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ARTICLE

Relationship between CYP2E1 Gene Polymorphism and Anti-tuberculosis Drug-induced Liver Injury

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ABSTRACT

Objective: To investigate the relationship between cytochrome P450 E1 (CYP2E1) gene polymorphisms and susceptibility to anti-tuberculosis drug-induced liver damage (ATDLI) in tuberculosis patients in the Chinese Han nationality. Methods: A retrospective analysis was performed on 360 patients with tuberculosis who had liver damage after tuberculosis treatment (case group) and 360 patients with tuberculosis who did not develop liver injury after treatment (control group). MassARRAY were used to detect CYP2E1 gene polymorphisms. Results: In a total of 8 tagged SNP loci selected, the rs8192773 locus failed to pass the test, and therefore, it is not included in subsequent analysis. At the remaining seven SNP sites, the difference in alleles was not statistically significant between the case group and the control group, suggesting that these sites may not be related to liver damage caused by anti-tuberculosis drugs. Three monomer domains were found in the seven tags SNP loci mentioned above. However, it was found that these haplotypes are not closely related to anti-tuberculosis drug-induced liver damage. Conclusion: The CYP2E1 gene polymorphism in the Chinese Han nationality is not related to the occurrence of anti-tuberculosis drug-induced liver injury.

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1. Introduction

The cytochrome P450 enzyme system is the main enzyme system that catalyzes the biotransformation of foreign compounds in the body. It was named so because of the specific absorption near the wavelength of 450 nm when it is in a reduced (ferrous) state and is bound to carbon monoxide (CO). Cytochrome P4502E1 (CYP2E1) is a member of the Phase I Metabolic Enzyme Cytochrome P450 Superfamily, which is expressed mainly in the liver and is involved in the metabolism of many drugs and carcinogens.[1] Recent studies have shown that CYP2E1 gene is polymorphic and different genotypes affect the expression of CYP2E1.[2] This difference may be related to genetic factors, and is one of the reasons for the difference in the metabolic capacity of the same substrate among different individuals and races. A number of articles have reported that the CYP2E1 gene polymorphism is associated with lung cancer, liver cancer, alcoholic cirrhosis and other diseases.[3-5] CYP2E1, as an important enzyme in the metabolic pathways of anti-tuberculosis drugs, has attracted the attention of many scholars at home and abroad due to the relationship between genetic polymorphisms and anti-tuberculosis drug-induced liver damage.[6-8] In this study, MassARRAY and case-control protocols were used to investigate the relationship between CYP2E1 gene polymorphisms and susceptibility to anti-tuberculosis drug-induced liver damage in the Chinese Han nationality.

2. Study Objects and Methods

2.1 Study Objects

Through retrospective analysis, from May 2010 to March 2016, 720 cases of primary or relapsed tuberculosis who met the inclusion criteria were selected. The patients were divided into two groups. The case group consisted of 360 patients who had liver damage after first-line anti-tuberculosis treatment (2HRZE/4HR). The liver damage induced by anti-tuberculosis drugs is defined as asymptomatic or symptoms of hepatic inflammation such as loss of appetite, nausea and vomiting, after taking anti-tuberculosis drugs, and diagnosed with the following conditions: (1) Serum AST and/or ALT is 2 times (or > 80 U/L) above the upper limit of normal (ULN), or (2) Any increase in ALT, AST, accompanied by gradual elevation of bilirubin (>2.5 mg/dl). The control group included 360 tuberculosis patients who took the same anti-TB drugs but no liver damage occurred. All patients need to meet the following conditions before they can be included in the study: Liver function is normal at the beginning of chemotherapy, and there are no factors that may cause liver damage, such as malnutrition, HIV infection, alcohol abuse, viral hepatitis, liver disease, cardiac insufficiency, and the use of other liver damage drugs, etc. Other conditions include that patients can be closely monitored for changes in liver function during treatment.

2.2 Methods

2.2.1 Extraction of Genomic DNA from Peripheral Blood Mononuclear Cells

1 ml of blood specimens of determining erythrocyte sedimentation rate (ESR) from patients with tuberculosis mentioned above were collected. DNA of the mononuclear cells in those samples was extracted using the whole genome DNA extraction kit (Tiangen Biotech, Beijing, China) according to the instructions and were immediately stored in a refrigerator at -20 °C.

2.2.2 Selection of NAT2 Gene SNPs and Detection of Their Polymorphisms

According to the gene polymorphism published in the public SNP database and related literature reports, 8 tag SNP loci from the CYP2E1 gene of the Chinese Han nationality were screened using haploview 4.2 software. See Table 1 for details. Mass spectrometry (MassARRAY, Sequenom, USA) was used to detect the genotype of each SNP locus.

2.2.3 Statistical Analysis

SPSS 13.0 and haploview 4.2 software, t-test, X2 test and other statistical methods were used to analyze the basic parameters of the case group and the control group. Pearson's chi-square test or Fisher's exact test was used to analyze the distribution of genotypes and alleles at each site in the case and control groups. The test level is α=0.05.

3. Results

3.1 Analysis of the Basic Characteristics of the Two Groups

There was no statistically significant difference in gender and age between the case group and the control group. The values of alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (DBIL) and total bilirubin (TBIL) before administration were all within the normal range. Hardy−Weinberg equilibrium in control group, MAF test of all data, the percentage of non-deletional genotypes in the locus, and other gene locus related information are shown in Table 1.

3.2 Association of Individual SNP and Anti-tuberculosis Drug-induced Liver Injury

The differences in the distribution of genotypes and alleles

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Table 1. The basic of the 8 SNP loci

Name	Position	ObsHET	PredHET	HWpval	%Geno	MAF	Alleles	Rating
rs3813865	135189234	0.332	0.343	1	98.8	0.22	G:C	
rs2031920	135189835	0.31	0.326	0.4073	95.8	0.205	C:T	
rs2070673	135190557	0.491	0.484	0.4236	99.2	0.41	T:A	
rs8192773	135195964	0	0	0	0	0	T:T	BAD
rs915908	135196949	0.229	0.243	1	98.3	0.142	G:A	
rs8192775	135198016	0.361	0.349	0.8664	99.8	0.225	G:A	
rs7092584	135198247	0.496	0.491	0.5121	99.8	0.432	C:T	
rs2515641	135201352	0.298	0.318	1	98.8	0.198	C:T	

Notes:
(1) Position: Position of the locus on the chromosome.
(2) %Geno: The percentage of non-deleted genotypes on the locus for all samples (the minimum value is 75%, less than this value is considered to have failed the test).
(3) MAF: The frequency of the last allele at this site (minimum value is 0.001, less than this value is considered to have failed the test)
(4) Alleles: Major and minor alleles at the locus
(5) Rating: Genetic sites that passed all tests will be entered for follow-up analysis. Sites that did not pass one or more tests were shown as BAD and excluded.

at each site between the case group and the control group were analyzed using the Pearson chi-square test or Fisher's exact test. The results are shown in Table 2. As can be seen from the table, there was no statistically significant difference in alleles between the case group and the control group at the seven SNP loci. The rs8192773 locus failed the test, so it does not enter the subsequent analysis.

Table 2. Differences in distribution of CYP2E1 genotypes and alleles between case group and control group

Site	Alleles	Case, Control Ratio Counts	Frequencies (case, control)	χ^2	P
rs3813865	G:C	542:150, 556:158	0.783, 0.779	0.042	0.838
rs2031920	C:T	562:132, 556:162	0.810, 0.774	2.686	0.101
rs2070673	T:A	430:286, 432:282	0.601, 0.605	0.030	0.863
rs915908	G:A	602:106, 626:92	0.850, 0872	1.389	0.239
rs8192775	G:A	562:158, 552:164	0.781, 0.771	0.190	0.663
rs7092584	C:T	416:302, 402:298	0.579, 0.574	0.038	0.846
rs2515641	C:T	570:142, 572:136	0.801, 0.808	0.122	0.727

3.3 Haplotype Analysis

A set of single nucleotide polymorphisms that are related to each other in a specific region of a chromosome and tend to be inherited globally to offspring, were known as haplotypes. The linkage disequilibrium between loci was examined by methods such as D'/r^2. In the seven sites of CYP2E1, three monomer domains were found, and the linkage disequilibrium occurred in each monomer domain. For the monomer domains calculated in the linkage disequilibrium analysis, the distribution ratios of the haplotypes in the case group and the control group in each monomer domain were calculated. Overall genetic haplotypes and disease associations were examined using the Pearson chi-square test ($p<0.05$). The results are shown in Table 3. No haplotype associated with anti-tuberculosis drug-induced liver damage was found in the 3 monomer domains consisting of 7 SNP sites.

Table 3. Relationship between haplotype and liver damage

Haplotypes	Frequency	Case, Control Ratio Counts	Frequencies (case, control)	χ^2	P
Block 3					
GC	0.573	417.5 : 296.5, 403.4 : 314.6	0.585, 0.562	0.767	0.3811
CC	0.222	164.8 : 549.2, 152.4 : 565.6	0.231, 0.212	0.715	0.3978
GT	0.206	133.4 : 580.6, 161.8 : 556.2	0.187, 0.225	3.245	0.0716
Block 4					
TG	0.447	311.8 : 406.2, 331.3 : 388.7	0.434, 0.460	0.974	0.3237
AG	0.411	296.4 : 421.6, 294.0 : 426.0	0.413, 0.408	0.030	0.8629
TA	0.142	109.3 : 608.7, 94.8 : 625.2	0.152, 0.132	1.248	0.2639
Block 5					
GCC	0.562	411.2 : 308.8, 397.2 : 319.8	0.571, 0.553	0.429	0.5126
ATC	0.224	154.8 : 565.2, 166.8 : 551.2	0.215, 0.232	0.621	0.4308
GTT	0.193	136.1 : 583.9, 141.1 : 576.9	0.189, 0.197	0.130	0.7188
GTC	0.015	10.9 : 709.1, 11.0 : 707.0	0.015, 0.015	0.001	0.9776

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4. Discussion

CYP2E1 is a member of the phase I metabolic enzyme cytochrome P450 superfamily. Through the metabolism of CYP2E1 in the liver, the drug produces some toxic products, such as free radical, pro electron group, oxygen group and so on. These toxic substances could covalently bind to large molecules in the liver cells, or cause lipid peroxidation in the cell membranes and organelles, resulted in the liver damage.[9-10]

Huang YS et al.[11] conducted a retrospective study of 318 cases of pulmonary tuberculosis or extrapulmonary tuberculosis that met the inclusion criteria in the Taipei Veterans General Hospital from May 1998 to August 2001. Among them, they found 49 cases (15.4%) had drug-induced hepatitis after taking anti-TB drugs. After digesting the CYP2E1 gene-specific amplified fragments of tuberculosis patients using the restriction endonuclease RsaI, they divided the CYP2E1 allele into the wild-type c1 and the variant c2 according to the results of the fragment electrophoresis, so that the genotype could be divided into the following three types: c1/c1, c1/c2, and c2/c2. After statistical analysis, it was found that the CYP2E1 c1/c1 genotype had a higher risk of liver damage (20.0% vs 9.0%) than other genotypes containing the mutation gene c2. Using CYP2E1 c1/c2 or c2/c2 genotypes and NAT2 fast acetylated genotypes as reference, Patients with CYP2E1 c1/c1 genotype plus NAT2 slow acetylation genotype had a significantly higher risk of hepatotoxicity than CYP2E1 c1/c1 plus NAT2 fast acetylation genotypes (risk odds ratio increased from 3.94 to 7.43). In a Swiss study, Vuilleumier N et al.[12] included 89 patients with tuberculosis latent infection treated with isoniazid for prospective studies. 26 cases (29%) of their patients were found to have abnormal liver function, of which 8 cases (9%) showed isoniazid-induced hepatitis. At the same time, CYP2E1*1A/*1A genotype was found to be associated with isoniazid-induced liver dysfunction. The CYP2E1*1A/*1A genotype had a positive predictive value and a negative predictive value for isoniazid-induced liver dysfunction of 39% and 84%, respectively. Therefore, they suggested that the CYP2E1 gene polymorphism might serve as a useful predictor of anti-tuberculosis drug-induced liver damage. Wang Tao and Soukaina Guaoua et al.[13-14] supported their conclusions through research and reported that the CYP2E1 RsaI polymorphism is closely related to the occurrence of anti-tuberculosis drug-induced liver damage, and c1/c1 genotype is considered as an independent risk factor for the development of anti-tuberculosis drug-induced liver damage. However, research results were not consistent. Teixeira RL et al.[15] studied 167 patients with tuberculosis and found that there was no correlation between the CYP2E1 gene polymorphism and the occurrence of anti-tuberculosis drug-induced liver damage in Brazilian population.

In this study, we did not classify CYP2E1 into c1/c1, c1/c2, and c2/c2 genotypes through restriction endonuclease RsaI recognition sites. We screened 8 target sites according to the gene frequency in Chinese SNPs reported in the public SNP database, and used the PCR-MassARRAY method to detect the frequency of all SNP loci in the case and control groups, and then the association of these SNP loci and their haplotypes with the anti-tuberculosis drug-induced hepatic impairment was analyzed. Our results did not reveal a correlation between CYP2E1 polymorphisms and anti-tuberculosis drug-induced hepatic impairment. The difference between this study result and other reports, first of all, may be related to ethnic differences. In different countries and regions, the distribution of CYP2E1 genotypes is quite different. Secondly, in the previously reported studies, the sample size was relatively small. In particular, the number of patients with hepatic impairment in these studies was small, and it was easy to make type II errors in the statistical analysis to obtain false-negative results. Thirdly, because the use of anti-tuberculosis drugs is reported to increase the incidence of drug-induced liver damage, in the study of Vuilleumier N et al.[12], isoniazid was used as the only chemotherapy drug, while in the present study, patients were simultaneously treated with two or more chemotherapeutic agents for the treatment of tuberculosis, which may also be an important reason for the difference in results. Finally, differences in diagnostic criteria and experimental design for drug-induced liver damage can also lead to discrepancies in the results of the study.

