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Blueberry Improves the Therapeutic Effect of Etanercept on Patients with Juvenile Idiopathic Arthritis: Phase III Study

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Juvenile idiopathic arthritis (JIA) is the most common arthritis in the adolescents under the age of 16. Etanercept, an inhibitor of tumor necrosis factor, is often used to treat JIA despite its significant side effects. Homeopathic remedies, such as blueberries, have anti-inflammatory properties with fewer unwanted effects and should be considered as a primary treatment. We aimed to explore the efficacy and safety of combination therapy of blueberry and etanercept for JIA. Two hundred and one JIA patients were selected, and randomly and evenly assigned to three groups: ETA (50 mg of etanercept twice weekly), ETABJ (matched etanercept and 50 ml blueberry juice daily) and ETAPJ (matched etanercept and placebo juice). The severity of JIA was measured using American College of Rheumatology scales (ACR) 20, 50 and 70. The levels of pro-inflammatory cytokines, interleukin-1 (IL1) alpha and IL1 beta, and interleukin-1 receptor antagonist (IL1RA) were measured by qRT-PCR and ELISA. After a 6-month follow-up, the ACR20, ACR50 and ACR70 in an ETABJ group were higher than those in other two groups ($P < 0.05$), suggesting clinically meaningful improvement in JIA. Meanwhile, the symptoms and side effects were reduced significantly or absent in an ETABJ group, including mental diseases, retrobulbar optic neuritis, gaining weight, infection, cutaneous vasculitis, diarrhea, uveitis and pancytopenia. Blueberries reduced the levels of IL1 alpha and beta, and increased the level of IL1RA. Thus, a combination therapy of blueberry and etanercept can reduce the severity of JIA and should be developed as a new method for JIA therapy.

Keywords: American College of Rheumatology scales; blueberry juice; etanercept; interleukin; juvenile idiopathic arthritis
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Introduction

Juvenile idiopathic arthritis (JIA) is one kind of autoimmune, non-infective and inflammatory joint diseases, and the duration is often more than three months in adolescent patients under 16 years of age (Hashkes and Laxer 2005). JIA affects approximately four in one thousand adolescents, with one in ten thousand having a more severe form (Foeldvari 2014). JIA may involve one or more joints and have a prolonged course, and result in considerable morbidity, including joint erosions and leg length discrepancy (Iesaka et al. 2006).

Medicine therapy is the main method for JIA treatment. Nonsteroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroid injections (IACIs) are mostly used medicine (Beukelman et al. 2008). However, the side effects of the medicine are significant (Brueggemann et al. 2010; Kawada et al. 2012; Foster et al. 2014; Mehta et al. 2015). Etanercept, an inhibitor of tumor necrosis factor, has been approved by Food and Drug Administration (FDA) to treat rheumatoid arthritis (RA), JIA and other arthritic diseases (Kerensky et al. 2012). However, etanercept can cause serious side effects: new infections (Chiang et al. 2014); nervous system problems including multiple sclerosis (Gomez-Gallego et al. 2008), seizures or eyes inflammation (Ozdemir et al. 2013; Saeed et al. 2014); blood problems (van Denderen et al. 2012); heart failure (Kerensky et al. 2012); allergic reactions (Kato et al. 2006); a lupus-like syndrome (Araki et al. 2011); and autoimmune hepatitis (Toulemonde et al. 2012). All the unwanted adverse effects limit its usage in JIA therapy.

Traditional Chinese medicine (TCM) has been practiced in China for thousands of years with a long-history clinical experience. Herbal products and acupuncture are the two most commonly used methods of TCM (Moudgil and Berman 2014). TCM is used in arthritis therapy based on traditional Chinese theories, the level of vital energy and other ancient criteria (Lu 2013). These oriental rules are quite different from that in the western system, such as the criteria of American College of

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Rheumatology (ACR). To improve the therapeutic results of TCM, it is necessary to integrate the parameters of the two systems for making full use of herbal treatment in arthritis patients.

