liver transplants.

- According to previous studies, humoral and cellular immunologic responses after the booster dose seem to be adequate only in the short term. A program of revaccination to increase immune responses and maintain immunity in the long term should be assessed.

- Studies about cellular response, especially the anamnestic response, in children after liver transplantation should be further evaluated.

Research questions

The following questions deserve in-depth study and further analysis;

- How common is incomplete immunization in children pre- and post-liver transplantation?
- How common is immunity loss from primary vaccination and VPIs in children with liver transplants?
- What are the humoral and cellular immune responses to hepatitis B revaccination in children after liver transplantation?
- Which revaccination regimen could increase and maintain longer immune protection after liver transplantation?
- What are the factors related with seroconversion and in maintaining long-term hepatitis B immunity after revaccination?

OBJECTIVES

Project 1

Primary objective

To evaluate the immunization status in Thai children at the time of liver transplantation and thereafter.

Secondary objective

To study the impact of VPIs and non-VPIs during hospitalization in children who had complete and incomplete vaccination.

Project 2

Primary Objective

To study and compare the safety and immunogenicity of the double-3-dose and standard-3-dose hepatitis B series in children with liver transplants with hepatitis B immunologic loss after liver transplantation.

Secondary Objective

To identify the factors related to humoral immune response in children with liver transplants who have hepatitis B humoral immune loss.

To determine the factors maintaining hepatitis B immunity in children with liver transplants after revaccination.

1.3 Scope of the study

Children in our liver transplant center who matched the inclusion and exclusion criteria for each project were recruited. In Project 1, the prevalence of VPIs and non-VPIs and history of immunization before and after liver transplantation at KCMH were retrospectively reviewed from patients' vaccination books and hospital records (inpatients and outpatients). In Project 2, we conducted the single-blind, randomized controlled trial with two vaccine regimens (standard-3-dose and double-3-dose regimens, intramuscular route) in children with liver transplants at KCMH. All of them were followed up five times (at 0, 1, 6, 7-9, and 9-12 months after revaccination), and

blood samples were collected for humoral and cellular immunologic study at each time point.

1.4 Clinical application of the study

The prevalence of VPIs after liver transplantation and the impact of incomplete immunization before and after liver transplantation will be valuable in learning how to prevent VPIs in children post-transplant and evaluating the revaccination program for each vaccine. With respect to hepatitis B infection after liver transplantation following immunologic loss or DNH that occurred in our center previously, we expect that this project could demonstrate a new strategy to prevent DNH in children after liver transplantation and change our immunization practices in these children. Moreover, the proposed vaccine regimen would have the potential to be used instead of the booster dose that we usually give to these children who have antibodies lower than the protective level. This study is also expected to advance knowledge of the cellular immune response to HBV vaccination in children after liver transplantation. It is hoped that this project will contribute new knowledge about both humoral and cellular immune responses to hepatitis B revaccination that could benefit clinicians and patients in developing an appropriate vaccination regimen and follow up.

Part 2

2.1 Immunization status and hospitalization from vaccine-preventable and non-vaccine preventable infections in liver-transplanted children

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ABSTRACT

BACKGROUND

Infections and associated morbidity and mortality may be more frequent in children who have undergone liver transplant than in healthy children. Immunization strategies to prevent vaccine-preventable infections (VPIs) can effectively minimize this infection burden. However, data on age-appropriate immunization and VPIs in children after liver transplant in Asia are limited.

AIM

To evaluate the immunization status and VPIs and non-VPIs requiring hospitalization in children who have undergone a liver transplant.

METHODS

The medical records of children who had a liver transplant between 2004 and 2018 at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) were retrospectively reviewed. Immunization status was evaluated *via* their vaccination books. Hospitalization for infections that occurred up to 5 years after liver transplantation were evaluated, and divided into VPIs and non-VPIs. Hospitalizations for

cytomegalovirus and Epstein-Barr virus (EBV) were excluded. Severity of infection, length of hospital stay, ventilator support, intensive care unit requirement, and mortality were assessed.

RESULTS

Seventy-seven children with a mean age of 3.29 ± 4.17 years were included in the study, of whom forty-one (53.2%) were female. The mean follow-up duration was 3.68 ± 1.45 years. Forty-eight children (62.3%) had vaccination records. There was a significant difference in the proportion of children with incomplete vaccination according to Thailand's Expanded Program on Immunization (52.0%) and accelerated vaccine from Infectious Diseases Society of America (89.5%) ($P < 0.001$). Post-liver transplant, 47.9% of the children did not catch up with age-appropriate immunizations. There were 237 infections requiring hospitalization during the 5 years of follow-up. There were no significant differences in hospitalization for VPIs or non-VPIs in children with complete and incomplete immunizations. The risk of serious infection was high in the first year after receiving a liver transplant, and two children died. Respiratory and gastrointestinal systems were common sites of infection. The most common pathogens that caused VPIs were rotavirus, influenza virus, and varicella-zoster virus.

CONCLUSION

Incomplete immunization was common pre- and post-transplant, and nearly all children required hospitalization for non-VPIs or VPIs within 5 years post-transplant. Infection severity was high in the first year post-transplant.

Keywords: Children; Hospitalization; Immunization; Liver transplant; Thailand; Vaccine-preventable infection

Core tip: Incomplete age-appropriate immunization in children waiting for a liver transplant was expected, and nearly half of them had not caught up with age-appropriate vaccinations post-transplant. Though there was no significant difference in hospitalization from vaccine-preventable infections (VPIs) and non-VPIs in children with complete and incomplete immunizations. At least 13.1% required hospitalization within 5 years post-transplant, and > 10% were admitted to the intensive care unit

and required respiratory support. The severity of infections was high during the first year post-transplant. Complete immunization and robust infection control should be prioritized in children both pre- and post-liver transplant.

INTRODUCTION

Infection after liver transplant is a serious concern due to the potential associated morbidity and mortality[2-5], as well as the standard complication and severe symptoms that can be experienced by immunocompetent patients. Such infections can give rise to graft rejection, thus affecting short- or long-term graft survival[5]. Accordingly, strategies to reduce overall post-transplant infection are warranted. Immunization is considered an effective, relatively noninvasive, and affordable way to reduce vaccine-preventable infections (VPIs)[6] such as measles, varicella, influenza, viral hepatitis A and B, among others. Infectious Diseases Society of America (IDSA)[7] and the American Society of Transplantation (AST) Infectious Disease Community of Practice[8] encourage accelerated vaccine, particularly with regard to live vaccines in immunocompromised children after solid organ transplant.

Children awaiting a liver transplant can be at a disproportionate risk of VPIs because they tend not to have undergone a complete series of age-appropriate immunizations, because their serious illness has taken medical priority over vaccination[18]. Verma and Wade[19] reported that in their experience at King's College Hospital, only 20-30% of children had undergone a complete series of age-appropriate immuniation prior to liver transplantation. Diana *et al*[20] reported that less than half of a cohort of children who underwent liver transplant at the Children's Hospital of Geneva in Switzerland had undergone a complete series of age-appropriate vaccinations with the rate of 43% for diphtheria-tetanus-acellular pertussis-polio vaccine, 44% for measles-mumps-rubella (MMR) vaccine, 13% for hepatitis B vaccine, and 5% for hepatitis A vaccine at the time of liver transplantation. Feldman *et al*[5, 9] investigated morbidity, mortality, and costs associated with VPIs in children after solid organ transplants, and reported a significantly higher rate of VPIs in these children than in the general pediatric population.

Studies conducted in the United States and other western countries in have highlighted the effect of VPIs in children after solid organ transplantation[6, 9, 21], but published data on VPIs in children after liver transplantation in the East are scarce. To improve the quality of life of liver-transplanted children by minimizing the serious infectious complications associated with post-liver transplantations, strategies to avoid VPIs based on strong evidence should be initiated worldwide, including in Asia. The aim of the present study was to evaluate immunization status in Thai children at the time of liver transplantation, and for up to 5 years post-liver transplantation. The prevalence and effects of VPIs and non-VPIs during hospitalization are also assessed.

MATERIALS AND METHODS

The current study was a retrospective review of all children who received a liver transplant at King Chulalongkorn Memorial Hospital in Thailand from January 2004 to August 2018. Demographic data, patient characteristics, and immunization records from vaccination books were collated. Hospitalization records pertaining to the liver transplant operation and admission due to infections for up to 5 years post-transplant were included. Hospitalizations for EBV and cytomegalovirus were excluded from the study. Infection etiology and source were investigated by the doctors in charge. Culture from specimens was available for all bacterial origins, and immunological and molecular techniques were available for the diagnosis of both viral and bacterial infections, including polymerase chain reaction panel analysis for respiratory tract infections and gastrointestinal infections, and antibody titers for hepatitis A/B/E, dengue, and measles.

Infections were divided into VPIs and non-VPIs. Length of hospital stay, severity of infections, and mortality from infections were collated and classified into three groups: intensive care unit (ICU) requirement, ventilator support, and death. Complete immunization was defined as that conducted in accordance with the Expanded Program on Immunization (EPI) in Thailand (Table 1) and the accelerated vaccination recommendations described in the 2013 IDSA Clinical Practice Guideline for Vaccination of the Immunocompromised Host [7] which notes: "... children aged 6-12 mo can

receive MMR and varicella vaccine and the second dose should be administered at 12 mo for MMR and ≥ 3 mo apart for varicella vaccine. However, the last MMR or varicella vaccine injection should not be within 4 wk of a liver transplant schedule." The present study received ethics approval by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Thailand (IRB number: 806/62)

Table 1 The immunization schedule in Thailand and accelerated vaccines by the Infectious Disease Society of America

	Vaccine	Birth	1 mo	2 mo	4 mo	6 mo	7 mo	9 mo	12 mo	18 mo	24 mo	4 yr	9 yr	11 yr
Thai's EPI vaccines	BCG	1												
	HBV	1	(For positive maternal HBsAg)	2		3								
	DTP, OPV/IPV			1	2	3				4		5		
	MMR					Acc[1]		1	Acc[1]		2			
	JE							1			2			
	Influenza					1	2							
	Tdap													1
	HPV												Acc	1-2[2]
Optional vaccine in Thailand	Rota			1	2	(3)								
	PCV			1	2	3			4					
	Varicella					Acc[1]		Acc[1]	1	2				
	HAV								1	2				
	Dengue												1-3[3]	

[1]Acc denotes accelerated vaccines from the 2013 Infectious Diseases Society of America Clinical Practice Guideline for Vaccination of the Immunocompromised Host in which measles-mumps-rubella (MMR) at 6 and 12 mo of age and varicella at 6 mo of age and 3 mo apart from the first dose; [2]Indicates 0 and 6 mo; [3]Indicates 0, 6, 12 mo. BCG: Bacillus Calmette-Guerin vaccine; DTP: Diphtheria-tetanus-pertussis; EPI: Expanded Program on Immunization; HAV: Hepatitis A vaccine; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B vaccine; HPV: Human papillomavirus vaccine; JE: Japanese encephalitis; OPV/IPV: Oral polio vaccine/inactivated polio vaccine; PCV: Pneumococcal conjugate vaccine; Tdap: Tetanus-diphtheria-acellular pertussis.

Statistical analysis

Continuous and categorical data are presented as the mean ± standard deviation, medians and interquartile ranges, proportions, or percentages as appropriate. The Mann-Whitney *U* test and unpaired *t*-test were used to compare continuous data, and Fisher's exact test and the χ^2 test were used to compare discrete data. $P < 0.05$ was considered statistically significant. Data analyses were performed using Statistical Package for the Social Sciences version 24.0.0 (SPSS, Inc.; Chicago, IL, United States). A biomedical statistician employed at the Department of Statistics Science, Kasetsart University (Bangkok, Thailand) reviewed the statistical analyses conducted in the study.