5. Conclusion

In summary, we used the MassARRAY method to analyze 8 SNPs in CYP2E1 and found no association between CYP2E1 polymorphisms and antituberculous drug-induced hepatic impairment.

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ARTICLE

Sequence Analysis of TNFRSF13b, Encoding TACI, in a Patient with Very Early Onset Inflammatory Bowel Disease: a Case Report

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ABSTRACT

Very early onset inflammatory bowel disease (VEO-IBD), IBD diagnosed before 6 years of age, frequently presents with increased severity, aggressive progression, and often poor response to conventional treatments. Although the cause of IBD is generally considered to be intestinal immune dysfunction induced by polygenic mutations and environment and other factors, VEO-IBD has a stronger genetic susceptibility specifically the neonatal- or infantile-onset IBD. Herein we report compound heterozygous mutations in the tumor necrosis factor receptor superfamily member 13b (TNFRSF13B) gene in a 3-year-old male that was admitted to our hospital with lasted jaundice, repeated fever and diarrhea in May 2014 at 2-month-old. He was diagnosed with VEO-IBD based on clinical, laboratory and histopathological examination. However, he was unresponsive to the conventional therapy, including the nutritional support therapy, antibiotic and immunosuppressive treatment, and surgical release of neonatal intestinal obstruction. Novel compound heterozygous mutations, c.[365G>A];[452C>T] (p.[R122Q];[P151L]), were discovered in TNFRSF13B, encoding TACI, for this patient.

1. Case Report

We describe the case of a 3-year-old male referred to our hospital (the Children's Hospital Affiliated to Zhejiang University) in May 2014 at 2-month-old. The patient was admitted to the gastroenterology department for lasted jaundice, repeated fever and diarrhea. During the period of hospitalization, he was found hepatosplenomegaly at 3-month-old and perianal abscess together with neoplasm at 2-year-old. He was G1P1 and full-term caesarean delivered with low birth weight (LBW) of 1.7kg. Mixed feeding after birth, he was added complementary food at the age of one. Physical examination showed poor reaction, severe malnutrition profile (height and weight -3SD), abdominal distention, hepatomegaly (subcostal 3cm and subxyphoid 2cm) and splenomegaly (line 1 was 5cm, line 2 was 6cm, line 3 was -4cm). Besides, several perianal neoplasms and abscess were visible without ulceration or pus. Laboratory data were the following; white blood cell count 4.78×10^9/L (normal: 4.0-8.0×10^9/L), hemoglobin 7.0 g/dL (normal: 12.0-16.0 g/dL), hs-CRP fluctuated between 10-200mg/L

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(normal: <8mg/L). CMV antibodies of IgM and IgG were both positive. The liver function test prompted significantly increased total bilirubin, mainly direct bilirubin which lasted for 3 months. Alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) and alkaline phosphatase levels were also elevated. Serum immunoglobulin showed only IgM mild decline, while IgG/IgA had no obvious abnormality (serum IgG 6.40g/l (normal: 4.0-5.0g/l), serum IgA 0.39g/l (normal: 0.1-0.4g/l), and serum IgM 0.43g/l (normal: 0.5-1.0g/l)). The activity of coagulation factors II, VII, IX, XI, XII, as well as the level of insulin and growth hormone remarkably decreased. Apart from those, several tests such as tuberculosis, fungi, cerebrospinal fluid and blood culture were negative. Because of lasted jaundice, laparoscopic bile duct exploration, cholangiography and cholangiography were performed which finally proved cholestasis. In the meanwhile, liver biopsy also showed hepatic cholestasis, punctate necrosis with a slight reduction in the interlobular bile duct. Repeated abdominal radiographs showed intestinal inflation and morphological rigidity. In the chest X-ray, the only findings were bronchiectasis in both lungs and osteoporosis. Total digestive tract radiography, abdomen CT and abdomen B ultrasound demonstrated hepatosplenomegaly and mesenteric vascular changes in spiral shape which indicated congenital malrotation of intestine. Plus, perianal B ultrasound showed perianal hypoechoic area of 0.8*0.3cm and cervical lymph node B ultrasound showed bilateral cervical lymph node enlargement. When applied biopsy of enlarged cervical lymph node, it revealed reactive hyperplasia. Then Small intestinal hydro-MRI was performed (Figure 1) and large intestine radiography (Figure 2) where there were signs compatible with inflammatory bowel disease at colonic, rectum, sigmoid colon levels. Colonoscopy showed multiple colorectal ulcers, partly longitudinal ulcers, and cobblestone change of the mucosa in the transverse colon (Figure 3); biopsies were taken at both levels. The anatomical-pathological examination of the samples reported fissure ulcer, focal crypt abscess, and chronic active inflammation of the mucosal epithelium, in which the goblet cells decreased while lamina propria lymphocytes, plasma cells, neutrophils and eosinophils increased (1-26/HP) (Figure 4). During the period of hospitalization, he underwent intestinal resection and colostomy for releasing of neonatal intestinal obstruction. In macroscopic examination (Figure 5), mucosa was hemorrhagic and hyperemic; multiple foci of ulcer and cobblestone change of the mucosa were seen. Genetic analysis revealed heterozygous mutations in TNFRSF13B, encoding TACI (Figure 6). Drugs such as antibiotics,antivirals, antifungals, intermittent intravenous immunoglobulin, hormone (Prednisone), aminosalicylic acid (Mesalazine), immunosuppressant (6-Mercaptopurine, Methotrexate, Thalidomide), biological treatment of anti TNF agents (which was withdrawed after anaphylactic shock occurring for second Ciyingfuli Infliximab therapy) were introduced and applied. The condition could take a favorable turn, but was of short duration. Less than 1-2 weeks at home, he would be admitted to our hospital due to recurrent infection.

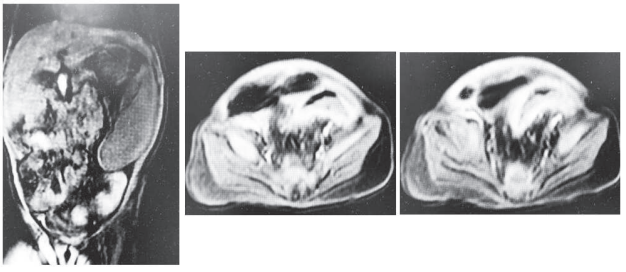

Figure 1

Notes: Small intestinal hydro-MRI image showed splenomegaly, intestinal morphological rigidity at the rectum, sigmoid colon and the right lower abdomen and significant enhancement in T1-weighted sequences after intravenous administration of paramagnetic contrast medium.

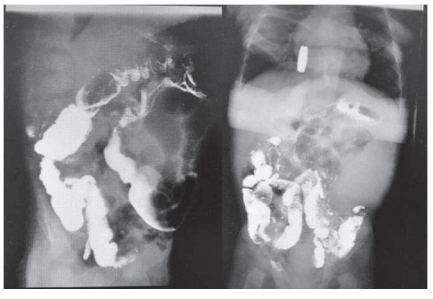

Figure 2

Notes: Large intestine radiography showed stiff morphology and rough wall of the intestinal canal, as well as slightly dilatation of sigmoid intestinal.

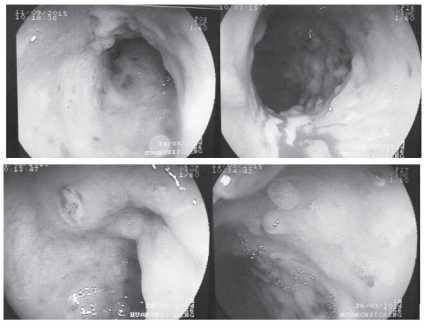

Figure 3

Notes: Colonoscopy image of multiple colorectal ulcers, partly longitudinal ulcers, and cobblestone change of the mucosa in the transverse colon.

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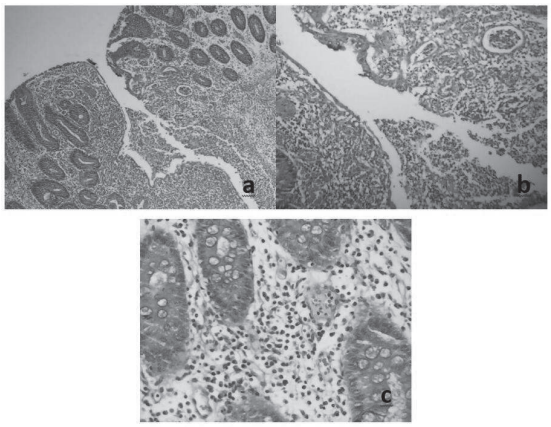


Figure 4

Notes: Pathological images showed fissure ulcer(a), focal crypt abscess(b), and chronic active inflammation of the mucosal epithelium, in which the goblet cells decreased while lamina propria lymphocytes, plasma cells, neutrophils and eosinophils increased(1-26/HP) (c).

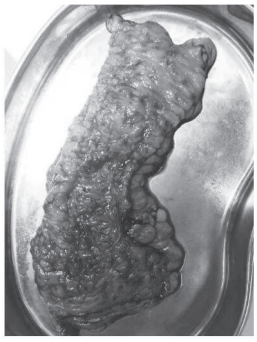
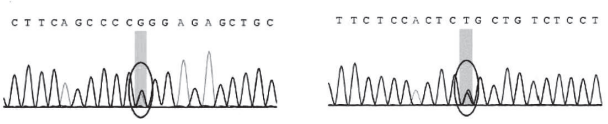
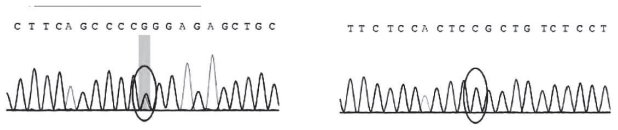


Figure 5

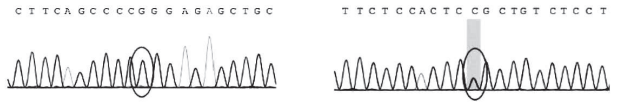
Notes: Macroscopic appearance of colectomy material. Mucosa was hemorrhagic and hyperemic; multiple foci of ulcer and cobblestone change of the mucosa were seen.



Child: chr17:16843819 and chr17:16852132 respectively exist a heterozygous mutation of c.452C>T and c.365G>A



Father: chr17:16843819 exists a heterozygous mutation of c.452C>T, while chr17:16852132 has no mutation



Mother: chr17:16843819 has no mutation, while chr17:16852132 exists a heterozygous mutation of c.365G>A

Figure 6

Notes: Sanger sequencing pictures. Two heterozygous mutations were found in the exon region of TNFRSF13B gene: c.365G > A (guanine > adenine) and c.452C > T (cytosine > thymine). It finally resulted in amino acid change: p.R122Q (arginine > glutamine) and p.P151L (proline > leucine). The double heterozygous mutations of TNFRSF13B gene, which are known as compound heterozygous mutations, were respectively from the parents and were consistent with autosomal recessive inheritance.

2. Discussion

Inflammatory bowel disease (IBD), including ulcerative colitis (UC), Crohn's disease (CD) and unclassified IBD (IBDU), is a group of chronic recurrent disease with complex pathogenesis and multiple factors related. Although the exact etiology of IBD is not yet completely known, recent studies have indicated that personal genetic susceptibility, environment, intestinal microbiota, and immune system are all involved.[1] With the technological progress in genetic testing and DNA sequencing, the most recent and largest genetic association study, which employed genome-wide association data for over 75,000 adolescent and adult-onset IBD patients and controls, identified 163 genes for IBD.[2] More recently, a trans-ethnic analysis including over 20,000 individuals of European and non-European ancestry identified an additional 38 new IBD genes.[3] While very early onset inflammatory bowel disease (VEO-IBD), IBD diagnosed before 6 years of age,[4] frequently present with increased severity, aggressive progression, and often poor response to conventional treatments, is supported to have a stronger genetic susceptibility specifically the neonatal- or infantile-onset IBD.[5] Especially when some related genes mutations are found in recent years, such as IL-10RA/B,[6] IL-10,[7] XIAP,[8] ADAM17,[9] etc., it makes some VEO-IBD become single gene diseases, many of which are classified as primary immunodeficiency disease(PIDs).

When it comes to this case, the child was then diagnosed as VEO-IBD based on clinical history, physical examination, endoscopic appearance, histologic findings, and radiologic studies, according to Porto criteria.[10] However, he was unresponsive to the conventional therapy, including the nutritional support therapy, antibiotic and immunosuppressive treatment, or surgical release of neonatal intestinal obstruction. Not until he was 2 years old, compound heterozygous mutations, c.[365G>A];[452C>T] (p.[R122Q];[P151L]), were discovered in the exon region of TNFRSF13B gene, encoding TACI, using whole exome sequencing (WES) analysis of DNA collected from the patient and his parents. And the two heterozygous mutations were respectively from the parents and were consistent with autosomal recessive inheritance.