The anti-inflammatory activities of many Chinese herbs have been proved in animal models of arthritis (Shin et al. 2003; Chang et al. 2005), as well as arthritis patients (Thomsen et al. 2005; Liu et al. 2007). However, there are still some problems for clinical trials of TCM because of the following reasons: a small population, complex backgrounds, no defined inclusion/exclusion criteria and inadequate statistical analysis so on. Thus, it is necessary to push the work forward using these criteria. Blueberry, a kind of small fruits with fewer side effects, may have perfect health effects on JIA due to its anti-inflammatory activities (Paulis et al. 2013; Johnson et al. 2013; Esposito et al. 2014). However, the role of blueberry for JIA therapy remains unknown, and the therapeutic effects are still needed to be determined. Here, we wanted to explore the possible molecular mechanisms for protective effects of blueberry on JIA by regulating the levels of inflammation-related genes, such as interleukin-1 (IL1) alpha, IL1beta and IL1 receptor antagonist (IL1RA).

Patients and Methods

Materials

Blueberry was purchased from Shenyang Shisheng blueberry company (Shenyang, China). Fresh blueberries were rinsed with cold, running water and drained on a fine mesh sieve or a colander lined with cheesecloth at room temperature. Fresh blueberry juice was prepared by homogenization. Blueberries were homogenized in a homogenizer (GYB60-65, Shanghai Donghua Co., Shanghai, China) with a speed at 60 l/h. Then, the blueberry juice was collected after centrifugation at 5,000 g for 10 min and stored at −20°C. Two liters of blueberry juice could be prepared from 3 kg of fresh blueberries.

Patients

Written, informed consents were obtained from all patients. A total of 450 males and females with JIA, under the age of 16 years, were selected if they met the criteria of ACR for RA for more than one year and were classified as Class I, II, or III (Verhoef et al. 2005). The three major types of JIA are oligoarticular JIA (OJIA), polyarticular JIA (PJIA) and systemic JIA (SJIA) (Kaalla et al. 2013).

The patients enrolled were categorized as the following criteria: OJIA, arthritis can affect from one to four joints for at least six months; PJIA, arthritis can affect five or more joints for at least six months; SJIA, arthritis develops for more than two weeks because of daily fever, which is greater than 39°C and returns to less than 37°C during the episodes of fever; and enthesitis-related arthritis (EJIA) that occurs at the attachment of a tendon or ligament to a bone, causing pain in the joints of lumbosacral spine and/or sacroiliac joints. All patients received non-steroidal anti-inflammatory drugs, corticosteroids (< 10 mg/day) for at least one month before randomization.

The following excluding criteria were considered during the recruitment: the JIA patients were diagnosed with severe diseases, such as cardiovascular, lung, liver, kidney, mental and blood disorders; the JIA patients had the following reasons, serious adverse events, a concomitant medication which was not permitted by the protocol, uncontrolled systemic symptoms, and familial and social situations interfering medical assessment.

Within half a month prior to randomization, all patients had a complete history including joint functions, biochemical functions and 18 symptoms. All these parameters were repeated once every 2 weeks in the study. The 18 symptoms include six joint-related symptoms: joint pain, hot or cold joint, joint tenderness, swollen and stiffness; and 12 non-joint related symptoms: thirst, nocturia, numb or cold and or heavy limbs, fatigue, vexation, fever, sore waist, dizziness, intolerant to cold, yellow and turbid urine. All the symptoms were scored as none, slight, medial, and severe in the number of 0, 1, 2 and 3 respectively.

Study design

After screening all participants with including and excluding criteria, 201 patients were selected, randomly and evenly assigned in three groups: ETA (receiving 50 mg of etanercept twice weekly, etanercept was purchased from Amgen, Thousand Oaks, USA) (Hooper et al. 2013) as a control group, ETABJ (receiving 50 mg of etanercept twice weekly, 50 ml blueberry juice daily) as a blueberry plus group and ETAPJ (matched etanercept and placebo juice) as a blueberry minus group. Since fifty ml blueberry juice was used for each patient (more than 50 kg) in an experimental group daily, the concentration of used blueberry juice was less than 0.1% (v/w). The duration of the whole follow-up was 6 months. All the protocols were approved by the Ethnical Committee of China-Japan Union Hospital of Jilin University. The study was performed based on the guidelines of the Declaration of Helsinki (Stockhausen 2000).