RESULTS

Patient characteristics and history of immunization

Seventy-seven children with a mean age of 3.29 ± 4.17 years were included in the study, of whom 41 (53.2%) were female. The indications for liver transplantation were biliary atresia (*n* = 63), indeterminate acute liver failure (*n* = 3), progressive familial intrahepatic cholestasis (*n* = 2), Alagille syndrome (*n* = 2), cryptogenic cirrhosis (*n* = 2), citrin deficiency (*n* = 1), Budd-Chiari syndrome (*n* = 1), hepatoblastoma (*n* = 1), autoimmune hepatitis (*n* = 1), glycogen storage disease type IV (*n* = 1), and bile acid deficiency (*n* = 1). The mean follow-up time was 3.68 ± 1.45 years, and 32 children were followed up for a full 5 years after liver transplantation. Vaccinations were noted in the vaccination books of 48/77 children (62.3%). Substantial proportions of children did not have complete vaccinations in accordance with Thailand's EPI (*n* = 25, 52%) (Table 1) or accelerated vaccinations in accordance with the IDSA recommendations (*n* = 43, 89.5%) ($P < 0.001$). Post-liver transplant, 23 children (47.9%) could not catch up with the appropriate immunizations for age. All children were revaccinated with hepatitis B vaccine if hepatitis B surface antibody was < 10 mIU/mL. Other vaccines they received after liver transplantation included those for influenza (*n* = 12), invasive pneumococcal disease (*n* = 10), Japanese encephalitis (*n* = 6), diphtheria/tetanus/pertussis-inactivated polio vaccine (*n* = 6), and hepatitis A (*n* = 3).

A minority of children were up-to-date with influenza vaccination (*n* = 18, 37.5%) and pneumococcal conjugate vaccine (*n* = 22, 45.8%) post-liver transplant compared with pre-liver transplant (*n* = 30, 62.5% for influenza and *n* = 36, 75% for pneumococcal conjugate vaccine) (*P* < 0.001; Table 2). With regard to live vaccines, three individuals were inadvertently vaccinated with MMR at their local hospitals without any serious side effects.

Table 2 Vaccination history in children at liver transplant and up to 5 years follow-up, *n* = 48

Vaccines	Incomplete vaccination for age at transplantation		Incomplete vaccination for age after liver transplant, *n* (%)
	Thai EPI program, *n* (%)	Accelerated vaccine from IDSA, *n* (%)	
DTP-OPV/IPV	12 (25)	N/A	6 (12.5)
HBV	6 (12.5)		0
MMR	12 (25)	30 (62.5)[b]	27 (56.3)[b]
JE	16 (33.3)	N/A	10 (20.8)
Varicella	16 (33.3)	34 (70.8)[b]	34 (70.8)[b]
HAV	26 (54)		23 (47.9)
Influenza	30 (62.5)		18 (37.5)[a]
PCV	36 (75)		22 (45.8)[b]
Rota	37 (77)	N/A	37 (77)
All	25 (52)	43 (89.5)[b]	23 (47.9)
	(not included rota vaccine)		(not included lived vaccine)

[a]*P* < 0.05 *vs* Thai Expanded Program on Immunization (EPI); [b]*P* < 0.001 *vs* Thai EPI program. DTP: Diphtheria-tetanus-pertussis; HAV: Hepatitis A vaccine; HBV: Hepatitis B vaccine; IDSA: Infectious Diseases Society of America; JE: Japanese encephalitis; MMR: Measles-mumps-rubella; N/A: Not applicable; OPV/IPV: Oral polio vaccine/inactivated polio vaccine; PCV: Pneumococcal conjugate vaccine.

Infections during and after liver transplant

Infection severity and mortality were highest during the first year post-liver transplant. The respiratory and gastrointestinal systems were the most common sites of infection (Table 3). Two children died within 3 mo after liver transplantation, and both had underlying post-transplant lymphoproliferative disorder. One of these two children had mixed infection with bocavirus, mycoplasma, and parvovirus B19. The other exhibited Epstein-Barr virus viremia that progressed to respiratory failure with an unidentified infectious origin. Of the 31 hospitalizations for VPIs recorded during the study period the median length of hospital stay was 6 d (range: 3-8 d), and in three cases ICU admission and ventilator support were required; two with influenza and one with *Streptococcus pneumoniae* infection. When the children were divided into complete and incomplete immunization groups based on Thailand's EPI, there were no significant differences in the numbers of hospitalizations for VPIs or non-VPIs (Table 4).

Table 3 Characteristics of hospitalization from VPIs and non-VPIs up to 5 years follow-up

Table 3.1 Type of infections

Time	Type of infections			
	VPIs		Non-VPIs	
	Times, n (%)	LOS (days)	Times, n (%)	LOS (days)
During transplant	4 (5.2)	51 (24,79)	73 (94.8)[b]	35 (27,49)
<3 months	2 (6.9)	3 (3,3)	27 (93.1)[b]	12 (7,28)[a]
3-6 months	5 (17.9)	8 (5,39)	23 (82.1)[b]	10 (4,15)
>6-12 months	3 (8.3)	5 (3,5)	33 (91.7)[b]	7 (6,17)
>12-24 months	6 (15)	5 (4,9)	34 (85)[b]	7.5 (5,10)
>2-5 years	11 (40.7)	6 (3,8)	16 (59.3)	5 (4,9)
Total	31 (13.1)	6 (3,8)	206 (86.9)[b]	8 (5,15)

Table 3.2 Organ specific infections

Time	Organ specific infections, n (%)					
	RS	GI	Blood	Renal	Skin	Others
During transplant	25 (35.2)	24 (31.2)	20 (26)	6 (7.8)	2 (2.6)	0
< 3 months	13 (44.8)	10 (34.5)	2 (6.9)	2 (6.9)	1 (3.4)	1 (3.4)
3-6 months	11 (39.3)	13 (46.4)	2 (7.1)	1 (3.6)	0	1 (3.6)
>6-12 months	15 (41.7)	11 (30.6)	6 (16.7)	0	2 (5.6)	2 (5.6)
>12-24 months	18 (45)	12 (30)	1 (2.5)	1 (2.5)	4 (10)	4 (10)
>2-5 years	7 (25.9)	10 (37)	1 (3.7)	0	6 (22.2)	3 (1.9)
Total	89 (37.6)	80 (33.8)	32 (13.5)	10 (4.2)	15 (6.3)	11 (4.6)

Table 3.3 The severity of infections

Time	The severity of infections, n (%)		
	ICU	Ventilator dependence	Death
During transplant	All	All	0
< 3 months	6 (20.7)	5 (17.2)	2 (6.9)
3-6 months	8 (28.6)	6 (21.4)	0
>6-12 months	10 (27.8)	6 (8.3)	0
>12-24 months	11 (27.5)	9 (22.5)	0
>2-5 years	5 (18.5)	1 (3.7)	0
Total	40 (16.9)	27 (11.4)	2 (0.84)

[a] p value < 0.05

[b] p value < 0.001

VPIs: vaccine-preventable infections; non-VPIs: non-vaccine preventable infections; LOS: length of stay; RS: respiratory system; GI: gastrointestinal; ICU: Intensive Care Unit

Table 4 Children with vaccination records who developed vaccine-preventable or unpreventable diseases

Table 4.1 Age-appropriate immunization followed the Thai's Expanded Program on Immunization

	Infection and hospitalization			
	None	VPI and non-VPI	Non-VPI	Total
Complete immunization	5	5	12	22
Incomplete immunization	5	6	15	26
Total	10	11	27	48

Table 4.2 Age-appropriate immunization followed the 2013 Infectious Diseases Society of America (IDSA)

	Infection and hospitalization			
	None	VPI and non-VPI	Non-VPI	Total
Complete immunization	9	9	25	43
Incomplete immunization	1	2	2	5
Total	10	11	27	48

Pathogens causing hospitalization in children post-liver transplant

A total of 237 infections requiring hospitalization were recorded during the study period. The most commonly identified bacterial pathogens were *Escherichia coli* (13.1%), *Salmonella* sp. (8.1%), and *Klebsiella pneumoniae* (6.8%), and the most

commonly identified viral pathogens were parainfluenza (5.9%), rotavirus (3.4%), and respiratory syncytial virus (3.4%). In cases of VPIs the most common pathogens were rotavirus (3.4%), influenza virus (2.5%), and varicella-zoster virus (2.1%) (Tables 5 and 6).

Table 5 Pathogen causing hospitalization in children after liver transplantation

Time	The rank of the pathogen, *n* (%)			
	Bacteria	Total	Virus, fungus, and unidentified	Total
During transplant	*E. coli* (*n* = 19, 24.7), *K. pneumoniae* (*n* = 12, 15.6), *A. baumannii* (*n* = 11, 14.3), *Enterococcus/Staphylococcus* (*n* = 4, 5.2), *Salmonella* (*n* = 3, 3.9), *P. aeruginosa* (*n* = 2, 2.6), *B. cereus/Corynebacterium/S. pneumoniae/Elizabethkingia meningoseptica/Stenotrophomonas/Streptococcus mirabilis/C. difficile* (*n* = 1, 1.3)	62	Rotavirus/adenovirus/bocavirus (*n* = 2, 2.6), parainfluenza/fungus/varicella-zoster virus (*n* = 1, 1.3)	9[b]
<3 mo	*E. coli/K. pneumoniae/Enterococcus/Salmonella/Aeromonas* (*n* = 2, 6.9), *Corynebacterium/C. difficile/Plesiomonas* (*n* = 1, 3.4)	13	Parainfluenza (*n* = 3, 10.3), coronavirus (*n* = 2, 6.9), rotavirus/bocavirus/RSV/dengue/fungus/ norovirus/rhinovirus/parvovirus B19 (*n* = 1, 3.4), unidentified (*n* = 6, 20.7)	19
3-6 mo	*Salmonella/E. coli* (*n* = 2, 7.1), *K. pneumoniae/Enterococcus/S. pneumoniae/Staphylococcus* (*n* = 1, 3.6)	8	RSV (*n* = 4, 14.3), influenza (*n* = 2, 7.1), rotavirus/parainfluenza/rhinovirus/measles/HHV6 (*n* = 1, 3.6), unidentified (*n* = 9, 32.1)	20
>6-12 mo	*E. coli* (*n* = 4, 11.1), *Salmonella* (*n* = 3, 8.3), *A. baumannii/Enterococcus*/mycoplasma/*C. difficile* (*n* = 2, 5.6), *Stenotrophomonas/Staphylococcus/Aeromonas/Pseudomonas/Plesiomonas/P. jirovecii* (*n* = 1, 2.8)	21	*P*arainfluenza (*n* = 3, 8.3), norovirus/herpes simplex virus (*n* = 2, 5.6), fungus/RSV/rhinovirus/influenza/measles (*n* = 1, 2.8), unidentified (*n* = 3, 8.3)	15
>12-24 mo	*Salmonella* (*n* = 8, 12.5), *E. coli* (*n* = 3, 7.5), *Aeromonas/Pseudomonas*/mycoplasma/*Plesiomonas* (*n* = 1, 2.5)	15	Parainfluenza (*n* = 6, 15), rotavirus (*n* = 2, 5), adenovirus/varicella-zoster virus/dengue/rhinovirus/influenza/measles/metapneumovirus/hepatitis E/coxakie AB (*n* = 1, 2.5) unidentified (*n* = 11, 27.5)	28

>2-5 yr	*Salmonella*/mycoplasma (*n* = 2, 7.4), *E. coli/K. pneumoniae/Staphylococcus/Vibrio cholera/B. cereus* (*n* = 1, 3.7)	9	Varicella-zoster virus (*n* = 3, 11.1), rotavirus/RSV/dengue/influenza (*n* = 2, 7.4), fungus/norovirus/herpes simplex virus/hepatitis B (*n* = 1, 3.7), unidentified (*n* = 3, 11.1)	18
Overall	*E. coli* (*n* = 31, 13.1), *Salmonella* (*n* = 20, 8.1), *K. pneumoniae* (*n* = 16, 6.8), *A. baumannii* (*n* = 13, 5.5), *Enterococcus* (*n* = 9, 3.8), *Staphylococcus* (*n* = 8, 3.3), mycoplasma (*n* = 5, 2.1), *C. difficile* (*n* = 4, 1.7), *Plesiomonas Shigelloides/Aeromonas* (*n* = 3, 1.3), *Corynebacterium/S. pneumononiae/Stenotrophomonas/P. aeruginosa/Aeromonas* (*n* = 2, 0.8), *Bacillus/Elizabethkingia meningoseptica/Streptococcus mirabilis/P. jirovecii/Vibrio cholera/B. cereus* (*n* = 1, 0.4)	128	Parainfluenza (*n* = 14, 5.9), rotavirus/RSV (*n* = 8, 3.4), influenza (*n* = 6, 2.5), varicella-zoster virus (*n* = 5, 2.1), dengue/norovirus/fungus/rhinovirus (*n* = 4, 1.7), adenovirus/bocavirus/herpes simplex virus/measles (*n* = 3, 1.3), coronavirus (n=2, 0.8), HHV6/metapneumovirus/hepatitis E/coxakie AB/hepatitis B (*n* = 1, 0.4), unidentified (*n* = 32, 13.5)	109[b]

[b]*P* < 0.001; virus *vs* bacterial causes of infections at each time point. *A. baumannii*: *Acinetobacter baumannii*; *B. cereus*: *Bacillus cereus*; *C. difficile*: *Clostridium difficile; E. coli*: *Escherichia coli*; HHV6: Human herpes virus 6; *K. pneumoniae*: *Klebsiella pneumoniae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *P. jirovecii*: *Pneumocystis jirovecii*; RSV: Respiratory syncytial virus; *S. pneumoniae*: *Streptococcus pneumoniae*.