TACI molecules, containing 293 amino acids, are mainly expressed in B cells or activated T cells. The cod-

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ing gene TNFRSFl3B is located on 17p and contains 5 exons. Gene mutations can be distributed in all regions of TACI. The function of TACI molecules are complex and diverse, including promoting isotype switching of mucosal IgA, playing an important role in the negative regulation of B cell activation and amplification, effectively participating in T cell dependent type two immune response molecules.[11-13] When the encoding gene mutation of TACI molecules arise, it will lead to T cells, B cells, antigen-presenting cells and the innate immune receptor deficiency.[14] There is an extremely convincing that mutations within the TACI gene resulting in amino acid substitutions are correlated with common variable immunodeficiency (CVID) and IgA deficient (IgAD) because extensive sequencing in healthy control subjects has failed to show the mutation.[16,17] In recently, TACI has been reported in about 8-10% of CVID patients and selective IgAD but sometimes found in healthy subjects who are not hypogammaglobulinemic.[17]

According to the diagnostic criteria for CVID published by the European Society for the prevention of immunodeficiency (ESID) and the national immunodeficiency group (PAGID), [18] CVID shall meet the following requirements: (1) The IgG level is lower than 5 g /L or 2.5 percentage points lower than peers. IgA is usually lower than normal level, while IgM can be normal or reduced; (2) There is no other definite cause of immunodeficiency; (3) Older than 4 years old. While selective IgAD is defined as a primary immunodeficiency characterized by an undetectable level of immunoglobulin A (IgA) in the blood and secretions but no other immunoglobulin deficiencies. In this case, the repeated blood immunoglobulin showed only IgM mild decline, while IgG/IgA had no obvious abnormality. That is, he can't be diagnosed neither as CVID nor selective IgAD at present. But it will still be interesting to follow this patient for the possible evolution of immunodeficiency because most patients with CVID do not present until adulthood.[19] Maybe, it is because TNFRSF13B defects alone do not cause CVID (or IgA deficient) and such an extremely heterogeneous immunodeficiency might be more likely related to additional, still unknown environmental and genetic factors as previous reports suggested.[15] Or maybe, these novel compound heterozygous mutations of TNFRSF13B, do not present as CVID (or IgA deficient), can lead to gut inflammation in some elusive way.

3. Conclusion

The key interest point in this report is that novel compound heterozygous mutations were discovered in TNFRSF13B, encoding TACI, for this patient with VEO-IBD. The relationship between the TNFRSF13B mutation and VEO-IBD is unclear, with further identification and follow-up of patients with such mutations needed to demonstrate the relationship between this biologic abnormality and its clinical manifestations. In terms of treatment, hematopoietic stem cell transplantation should be the best radical treatment for IBD with gene mutation, especially for severe and poorly controlled IBD patients. The potential mechanism is to repair the damaged intestinal mucosal barrier through differentiation and proliferation of intestinal stem cells. In principle, if TNFRSF13B deficiency is identified as a cause of primary immunodeficiency and early-onset IBD, it might be amenable to allogeneic hematopoietic stem cell transplantation to correct for the disease.

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ARTICLE

Polysaccharide from Fruits of Physalis Alkekengi L. Enhances Antitumor Efficacy by a DNA Vaccine

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ABSTRACT

Physalis. alkekengi fruit has long been used in traditional Chinese medicine for tumor therapy. In the present study, using plasmids that encode ovalbumin (OVA) gene, we investigate the adjuvant activity of a polysaccharide fraction (PPSB) isolated from P.alkekengi fruit. Formulation by simple procedures of mixing of the OVA-encoding pCI-neo-sOVA plasmid with PPSB not only induced specific antibody responses, but also induced antigen-specific cytotoxic T lymphocyte (CTL) responses (Graph abstract). Furthermore, immunization using this vaccine prevented the growth of OVA-expressing B16-OVA tumor cell growth in the immunized mice. Thus, we provide evidence supporting the adjuvant activity of PPSB in DNA vaccine against tumor.

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1. Introduction

DNA vaccines are cost effective, heat-stable and safe and have been extensively investigated within the last two decades for the prevention or therapy of infectious diseases, allergy and tumors. Despite these advantages, the rather poor immunogenicity and inefficient delivery of DNA vaccines hindered its exploitation.[1] A promising strategy to augment potency of DNA vaccines is through improvement of adjuvants. Although a variety of chemical adjuvants or immunomudulatory molecules have been formulated into the DNA vaccines, different disadvantages with these adjuvants have also been observed.[2] In this study, we accessed the adjuvancity of the polysaccharide fraction (designated PPSB) isolated from fruit of Physalis alkekengi L. of the family of Solanaceae, which has been used historically in traditional Chinese medicine for its anti-inflammatory and anti-tumor activity.[3-5] This herbaceous perennial plant is native in southern Europe and Asia. The polysaccharide fraction isolated from P. alkekengi fruit has been characterized structurally and its anti-diabetic effect has also been evaluated by Tong etc. It is an acid heteropolysaccharide of 27kDa consisting of Ara, Gal, Glc and GalA in ratio of 2.6:3.6:2:1 and demonstrates potential of adjuvant in an anti-fungal vaccine formulation.[6] The present study was designed to test whether the polysaccharide molecule can enhance cellular immune responses as well. Moreover, we evaluated the anti-cancer potential of the molecule.

2. Materials and Methods

2.1 Preparation of Polysaccharide PPSB from Fruits of P. Alkekengi

The fresh fruits of P. alkekengi were identified by Prof. Dafang Cui of South China Agriculture University. The polysaccharide PPSB was isolated from fruits of P. alkekengi as described by Wang et al.[6] Briefly, fresh fruits of P. alkekengi were decocted in distilled water at 100◦C for 3 h. The crude polysaccharide (CP) was precipitated from the decoction with 85% ethanol. CP dissolved in distilled water was frozen at −20℃ and thawed for at least 3 times, during which the insoluble materials were removed by centrifugation. CP was further precipitated with 50% ethanol. The residue was discarded, and the supernatant was further precipitated with 70% ethanol to obtain precipitate (PPSA). The proteins in PPSA were removed by treatment with a combination of proteinase and followed by Sevag method. Then the PPSA was further purified through a Sepharose CL-6B column eluted with 0.15 mol/L NaCl, and the main polysaccharide fraction (PPSB) was collected, dialyzed and lyophilized. The solution of PPSB was filtered by 0.20 m filter. The endotoxin level was less than 10 pg/mL measured by Limulus amoebocyte lysate assay on a microbiology kinetic reader MB-80 (Goldstream, China).

2.2 Plasmids

The pCI-neo-sOVA plasmid, which encodes soluble chicken egg ovalbumin (OVA), was from Addgene (plasmid # 25098, Cambridge, MA). Plasmids were purified using a QIAGEN Midiprep kit according to the manufacturer's instruction (Valencia, CA). Large scale plasmid preparation was performed by Feiyang (Guangzhou, China).

2.3 DNA Immunization

The institute guidelines for animal use and care were followed in all animal studies. Female C57BL/6 mice, 6-8 weeks of age, were purchased from Medical Experimental Animal Center of Guangdong (Guangzhou, China). DNA immunization was completed under diethyl ether anesthesia. Plasmid DNA (pCI-neo-sOVA, 10 μg) mixed with or without 30 μg PPSB in 100 l of PBS was injected subcutaneously around the base of the tail. In the preliminary experiments, 30 μg PPSB as adjuvant resulted in the highest antibody titer among different doses and the peak of antibody titer occurred one week after the last immunization (data not shown). Thus, 30 μg PPSB/mouse was used in all animal experiments. Mice in the negative control group were left untreated. Mice were dosed three times, at an interval of 2 weeks.

2.4 Enzyme-linked Immunosorbent Assay (ELISA)

OVA-specific antibodies (IgG, IgG1, and IgG2b) in serum samples of the immunized mice were measured by an indirect ELISA as previously described.[7] Briefly, EIA/RIA flat bottom, medium binding, polystyrene, 96-well plates (Corning-Costar, Corning, NY) were coated with 5 g/ml of OVA proteins in 100 μl carbonated buffer (0.1 M, pH 9.6) at 4°C overnight. The plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20) twice and then blocked with 4% (w/v) bovine serum albumin (BSA) in PBS/Tween 20 for 1 h at 37°C. Serum samples were diluted two or ten-fold serially in 4% BSA/PBS/Tween 20 and added to the plates in triplicate wells following the removal of the blocking solution. The plates were incubated for an additional 4 h at 37°C. The serum samples were removed, and the plates were washed 5 times with PBS/Tween 20. Horseradish peroxidase-labeled goat anti-mouse immunoglobulin (IgG and IgG1 from Shrbio, Nanjing, China, or IgG2a from Bio-Tc, Luoyang, China, 5000-fold dilution in 1% BSA/PBS/Tween 20) was added into the wells and the plate was incubated for another hour at 37°C. Plates were again washed four times with PBS/

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Tween 20, and 100 ml of 3, 3', 5, 5'-tetramethyl benzidine solution (TMB, Maibio, Shanghai, China) was added in each well at room temperature, followed by termination with the addition of 0.2 M sulfuric acid. The plate was read at 450/630 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT). Antibody titers were expressed as Log2 value of the highest dilution of serum that gave an absorbance value which exceeded an optical density value plus 2x standard deviation (S.D.) of the untreated mice.

2.5 Splenocyte Stimulation and Measurement of Cytokine Production

Two weeks after the last immunization, mice were sacrificed, and single-cell suspensions of splenocytes were prepared as previously described.[7] Briefly, spleens from each group of mice were collected aseptically and pooled together into 5 ml of HBSS (Hank's Balanced Salt Solution, 1x). Spleens were homogenized in RPMI1640 medium (Invitrogen, Carlsbad, CA) containing 2% fetal bovine serum (FBS, Thermo Scientific) with the plunger of a 1-mL syringe and followed by passing through a sterile 70 mm cell strainer for removing connective tissues and other debris. Red blood cell lysis was achieved by treating the cell suspension on ice for 5 min with Tris-NH4Cl buffer (0.75% NH4Cl and 0.205% KHCO3 in H2O, pH 7.2), followed by washing. The suspension was centrifuged at 800 rpm for 4 min at 4°C. After pouring off the supernatant, the cell pellet was re-suspended in of RPMI1640 medium supplemented with 10% FBS, 100 units/ml of penicillin (Invitrogen), 100 mg/ml of streptomycin (Invitrogen), 1% insulin-transferrin-selenium medium (Invitrogen) and 40 M of 2-mercaptoethanol (Sigma-Aldrich). Prepared splenocytes (5×106/well) were seeded into a 48-well plate in triplicate (n=3) and stimulated with 0 or 0.2 μM of SIIFEKL peptide (Genscript). After incubation at 37°C with 5% CO2 for 94 h, 80 μl of 5 mg/ml MTT (Thiazolyl blue tetrazolium bromide, Sigma-Aldrich) solution was pipetted into each well. After further 3 h of incubation at 37°C with 5% CO2, the plate was read at 490 nm. The cell proliferation index was reported as the ratio of the OD490 value of the stimulated cells (i.e. 0.2 μM of SIIFEKL peptide) over the OD490 value of un-stimulated cells. In addition, splenocytes (3 x 106 cells in 500 μl, n=4) were stimulated with 0 or 0.2 μM of SIIFEKL peptide for 48 h. The cells were spun down, and the supernatant was harvested for detectiong of IL-4 and IFN-levels using ELISA kits from Raybiotech (Atlanta, GA).

2.6 Tumor Prevention Assay

C57BL/6 mice were immunized with pCI-neo-sOVA plasmid s.c. for 3 times at an interval of 2 weeks as described above. One week after the last immunization, mice (n=5) were grafted s.c. in the right flank with 5 x 105 of OVA-expressing B16-OVA melanoma cells. Tumor volume was monitored every two days. Tumor size was calculated using the following formula: Tumor volume (mm3)= ½ [length x (width)2].

2.7 In Vivo Cytotoxic T Lymphocyte (CTL) Assay

An in vivo CTL assay was carried out as previously described[8] with a little modification. Briefly, C57BL/6 mice were immunized as described above. One week after the last immunization, splenocytes isolated from naïve C57BL/6 mice were pulsed with 0.2 μM SIINFEKL peptide (GenScript) in PBS for 45 min or left unpulsed. The pulsed cells were labeled with 10 μM of CFSE (CFSEhigh), while the unpulsed population was labeled with CFSE at a lower concentration of 1 μM (CFSElow). The two populations of cells were pooled together at a 1:1 ratio, from which, ten million cells were injected intravenously into the immunized mice. Mice were euthanized 16 h later, and the splenocytes were prepared and analyzed with a flow cytometer (BD, FACSCalibur) to determine the relative frequences of CFSEhigh and CFSElow populations. Specific lytic activity was calculated using the following equation:

$$\%\ \text{specific cell lytic activity} = \left(1 - \frac{\frac{CFSE^{low}_{untreated}}{CFSE^{high}_{untreated}}}{\frac{CFSE^{low}_{treated}}{CFSE^{high}_{treated}}}\right) \times 100$$

2.8 Statistical Analysis

Unless otherwise indicated, statistical significance was determined with a one-way analysis of variance followed by pair-wise comparisons with Fisher's protected least significant difference procedure. A p value of < to 0.05 (two-tail) was considered significant (*, $p < 0.05$; **, $p < 0.01$).