Efficacy evaluation

Outcome was measured by ACR20, ACR50 and ACR70 (The American College of Rheumatology Criteria) to assess the efficacy of each group before and after a 6-month follow-up. The improvement of JIA was defined using ACR20, ACR50 or ACR70 to reflect the 20%, 50%, or 70% improving levels in these parameters, which include patient and physician assessment, erythrocyte sedimentation rate (ESR), pain scale and health assessment questionnaire (HAQ). The efficacy was evaluated by intramural judgment.

Safety analysis

The safety of different methods was measured by adverse events, clinical chemical measurements and vital signs. The safety was assessed at a baseline and at every 2 weeks during the treatment. Safety analyses included all randomized patients who received more than one dose of study medication and underwent more than one safety assessment after baseline.

The adverse events were investigated among three groups. According to a previous report, the following main side effects were assessed: mental diseases, retrobulbar optic neuritis, gaining weight, infection, cutaneous vasculitis, diarrhea, uveitis and pancytopenia. All the symptoms were diagnosed by the experts from China-Japan Union Hospital of Jilin University.

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The discoveries of tumor-derived RNA in the serum offer a noninvasive way for investigating gene expression in patients (Tsui et

al. 2002). Furthermore, qRT-PCR makes serum RNA detection assays more sensitive. Thus, the serum RNA was measured by qRT-PCR since RNA was more reliable than DNA in blood for reflecting the changing expression patterns of target genes. Five ml of blood samples were withdrawn from forearm. Serum was collected by centrifugation at 1,000 *g* for 5 min. Total RNA was extracted by miRNeasy kit (#217004, Qiagen, CA, USA). The RNA was reversely transcripted into cDNA by M-MuLV reverse transcriptase and oligo (dT) primers. The SYBR Green DNA PCR kit (#0804104, Applied Biosystems, Foster City, CA, USA) was used for qRT-PCR analysis. PCR primers were synthesized as follows: IL1 alpha (GenBank No: NM_000575.3), F: 5′-accgtgattctaagaatctc-3′ (positions 541 to 560) and R: 5′-gttggtctcactacctgtg-3′ (641 to 660), with the PCR product of 120 bp from two exons; IL1 beta (GenBank No. NM_000576.2), F: 5′-aagaatctgtacctgtcctg-3′ (541 to 560) and R: 5′-tatcttgttgaagac aaatc-3′ (641 to 660), with the PCR product of 120 bp from two exons; IL1RA (GenBank No. XM_005263661.3), F: 5′-tcttgg gaatccatggaggg-3′ (143 to 162) and R: 5′-tttctgttctcgctcaggtc-3′ (246 to 265), with the PCR product of 120 bp from two exons; GAPDH (GenBank No. NM_001289746.1), F: 5′-gatccctccaaaatcaagtg-3′ (241 to 260) and R: 5′-tcagcagaggggcagagatg-3′ (361 to 380), with the PCR product of 140 bp from two exons. The mRNA levels were calculated as relative increases compared to GAPDH sets as 100%.

ELISA analysis

Serum levels of IL1 alpha, IL1 beta and IL1RA were test using the following kits: Human IL1 alpha ELISA Kit (ab46028), Human IL1 beta ELISA Kit (ab46052) and Human IL1RA ELISA Kit (ab100565) (Abcam Trading (Shanghai) Company Ltd., Shanghai, China).

Statistical analysis

All data were analyzed using SPSS20.0 software (SPSS, Inc., Chicago, Illinois, USA). The efficacy of therapies was tested using a standard Per-Protocol method. The proportions of ACR20, ACR50 and ACR70 responses and adverse effects in three different groups, were examined by the Chi square analysis. The improvement on symptoms was measured using the scores before the therapy minus the scores after the therapy. The improvements of symptoms were analyzed by Mann-Whitney U analysis with a non-parameter test.