Table 6 Vaccine preventable infections causing hospitalization in children after liver transplant

Time	During transplant	< 3 months	3-6 months	>6-12 months	>12-24 months	>2-5 years	Overall
Rota	2	1	1	0	2	2	8
Influenza	0	0	2	1	1	2	6
Varicella	1	0	0	0	1	3	5
Dengue	0	1	0	0	1	2	4
Measles	0	0	1	1	1	0	3
S. pneumoniae	1	0	1	0	0	0	2
Hepatitis B	0	0	0	0	0	1	1
Hepatitis E	0	0	0	0	1	0	1
V. cholera	0	0	0	0	0	1	1

DISCUSSION

In this study, incomplete age-appropriate immunization before liver transplantation in children was common, particularly with regard to live vaccines that can be accelerated before liver transplantation. Post-liver transplant in nearly half of the children in the study did not catch up with all age-appropriate vaccines. At least 13.1% of the children in the study required hospitalization for VPIs during the 5 years post-liver transplant, and in these cases, the lengths of hospital stays were up to 1 wk. More than 10% of the children required admission to the ICU and respiratory support from VPIs, reflecting the burden of VPIs during the post-transplant period. With regard to non-VPIs, both bacterial and viral infections of the respiratory and gastrointestinal systems played major roles in hospitalizations with severe infections and mortality, especially during the first year post-transplant.

To the best of our knowledge, the current study is the first to investigate immunization status and infections requiring hospitalization in Asian children who underwent a liver transplant. Comparing to previous studies in Europe[9,10] and the United States[4], in the present study, there was a higher rate of incompete age-appropriate immunization before liver transplantation, particularly with respect to the accelerated MMR and varicella vaccination. However, the number of hospitalization with VPIs (13.1%) was comparable to that in a study conducted in the United States by Feldman *et al*[4] (11.3%). Moreover, the VPIs in that study was more severe and required longer hospital stays than those in the current study. Genetic risk factors may explain this phenomenon, as with the more contagious and severe coronavirus disease 2019 infections in Europe and United States than in Thailand.

Prior to liver transplant, the physicians frequently do not offer patient immunization, particularly with respect to live vaccines[7, 22-24]. There is solid evidence of adequate immune response to varicella and measles vaccination in children aged < 1 year; hence, the policy to promote accelerated vaccination in children before immunosuppressant was initiated[25-28]. It is probable that this is not standard practice

in normal children. Moreover, children waiting for a liver transplantation may have had complex and serious illness that needed to be given priority. Some physicians may not be familiar with the accelerated immunization program [21], and therefore, they decide to postpone it. Consequently, a specific protocol and concerted focus on educational interventions, or the development of specialized team care that is responsible for these issues is crucial to ensure that all candidates receive appropriate vaccinations to minimize complications associated with VPIs [7]. One great benefit of pre-liver transplant vaccination is higher immunogenicity compared with revaccination post-liver transplant[28]. Moreover, pre-transplant vaccination of children will likely lead to herd immunity that will be beneficial for other transplant children in outpatient and inpatient clinics during their visits[23].

In the present study, the rate of incomplete age-appropriate immunization after liver transplantation was high and there was no significant difference between the pre-transplant rate (52.0%) and the post-transplant rate (47.9%). In theory, children's vaccination schedules should be postponed for more than 2 mo after liver transplantation because of the possibility of an inadequate immune responses[21]. The high level of immunosuppressants is another factor to consider. In the present study almost half of the children were not up-to-date with their age-appropriate immunization during up to 5 years of follow-up. The reasons might be relatively low concern over children in a stable condition post-transplant and a level of immunosuppression that is not low enough to warrant immunization. Notably, only 62.3% of the children's guardians brought vaccination books to visits to the doctor. As well as unawareness, financial problems would likely be a major concern for the children's guardians, especially with regard to vaccines that are not included in Thailand's EPI such as pneumococcal conjugate vaccine, influenza vaccine, hepatitis A vaccine, and varicella vaccine. Fortunately the infectious diseases unit in our department conducted a campaign to promote the administration of pneumococcal conjugate vaccine and influenza vaccine to all immunocompromised children every year at no charge. This afforded the children in the present study the opportunity to access these vaccines, and there was a significant increase in the proportion of children that received these vaccines post-transplant ($P < 0.001$). Long-term provision of these

high-cost vaccines by the authorities would be a worthwhile venture. With respect to live vaccines, there has been controversy about whether they should be administered to children after liver transplantation [27, 29-33]. Thus, further reports and large cohort studies are required in order to clarify the safety of live vaccines in these vulnerable patients, before they are routinely vaccinated post-transplant.

In this study, the rate of hospitalization for VPIs up to 5 years post-transplant was similar to those reported in previous studies [5, 6, 9, 21], but significantly higher than that in the normal population[19]. There was the mortality report of VPIs in children with immunocompromised hosts [3, 4, 32, 34-36], but in this study, there was no mortality from VPIs. The VPIs requiring hospitalization in the current study were due to rotavirus, influenza, varicella, dengue fever, measles, *Streptococcus pneumoniae*, hepatitis B/E, and *Vibrio cholera*. These data should emphasize the value of complete immunization and robust infection control to physicians.

Viral hepatitis is endemic in Thailand, but interestingly in the present study there were no reports of hospitalization for hepatitis A post-liver transplant, and only one case of hepatitis E infection that required hospitalization. Viral hepatitis can be symptomatic and severe in older children and adults, and older children and adults may ingest more contaminated food and water than young children. Consequently, serology testing and immunization may be valuable in these groups. There is a reported case in which *de novo* hepatitis B infection was diagnosed 3 years after a liver transplant despite the recipient having undergone complete hepatitis B immunization pre-transplant[26]. This demonstrates that complete hepatitis B immunization pre-liver transplant does not guarantee post-transplant protection. That case prompted us to instigate a protocol for reimmunization and hepatitis B surface antibody monitoring every 3-6 mo to maintain a protective level of > 100 mIU/mL. *De novo* hepatitis B in the aforementioned boy who had hepatitis B surface antibody > 1000 mIU/mL pretransplant [35] may reflect waning immunity post-liver transplant. As well as vaccination, research evaluating the humoral and cellular immunity evoked by each vaccine should be conducted to determine vaccination schedules and the antibody parameters required to prevent VPIs more effectively. In the present study, the overall

infection rate was high in the first year post-transplant, hence vaccination should be initiated as soon as possible after liver-transplanted children are sufficiently stable. Predictors of high immunogenic responsivity to vaccination are needed to enable physicians to decide on optimal timepoints for reimmunization.

The current study had some limitations. It was a single-center study with a relatively small sample size. The true prevalence of VPIs may be lower than the frequency in the study, because the study only included children with severe enough illness to require hospitalization. Almost all children in the present study were referred from distant and rural areas, and it is possible that some of them subsequently attended more local hospitals due to infections. The main strength of the study was the reliable vaccination records obtained directly from the patients' vaccination books, which facilitated comparisons of vaccination status pre-transplant and post-transplant.

CONCLUSION

Incomplete immunization was common in children pre-liver transplant and post-liver transplant. Almost all of the children in the study required hospitalization due to VPIs or non-VPIs within 5 years post-liver transplant. The severity of infections was highest in the first year post-liver transplant.

ARTICLE HIGHLIGHTS

Research background

Infection after liver transplantation is a serious concern due to potential morbidity and mortality, thus strategies to reduce overall post-transplant infection are warranted. Immunization is an effective and relatively noninvasive and affordable way to reduce vaccine-preventable infections (VPIs).

Research motivation

There is strong evidence that VPIs and non-VPIs post-transplant cause high fatality and increase graft rejection, but published data on VPIs and their effects in children post-liver transplant in Asia are scarce.

Research objectives

To investigate immunization status in children at the time of liver transplantation and up to 5 years thereafter. The prevalence and impact of VPIs and non-VPIs during hospitalization were also evaluated.

Research methods

The current retrospective study included 77 children who underwent liver transplantation and were followed up for up to 5 years thereafter. Demographic data, patient characteristics, immunization details derived from vaccination records, and hospitalizations for VPIs and non-VPIs were analyzed.

Research results

The mean follow-up duration after liver transplantation was 3.68 ± 1.45 years. Of the 77 children in the study, 48 (62.3%) had vaccination records in their vaccination books. There was a significant difference in the proportion of children with incomplete vaccination according to Thailand's Expanded Program on Immunization (n = 25, 52%) and accelerated vaccine from Infectious Diseases Society of America recommendations (n = 43, 89.5%) ($P < 0.001$). Post-liver transplant almost half of the children in the study did not catch up with appropriate immunizations for age. There were 237 infections requiring hospitalization during up to 5 years of follow-up post-liver transplant at our hospital. The risks of VPIs and non-VPIs were highest during the first year after liver transplantation, and 2 children died. Respiratory and gastrointestinal systems were common sites of infection. The most commonly identified pathogens that caused VPIs were rotavirus, influenza virus, and varicella-zoster virus.

Research conclusions

Incomplete age-appropriate immunization in children pre-liver transplant and post-liver transplant were common. At least 13.1% of the children in the study required hospitalization for a VPI during a follow-up period of up to 5 years post-transplantation. There was high morbidity, especially during the first year after

transplantation. Hence, complete immunization and robust infection control should be considered in such children.

Research perspectives

The current study suggests that incomplete age-appropriate immunization is a major concern, because a large number of patients with VPIs requiring hospitalization were recorded. Interestingly, waning immunity post-liver transplant can evidently lead to VPIs, as evidenced by a case in which *de novo* hepatitis B infection developed 3 years post-liver transplantation in a child who had a hepatitis B surface antibody titer of > 1000 mIU/mL pre-liver transplantation. As well as policies to increase pre-transplant immunization rates, studies investigating humoral and cellular immunity induced by vaccination after liver transplantation are needed.

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2.2 Safety and immunologic response of double- and standard-dose hepatitis B revaccination in children with liver transplants

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On process of submission

ABSTRACT

Background

High prevalence of hepatitis B (HB)-antibody loss after liver transplantation (LT) and *de novo* HB infection were documented. This study aimed to compare the effectiveness of two revaccination regimens in inducing protective immunity in children with liver transplants.

Methods

Children who underwent liver transplantation at King Chulalongkorn Memorial Hospital were recruited. All received primary HB immunization but anti-HBs antibodies after LT if HBs antibodies ≤100 mU/mL. Children were stratified by age at transplantation and then allocated with block of four randomization into two groups; standard (0.5 ml) 3-dose and double (1 ml) 3-dose HB vaccine intramuscularly at 0, 1, and 6 months. The parents were blind with respect to the vaccine regimen. Anti-HBs titers were assessed at 0, 1, 6, 7-9, and 9-12 months. A participant was categorized as a responder if the participant had anti-HBs levels <10 mU/mL before revaccination but had seroconversion (anti-HBs >10 mU/mL) after the 3-dose vaccination regimen. Participants were defined as nonresponders if they had anti-HBs levels <10 mU/mL before revaccination and had no seroconversion after the 3-dose vaccination regimen.

Peripheral blood mononuclear cells (PBMCs) were extracted for cellular immune study by the enzyme-linked immune absorbent spot assay (ELISpot) and flow cytometry. The primary outcome was the percentage of responders and geometric mean titer (GMT) of anti-HBs levels at 7-9 months. The trial was registered in Thai Clinical Trials Registry with study number TCTR 20180723002.