3. Results and Discussion

3.1 PPSB Augmented Both Antibody and Cellular Responses against OVA in Mice Immunized with OVA-encoding pCI-neo-sOVA Plasmid

Wang group has shown previously the adjuvant effect of PPSB in a DNA vaccine against systemic candidiasis which elicited a strong specific antibody response against a 47-kDa antigen, a breakdown product of heat shock protein 90 (HSP90). Thus, the protective efficacy against systemic candidiasis was also elevated by PPSB.[4] Whether this approach can enhance antigen-specific CD8+ T cell responses as well remains unresolved. We show herein that PPSB can augment both antibody and CTL responses. Mice were immunized subcutaneously with OVA-en-

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coding pCI-neo-sOVA plasmid with PPSB as adjuvant (designated plasmid/PPSB) as described above and boosted twice with the same regimen. Mice immunized with OVA-encoding pCI-neo-sOVA plasmid without PPSB (designated plasimid) or left untreated (UT) were used as controls. Blood samples were collected two weeks after the last immunization. The serum OVA-specific total IgG titier detected in the plasmid/PPSB group was significantly higher than that in the plasmid group (Figure 1A). Both IgG1 and IgG2a levels in mice serum were significantly elevated in plasmid/PPSB group compared with those in plasmid group (Figure 1B, 1C). This adjuvant effect is independent of the type of antigen encoded by the plasmid because the similar effect was also observed when PD-HSP90C plasmid with PPSB was immunized as previously reported.[4] No specific IgE antibody was detected (data not shown), indicating the lack of allergic responses.

To investigate whether PPSB affects the cellular immune responses induced by pCI-neo-sOVA immunization, splenocytes isolated from the immunized mice were re-stumulated in vitro with SIINFEKL peptide. Significant enhancement of both IFNg and IL-4 levels in culture supernatant of plasmid/PPSB group were detected in comparison with those of plasmid group (Figure 1D, 1E). In the classical Th1/Th2 paradigm, while IFNg elevation suggests the development of a type 1 CD4+ T helper cell (Th1) response, an elevation in IL-4 level is generally thought to indicate a Th2 response. But this useful paradigm is not able to account for results of many studies and thus cause controversial.[9] In addition, while IFNg plays a role in the activation of monocyte/macrophages, IL-4 is also a B cell growth and differentiation factor. When re-stimulated in vitro with SIINFEKL peptide, the proliferation of splenocytes from the mice of plasimid/PPSB group was significantly augmented compared with that of plasmid group (Figure 1F). Taken together, PPSB as adjuvant enhanced both the specific antibody and cellular responses in the present DNA vaccine formulation with pCI-neo-sOVA plasmid.

3.2 PPSB Enhanced the Antigen-specific CTL Responses and Prolonged the Survival of Tumor-bearing Mice

It is well accepted that cellular immune responses, especially CD8+ response, are major players in the anti-tumor defense. The present DNA vaccine also induced antigen-specific CTL responses. Cytotoxic CD8+ T cells are considered the principle effectors in protective immune responses against intracellular pathogens and tumor. Significantly higher CTL activity was detected in mice of plasmid/PPSB group than that of plasmid group (Figure 2).

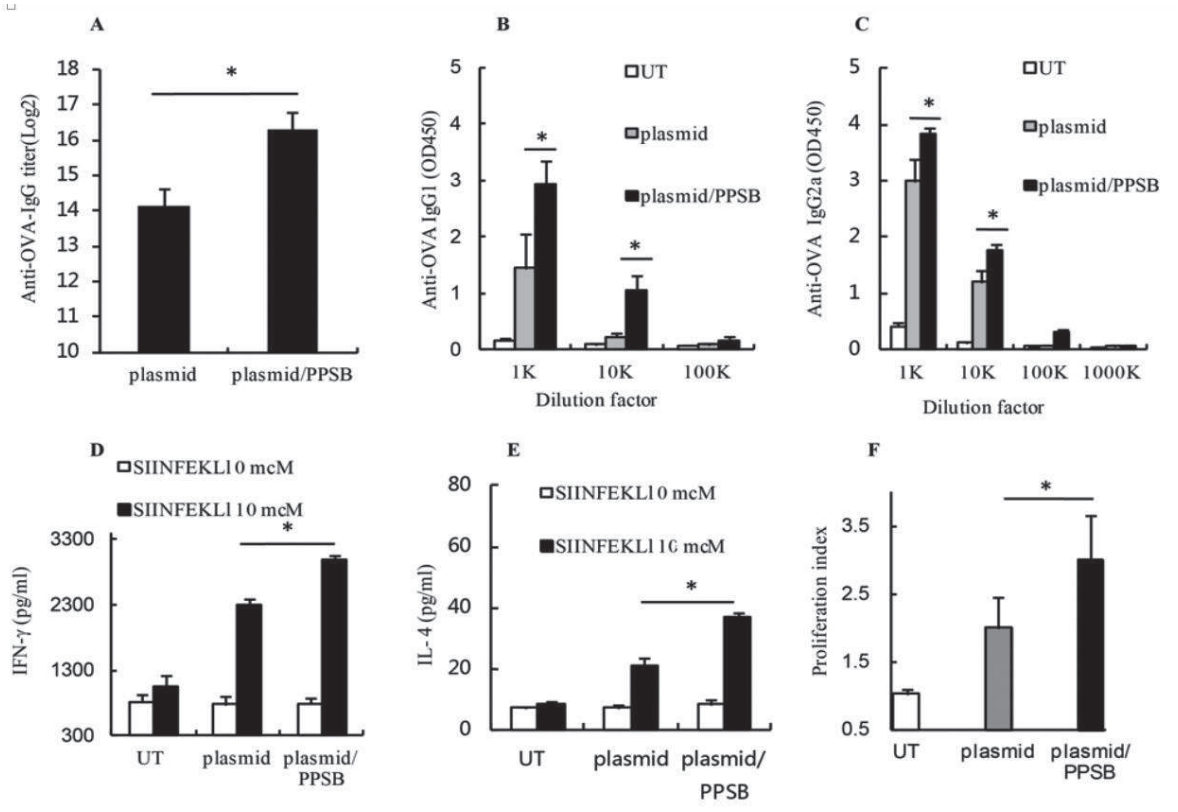

Figure 1

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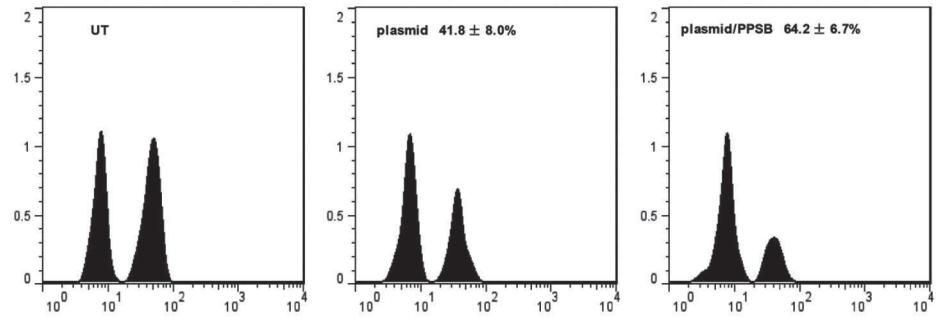

Figure 2

To determine the protective effects of the DNA vaccine combining pCI-neo-sOVA plasmid and PPSB, the immunized mice were challenged with B16-OVA tumor cells. The PPSB containing DNA vaccine formulation induced immune responses strong enough to prevent the growth of OVA-expressing B16-OVA cells (Figure 3). Thus, PPSB as adjuvant may potentially be used to elicit protective immunity against intracellular pathogens or tumors. Each time when the blood samples were collected, complete blood count was performed on XT-1800i heamatology analyzer (Sysmex, Japan), blood urea nitrogen and alanine aminotransferase were detected on Cobas c 702 chemistry analyzer (Roche, Switzerland) and no significant difference was found among groups of animals (data not shown), indicating good tolerability of the vaccine.

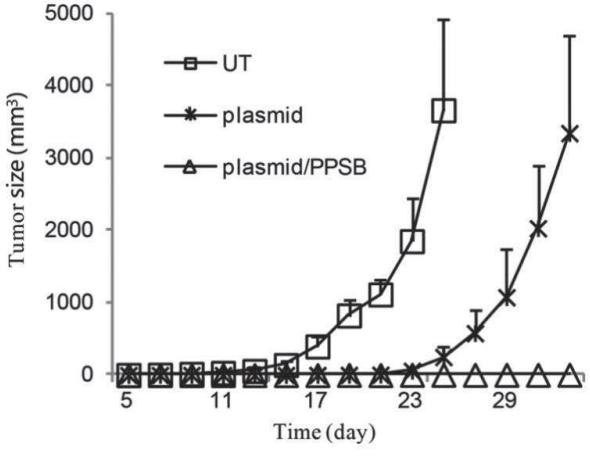

Figure 3

4. Discussion

DNA vaccines differ from other approaches of gene therapy in that they elicit tumor-specific immune responses, rather than directly kill tumor cells. Over the last decades, intensive preclinical studies have been performed using the DNA-based vaccine preparations. However, while most DNA-based antitumor vaccine formulations are well tolerated by animals and cancer patients, they often fail to generate therapeutically satisfied clinical responses.[10] And no DNA vaccine has been approved for human use by FDA so far. Improvement of adjuvant in the DNA vaccine formulations might be an attractive approach. Unfortunately, limited number of choices of adjuvant is available currently. Adjuvants often fail because of manufacture difficulties, lack of stability or effectiveness, tolerability or safety concerns. Adjuvants currently licensed for human use in the US and/or Europe includes aluminum salts, oil-in water emulsions (MF59, AS03 and AF03), virosomes and monophosphoryl lipid A (MPL, AS04). The inappropriate selection of adjuvant may render the vaccine inadequate. The classic aluminum hydroxide adjuvant is generally not optimal for eliciting cellular responses.[11] The oil-in-water emulsion preparations are not adequate for DNA vaccines. T cell responses are not optimally induced by these most commonly used adjuvants approved for use in human.[12] Thus, novel adjuvants appropriate for DNA vaccines are urgently needed. It is essential to avoid using undefined components in adjuvant formulations during vaccine development. Natural polysaccharides such as glucan from fungi or plants have been reported to be immunostimulatory.[13] And they are generally well-tolerated.[14] P. alkekengi grows in countries include China, Russia and Japan and has long been used in traditional Chinese medicine and as food in China. A polysaccharide PPSB isolated from P. alkekengi has been characterized by Wang group.[6] We are interested in the adjuvant activity of PPSB also because of the easy availability of the plant and low manufacturing cost of PPSB. Moreover, simple formulation procedures with PPSB are enough to create a vaccine which elicits considerable humoral and cellular immune responses. In the case of event of pandemic, large number of doses of vaccine is needed to cover the target population. For example, it is estimated approximately 1 billion doses of pandemic influenza vaccine could be produced currently, which is insufficient to cover the worldwide population.[15] In the future study, we plan to test the adjuvant activity of PPSB paired with protein antigen to

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explore its ability to increase manufacturing capacity by reducing the amount of antigen needed to induce immune responses desired.

5. Conclusion

The present study provides direct evidences for the adjuvant effect of PPSB which elevated both the humoral and cellular immune responses elicited by DNA vaccines. Antigen-specific CTL activity was enhanced by PPSB and the survival time of tumor-burden mice was significantly prolonged. In conclusion, PPSB is a potential adjuvant for DNA vaccines against tumor.

Abbreviations

OVA: ovalbumin;
CTL: cytotoxic T lymphocyte;
PPSB: polysaccharide isolated from fruits of P. alkekengi.

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ARTICLE

Longevity Internal Elixir—Tradition of Yue School in East Sichuan for Warding off Disease, Keeping in Good Health and Cultivating Mind

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ABSTRACT

With the continuous improvement of the national economy and people's quality of life, people's attention to human health and health is increasing. Taking exercise and reasonable diet and good living habits to adjust the body's meridian operation and promote the continuous optimization of human body function is an important way to achieve healthy development and optimize people's lifestyle. This paper describes the formation and development of Longevity Internal Elixir form Yue School in East Sichuan and its positive effects on human health. It analyzes the implementation methods and steps of Longevity Internal Elixir and points out the precautions and safeguards for the implementation of Longevity Internal Elixir. Through the analysis of this paper, it is clear that Longevity Internal Elixir plays an important role in the health of the people, exerts its role in promoting bodily functions. The implementation methods and safeguard measures of Longevity Internal Elixir are also clarified in this paper.

1. Introduction

The accelerated pace of life in modern society has made people's physical and mental pressures increasingly increasing, people's physical health has been seriously affected, and the demand for health training is increasing. Internal elixir refers to the practice of cultivating the body in itself with the essence of the energy and spirit, as the medicine under the guidance of the idea of unity of nature and man. Internal elixir has a long history of development in China. Longevity Internal Elixir can be used to eliminate diseases and health, and play its dominant role in promoting the improvement of people's physical functions.

2. The Formation & Development and Function of Longevity Internal Elixir

Longevity Internal Elixir (The Longevity Yin Elixir) was introduced to Yue School more than 800 years ago. In the process of continuous dissemination and development, through the study and practice of the school inheritors, the practice experience and sentiment of the Elixir are integrated into it, which makes the function further improved and optimized, and forms a complete health keeping method and the theory system of disease eliminating and life prolonging.[1] Longevity Internal Elixir has been circulating in the Taoism and Yue School in East Sichuan since it emerged from YIN-Changsheng in the Han Dy-

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nasty. It belongs to China's excellent national quintessence essence; its spread and development has positive significance for improving the physical fitness of the people.

Moving through the tip of the tongue against the upper root (the top of the tongue), Longevity Internal Elixir can effectively promote the regulation of the human immune system through the combination of skill, technique and medicine under the guidance of long-term and regular exercise, so that the immune function can be significantly improved. At the same time, it can optimize the proliferation function of normal human cells, and lay a good foundation for eliminating pain and prolonging life. Longevity Internal Elixir is not simply exercise. It combines the theory of yin and yang and five elements with the operation of the human meridians. Through the combination of Internal Elixir exercise, acupressure and Chinese herbal medicine, it can improve the function of human body, which not only promotes the regeneration of normal cells, but also effectively inhibits tumor cells;

In the treatment of chronic diseases such as hypertension, diabetes, prostate and breast hyperplasia, it can play an effective role[2] and play an effective role in promoting the cure of the disease. Longevity Internal Elixir combines exercise, massage relaxation and Chinese herbal medicine treatment to regulate the body's yin and yang balance, promote metabolism, and make the effect of urination and perspiration more significant. Longevity Internal Elixir regulates the operation of the human meridians under the guidance of the traditional Taoist principles of skills and regulations and traditional Chinese medicine theory to achieve the purpose of preventing diseases and health treatment.

3. The Implementation Methods and Steps of Longevity Internal Elixir

3.1 Lie Down

The practitioner should lie down on a clean bed in a well-conditioned room with fresh air, and the height of the pillow is slightly higher than the height of normal sleep. Then open Hukou, an acupoint at the part of the hand between the thumb and the index finger. Place the fingertips of the two palms on the outer edge of the kidneys of the lower back to ensure that the palm is in the state of fitting the sheets; The two feet are in a state of closeness, and the knees are slightly raised to form a right angle, and the two soles of the feet are flat on the bed in a natural state. Maintain the above position without moving, put the tongue on the upper jaw, and close both eyes to ensure the peace of mind, keep this state and smoothly breathe for 81 times.[3] After the above steps are completed, the state is maintained, and the body rotates to the right side, and is in a sleeping position. The upper limb movement is changed to the state of the right arm fitting bed, the elbow is slightly curved and the acupoint Hukou is opened, the palm is placed on the left shoulder, and the thumb is close to the left side of Jianjing, an acupoint between shoulder and ta chuei;

At the same time, the left-hand movement is changed, and the four fingers are inserted into the two crotches, close to foot-Taiyin acupoint Qihai, and the acupoint Hukou is opened, and the thumb is close to the foot-Shaoyang gallbladder meridian, in this position, breathing 36 times. After the above steps are completed, the body is adjusted to the left sleeping position, and the above steps are also performed to complete another 36 breaths. In the final stage, the practitioner turns into a flat state, and the elbows are slightly curved. Place the two palms together and keep the palms down at the position of Dantian, three cross fingers under the navel. At the same time, the legs are straight and the heels are fitted to the bed. In this position, the movement is completed after 27 breaths. During the implementation of this step, for people with weak constitution and bed-ridden disease, exercise can be repeated, and the practice time is more than 30 minutes. After this situation is completed, it is necessary to massage and relax on the abdomen, chest, and head and back neck; those with good limb function should take the action and massage. Allow the patient to sit on the bed with the knees or the wooden chair, keep the shoulders wide and shoulder wide, the back is straight forward and perpendicular to the ground, close the eyes, and the palms are naturally placed on the knees. In this position, the transition exercise is carried out. In this way, for the elderly who have high blood pressure and vascular aging diseases, the unsafe factors caused by sleeping posture can be avoided and effective control and avoidance can be avoided to avoid safety accidents. In addition, the quilts and sheets that used in the lie-down exercise need to be pat with a stick outdoors to avoid the dust being sucked into the body.

3.2 Sit Cross-legged Meditation

This step can be carried out indoors or outdoors. During the implementation process, the practitioner's body faces the south. The hand movements are: hands cross and overlap, palms up, the outside of the hands is close to Dantian (three cross fingers under the navel), and the female uses the left hand in the lower right hand position, while the male is the opposite. In this position, the practitioner relaxes the whole body muscles, and the spine leans forward slightly, while the lower jaw is slightly forwarded and breathed 81 times.[4] The above steps need to be repeated more than three times for the beginner, and the time is about 45 minutes. In a state of physical and mental relax-

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ation, "sit like a clock bell" (the head is the top of clock bell, the shoulder ring is the middle end, and the circle formed by the knee tip and the waist is the lower edge of the clock bell), meditate in the state of ignoring all the distractions in the heart; to achieve micro-closed eyes, concentrated, eye-view nose, nose-view mouth, mouth-view heart, heart-view perineum, through this state guidance, the Qi is naturally raised and then lowered to ensure that the Qi cycle is constantly running in the human body.[5] This type of meditation has high requirements on people's mentality and character. The practitioners must eliminate distracting thoughts, be compassionate and helpful in life, and nurse their own character and spirit to ensure the effect of meditation. After this step is completed, the practitioner needs to rub the palms and dry the face with both hands, while pressing the outside of the palm of the hand through the nose clip to the acupoint Baihui to the back of the neck to press; then use the right hand to rub the left foot acupoint Yongquan and the left hand to rub the right foot acupoint Yongquan.

3.3 Open and Close Peiyuan Pile

In this step, the practitioner stands, feet and shoulders are the same width, slightly bent on both knees, ten toes grip, and both eyes glare at the front, the two palms are separated by ten fingers, and the fingertips are slightly buckled to ensure that the acupoint Laogong is at the empty state. The two palms are diagonally opposite to the each other. The distance between the two palms should be 5/3 decimeters apart. Place the two palms in front of the chest, the fingers and shoulders are flat at the same height, and the two arms swing inward from 1 decimeter to 5/3 decimeters while inhale; exhale when the back swings forward, after repeated swinging for 49 breaths, the two arms move left and right again and again from 1 decimeter to 5/3 decimeters, and the breathing state is open and close, and the same repeated 49 breaths.[6]

3.4 Whirl and Exercise (Zhuantian Crouch)

The practitioner stands in front of the flat, two cypress trees one meter apart, for aerobic exercise; if there is no tree, walk naturally, and carry out the positive and negative whirl movements with bare feet or round-mouth cloth shoes. This movement originated from the long-term self-meditation practice of the Taoist priests from The Way of the Five Pecks of Rice in East Sichuan in Han Dynasty. They knew that meditation is only part of the exercise of Inner elixir, after observing the action of grinding the mill by working people; they realized the concept of keeping fit that "every laws of nature comes from circle". Practical experience has shown that the movement of the knees, the buckle shoulders, the shock feet and the rotation of the spine as the axis during the transition can promote the meridian movement of the lower limbs, hands and neck. It also stimulates the opening and closing movement of the two lungs, the two kidney, the two eyeballs, and the two testicles.[7] In the process of implementing the posture change, when the practitioner is required to change the posture, when the left foot is buckled and the foot is kicked, the upper body turns right and the right foot abduction; if the right foot is buckled, the upper body needs to turn left and the left foot is outreached. If time and environmental conditions are limited, the practitioner can only use his tongue to hold the upper jaw. Then walk in the fresh air, take beat and relax two steps to ward off disease and keep in good health.

3.5 Flap and Massage to Relax

After the Longevity Internal Elixir exercise step is completed, an effective massage relaxation exercise is required to relax the limb muscles and meridians. After practicing, the practitioner can relax by hot water soaking feet or hot towel on the neck; if the practitioner practices in the winter with ice and snow, he cannot immediately warm by fire; if it's summer, he cannot wear sweaty clothes for long time, and avoid the invasion of wind evil. The most important thing is to ensure that the breathing is natural and slow during the process. It is better to feel comfortable by the practitioner himself.[8] In addition, the practitioner can use the reverse abdominal breathing method and the normal breathing method to practice. In the early stage of practice, there is a large increase in appetite, snoring, tears in the eyes, fart, or fever and numbness in a certain part of the body, all of which are normal reactions of elixir practice.

4. The Precautions and Dietary Safeguards of Longevity Internal Elixir

Longevity Internal Elixir has an important regulatory effect on the human body, but there are some contraindications in the practice that need to be noted. This elixir exercise method is not suitable for pregnant women or pre-pregnant women, and mental illness patients are also not suitable for exercise practice. In the exercise, it is necessary to ensure that the tongue must hold to the upper jaw in the whole process. When the mouth is full of saliva, it should be swallowed by 3 times, and the Dantian field will be sunk along the front line, and the anus should be tightened. 30 minutes before and after Longevity Internal Elixir exercise, ban on urine and diet; beginners can do the next action after 15 days[9], and for 81 days, sex is strictly forbidden, what's more each time for Longevity Internal Elixir exercise should last more than one hour. In addition, the exercise should start from the summer

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solstice or the winter solstice. Every day before or after the daylight, or every evening, a flapping and relaxing action must be performed after each exercise. In daily life, the practitioner needs to be abstinent and ensure adequate sleep. The ancients said, "If the oil is exhausted, the lamp will be dry, and if a person is exhausted, he will die", which requires that the practitioners should be based on sufficient energy and achieve the goal of warding off disease, keeping in good health and cultivating mind by elixir exercise of energy and Qi.

In the daily diet and life, it is necessary to use the amount of plantain, houttuynia, dandelion and clover in the daily diet and life, as a tea; in the right season, the roots of fresh mulberry, purslane, dandelion, houttuynia, and chicken grass are eaten. For middle-aged and elderly people, you can put the dodder, yellow essence, black bean and black sesame in a ratio of 1:2:3:4, steam and cook, then dry and develop into powder, then add honey to make pills, take less than one day. Drinkers can take a spoonful of replenishing medicinal liquor made with traditional Chinese medicine such as ginseng, jujube, mulberry, rehmannia, radix astragali, medlar, and sakura. If not drinking, the practitioner can take one gram of Sanqi Danshen powder, or drink three lips of warm water before he start the elixir exercise, and if he does the elixir exercise in the morning, he should drink ginger water.

5. Conclusion

In summary, Longevity Internal Elixir has a positive effect on normal cell proliferation and immune system regulation. The combination of exercise, acupressure and Chinese herbal medicine can effectively regulate and optimize bodily functions. The Longevity Internal Elixir exercise under the guidance of scientific concepts plays an important role in warding off disease, keeping in good health and cultivating mind of the people.

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ARTICLE

Aloe Royal Jelly Powder Contributes to Maintain Normal Immunity: a Randomized Clinical Trial

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ABSTRACT

Objective: To study the safety and the function of maintaining normal immunity of product with royal jelly lyophilized powder and whole-leaf aloe drying powder as main raw materials. Methods: Selected 125 subjects qualified for inclusion/exclusion criteria for a trial test. (The trial group lost 9 subjects. The control group lost 6 subjects.54 effective subjects of trial group includes 12 men and 42 women. 56 effective subjects of control group including 9 men and 47 women.) The trial group was evaluated by self-control and inter-group comparison, after 90 days of continuous use of aloe royal jelly. Results: There are significant differences in the overall feeling, physiological feeling, psychological feeling and comprehensive evaluation of the individuals in the trial group after 90 days ($P<0.001$), which are higher than before. The control group has no statistically significant difference in the comprehensive evaluation before and after the trial ($P>0.05$). The ratio of CD4/CD8, IgG, IgA and IgM in the trial group and the placebo control group are above the normal low-limit. There is no obvious abnormality in indicators of blood test, blood biochemistry, liver and kidney function and other clinical tests. Conclusion: Aloe royal jelly powder contributes to maintain normal immune function and has no harmful effect to the health of subjects.

1. Introduction

Royal jelly has very high nutrition and immune value. It also has immune value in its pharmacological actions.[1-4] This paper investigates a cohort study on the health food which uses the royal jelly lyophilized powder and whole-leaf aloe drying powder as main materials, in order to investigate its safety and the function of maintaining the normal immunity.

2 Materials and Methods

2.1 Sample and Placebo

Aloe royal jelly powder used the royal jelly lyophilized powder and whole-leaf aloe drying powder as main materials. It had passed animal toxicology safety assessment and hygienic examination. Placebo's size and appearance were the same as the functional sample.

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2.2 The Subjects

The trial program was approved by the ethics committee of Tianjin Third Hospital. The subject inclusion criteria: the comprehensive evaluation score of the immune-related health score table was less than 80. The ratio of CD4 and CD8 to the peripheral blood T lymphocyte subsets, the ratio of CD4/CD8, the content of serum immunoglobulin IgG, IgA, IgM, IgE and the content of interleukin IL-2 and IL-4 were within the normal range. The subject exclusion criteria:

(1) Those who could not eat orally or who could not take this health food according to the rules.

(2) Those who were over 70 years old, pregnant or lactating women or who were intolerant or allergic to this health food.

(3) Those whose chief complaint was not clear.

(4) Those who had severe occupational disease or other severe diseases.

(5) Those who had serious diseases such as heart, brain, liver, kidney and hematopoietic system diseases or who had psychotic disease.

(6) those whose the ratio of CD4 and CD8 in peripheral blood T lymphocyte subsets, the ratio of CD4/CD8, the content of serum immunoglobulin IgG, IgA, IgM, IgE or the content of serum interleukin IL-2 and IL-4 were higher than the upper limit of normal value or lower than the normal value.

(7) Those who had immune-related diseases such as systemic lupus erythematosus, rheumatoid arthritis, systemic vasculitis, scleroderma, pemphigus, dermatomyositis, mixed connective tissue disease, primary thrombocytopenic purpura, autoimmune hemolytic anemia, Hashimoto's thyroiditis, primary myxedema, hyperthyreosis, ulcerative colitis and so on.

(8) Those who did not take this health food according to the regulations or whose incomplete information affected the efficacy or safety judge.

(9) Those who took food or medicine related to the function of this health food in the short term, affecting the judgment of the result.

2.3 Experiment Design and Grouping

The experiment was designed between groups and self-control. The subjects were randomly divided into trial and control groups according to the immune-related indexes. Reduce the gap between sex, age, diet and other factors as far as possible, and reduce the impact on the results of the experiment. Ensure the comparability between the groups through the balance test.