Findings

Sixty-six children were recruited and randomly assigned into two groups with 33 participants in each group. At the end point, three in the standard-dose and four participants in the double-dose group dropped out. Thirty and 29 participants from standard-dose and double-dose regimens, respectively, were included per protocol analysis. At months 7-9, the percentage of seroconversion was 23 (92.0%) of 25 (95% CI: 73.9-99.0) in the standard-dose group and 16 (88.9%) of 18 (95% CI: 65.3-98.6) in the double-dose group. Regarding the GMT of anti-HBs antibodies, there was no significant difference between the two groups at all five time points. However, the GMT of anti-HBs antibodies at time point 4 (1372.4 [95% CI: 650.2-2896.7] in the standard-dose group and 730 [95% CI: 262.7-2031.6] mU/mL in the double-dose group) was significantly higher than at time point 2 (241.3 [95% CI: 90.9-641.0] in the standard-dose group and 181 [95% CI: 63.8-516.1] mU/mL in the double-dose group) in both groups ($P< 0.05$). No serious adverse reactions to the HB vaccine were reported. After time point 5, the GMT of anti-HBs levels in the double-dose group was significantly higher than after a booster dose (time point 2) (969 [95% CI: 328.2-2861.4] and 181.5 [95% CI: 63.8-516.1] mU/mL). IFN-γ at time point 4 was significantly higher than at time point 1 (32 [4,68] and 14 [0,23] spot-forming cells/10^6PBMCs, $P<0.05$). There was no significant difference in the subpopulations of T-reg, CD4 T cells, CD8 T cells, B cells, and NK cells. In comparing responders (n=38) and nonresponders (n=4), the time of revaccination after LT and the tacrolimus level were the significant factors in seroconversion. The time of revaccination after liver transplantation in responders and nonresponders was 1.95 (0.66, 4.95) and 0.58 (0.54, 0.65) years, respectively. The tacrolimus levels in responders and nonresponders were 3.6 (2.6, 5.7) and 6.7 (5.8, 7.8) ng/mL, respectively.

Interpretations

The 3-standard-dose and 3-double-dose HB regimens were highly effective and safe for children with liver transplants, and the double-dose regimen maintained the high anti-HBs level at short-term follow up. For successful reimmunization with a robust humoral response, anti-HBs antibodies should be monitored post-liver transplant and HB revaccination should be introduced not earlier than 6 months after LT when the immunosuppressant level is still high.

INTRODUCTION

Hepatitis B virus (HBV) infection is a great burden worldwide. More than 2 billion people are infected with HBV and about 12.4% of them have chronic disease. Unfortunately, approximately 15-20% of chronic carriers die from cirrhosis or hepatocellular carcinoma. HBV infection is endemic in the sub-Saharan African and Asian regions with a prevalence up to 15%[37]. Antiviral therapy may not eradicate HBV from the body and long term use can lead to drug tolerance and toxicity[38]. Since the HBV universal vaccination programs stated in the 1990s, the prevalence of HBV infection has rapidly decreased. However, non-vaccinated people who were born in the era when the vaccine was not available still have a high rate of HBV infection. Moreover, immunocompromised people, particularly patients who have undergone liver transplantation, could have *de novo* hepatitis B infection (DNH).

DNH is defined as the development of new hepatitis B surface antigen (HBsAg) positivity after liver transplantation in recipients previously negative for HBsAg. This condition was recognized and reported in the 1990s[39, 40]. Because the mechanism of DNH was not clearly identified, Skagen et al[5] performed a systematic analysis in 2001 and found the potential risk for DNH in recipients who received hepatitis B core antibodies (HBcAb) from a positive donor depended on the recipients' immune status with respect to hepatitis B and type of prophylaxis therapy given. The risk for DNH was highest in HBV naïve liver recipients from HBcAb-positive donors; the incidence of DNH could be high up to 58% without any prophylaxis regimen after liver transplantation. However, prophylaxis with antiviral hepatitis B immunoglobulins (HBIGs) or HBV vaccine could decrease the incidence to 1.7%[41].

With respect to DNH in recipients who received livers from HBcAb-negative donors, there is a hypothesis that the loss of HBV protection after transplantation and immunosuppressant administration might be a major cause of DNH. Many studies report DNH after liver transplantation even though children had high anti-HBs levels >100 mU/mL before and/or after liver transplantation[1, 12, 13]. It is possible to infer from these cases that DNH might occur at the time of the antibody loss that leads to the loss of protection. DNH is of serious concern not only because of long-term liver consequences but also the increasing risk of acute graft rejection[41]. Consequently, a strategy to prevent DNH is crucial.

There is evidence that in antivirals and HBIGs can prevent DNH in recipients who received liver from HBcAb-positive donors[42]. Recently, many studies have postulated that HBV vaccination can prevent DNH in recipients from both HBcAb-positive[43] and HBc-Ab-negative donors[12, 14, 44]. In countries as Taiwan where hepatitis B is endemic, hospital policy requires a high level of anti-HBs antibodies after transplantation to prevent DNH[12, 14]. Lin *et al*[44] demonstrated that maintaining high anti-HBs levels (>1000 mIU/mL) without antivirals or HBIGs could prevent DNH in children who received HBcAb-positive livers. This practice is very cost-effective; however, children had to receive frequent boosters during their hospital stay. In immunocompromised patients, including children, anti-HBs will decrease rapidly after liver transplantation and DNH can occur[1]. International guideline recommend booster vaccines to keep anti-HBs antibodies >100 mIU/mL[45]. However, there is no strong evidence supporting this recommendation in children after liver transplantation. Many studies mentioned above reported DNH in children after liver transplantation who had anti-HBs levels of more than 100 mIU/mL[1, 44]. Hence, strategies to prevent DNH in the pediatric population after liver transplantation might be different from the general recommendations from international organizations.

In one study, immunogenicity was assessed in healthy nonresponders after four hepatitis B vaccine regimens. A meta-analysis compared the four approaches with different doses, amount of vaccine, and route.[46] The authors did not find any evidence to support the use of the double-dose regimen intramuscularly in healthy adults who

were defined. In HIV-infected patients, however, a recent systematic review and meta-analysis reported multiple-double-dose hepatitis B vaccination was more effective than the multiple-standard-dose regimen with respect to seroconversion and higher immunogenicity after 4-6 weeks and >12 months after completion of the vaccination. After liver transplantation, children might have T-cell suppression from immunosuppressants similar to the immune defect in HIV-infected patients. In these latter patients, there was a high rate of seroconversion after a booster dose but the rapid decline lead to multiple booster doses to maintain high anti-HBs levels[44]. Humoral and cellular immunologic responses after the booster dose seem to be adequate in only the short term, and better ways to achieve and maintain protection against hepatitis B in children after liver transplantation are needed. Therefore, the aim of this study was to evaluate the safety and immunogenicity of the double-dose compared to standard-dose hepatitis B vaccination series in children with liver transplants with hepatitis B immunologic loss. T-cells targeted by immunosuppresants might be the main factors in immunologic loss and poor response to vaccine in children after liver transplantation. Therefore, the additional aim of this study was to study immune cellular frequency and function, particularly in T cells, after vaccination and to determine the factors for seroconversion after completion of revaccination in children with liver transplants.

METHODS

Study design and participants

The current study was a randomized, single-blind clinical trial conducted in children who underwent liver transplantation at the King Chulalongkorn Memorial Hospital in Thailand between January 2003 and May 2019. The study protocol received approval by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Thailand (IRB number: 142/60) and was registered at Thai Clinical Trials Registry (TCTR) with study number TCTR 20180723002. Eligible participants were children aged 1-18 years who underwent liver transplantation more than 6 months previously, in stable clinical status, had received hepatitis B immunization before liver transplantation, but

had anti-HBs antibodies less than 100 mU/mL post-liver transplantation. Children with liver transplants were excluded if they had a temperature above 38ºC on the day of enrollment.

Participants were seen at day 0, 1 month, and 6 months for vaccination and blood sample collection before vaccination. After that, blood samples were collected at 7-9 months and 9-12 months, according to the time for patients' routine appointments. Accepted intervals between the first dose and second dose were 3 to 6 weeks; between second dose and third dose, 18 to 22 weeks; and between the first dose and third dose, 24 to 28 weeks. Anti-HBs were measured from 4 weeks to 3 months after completion of revaccination. At the endpoint, the participants were classified into two groups, responders and nonresponders. A responder was defined as a participant who had seroconversion (anti-HBs levels ≥ 10 mU/mL) after the third dose hepatitis B vaccination. Slow and rapid responders were defined as participants who had seroconversion after the first dose or the third dose hepatitis B vaccination, respectively. Nonresponders were defined as participants who had no seroconversion (anti-HBs levels ≥10 mU/mL) after the third dose hepatitis B vaccination. The anti-HBs antibodies of responders and nonresponders were measured before revaccination and anti-HBs levels were less than 10 mU/mL.

Guardians and the patients aged more than 12 years gave written informed consent, and the patients aged 7-12 years gave informed assent at the time of enrollment in accordance with the Declaration of Helsinki.

Randomization and masking

Children with liver transplants who matched with inclusion and exclusion criteria were first ranked by the time since liver transplant, then were individually randomly allocated in the ratio of 1:1 by block of 4 to one of the following groups; 3-standard-dose hepatitis B vaccine (at a dose of 10 μg) or 3-double-dose hepatitis B vaccine (at a dose of 20 μg) at day 0 and months 1 and 6. Recombinant hepatitis B vaccine was administered intramuscularly in the left or right lateral thigh. The participants and guardians were blinded for the vaccine dosage assignments.

Procedures

After enrollment, the data for outcome assessments included:

Demographic data, patient characteristics, and laboratory measurements

1) Demographic data and patient characteristics
- Age, sex, and comorbidity
- Etiology of liver disease prior to liver transplantation
- Medications, type and dosage or level
- The time after liver transplantation
- Complications after liver transplantation: surgical complications (bile duct stenosis, hepatic artery stenosis), medical complications (acute or chronic rejection, cytomegalovirus infection, renal insufficiency, post-transplant lymphoproliferative disorder (PTLD)
- Physical examination (liver and spleen size, lymph node enlargement, weight, and height)

2) Laboratory measurements: CBC, liver function tests, coagulogram, electrolytes, BUN/creatinine

Humoral response and safety assessments

Determination of HBV infection status was done before vaccination by measuring levels of anti-HBs, HBsAg, and anti-HBc immunoglobulin M (IgM). Then anti-HBs levels were measured at day 0, 1 month, and 6 months before vaccination and at 7-9 months and 9-12 months by the local laboratory. Additional blood samples were sent to the central laboratory for further evaluation. Accepted intervals between the first dose and second dose were 3 to 6 weeks; between second dose and third dose, 18 to 22 weeks; and between the first dose and third dose, 20 to 28 weeks; the interval between 4 weeks and 3 months after the third dose.

Any adverse reactions from vaccination were assessed by telephone call at 72 hours after each vaccination and recorded. If there was an adverse reaction, monitoring was extended up to 4 weeks.

Participants were asked about side effects of the vaccine (1 hour after vaccination and telephone call at 72 hours):

- Local side effects: pain, erythema, induration, edema, pruritus, and hematoma.
- Systemic side effects: fever, headache, fatigue, arthralgia, asthenia, diarrhea, nasopharyngitis, and other complaints.

Apart from the routine laboratory work-up ordered by the physician in charge and anti-HBs antibody measurement at every visit, blood samples at each visit were collected and sent to the central laboratory for extraction into PBMCs and serum. The sera were stored at -80ºC until the study was completed. Anti-HBs levels were measured in these sera again by an automated enzyme-linked immunosorbent assay performed with the ARCHITECT analyzer (Abbott, Germany) according to the manufacturer's instructions. The lower limit of detection of the assay was 0 mU/mL. All values below 1 mU/mL were transformed to $\log_{10}$ for computing the geometric mean titer for anti-HBs values of 0 mU/mL.

Cellular response

- *In vivo* cellular immune response to vaccination was measured with DTH skin testing.

At first visit, all participants were asked to participate in the DTH skin test after informed consent and/or informed assent from guardians and/or participants. This test was done by Mantoux method; 0.1 ml of hepatitis B vaccine and normal saline were intradermally injected at the volar surface of the forearm with a double-blind technique (both participants/guardian and investigator). Skin induration was measured by parents themselves. Furthermore, parents had to take a photograph of skin induration with a scale and send it back to the investigator for measurement. The investigator recorded the skin induration separately from parents. An induration size ≥ 5 mm and larger than the control (normal saline) was considered a positive result.

- *In vitro* cellular immune response to vaccine

Blood samples from participants were collected at five time points for PBMC extraction and stored at -80ºC for an *in vitro* study of cellular response to vaccine.