2.4 Dose and Time

The trial group took the samples 2 times a day per person, 5g each time. Pour cool boiled water to drink. The control group was given placebo. And the same method was taken with the trial group. Samples and placebos were taken continuously for 90 days.

2.5 Observation Indicators

2.5.1 Safety Indicators

General indicators were observed including sleep, diet, mental status, stool and so on. Before the experiment, Chest X-ray, electrocardiogram and abdominal B-ultrasound examination were performed. Blood routine examination, blood biochemical examination, blood pressure, heart rate and liver and kidney function were performed before and after the trial.[5]

2.5.2 Functional Indicators

Criteria: Two or more indicators, including overall feeling, physiological feeling, psychological feeling and comprehensive evaluation, had a statistically significant difference in the trial group ($P<0.05$). Meanwhile, indicators had no statistically significant difference in the control group. If all of indicators in the trial group and control group, including ratio of CD4/CD8, IgG, IgA, IgM, were above the low limit of normal value after the experiment, the health product was judged to have the function of maintaining normal immunity.

2.6 Data Statistics Method

The matched t test was used to compare the self-control data, and the group t test was used to compare the means of two groups. The group t test needed the variance homogeneity test. The data of the non-normal distribution or the inhomogeneous variance were converted properly. After the normal variance was satisfied, the converted data was used for t test. If the converted data could still not satisfy the requirement of homogeneous normal variance, the t' test or rank sum test should be used. But the data with large coefficient of variation (such as $CV>50\%$) should be tested by rank sum test.

2.7 Result Determination Method

Two or more indicators, including overall feeling, physiological feeling, psychological feeling and comprehensive evaluation, had a statistically significant difference in the trial group ($P<0.05$). Meanwhile, indicators had no statistically significant difference in the control group. If all of indicators in the trial group and control group, including ratio of CD4/CD8, IgG, IgA, IgM, were above the low limit of normal value after the experiment, the health product was judged to have the function of maintaining normal immunity.

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3 Results

3.1 General Situation

At the beginning of the experiment, the volunteers who satisfy the criteria are divided into the trial group (63 cases) and the control group (62 cases). Before the experiment, the general conditions of two groups (age, mental state, sleep status and diet) are generally the same. The inspection results of volunteers in two groups shows that there are no obvious abnormalities in inspections of abdominal B-ultrasound, electrocardiogram and chest X-ray. There is no significant difference in CD4, CD4/CD8 and IgG (P>0.05). The number of loss is 15 cases. The final effective statistics numbers are 54 cases in the trial group and 56 cases in the control group. The loss rate is 12%. The basic situation of two groups is shown in Table 1.

Table 1. Comparison of the balance between two groups before the experiment

Items	Trial Group	Control Group
Cases	54	56
Male/Female	12 / 42	9 / 47
Age(Years)	55.04±9.90	55.23±9.66
Immune-related health rating score	68.56±8.47	65.32±12.31
CD4	31.79±5.09	32.38±4.57
CD4/CD8	1.65±0.57	1.84±0.59
IgG	12.97±4.62	12.61±3.71

3.2 Effect of Samples on Safety Indicators

The heart rate and blood pressure of volunteers in two groups are in the normal range. There is no significant difference of volunteers in trial group before and after experiment (P>0.05). There are no obvious abnormalities in two groups' blood, urine and stool routine examination and blood biochemical examination before and after experiment. It indicates that samples have no harmful effect to volunteers' health. See Table 2 for results.

3.3 Effect of Samples on Functional Indicators

3.3.1 Health Scores

There are statistically significant differences in the overall feelings, physiological feelings, psychological feelings and comprehensive evaluation before and after the experimental diet (P<0.001). The score after experiment is higher than it before experiment. The differences in overall feelings, physiological feelings, psychological feelings and comprehensive evaluation are statistically significant between two groups (p<0.01). See Table 3, 4 for results.

3.3.2 Cellular Immunity Indicators

There is no significant difference in cellular immunity indicators of two groups before and after experiment (P > 0.05), as shown in Table 5.

Table 2. Comparison of safety indicators before and after experiment

	Trial Group (n=54)		Control Group (n=56)	
	Before	After	Before	After
Leukocyte ($\times10^9$/L)	6.03±1.27	6.21±1.74	6.01±1.38	5.95±1.43
Erythrocyte ($\times10^{12}$/L)	4.72±0.39	4.66±0.40	4.70±0.39	4.70±0.36
Platelet ($\times10^9$/L)	234.54±43.21	237.72±44.85	251.52±48.11	250.84±48.00
Hemoglobin (g/L)	142.26±10.85	139.94±13.41	141.46±10.55	139.86±9.88
Total Protein (g/L)	74.04±3.66	73.17±4.51	75.55±3.52	74.81±4.16
Albumin (g/L)	47.45±2.22	46.79±2.61	47.39±4.83	46.55±2.47
Glutamic-pyruvic Transaminase (U/L)	21.26±10.97	19.87±10.22	27.93±17.92	24.23±17.26
Glutamic-oxalacetic Transaminase(U/L)	19.81±6.62	19.61±6.24	22.82±9.15	20.82±7.98
Urea (mmol/L)	5.08±1.05	5.25±1.14	4.84±1.18	5.16±1.36
Creatinine (μmol/L)	69.93±14.90	67.78±15.65	65.46±14.79	66.13±18.10
Glucose (mmol/L)	5.49±0.99	5.41±1.02	5.36±0.80	5.24±0.98
Total Bilirubin (μmol/L)	11.52±3.18	10.95±2.72	11.41±3.12	10.57±4.73
Total Cholesterol (mmol/L)	5.18±1.11	5.28±1.02	5.14±0.97	5.25±0.84
Triglyceride (mmol/L)	1.37±0.74	1.54±0.84	1.70±1.08	1.85±1.05
Heart Rate (Times/min)	73.56±10.58	73.24±9.65	74.77±11.59	74.77±10.79
Systolic Pressure (mmHg)	130.07±19.22	129.96±18.60	135.16±19.32	135.48±18.54
Diastolic Pressure (mmHg)	77.26±9.23	77.13±8.40	78.80±14.84	78.61±13.60
Urine Routine	normal	normal	normal	normal
Stool Routine	Normal	normal	normal	normal

Notes: All of volunteers of trial group have no adverse reactions during the experiment, such as nausea, flatulence, diarrhea and allergy and so on.

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Table 3. Comparison of health scores in trial group before and after experiment

	Cases	Before	After
Overall feeling	54	22.17±3.17	23.52±2.63***##
physiological feeling	54	22.76±4.12	24.28±4.07***##
psychological feeling	54	23.63±4.11	25.20±3.72***##
comprehensive evaluation	54	68.56±8.47	73.00±7.65***##

Notes: Self-comparison *** P<0.001; Comparison between two groups ## P<0.01

Table 4. Comparison of health scores in control group before and after experiment

	Cases	Before	After
Comprehensive Evaluation	56	65.32±12.31	64.73±12.19###

Notes: Self-comparison p>0.05; Comparison between two groups ### p<0.001

Table 5. Changes of cellular immune indicators before and after experiment

	Trial Group(n=54)		Control Group(n=56)	
	Before	After	Before	After
CD4/CD8	1.65±0.57	1.78±0.71	1.84±0.59	1.90±0.72
CD4	31.79±5.09	31.36±6.72	32.38±4.57	32.58±7.23
CD8	21.41±7.59	19.93±7.79	19.26±6.10	18.88±5.90

Notes: Self-comparison P>0.05; Comparison between two groups P>0.05

3.3.3 Humoral Immune Indicators

The mean values of two groups are in the normal range before and after experiment. See Table 6 for results.

Table 6. Changes of humoral immune indicators before and after experiment

	Trial Group(n=54)		Control Group(n=56)	
	Before	After	Before	After
IgE	0.04±0.09	0.19±0.21***	0.05±0.11	0.21±0.23***
IgG	12.97±4.62	22.04±5.73***	12.61±3.71	22.02±8.30***
IgA	1.82±0.83	2.01±0.88	2.00±1.00	1.90±0.96
IgM	1.80±1.85	0.90±0.40**#	1.67±1.61	0.73±0.35***
IL-2	33.54±5.91	6.70±3.28***	35.00±7.45	7.97±6.59***
IL-4	13.00±6.16	22.41±51.86	13.76±6.94	25.16±52.93

Notes: Self-comparison *** p<0.001, ** p<0.01; Comparison between two groups # p<0.05

4. Conclusion

The volunteers who are in accordance with the experimental standard are tested for 110 cases (including 54 cases in trial group, 12 men and 42 women, 56 cases in control group: 9 men and 47 women). The control group is given placebo. The control group has no statistically significant difference in their comprehensive evaluation before and after experiment (P > 0.05). And the trial group is given aloe royal jelly mineral powder. After 90 days, there are significant differences in overall feelings, physiological feelings, psychological feelings and comprehensive evaluation (P<0.001). The test is higher than before. The ratio of CD4/CD8, IgG, IgA and IgM in trial group and control group are all above the low-limit of the normal value. According to the revision of "enhanced immunity function evaluation test method", the results show that aloe royal jelly powder has the function of maintaining normal immunity.[6,7]

Before and after experiment, the results of blood, urine, stool routine examination and blood biochemical examinations are in the normal range. They show that the sample has no adverse effect on the volunteers' health. No adverse reactions such as nausea, flatulence, diarrhea and allergic reactions are found during the experiment, indicating that the safety of sample satisfies the requirements.

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ARTICLE

Clinical Application of Percutaneous Transluminal Angioplasty and Stent Implantation in Acute Lower Extremity Deep Venous Thrombosis

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ABSTRACT

Purpose: To analyze the application of percutaneous transluminal angioplasty and stenting in acute deep venous thrombosis of lower extremities. Methods: 70 patients were divided into two groups according to the presence or absence of percutaneous transluminal angioplasty and stenting. Results: The mean circumferential diameter difference between the affected limbs and the healthy limbs and the knees at 15 cm was statistically significant. The cure rate and effective rate of the research group were higher than those of the control group (P<0.05). Conclusion: Percutaneous transluminal angioplasty and stenting are of high value in acute lower extremity deep venous thrombosis.

1. Introduction

Deep vein thrombosis of the lower extremity, also known as deep vein thrombosis of the lower extremity, is a common disease, which refers to the clotting of venous blood in the deep veins of the lower extremities. This disease is prone to residual varicose veins, lower extremity edema, hyperpigmentation, stasis, ulcers, dermatitis and other diseases.[1] A large number of literature reports that anticoagulation and transcatheter endovascular thrombolysis are effective methods for the treatment of deep vein thrombosis, but not all patients can achieve therapeutic goals. In recent years, academic research on the deep venous thrombosis of the lower extremities has continued to deepen, and with the rapid development of interventional therapy, some scholars have begun to propose a comprehensive intervention program for the treatment of deep venous thrombosis of lower extremities, and put it into practice. In this paper, the authors conducted a group research of 70 patients to explore the clinical application of percutaneous transluminal angioplasty and stenting in acute lower extremity deep venous thrombosis. The research is summarized as follows.

2. Data and Methods

2.1 Basic Data

From March 2015 to November 2017, 70 patients with acute deep venous thrombosis of the lower extremity admitted to our hospital were selected as subjects of this research. All subjects had complete clinical data. The affected limb was diagnosed by color Doppler or deep

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venous angiography of the lower extremity, and the first onset was clinically manifested as lower extremity swelling, pain, bruising, etc. The patients are informed of the research, willing to participate in the research, and signed the informed consent forms. The research was approved by the Medical Ethics Committee. Among them, patients who did not want to participate in the research were excluded, and patients with severe heart, liver and kidney dysfunction, mental disorders, severe disability and inability to communicate properly were excluded. Females during pregnancy or lactation were also excluded. According to the presence or absence of percutaneous transluminal angioplasty and stenting, the 70 patients were divided into two groups: control group (n=32) and research group (n=38). Control group: 19 males and 13 females, the youngest age was 18 years old, the maximum age was 74 years, the average age was (51.4±5.98) years old, the course of disease was 1-7 days, and the average disease duration was (4.5±0.86) days. Research group: 22 males and 16 females, the youngest age was 19 years old, the maximum age was 71 years old, the average age was (50.9±5.14) years old, the course of disease was 1-9 days, and the average disease duration was (4.7±1.35) days. Objective comparison of the basic data of the above two groups of patients, the difference is not large, no statistical significance, P> 0.05, but comparable, can be grouped.

2.2 Methods

In the control group, percutaneous transluminal angioplasty and stenting were not performed. The research group underwent percutaneous transluminal angioplasty and stenting. The specific treatment plan is as follows: (1) the inferior vena cava filter was placed through the contralateral femoral vein. According to the actual situation of the patient, 57 cases were selected Braun filter, and 13 cases were selected with Cordis filter. Take the prone position, after successful puncture of the iliac vein, the conventional indwelling sheath, using catheter pulse technology, give 3000 U heparin, and inject 50-100 million U urokinase. Thrombus was still seen in 41 patients after thrombolysis and was treated with thromboablation. Based on the fluoroscopy, a 7F thrombus ablation (ATD) catheter was selected, and the head end was placed in close contact with the thrombus to perform ablation. At the time of ablation, an appropriate amount of contrast agent was injected through the catheter sheath bypass to evaluate the thrombus ablation effect. After the above treatment, 38 patients with residual stenosis >30%, residual segmental irregular stenosis or occlusion of the vessel segment were performed by angiography, and percutaneous transluminal angioplasty and stenting were performed. During operation, the guide wire is exchanged and delivered to the inferior vena cava, through the filter, to the proximal end; a 6-10mm Nylon balloon produced by American Bard International Co., Ltd. was used for preoperative percutaneous transluminal angioplasty with a lesion of 4-8 atm. Before placing the stent, give 3000 U heparin. According to the specific condition of the diseased blood vessel, choose a suitable self-expanding stent (8-12 mm in diameter and 6-10 cm in length). Lum inexx W allstent stents are generally used, which are implanted in the order of the proximal end - the distal end. In patients with stenosis of the common iliac vein, the head end of the stent enters the inferior vena cava, about 0.5-1.0 cm. During the release of the stent, the position is slightly adjusted to ensure good adherence and smoothness. After the sheath is retracted to the end mark, the stent is completely released. The same method is used to perform the next stent implantation operation. Under normal circumstances, the distal end of the distal end stent should not exceed the proximal segment of the femoral vein. (2) 500,000 urokinase was pumped daily through the indwelling sheath for 3-5 days; subcutaneous injection of low molecular weight heparin, i.e. speed Bilin, 0.4ml/time, once a day; oral aspirin, 100mg/time, 1 Times / d; from the 5th day, add warfarin, starting from 8d, use warfarin alone. Among them, warfarin anticoagulant therapy lasts for 6-9 months. According to the patient's condition, the dosage of warfarin is adjusted appropriately to ensure that the international normalized ratio (NR) is within the controllable range.

2.3 Observation Indicators and Efficacy Evaluation

2.3.1 Observation Indicators

The average circumferential diameter difference between the affected limbs and the healthy limbs and the knees at 15 cm was observed and compared.

2.3.2 Evaluation Criteria for Efficacy

The efficacy evaluation criteria of this research included four indicators: cure, markedly effective, effective and ineffective: (1) cure. After treatment, the blood flow recovered completely, or basically recovered, no contrast agent retention, the residual stenosis of the lumen was less than 30%, the residual stenosis of the stent implantation was less than 20%, and the clinical symptoms and signs basically disappeared. (2) Markedly effective. After treatment, the patient's blood flow recovered most, no contrast agent retention, lumen residual stenosis 30%-70%, clinical symptoms and signs basically disappeared. (3) Effective. After treatment, the patient's blood flow recovery part, accompanied by mild contrast agent retention, occlusion of the lumen has been opened, but the residual stenosis is greater than 70%, or the vascular part of the obstruction section is opened, the collateral circulation is significantly increased compared with before treatment, clinical symptoms And the signs have improved. (4) Invalid. After

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treatment, the above criteria were not met, and there was obvious contrast agent retention.[2] Total effective rate of treatment = (number of cures + number of effective cases + number of effective cases) / total number of cases 100%.

2.4 Statistical Methods

The data of this research were processed by SPSS20.00 software. The mean plus or minus standard deviation ($\bar{x} \pm s$) and the case (n) and percentage (%) indicate the measurement data and the count data. The T value and the X2 test were performed. The test value P was less than 0.05. The difference was statistically significant.

3. Results

3.1 The Average Circumferential Diameter Difference between the Affected Limb and the Healthy Limb on the Knee and 15cm below the Knee

The average circumferential diameter difference between the affected limbs and the healthy limbs and the knees at 15 cm was compared between the two groups. There was no significant difference before surgery. However, the difference was significant after the next day and the 5th day after surgery, which was statistically significant (P <0.05). See Table 1 for details.

3.2 Comparison of Clinical Efficacy Analysis

The clinical treatment effect of the two groups of patients was evaluated and compared. The total effective rate of the research group was 96.9%, and the total effective rate of the control group was 100%. There was no significant difference between the groups (P>0.05), but the cure rate and marked efficiency of the research group were significantly higher than the control group (P<0.05). See Table 2 for details.

4. Discussion

Deep venous thrombosis (DVT) is a disease of limb venous reflux. It refers to abnormal blood clotting in deep veins. Slow blood flow, damage to the vein wall and hypercoagulability are the key causes of the disease. Once a thrombus is formed, a small number of cases can be ablated or confined to the site of occurrence. Most patients will slowly spread to the deep vein trunk of the entire limb. If not diagnosed and treated in time, it may cause thrombosis and form sequelae, which have different effects on patients' daily life and work and research, greatly reducing the quality of life of patients, and even inducing pulmonary embolism and other diseases, causing serious consequences.[3-4] In recent years, a large amount of data indicates that the incidence of acute deep venous thrombosis of the lower extremities is increasing year by year. The clinical diagnosis and treatment has attracted the attention and attention of the public and clinicians, experts and scholars, and has become one of the important topics for academic research.

It has been reported that the anatomical features of the left iliac vein make it easier for the iliac vein system to form a thrombus. For most patients, the single treatment of blood flow is not effective, which easily leads to incomplete lumen recanalization or thrombus re-formation.[5] At the same time, deep venous blood flow cannot be recovered for a long time, may affect the function of venous valve, induce post-thrombotic embolism syndrome.[6] A total of 70 patients in this research were unilateral deep vein thrombosis. After anticoagulant thrombolysis and thromboablation, the patient's affordability was considered. 38 patients underwent percutaneous transluminal angioplasty and stenting. Table 1 shows that there was no significant difference in the mean circumferential diameter difference

Table 1. Analysis and Comparison of the Average Circumferential Diameter Difference between the Affected Limbs and the Healthy Limbs at 15 cm at Different Time Points in the Two Groups of Patients [$\bar{x} \pm s$, cm]

Time	Position	Research Group (n=38)	Control Group (n=38)	T-value	P-value
Before operation	Above knee	7.5±2.94	7.3±2.35	0.310	0.757
	Below knee	5.9±1.89	5.8±1.97	0.216	0.829
Next day after operation	Above knee	4.7±1.72	5.6±1.82	2.123	0.037
	Below knee	3.5±1.17	4.1±1.25	2.154	0.034
5th day after operation	Above knee	0.9±0.31	1.8±0.69	7.227	0.000
	Below knee	0.7±0.25	1.5±0.51	8.536	0.000

Table 2. Evaluation and Comparison of Clinical Efficacy of Two Groups of Patients [n, %]

Group	Number of cases	Cure	Markedly effective	Effective	Invalid	Total effective rate
Control Group	32	9（28.1%）	8（25%）	14（43.8%）	1（3.1%）	31（96.9%）
Research Group	38	20（52.7%）	15（39.4%）	3（7.9%）	0（0）	38（100%）
X^2- value	/	12.566	4.749	33.619	3.148	3.148
P-value	/	0.000	0.029	0.000	0.075	0.075

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between the research group and the control group before the operation of the affected limb and the limbs and the knees at 15 cm (P>0.05). However, on the second day after surgery and on the fifth day after surgery, the mean circumferential diameter difference between the affected limbs and the healthy limbs and the knees at 15 cm was lower than that of the control group (P<0.05). Table 2 shows that there was no significant difference in the total effective rate between the research group and the control group (100% vs 96.9%, P=0.075). However, the cure rate and effective rate of the research group were significantly higher than those of the control group (52.7% vs 28.1%; 39.4% vs 25%, P < 0.05). According to the data in Table 1 and 2, percutaneous transluminal angioplasty and stenting are effective in improving clinical symptoms of patients.

Regarding the implantation of intravascular stents, the key is to determine that blood flow obstruction is caused by localized stenosis before surgery.[7] For percutaneous transluminal angioplasty and stent implantation, the following points should be noted during the operation: (1) Fully expand the stenotic vessels of the stent, and minimize residual stenosis. (2) There are many venous valves in the middle and distal femoral veins. When the stent is placed, it is easy to cause venous valve injury and cause sequelae of deep vein thrombosis. It is necessary to pay attention to it. Under normal circumstances, the end of the stent should not exceed the proximal end of the femoral vein. (3) The stent should not be connected to the joint as much as possible to prevent the joint from shifting. (4) The wall of the venous blood vessel is thin and the elasticity is poor. Once the pressure is applied, the collapse phenomenon is easy. For this, when the stent is selected, the diameter should be slightly larger than the diameter of the adjacent normal blood vessel, generally 2-3 mm. Ensure that the tension is appropriate and ensure that the blood vessels are unobstructed. (5) After placing the stent, slowly retract the catheter based on the perspective condition to prevent the position of the stent from changing.[8]

5. Conclusion

Through this research, the authors found that percutaneous transluminal angioplasty and stenting have a high application value in acute lower extremity deep venous thrombosis. For the acute phase of iliofemoral venous thrombosis, combined with percutaneous transluminal angioplasty and stenting, on the one hand, the meridian blood flow can be cleared as soon as possible, and the blood supply can be completely reconstructed. On the other hand, to eliminate the remaining problems of venous wall stenosis, to prevent increased pressure on the distal end of the stenotic blood vessels, slow blood flow to form new venous thrombosis, to prevent the formation of venous thrombosis. For high risk factors, certain measures can be taken to prevent deep vein thrombosis of the lower extremities. For example, preoperative and postoperative drug prevention, intraoperative operation, gentle movements, so as not to damage the intima, to avoid postoperative lower leg occipital to affect the deep venous return of the calf, encourage patients to carry out simple foot and toe active activities. Do more deep breathing, coughing movements, conditions to allow, early out of bed activities, if necessary, wear medical elastic stockings under the lower limbs, especially elderly patients, cancer, femur fractures, and postpartum women should pay attention.

In summary, percutaneous transluminal angioplasty and stenting for the treatment of acute deep venous thrombosis of the lower extremity, the effect is significant, it is recommended to promote the use.

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REVIEW

Latest Advances in the Treatment of Post-stroke Limb Spasm with Botulinum Neurotoxin

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ABSTRACT

Objective: To describe the latest progress in the use of botulinum neurotoxin for post-stroke limb spasm. Methods: This paper looks up the relevant research literatures in recent years in PubMed, Web of Science, Springer, Ovid, CNKI, WanFang databases and summarizes them. Results: The latest progress in the use of botulinum neurotoxin for post-stroke limb spasm was studied from the following aspects: the action mechanism of botulinum neurotoxin; efficacy evaluation; injection dose; target muscle selection; guiding technology; combination therapy. Conclusion: Botulinum neurotoxin is the first-line treatment for post-stroke limb spasm. We need to make continuous improvement and progress from the treatment period, injection dose, target muscle selection, guiding technology and efficacy evaluation to improve the quality of life of the majority of post-stroke survivors in China.

1. Introduction

Stroke is the leading cause of upper and lower limb spasm (the ratios are respectively about 66.2% and 62.1%), and the incidence of limb spasm in post-stroke survivors is 20-40%, which is a large part of the cause of disability.[1] Regarding the mechanism of post-stroke spasm, most researchers believe that excessive spinal cord excitement leads to spasm when the high-grade cortex is damaged. Some scholars have also suggested that the reticular spinal cord and the vestibular spinal cord mainly regulate excitation and inhibition. Under normal circumstances, it is in a state of equilibrium. When the nerve function is impaired, the balance is broken, and the cortex and spinal cord can be remodeled. The spasm is a milestone in the functional recovery process, since it will appear or disappear as the function recovers.[2] Post-stroke stroke Limb spasm can cause a serious decline in the quality of life of patients, increasing care and social and economic burden. For post-stroke spasm, treatment goals

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should aim to improve dysfunction, task performance, handling and self-care, reducing the burden of care and maintaining joint mobility, and avoiding skin complications. Botulinum neurotoxin (BoNT) is the focus of attention in the treatment of spasm in recent years. It began in 1989 when Das et al. successfully used botulinum neurotoxin for the treatment of spasm and myotonia after cerebral infarction. Subsequent randomized controlled trials have been reported. The results of recent studies on the use of BoNT for post-stroke spasm are summarized below.

2. The Pharmacology of Botulinum Neurotoxin

The botulinum neurotoxin consists of a 100kDa heavy (H) chain and a 50kDa light (L) chain linked by a disulfide bond, which mainly binds to cholinergic neuromuscular and blocks the endplate acetylcholine release causing paralysis. Botulinum neurotoxins are classified into seven types of A-G according to the serotype. The main types used in humans are type A and type B. There is no clear conversion formula between the dosage forms. Recently, the amino acid sequence of the new botulinum neurotoxin has been changed slightly but its activity and toxicity have changed greatly (study from rodents).[3] The botulinum neurotoxin currently used in clinical practice has the following three characteristics:

(1) Botulinum neurotoxins have a short duration and require repeated treatment every 3-4 months. In an attempt to solve this problem, researchers have extended their action time by using polyclonal antibodies to neural cell adhesion molecules.[4] And the botulinum neurotoxin is mixed with spheroidal chitosan,[5] although the above two studies are from animal experiments, it may provide some ideas for the development of botulinum neurotoxin stabilizer.

(2) Botulinum neurotoxin has the characteristic of being impossible to reverse, once the toxin has reached the cholinergic endplate, its activity cannot be reversed until it stops naturally.

(3) The botulinum neurotoxin only acts on the peripheral nerve muscles, and the existence of ultrastructural connections with the central nervous system has not been confirmed. Despite the findings of functional magnetic resonance studies, botulinum neurotoxins are used to treat post-stroke spasm, in terms of fMRI, the intensity of BOLD (blood oxygen level dependent) decreased significantly and increased cerebellar activity,[6,7] but the mechanism is unknown. Botulinum neurotoxin has been developed for 30 years, and its research field has gradually involved multiple disciplines. The power of function is obvious.