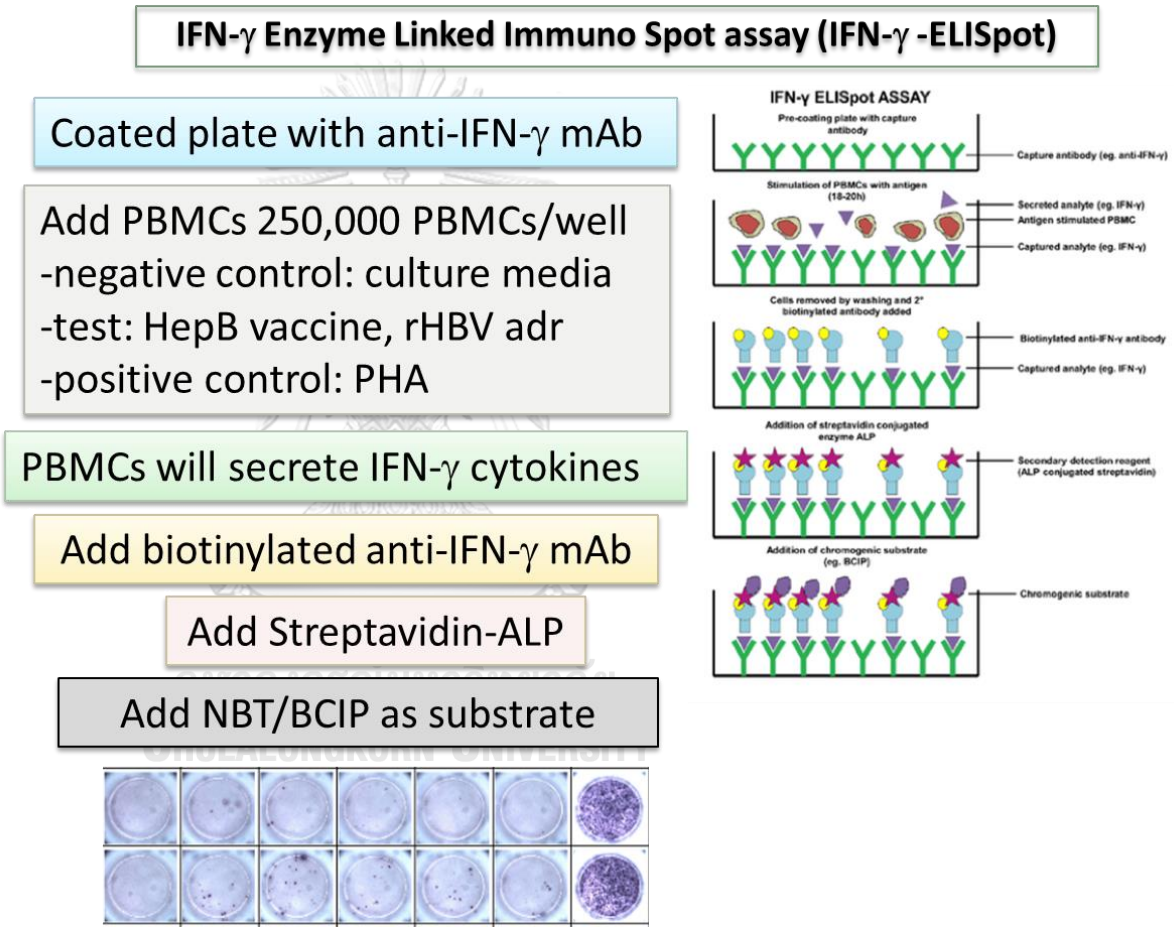
PBMCs were isolated from fresh acid citrate dextrose (ACD) blood by Ficoll-Hypaque density gradient centrifugation. Cells were resuspended in T-cell medium (RPMI 1,640 medium, supplemented with 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 U/mL streptomycin, and 10% fetal bovine serum (FBS, Gibco, USA). Cell were kept frozen (for co-culture experiments) with liquid nitrogen until needed. For cryopreservation, cells were resuspended in freezing medium (fetal bovine serum containing 10% dimethyl sulfoxide) to a concentration of $1X10^7$ cells/mL. Sample PBMCs from the first 22 complete collected samples were analyzed for T-cell-specific response to hepatitis B vaccine, subpopulation frequency of regulatory T cells/B cells, and NK cells.

HBsAg-specific cytokine production using ELISPOT assay to quantification of IFN-γ secreting T cells

HBV-specific T cell responses were evaluated in peripheral blood mononuclear cells (PBMCs) using the human interferon-gamma enzyme linked immunospot (IFN-γ ELISpot) assay. All assays are performed with duplicate. Briefly, 96 well nitrocellulose membrane plates (MAIPS45; Millipore, Bedford, MA, U.S.A.) were coated overnight at 4°C with 5μg/ml anti-human IFN-γ (1-D1K) monoclonal antibodies (mAb) (Mabtech, Stockholm, Sweden). Then, the plates were washed and blocked with culture medium (RPMI1640 with 10% FBS) for 1 hour at room temperature (RT). Next, a quantity of 250,000 PBMCs per 100 µL per well were cultured with HBsAg adr subtype recombinant protein (MyBiosource, USA) at a final concentration of 5 μg/ml at 37°C with 5% CO_2 for 40 hours. Culture medium alone served as a negative control and phytohemagglutinin (PHA) as a positive control. After incubation, the plated were washed with 1xPBS and added 1 μg/ml anti-human IFN-γ-biotinylated mAb (7-B6-1 biotin; Mabtech, Stockholm, Sweden) in PBS for 3 hours at RT. Following wash steps with PBS, a 1:1000 dilution of 100 µL, streptavidin-ALP in PBS were added to each well and incubated for 1 hour at RT. After washed, the substrate solution (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; BCIP/NBT) were added 100 µL into

each well. The spots were developed until distinct spots emerge. The reaction was stopped by washing extensively in tap water and rinse the underside of membrane. Leave the plate to dry. The spots are analyzed by using ELISpot reader (Carl Zeiss, Germany). Mean numbers of IFN-γ-producing and spot-forming cells (SFC) are calculated from duplicate assays. HBsAg-specific responses are calculated by subtracting the negative control and expressed as SFU per 10^6 PBMCs (Figure 1).

Figure 1 Demonstrate the IFN-γ Enzyme Linked ImmunoSpot (ELISpot) assay

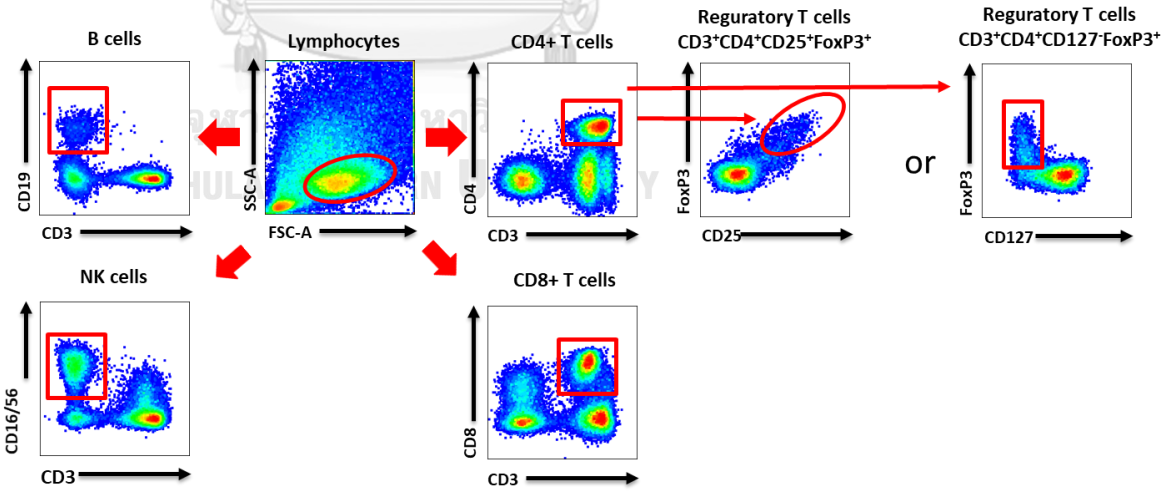


Flow cytometric staining protocol for Regulatory T cells, B cells and NK cells subpopulations

The PBMC were stained immune cells based on cell-surface markers, as follows: $CD127^{-}FoxP3^{+}CD4^{+}$ and $CD25^{+}FoxP3^{+}CD4^{+}$ (Regulatory T cells; Treg), $CD19^{+}$ B cells, $CD3^{-}$

$CD56^+CD16^+$ (NK cells). The percentage of Treg, B cells and NK cells were measured from lymphocytes. In brief, one million PBMCs were stimulated with HBsAg adr subtype recombinant protein (MyBiosource, USA) at a final concentration of 5 μg/ml at 37°C with 5% CO_2 for 2 days. After stimulation, cells were washed with phosphate-buffered saline with 2% fetal bovine serum (FBS; FACS buffer) and stained with cell surface markers for 20 min at 4°C. The cell-surface contained CD25 PE (BC96), CD16 PE-DZ594 (3G8), CD56 PE-DZ594 (5.1H11), CD19 PerCP-Cy5.5 (HIB19), CD3 PE-Cy7 (UCHT1), CD127 AF647 (A019D5), CD8 AF700 (SK1), CD4 APC-Cy7 (RPA-T4) (Biolegend, San Diego, CA, USA). After washing the cells twice in FACS buffer, cells were fixed/permeabilized and stained with FoxP3 AF488 (PCH101) (eBioscience, San Diego, CA, USA) antibodies for 30 min at RT. After washed, cells were fixed with 2% paraformaldehyde and analyzed by flow cytometry. Eight-color flow cytometric immunophenotyping will perform on a LSRII and data will be analyzed using Flowjo software (Figure 2).

Figure 2 shows an example of the gating strategies used in flow cytometry for measuring $CD127^-FoxP3^+CD4^+$ and $CD25^+FoxP3^+CD4^+$ for regulatory T cells, $CD3^+CD4^+$ for T helper cells, $CD3^+CD8^+$ for cytotoxic T cell, $CD3^-CD16^+CD56^+$ for NK cells and $CD3^-CD19^+$ for B cells



Outcomes

The primary endpoint of this study was the comparison of the percentage of responders and nonresponders at 4 weeks to 3 months after completion of the two revaccination regimens. The GMT of the anti-HBs antibody level was measured at five time points (0, 1, 3, 7-9, and 9-12 months). Comparisons among each time point in

each vaccination regimen and between two revaccination regimens were considered the secondary outcome. Additional secondary outcomes were all adverse events reported in the 72 hours and up to 2 weeks in cases when adverse events happened after each revaccination and analysis of the factors related to poor humoral immune response after a completion of revaccination regimens.

Statistical analysis

The sample size was calculated for a randomized controlled trial with binary outcome with the assumption that children in the 3-standard-dose and 3-double-dose hepatitis B vaccine groups will have seroconversion or anti-HBs levels > 10 mU/mL after the complete 3-dose hepatitis B vaccine of 30% and 70%, respectively, with alpha (α) = 0.05 and test power of 80%. The ratio of participants in both groups was 1:1 using two independent proportions without a continuity correction formula. At least 48 children were needed for the study (24 participants in each group). Considering a 20% dropout rate, the total number of participants needed for recruitment in the present study was 58.

Data analyses were performed using Statistical Package for the Social Sciences (SPSS) version 24.0.0 (SPSS, Inc., United States), Stata version 15.1 (Stata Corp., USA) and GraphPad Prism 5.03, as appropriate. Continuous and categorical data were presented as mean (± SD) or median (IQR), and proportion or percentage as appropriate, respectively. The Mann-Whitney *U* test and unpaired *t*-test were used to compare continuous data between groups as appropriate. The Wilcoxon Signed Rank test and paired *t*-test were used to compare continuous data within groups at different time points as appropriate. Fisher's exact test and the Chi-square test were used to compare discrete data as appropriate. Univariate analysis was performed for the independent factors of anti-HBs seroconversion after revaccination. A P value < 0.05 was regarded as being statistically significant. The per-protocol analysis was used for the anti-HBs antibody result. The GMT was calculated and represented logarithmically ($\log_{10}$scale) in which anti-HBs titers <1 mU/mL were transformed to the value of 0. The statistical review of the study was performed by a biomedical statistician at Department of Statistics Science, Kasetsart University, Bangkok, Thailand.

Role of the funding source

The funders did not participate in this study design, data collection, data analysis, and interpretation. The corresponding author and the first author had full access to all data in this study.