3. Post-stroke Upper Limb Spasm

The use of botulinum neurotoxin to treat post-stroke spasm is common in Europe and the United States. The investigation of the use of botulinum neurotoxin in Italy suggests that 80% of physicians use botulinum neurotoxin to treat adult spasm.[8] The 2016 US post-stroke spasm treatment guidelines also suggest that botulinum neurotoxin type A is effective in adult spasm (A grade evidence).[9] There are no relevant data reports in China. The main influencing factors of treatment outcome are as follows:

3.1 Treatment Period

The Early treatment is better. Fheodoroff et al.[10-12] found that the former was better in comparing the therapeutic effects in the two subacute and chronic phases. However, there are not many patients who actually get early treatment. Nalysnyk et al.[1] systematically reviewed 24 randomized controlled trials, 7 non-randomized, and 37 single-armed studies and found that the average time of upper limb spasm began to be treated after 46.9 months of onset, so there was a global delay in the treatment of post-stroke spasm.

3.2 Target Muscle Selection

The choice of muscle directly affects the therapeutic effect, and only the accurate positioning of the target muscle can be targeted. Nalysnyk et al.[1] conducted a systematic retrospective analysis to find the most frequently injected muscles: the radial flexor digitorum (64.0%) and the ulnar wrist flexor (59.1%), followed by the superficial flexor, the deep flexor, the biceps and the flexor hallucis longus. Spasm often causes severe joint deformities. Heikkila[13] boldly envisages the effect of intra-articular injection of botulinum neurotoxin, and studies the side effects of dogs as subjects. The results are not significantly different. Therefore, it is estimated that intra-articular injection of botulinum neurotoxin is not effective.

3.3 Dosage Requirement

If the dose is too small, the treatment is ineffective. If the dose is too large, it will cause toxic side effects. At present, there is still no research confirmed the safety of using the dosage range beyond the guideline. Nalysnyk et al.[1] obtained a systematic review of the average dose of upper limb spasm: shoulder muscles (40–100U), elbow flexors (41.1–95.4U), wrist flexors (23.8–72.8U), finger flexor (11.3–97.8U), forearm (5.0–33.3U), thumb muscle (18.9–30U), average total dose between 5-200U, it can be seen that there is a significant difference in the dose of each study. In order to determine whether dose differences affect treatment outcomes, Yablon et al.[14] included 7 randomized double-blind controlled studies

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for centralized data analysis: as measured by the greatest decrease in muscle tone (Ashworth drops by 1 point), the optimal dose for certain specific muscle groups: 22.5 U of the lateral flexor, 18.4 U of the ulnar flexor, 66.3 U of the superficial flexor, and 42.5 U of the deep flexor; there is a positive correlation between the dose effects within a certain range, and consistent with the results of Wissel et al.[15], Wissel will improve the total dose from 400U to 800U, better improvement in limb muscle tension and target achievement.

3.4 Efficacy Assessment

The most commonly used evaluation methods in clinical practice are MAS (Modified Ashworth Scale), SFS (Spasm Frequency Score), MTS (Modified Tardieu Scale), Brunnstrom, comprehensive spasm scale, and medial muscle tension scale. The assessment method is subjective, with a rough division, large individual differences, and lack of standardization. A large number of randomized controlled trials using MAS confirmed that BoNT can improve muscle tone, while decreased muscle tone only indicates improvement in passive function and does not explain the improvement in active function. Therefore, researchers began to use BI and FIM (Barthel Index and the Functional Independence Measure) to evaluate the efficacy, and the evaluation did not make a slight evaluation of the local function of the limb. For this reason, Fridman[16] designed the method of gripping the upper limbs. 8 patients with FIM scores of 126 were enrolled. The average total dose of 305 U (Botox) was injected by body surface markers and EMG guidance. The grasping speeds before and after injection were significantly different. However, there are shortcomings in the study that the control group is not established and the sample size is too small. The effect of the botulinum neurotoxin or the self-recovery of the patient itself cannot be judged. Therefore, the results need to be confirmed by more data, but this method is more detailed than BI and FIM evaluation, and it is worthy of clinical promotion. In addition, the researchers used the GAS (Goal attainment scaling) scoring initiative, a representative international, prospective; cohort study is the ULIS-II (the Upper Limb International Spasticity Study-II, ULIS-II for short), which was completed by 84 centers, 22 countries, and 355 patients, mainly referring to the long flexor as the target muscle, followed by the biceps and diaphragm, and the total dose range of BoNT-A (Dysport: 400-1900U; Botox: 50-500 U; Xeomin: 100-600 U), using EMG and electrical stimulation as a guiding method. During a follow-up period, the GAS score changed by an average of 17.6 (95% CI 16.4 to 18.8; $P < 0.001$),[17] Therefore, it is inferred that BoNT is beneficial to improve the active function. However, Fheodoroff et al.[18] subgroup analysis of the study found that the degree of hemiplegia, age, and post-stroke time all affected the GAS score, and each patient's target setting was inconsistent, so there are many defects in GAS evaluation. In order to make up for the above shortcomings, in the ULIS-III research plan (currently under study), a GAS-neous scale was designed to incorporate patient subjective feelings and look forward to relevant research results. Throughout the recent research on the upper limbs spasm after stroke, although the reduction of muscle tone after botulinum neurotoxin treatment makes the patient's movement more convenient and partially relieves the burden of care, however, the active function and muscle strength of the limbs have not been significantly improved. Maybe we need to find answers from the aspects of spasm mechanism, muscle positioning, dose selection, guiding technology, and evaluation methods.

4. Post-stroke Lower Limb Spasm

Compared with the post-stroke upper limb spasm, the study of post-stroke lower limb spasm is less, and the treatment is more challenging. The reasons are as follows:
(1) The lower limb muscles are deep and the range is large, so positioning is difficult.
(2) The dosage requirements are large, and the consideration of high dose safety is more than the treatment of the upper limbs.
(3) Lack of standardized evaluation methods: commonly used MAS, GAS and other scales, but subject to the evaluation of factors, and the degree of hemiplegia and post-stroke time, gait, pace measurement is subject to subjective factors, measurement is difficult.
(4) The improvement of active function is not clear: a multicenter randomized double-blind blank control study from Japan (120 patients in the group), the experimental group was injected with BoNTA300U in the ankle joint flexor, using MAS, gait mode, pace as an evaluation method, at 12 weeks of follow-up, the extent of MAS decline at 4, 6, and 8 weeks was significantly better in the test group than in the control group, while there was no significant difference in gait pattern and stride.[19] Another large multicenter, randomized, double-blind, blank-blind, controlled study (52 centers involved) selected 381 patients with limb spasm after half a year of stroke and set up 3 groups: Dysport1000U, 1500U and blank control group were injected. The treatment was repeated for 4 cycles and followed up for 1 year. It was found that a single treatment can only improve muscle tone, and the pace can be improved after one year of repeated treatment. Dashtipour et al.[20] confirmed the efficacy of muscle tension improve-

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ment by meta-analysis, consistent with the results of these two multicenter studies. Gupta et al.[21] also confirmed by meta-analysis that there was no significant difference in walking speed and quality of life before and after injection of botulinum neurotoxin in patients with lower limb spasm. Therefore, it can be seen that the improvement of muscle tension is clear, but the active function and improvement are not clear.

For this reason, the researchers looked for the cause from the dose and concentration. 104 patients with spasm were randomly divided into four groups. The target muscles were the posterior tibial muscle and the soleus muscle. Two different doses (200U and 400U) and two different concentrations (50U/mL or 100U/mL) of botulinum neurotoxin were injected, which was evaluated by MAS and 10m speed. The follow-up of 12 weeks showed the effect, and the dose of 400U and the concentration of 50U/mL group was better.[22] Kim[23] found the main medial calf muscle affecting pace by healthy human studies, so it may be desirable to use the pace to evaluate the efficacy of this intramuscular injection. Analysis of the above studies: the current inclusion of assessment methods are inconsistent, botulinum neurotoxin treatment of lower extremity spasm after stroke to improve muscle tension is clear, repeated treatment effect and safety is affirmed, active function improvement is not clear. We also need to find the reasons from the evaluation methods, dose and concentration, target muscle positioning methods.

5. The Treatment Safety Botulinum Neurotoxin

It is safe for post-stroke spasm treatment according to the FDA-approved drug dose range of onabotulinumtoxinA/incobotulinumtoxinA 600U and abobotulinumtoxinA 1500U.[24] Severe adverse events were reported from the case (myasthenia gravis, Lambert-Eaton syndrome, anterior horn dysfunction).[25,26] According to the data analysis, the probability of antibody production is very small: 0.5% (Botox), 0 (Abobotulinumtoxin A, Incobotulinumtoxin A, Rimabotulinumtoxin B).[27] To understand the high-dose effects and side effects, Santamato et al.[28] reviewed eight studies that found that treatments beyond the recommended dose of 300 U were more effective in treating spasm with no significant side effects. At present, there are no clear big data confirmations about the adverse effects beyond the scope of the guidelines. However, as can be seen from the above description, botulinum neurotoxin is highly safe, and the clinician does not have to worry too much about the adverse reaction as long as it is within the prescribed dosage range and skillful operation.

6. Guiding Methods

The existing guiding methods include sports endplate area, palpation, manual needle marking, electromyography, electrical stimulation, and ultrasound guidance. In theory, the endplate region is the best method, but it is difficult to accurately position the endplate region on the living body. There are few studies on the comparison of different guiding techniques. The muscles with clear body signs are usually palpated, manual needles or EMGs are quickly and accurately positioned. For deep muscles with difficult positioning (such as piriformis), ultrasound positioning is more accurate to reduce peripheral vascular damage. Electrical stimulation is suitable for thinner and superficial muscles, in order to compare the accuracy of different positioning methods, a prospective study of 81 patients compared manual needle localization, electrical stimulation localization, and ultrasound localization. It was found that electrical stimulation was more accurate for the lateral muscle of the gastrocnemius than for the medial muscle of the gastrocnemius. Because the lateral muscle is thinner than the medial muscle, ultrasound alignment was found to be the most accurate after comparison of the three methods.[29] Therefore, different positioning methods should be selected clinically according to different muscles.

7. Combination Therapy

Existing rehabilitation methods include electrical stimulation, magnetic stimulation, bioelectric stimulation, acupuncture, shock wave therapy, adhesives, splinting, exercise therapy, and robotic rehabilitation. In a randomized, single-blind, crossover study of electro-assisted exercise therapy, 11 patients who underwent severe stroke for the first half of the year participated in 90 minutes of follow-up and 18 weeks of follow-up. The results showed an increase in the number of upper limb activities in daily life.[30] Functional electrical stimulation improves the spasm state of the hemiplegic wrist and finger flexor.[31] Station-sitting alternating action combined with percutaneous electrical stimulation can improve the spasm state and balance function of the limb.[32] Studies have found that peripheral magnetic stimulation does not improve spasm status, but can improve sensory function.[33] Extracorporeal shock wave therapy can improve the spasm state of the plantar flexor digitorum after stroke and improve the passive movement of the ankle joint dorsiflexion.[34] Compared with the above methods, robotic rehabilitation combined with botulinum neurotoxin treatment can obtain better clinical efficacy [35,36], but the cost is too high and it is difficult to popularize. Splint fixation and adhesive combination with botulinum neurotoxin treatment can improve the symptoms of spasm. In contrast, the adhesive is even better. A single-blind randomized

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controlled trial enrolled 70 patients with upper extremity spasm after stroke. After injection of botulinum neurotoxin into the flexor digitorum of the wrist, they were divided into two groups, Group A and Group B. Group A was treated with adhesive and schedule muscles, and Group B was fixed with splint. Both groups were able to reduce the excessive activity of spasm, and the efficacy of Group A was better than that of Group B.[37] Exercise therapy is also effective, especially in children, a randomized controlled trial of perinatal stroke children showed that botulinum neurotoxin combined with early intensive leg function training improved walking ability.[38] In the use of botulinum neurotoxin, it is necessary to combine rehabilitation exercise training to maximize the treatment effect.

8. Challenges Ahead

Although botulinum neurotoxin is recommended as a first-line treatment for post-stroke spasm, there are still many difficulties for clinicians, mainly as follows:

8.1 The Prediction of Spasm

Whether it is based on the degree of hemiplegia and deep sensory disturbance, or using MAS, GAS score and robot evaluation, the subject and the tester are subjective and lack quantitative measurement methods, which ignore the patient's subjective perception to assess the extent of spasm. Zorowitz et al.[39] considered that a 13-item spasm screening scale could make up for the shortcomings.

8.2 Clinical Efficacy Evaluation

All clinical studies found that passive function improvement and active function improvement were not clear. Perhaps the mechanism of spasm and the mechanism of recovery of motor function are not the same.[40] Some questionnaires found that cold, fatigue, stress, and anxiety can increase the severity of spasm status,[41] but our assessment often ignores these contents.

8.3 Individualized Treatment

Patient satisfaction is gradually reduced with factors such as treatment cycle, peak incidence, and low-efficiency treatment, so the same patient needs individualized strategies at the beginning of treatment and during treatment,[42] however, the formulation of individualization is a very big problem.

8.4 Invalid Treatment Attribution

How to identify whether it is caused by antibody production, injection methods, interval time, disease outcomes, dosage forms, etc. and how to convert different dosage forms.[43]

9. Future Prospects

The number of strokes is still rising, and the number of disabled people is increasing year by year. In the face of post-stroke spasm, botulinum neurotoxin is a safe and effective local injection treatment, and should be carried out as soon as possible. Compared with foreign countries, China's use and understanding of botulinum neurotoxins is still insufficient, so there is still a large amount of clinical data to be explored. Based on reasonable spasm prediction and efficacy evaluation methods, a comprehensive treatment with botulinum neurotoxin as a core drug and functional physiotherapy can be established to bring more benefits to patients with post-stroke spasm disability.

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Some examples:

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- This result was later contradicted by Becker and Seligman[5].
- This effect has been widely studied[1-5,7].

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