RESULTS

Study populations

Ninety-four children underwent liver transplant between 2003 and 2019, and 66 were eligible for this study. There were 33 participants in each group. However, anti-HBs levels >100 mU/mL were detected in two participants in the standard-dose group and double-dose group, respectively. One participant in the double-dose group was diagnosed with *de novo* hepatitis B after enrollment. After vaccination, one participant in the standard-dose group could not come to follow up at the appropriate time, and one in the double-dose group was diagnosed with PTLD that progressed to B-cell lymphoma. Therefore, 59 participants received the 3-dose vaccinations according to the study protocol (30 children in standard-dose group and 29 children in double-dose group) (Figure 3).

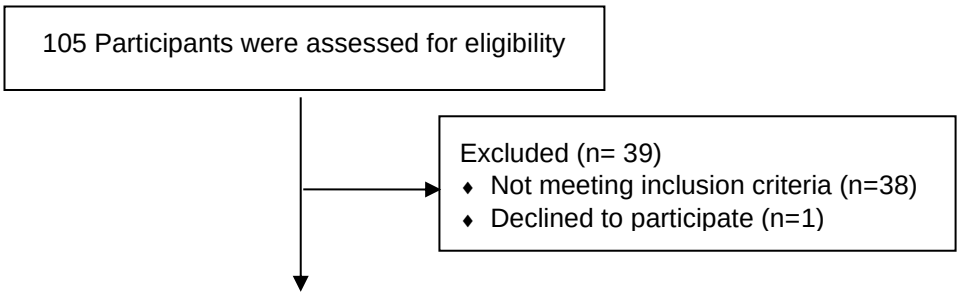
The differences among demographic data, patient characteristics, immunosuppressant levels, and basic laboratory results for the 3-standard-dose and 3-double-dose vaccination regimens were not statistically significant (Table 1).

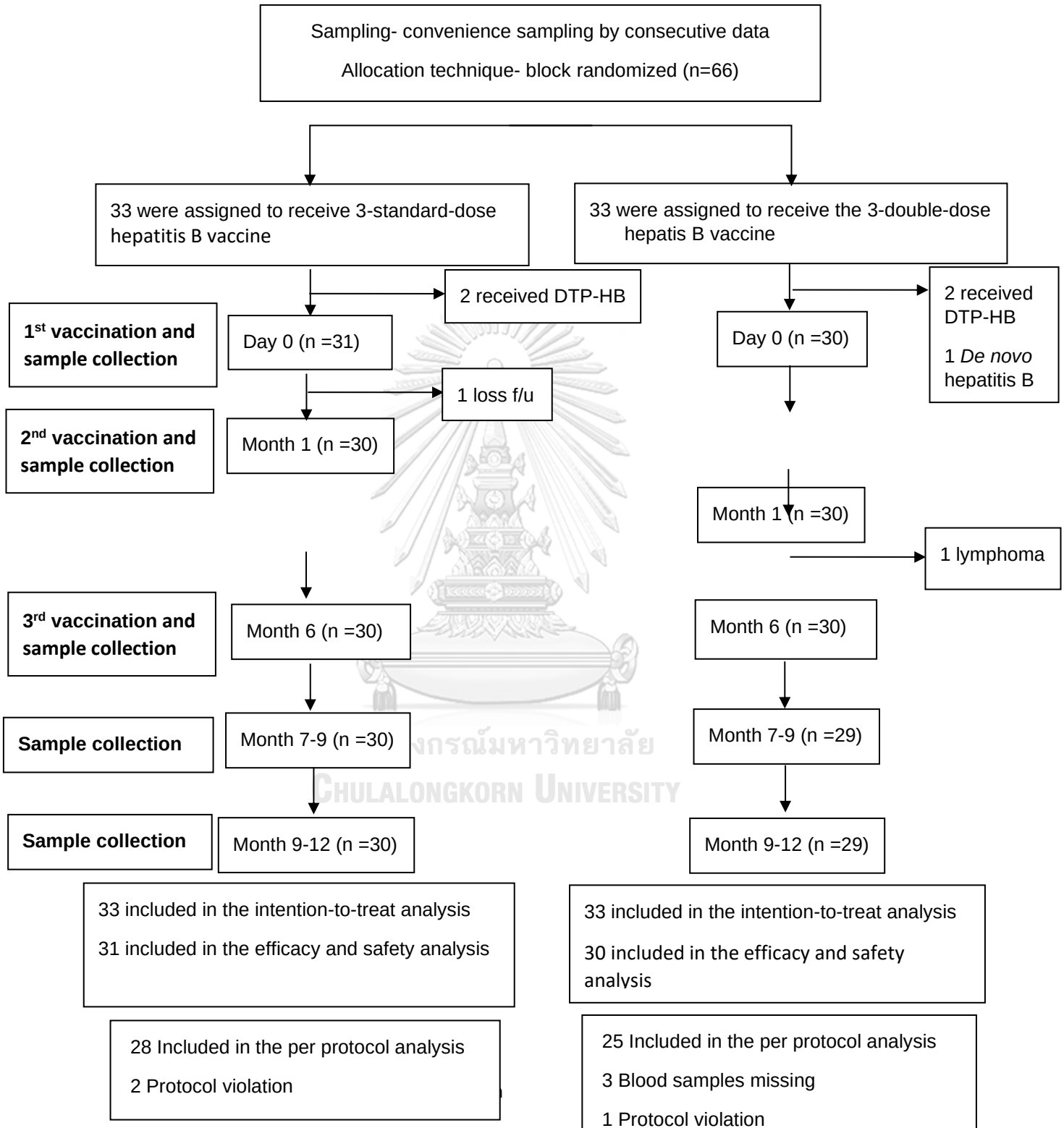
Table 1 Patient characteristics before hepatitis B revaccination

Parameters	Standard dose (N=33)	Double dose (N=33)
Age at transplantation (years)	1.03 (0.83, 2.83)	1.83 (1.23, 7.01)
Male (n, % male)	15, (45.5)	17, (51.5)
Age at HB re-vaccination (years)	1.02 (0.66, 4.83)	1.46 (0.63, 4.6)

Number of immunosuppressant (n, %)		
- None	1 (3.0)	0
- One	15 (45.5)	16 (48.5)
- Two	14 (42.4)	13 (39.4)
- Three	3 (9.0)	4 (12.1)
Type of immunosuppressant (n, %)		
- Tacrolimus	12 (36.4)	12 (36.4)
- Cyclosporin -	7 (21.2)	4 (12.1)
More than one	14 (42.4)	12 36.4)
Immunosuppressive level (ng/mL)		
- Tacrolimus	3.2 (2.45, 5)	3.7 (2.95, 5.6)
- Cyclosporin	217 (123, 629)	386 (106, 937.5)
Disease pretransplant; BA (n, %)	23 (79.3)	20 (68.9)
Complications (PTLD, Rejection, surgical conditions)	16 (55)	12 (41)
Anti-HBs level (mIU/mL) at beginning	2.3 (0.75, 12.7)	1.7 (0.5, 8)
Laboratory investigation		
- SGOT (IU/L)	41 (35.5, 51)	41 (32, 55)
- SGPT (IU/L)	31 (19, 42)	26 (18, 49.5)
- GGT (IU/L)	24 (18, 63)	34 (22, 83.5)
- Albumin (g/dL)	4.2 (4, 4.4)	4.1 (3.85, 4.2)
- Hb (g/dL)	12 (10.8, 12.7)	10.8 (9.9, 12.2)
- WBC (10^6/L)	8290 (6840, 12150)	7760 (5605, 9835)
- Lymphocyte count (x10^6/L)	578 (267, 6285)	3100 (580.5, 4915)
- Platelet count (x10^9)	244 (203.5, 313)	230 (178.5, 320)

Figure 3. Enrollment, randomization and follow-up





Baseline anti-HBs titers after enrollment were 19.42 ± 24.24, and 26 participants had anti-HBs levels between 10-100 mU/mL (7 and 11 participants in single- and

double-dose groups). Anti-HBs titers were measured with a mean follow-up period of 13.26 ± 2.46 months and 4.01 ± 2.34 months from enrollment and completion of 3-dose vaccination regimen, respectively.

Seroconversion or anti-HBs antibodies <10 mU/mL increased to >10 mU/mL in 66.7% (14/21) and 91.3% (21/23) of standard-dose group *vs* 55.6% (10/18) and 88.9% (16/18) of the double-dose group had seroconversion after the first vaccination (mean time from vaccination to measurement was 32 ± 6 days) and third vaccination (mean time from vaccination to measurement was 96.8 ± 60.3 days), respectively. There was a higher rate of seroconversion after the third vaccination in both groups (P > 0.05) and seroconversion in the standard-dose group was higher than in the double-dose group (P > 0.05) (Figure 4).

With regard to the GMT of anti-HBs levels, there was no significant difference between two groups at all five time points. However, the GMT of anti-HBs at time point 4 (1372.4 mU/mL [95% CI: 650.2-2896.7] in the standard-dose group and 730 mU/mL [95% CI: 262.7-2031.6] in the double-dose group) was significantly higher than at time point 2 (241.3 mU/mL [95% CI: 90.9-641.0] in the standard-dose group and 181 mU/mL [95% CI: 63.8-516.1] in the double-dose group) in both groups (P < 0.05). No serious adverse reactions to HB vaccine were reported. After time point 5, the GMT of anti-HBs antibodies in the double-dose group was significantly higher than after a booster dose (time point 1) (969 mU/mL [95% CI: 328.2-2861.4] and 181.5 mU/mL [95% CI: 63.8-516.1]) (P < 0.05) (Figure 5).

Figure 4 Seroconversion after vaccination in participants with baseline anti-HBs antibodies <10 mU/mL

Figure 4.1 Standard-dose group

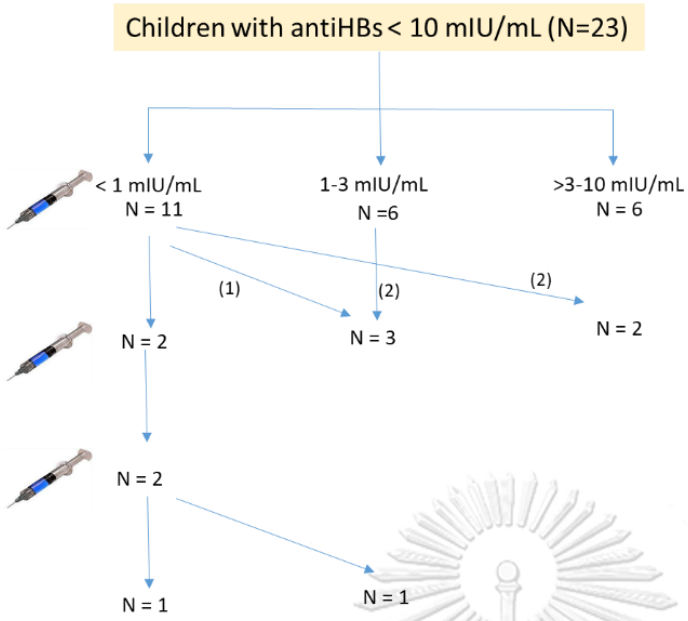


Figure 4.2 Double-dose group

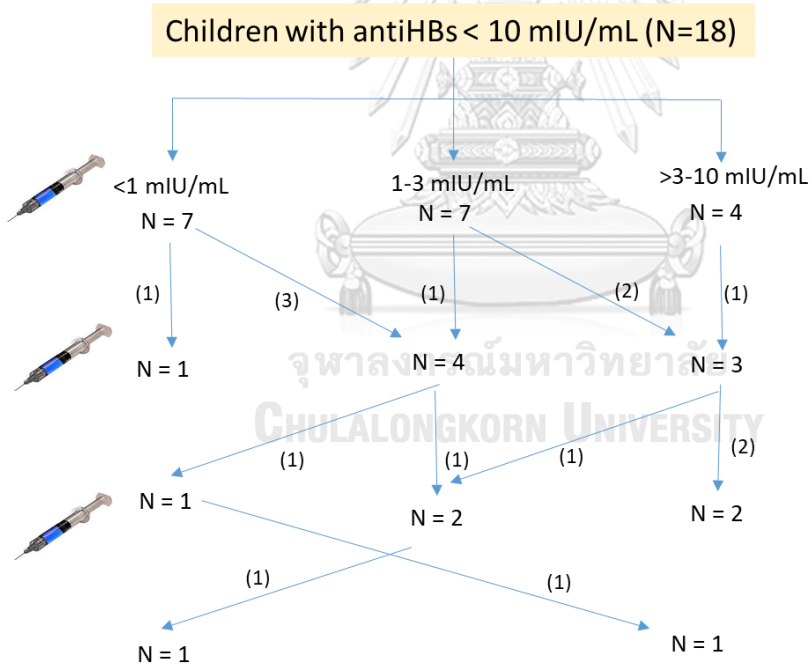
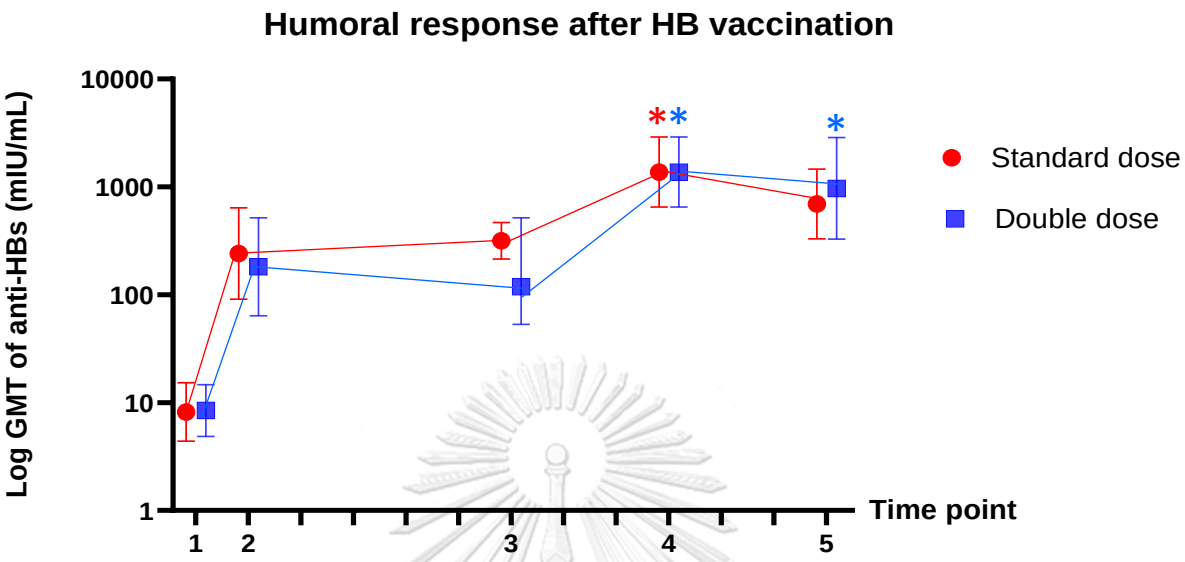


Figure 5 Log GMT of anti-HB antibodies after vaccination at 5 time



*P value < 0.05 vs time point 2

Nonresponders

After completion of the vaccination regimen, there were two nonresponders in each group. Three of the four nonresponders received a series of revaccinations. All of them seroconverted after the fifth (N=1) and the sixth (N=2) revaccination dose (Figure 6). The characteristics of the four nonresponders are shown in Table 2.

Figure 6 Anti-HBs antibodies of nonresponders after the second course of revaccination

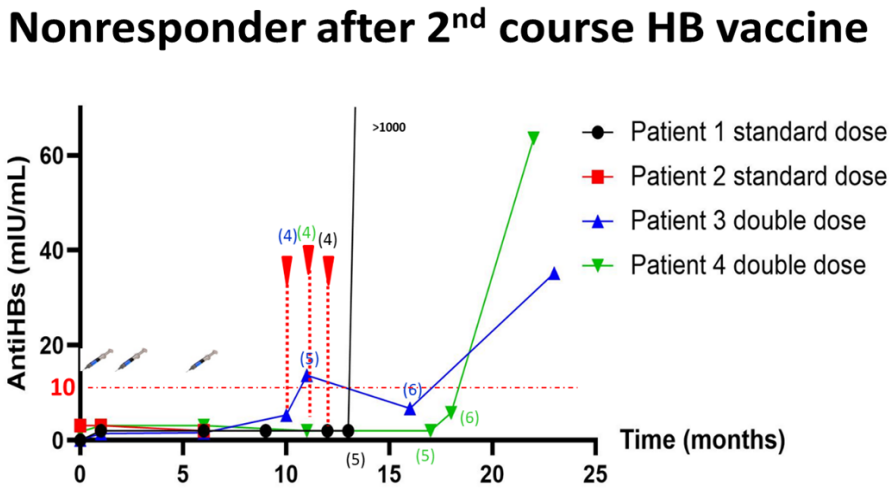


Table 2 Characteristics of four nonresponders after completion of revaccination regimen

No	Sex	Dose	Age at LT (yr)	Time to vac (yr)	DTH skin test	Dis.	Donor	Anti-HBc	CMV VL	EBV VL	Drug	Time point of Anti-HBs and level (mIU/mL)				
												1	2	3	4	5
1	M	S	0.83	0.58	Neg	BA	living	Neg	neg	Neg	tacrolimus, azathioprine	0	2.2	3.1	0.22	0.3
2	F	S	16.09	0.53	Neg	BA	cardeveric	Neg	neg	Neg	tacrolimus, azathioprine, prednisolone	0	0	2.01	2	2
3	F	D	0.67	0.57	Neg	BA	living	Neg	2350	67	tacrolimus, MMF, prednisolone	3.1	3.1	0.51	0.45	0.4
4	M	D	6.07	0.67	pos	ALF	cardeveric	pos	neg	neg	tacrolimus, lamivudine	0.1	1.4	1.6	5.3	5.0

S; standard-dose group, D; double-dose group, LT; liver transplantation, vac; vaccination, Dis; disease, BA; biliary atresia, CMV; cytomegalovirus, EBV; Epstein-Bar Virus, MMF; mycophenolate mofetil

Safety of hepatitis B vaccination

There were 17 adverse reactions in a total of 198 injections (0.9%) (12 and 5 adverse reactions in the standard-dose and double-dose groups, respectively). Pain at the injection site was the most commonly reported (n=10) but subsided within 72 hours without any medication. Other adverse reactions were itching at injection site (N=2), fever within 72 hours after injection (N=4), and diarrhea (N=1). None of the participants had transaminitis or graft rejection within 2 weeks of the vaccination.

Cellular response after revaccination

***In vivo* study for specific T helper$_1$ cells using the DTH skin test with hepatitis B vaccine**

Of the participants, 57 (96.7%) had DTH skin testing with hepatitis B vaccine, and induration that was not less than 5 mm in diameter after 48 or 72 hours was considered a positive result. Comparing the results of DTH skin testing with seroconversion of anti-HBs antibodies after the first and third vaccinations, the sensitivity, specificity, negative predictive value, positive predictive value, and accuracy were 75%, 53.6%, 84.6%, 38.9%, and 70.18% vs 60.3%, 79%, 97.4%, 10.7%, and 61.2%, respectively.

***In vitro* study T-cell specific response to hepatitis B vaccine and antigen by using interferon-gamma enzyme-linked immunospot assay (IFN-γ ELISpot)**

We analyzed blood samples from 22 participants (13 standard- and 9 double-dose groups) that had been collected at 4 time points. They were divided into responder (N=21) and nonresponder (N=1) groups. Comparing the IFN-γ-secreting cells between responder and nonresponder groups, there was a higher mean number of IFN-γ secreting cells after vaccination in the responder group. There was a statistically significant higher mean number of IFN-γ-secreting cells before vaccination and after the third vaccination in the responder group (p = 0.019) (Table 3).

Table 3 T-cell specific responses to hepatitis B antigen using IFN-γ assays compared between responder and nonresponder groups

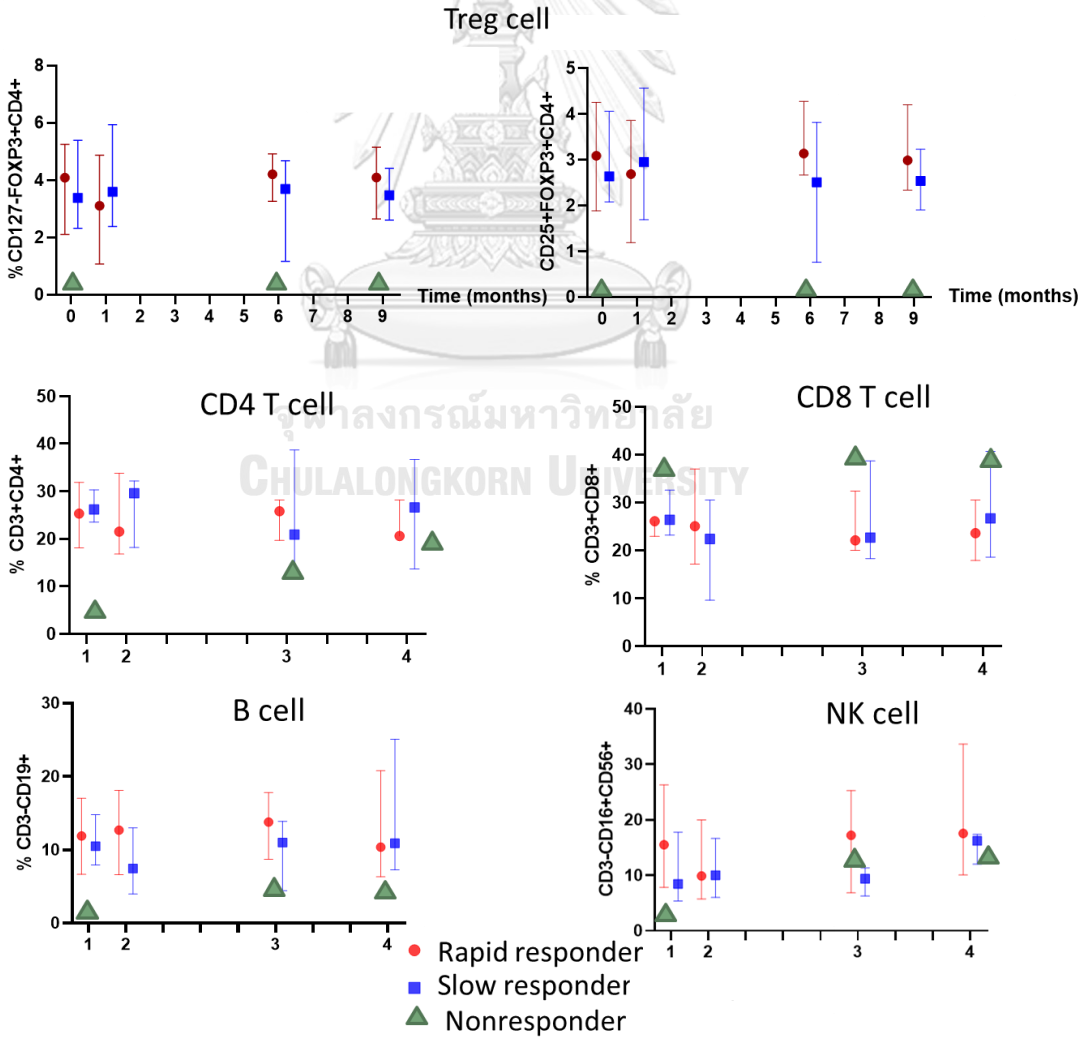
Time point	Responder (n=21)	Hyporesponder (n=8)	P value
1 (day 1) (SFCs/10^6 PBMCs)	14 (0, 23)	0	0.300
2 (month 1) (SFCs/10^6 PBMCs)	2 (0, 12)	-	-
3 (month 6) (SFCs/10^6 PBMCs)	8 (0, 44)	12	0.930
4 (month 7 up) (SFCs/10^6 PBMCs)	32 (4, 68)*	0	0.138

*P value < 0.05 vs time point 1; Present as median (IQR)

Subpopulation analysis of regulatory T cells, cytotoxic T cells, B cells, and NK cells

Subpopulations of immune cells were studied by measuring the expression of the following marker combinations: CD127-FoxP5+CD4+ and CD25+FoxP3+CD4+ for regulatory T cells, CD3+CD4+ for T helper cells, CD3+CD8+ for cytotoxic T cells, CD3-CD16+CD56- for NK cells, and CD3-CD19+ for B cells. There was no difference in T cells, B cells, and NK cells between the rapid responder, slow responder and nonresponder groups (Figure 7).

Figure 7 Frequency of cellular subpopulations analyzed by flow cytometry at 4 time points



Factors related to hepatitis B humoral immune response after revaccination

Baseline characteristics of the responder (N=38) and nonresponder (N=4) groups were compared to determine the factors associated with seroconversion after completion of revaccination (Table 4). The time after liver transplant and revaccination and tacrolimus level were the significant factors differentiating responders and nonresponders ($P < 0.05$).

Table 4 Univariate analysis the baseline characteristics between responder and nonresponder after 3-dose hepatitis B vaccination

Parameters	Responders (N=38)	Nonresponder (N=4)	P value
Age at transplantation (years)	1.35 (0.83, 3.01)	3.45 (0.71, 13.58)	0.797
Male (n, % male)	20 (52.6)	2 (50)	0.920
Age at HB re-vaccination (years)	1.95 (0.66, 4.95)	0.58 (0.54, 0.65)	0.030
Number of immunosuppressant (n, %)			
- None	1 (2.6)	4 (100)	
- One	31 (81.6)	-	0.527
- Two	6 (15.8)	-	
- Three	-	-	
Type of immunosuppressant (n, %)			
- Tacrolimus	31 (81.6)	4 (100)	
- Cyclosporin	6 (15.8)	-	0.527
- None	1 (2.6)	-	
Immunosuppressive level (ng/mL)			
- Tacrolimus	3.6 (2.6, 5.7)	6.7 (5.8, 7.8)	0.028
- Cyclosporin	209 (94, 941)	-	-
Diseases (n, %)			
- BA	28 (73.7)	3 (75)	0.955
Complications (PTLD, Rejection, surgical conditions)	20 (52.6)	1 (25)	0.293
Anti-HBs level (mIU/mL)			
At beginning	1.45 (0.48, 3.7)	0.5 (0.0, 1.38)	0.059
Laboratory investigation			
- SGOT (IU/L)	41 (33, 54)	42 (28, 50)	0.932

- SGPT (IU/L)	30 (19, 46)	28 (18, 56)	0.238
- GGT (IU/L)	30 (21, 66)	113 (28, 181)	0.635
- Albumin (g/dL)	4.1 (3.9, 4.3)	3.9 (3.6, 4.4)	0.428
- Hb (g/dL)	11.8 (10.4, 12.7)	10.2 (8.8, 14.2)	0.983
- WBC ($x10^6$/L)	7810 (6332,10862)	8675 (3642, 9627)	0.507
- Lymphocyte count ($x10^6$/L)	619 (266, 3808)	4020 (941, 5527)	0.314
- Platelet count ($x10^9$)	232 (174, 313)	298 (204, 425)	0.103

DISCUSSION

This study was a randomized, single-blinded, controlled trial of the efficacy of the 3-standard-dose and 3-double-dose hepatitis B vaccination regimens in children with liver transplants. There was a high seroconversion rate in both vaccination groups without any serious adverse reaction after revaccination. The seroconversion rate was higher after the third dose compared to the first dose, and the anti-HBs level after the third dose was significantly higher than after the first vaccination in both groups. Moreover, the participants who received the 3-double-dose regimen had significantly higher anti-HBs levels at 6-month follow up as compared with after the first dose. With regard to the factors related to seroconversion, time from liver transplant to revaccination and level of tacrolimus were significantly different between responders and nonresponders.

Previous cohort studies[12, 14] mentioned giving a booster dose of hepatitis B vaccination in children with liver transplants to keep anti-HBs above 10 and 1000 mIU/mL in order to prevent DNH while the international guidelines[45, 47] suggest maintaining anti-HBs titers above 100 mIU/mL by a booster dose in patients with solid organ transplants. Lin *et al*[12] reported a number of vaccination 1-19 times to keep the very high anti-HBs level from a booster strategies. This is the first randomly controlled trial (RCT) in children with liver transplants that compared the 2-dose-regimen for hepatitis B reimmunization as an alternative to multiple repeated booster vaccines. Because there were no data on other hepatitis B regimens for reimmunization, a number of hepatitis B regimens were assessed in HIV-infected people. Several non-

randomized studies[48-52] and one RCT study[53] found the highest response and the greater geometric mean of anti-HBs with the double-dose regimen after 72 weeks' follow up (months 0, 1, 6). With respect to these results, the international guidelines recommend 3-double-dose rescue hepatitis B vaccinations in the HIV-infected population with anti-HBs antibody loss[18, 54-56]. Similarly, the current study also demonstrated the great benefit of the 3-dose regimen of hepatitis B vaccine for seroconversion and the higher level of anti-HBs antibodies following the third dose compared to only one dose, without any serious adverse reactions. The rate of seroconversion in the standard- and double-dose regimen was high and GMT of anti-HBs levels after 6-month follow up seemed to be higher in the double-dose group, consistent with the results from the studies in the HIV-infected population[53].

The HIV-infected population might have had a T-cell defect such as in the children with liver transplants who received mainly T-cell immunosuppressive agents, and the response to the double-dose hepatitis B regimen was similar in both. T cells might play a major role in humoral response after revaccination. Consequently, the current study analyzed cellular immunogenicity to hepatitis B vaccine focusing on T helper$_1$ cell response and found a significantly higher level of IFN-γ-secreting cells with the ELISpot assay compared with the baseline in the responder group. The current study was not in accord with the study of Carollo *et al*[57] but had similar results to those of Ni *et al*[14] who found higher surrogate markers for both T helper$_1$ and T helper$_2$ after hepatitis B revaccination. With respect to other types of cellular immunogenicity to hepatitis B revaccination, there was only one study of T-reg cells in liver transplant recipients. Bauer *et al*[58] found the specific induction of T-reg cells could contribute to the poor humoral response after hepatitis B revaccination in liver transplant recipients similar to T-reg cells inducing immune tolerance in hepatitis B-infected people who could not clear hepatitis B infections. With regard to B-cell function in hepatitis B revaccination, recently; Bolther *et al*[59] studied both regulatory B cells (B-reg) and IFN-γ-positive T cells to predict humoral response in healthy students and workers. They found no correlation of the surrogate markers for B-reg/T helper$_1$ cells and anti-HBs antibodies in this healthy population. The reason might be

other cellular responses that cooperate with B cells in producing anti-HBs in the final step. So far, there has been no study on the role of B cells in liver transplant recipients. The current work studied these subpopulations by flow cytometry and found no changes in frequency of B cells, T cells, T-reg cells, and natural killer (NK) cells with this method. Further studies that investigate both quantity and quality of involved cells including the specific B cells (transitional, mature naïve, IgM memory, and switched memory B cells), T-reg cells, T follicular helper cells, long-lived plasma cells, and the humoral response to hepatitis B vaccination should be encouraged.

An *in vivo* study of cell-mediated immunity (CMI) by the DTH skin test was used for screening patients suspected of having a T-cell defect[60]. Again, this is the first study that used the DTH skin test to predict the humoral response to hepatitis B revaccination in children with liver transplants. However, HBV was not the best marker to predict T-cell immunity compared to other antigens[17]. Krittaecho *et al*[17] found three suitable antigens for DTH testing for adults in Thailand, including purified protein derivative (PPD), tetanus toxoid (TT), and *Candida albicans*. They reported only a 5.3% positivity rate for hepatitis B vaccine. The possible explanations for these low positive results may be a suboptimal amount of antigen[61], less exposure to hepatitis B virus, or no previous hepatitis B immunization. In this study, we avoided using multiple antigens in these young children and chose only hepatitis B vaccine to determine the hepatitis B-specific T-cell response, not an overall CMI defect. In addition, the pure antigens for PPD, TT, and *Candida albicans* were not available at the time. Surprisingly, we found a higher rate of positive hepatitis B DTH skin tests compared to previous studies[17]. The clinical usefulness of the DTH skin test to predict slow responders and nonresponders after revaccination was high, with a negative predictive value of 84.6% and 97.4% after the first and third vaccination, respectively. Hence, it might be a valuable bedside test to educate patients to be aware of hepatitis B infection even if they are receiving 3-dose hepatitis B revaccination. Ongoing research also needs to identify how to increase the humoral response for these patients other than using a 3-dose vaccination regimen.

In the present study, the rate of seroconversion was high. However, rapid decline of anti-HBs levels might occur in children who have undergone liver transplant[1, 10]. The present study investigated the differences between revaccinated children who were responders and nonresponders. We found the time from liver transplantation to revaccination and the immunosuppressive level were the significant factors for seroconversion. In theory, immunosuppressants that suppress mainly T-cell function are responsible for seroconversion in these immunocompromised children. However, we could not demonstrate the cellular defect in our pilot investigation. The explanation could be the low number of nonresponders, the variation in the time the participants were enrolled, and the immunosuppressant protocol the participants received; only participants who were stable and underwent liver transplantation more than 6 months previously were recruited for the study. Hence, we are going to follow up anti-HBs levels long term to study the factors that could maintain anti-HBs immunity. In the meantime, cellular immunity in patients who had anti-HBs loss or a rapid decline after the revaccination regimen will be further studied and compared with participants who maintained high anti-HBs titers after completion of the revaccination regimen.

The strength of this study is that it is a single-blind RCT that compared the potential of vaccination regimens to effectively increase humoral response after revaccination in children who had a liver transplant. Moreover, we also collected blood samples at each time point of vaccination and short-term follow up (up to 6 months) and studied aspects of cellular immunogenicity to the vaccine, both *in vivo* and *in vitro*. However, the high response to the hepatitis B vaccination regimen limited the study in analyzing cellular immunogenic responses and potential factors in seroconversion because of the low number of participants who were nonresponders in this study. Genetic factors may be one explanation for seroconversion after revaccination but were not investigated in this study.

In conclusion, the present study demonstrated that the 3-standard-dose and 3-double-dose hepatitis B vaccination regimens were highly effective and safe for children with liver transplants who were previously immunized but lost hepatitis B antibodies after transplant. Anti-HBs levels at short-term follow up or up to 6 months

after complete vaccination in the double-dose group were higher than in the standard-dose group. The negative results from the DTH skin test could predict slow responders and nonresponders in children with liver transplants after the 3-dose hepatitis B regimen. IFN-γ-secreting cells identified by the ELISpot assay were surrogate markers for humoral responses after revaccination. For successful reimmunization with a robust humoral response, anti-HBs levels should be monitored and revaccination should be introduced earlier before the antibody level is too low.

Part 3

3.1 Conclusion and implication

The previous study[1] and this ongoing research found a high prevalence of anti-HBs antibody loss in children who underwent liver transplantation and two cases of DNH in our pediatric liver center. This is one of the VPIs that occurred after liver transplant even though there was complete immunization prior to liver transplant. Revaccination after liver transplant should be considered at least for hepatitis B. The potential factors involved in humoral loss of hepatitis B protection were low titer of anti-HBs antibodies, hypoalbuminemia, and jaundice[1] prior to liver transplant. The potential factors for seroconversion after liver transplant included the time from liver transplant to revaccination and immunosuppressive level. In this study, children after liver transplantation had a good humoral and T-helper$_1$ response after revaccination and could maintain an adequate antibody level for more than 6 months with either the 3-standard or 3-double-dose regimen. The DTH skin test might be useful in clinical practice for predicting seroconversion after revaccination. To sum up, the strategies to prevent hepatitis B infection in children after liver transplantation should include the complete hepatitis B vaccine. EPI is crucial and a booster dose at least 2 weeks before the liver transplant operation should be considered because high antibody levels before liver transplant could delay the rapid anti-HBs antibody loss post-transplant. For 6 months after liver transplant, anti-HBs levels should be monitored regularly every 3 months, and revaccination with the 3-standard or 3-double dose regimen is recommended.

For other VPIs, revaccination data are limited. In the present study, there was a high rate of incomplete immunization before liver transplantation but the rates of VPIs in children with complete and incomplete immunizations showed no significant difference. Loss of these VPIs might occur in children after liver transplant, and revaccination could be considered. Additional study is need on immunity to protect against other VPIs, such as hepatitis A, influenza, and pneumococcal infection.

3.2 Limitation of the study

Because the study was conducted in a single center, the number of participants was limited. There were too few nonresponders to study the cellular immune response in this population. Healthy and age-matched children who had anti-HBs antibody loss should be recruited to compare the cellular immune response in children with liver transplants who were responders and nonresponders. Long-term follow up is needed to determine the effectiveness of the proposed hepatitis B regimen in children after liver transplantation.

3.3 Suggestion in research perspectives

Further research in a multicenter study on aspects of other hepatitis B vaccine regimens for children who did not response to 3-dose regimen is needed, and the cellular response in children who did not have seroconversion after the first dose should be evaluated. The comparison of cellular immune responses in responders and nonresponders in children with liver transplants with those in healthy, age-matched controls might reveal some cellular defect in children with liver transplants who had humoral responses. We also suggest research on an animal model or cell lines with hepatitis B virus exposure to see whether a robust immunologic response can clear infection instead of using hepatitis B vaccine should be explained more how children who had anti-HBs loss, could infected with DNH B.

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