Results

Baseline characteristics of all subjects

A total of 450 subjects who underwent randomization, 180 patients were excluded for interfering the inclusion criteria, and 69 patients were excluded for refusing to assign the agreement. Finally, 201 were included in the study, and evenly assigned into ETA, ETABJ and ETAPJ groups. Baseline characteristics were similar among the three groups for all the parameters (Table 1). OJIA, PJIA, SJIA, and EJIA were about 50%, 30%, 10%, and 10% of all JIA cases (Table 1). There was no significant difference among three treatment groups ($P < 0.05$).

ACR20, ACR50 and ACR70 measurement

In the 6-month follow-up, in ETA group, 10 patients dropped out because of serious side effects; In ETABJ and ETAPJ groups, 2 and 4 patients dropped out because of discontinuation. The ACR evaluation was shown in Table 2. The rate of withdrawal from therapy was the highest in ETA group and the lowest in ETABJ group. The elevated levels of alanine aminotransferase and other unwanted adverse such as rash, infection, short breathing, headache, palpitation, insomnia, allergic action and back pain, were higher in

Table 1. Baseline characteristics of the participants.

Characteristics	ETA	ETAPJ	ETABJ	P value
Male/Female	26/41	27/40	28/39	0.75[a]
Age (year)	13.33 ± 3.12	13.83 ± 3.49	13.24 ± 3.20	0.92[a]
Cases of JIA classification				
OJIA (% of all cases of JIA)	33 (49.25)	34 (50.75)	32 (47.76)	0.87[a]
PJIA (% of all cases of JIA)	21 (31.34)	20 (29.85)	22 (32.84)	0.85[a]
SJIA (% of all cases of JIA)	7 (10.45)	6 (8.96)	6 (8.96)	0.77[a]
EJIA (% of all cases of JIA)	6 (8.96)	7 (10.45)	6 (8.96)	0.77[a]
Duration (months)	2.69 ± 1.58	2.99 ± 1.46	2.76 ± 1.53	0.54[a]
Patients receiving NSAIDs (%)	27 (40.3)	28 (41.8)	29 (43.3)	0.65[a]
Patients receiving corticosteroids (%)	8 (11.9)	9 (13.4)	8 (11.9)	0.43[a]
RF positive No. (%)	195 (68.7)	49 (73.1)	47 (70.1)	0.53[a]
Rest pain (0-100 mm ruler)	43.17 ± 19.06	42.18 ± 20.01	45.22 ± 18.56	0.78[a]
Morning stiffness (min)	59.61 ± 80.12	61.23 ± 43.65	69.61 ± 59.09	0.28[a]
Hands grasp force (mmHg)	55.17 ± 42.45	56.72 ± 42.36	55.37 ± 41.34	0.91[a]
Walking time in 20 meters (s)	21.97 ± 20.56	22.81 ± 23.02	23.97 ± 21.16	0.86[a]
Patient global assessment (0-100 mm ruler)	64.76 ± 19.01	65.28 ± 19.12	64.55 ± 18.35	0.95[a]
Physician global assessment (HAQ form)	65.21 ± 18.77	66.67 ± 18.25	65.43 ± 18.12	0.92[a]

OJIA, oligoarticular JIA; PJIA, polyarticular JIA; SJIA, systemic-onset JIA; EJIA, enthesitis-related arthritis. [a]$P > 0.05$ among three groups.

Table 2. ACR20, ACR50 and ACR70 among three groups after 6-month follow up.

ACR	ETA effective No. (%)	ETAPJ effective No. (%)	ETABJ effective No. (%)	P-value
ACR20	31 (54.4)	32 (50.8)	49 (75.4)	0.001[a]
ACR50	20 (35.1)	22 (34.9)	34 (52.3)	0.001[a]
ACR70	26 (45.6)	28 (44.4)	30 (45.4)	0.001[a]

[a]$P < 0.05$ after the treatment for 6 months.

Table 3. Symptom improvements in ACR20 responded cases.

	ETA			ETAPJ			ETABJ		
	Non-effective	Effective	P-value	Non-effective	Effective	P-value	Non-effective	Effective	P-value
Joint pain[a]	57.50	23.47	0.001	48.92	26.84	0.001	27.01	38.15	0.001
Swollen joint[a,b]	37.42	13.49	0.001	33.19	12.95	0.001	20.70	38.44	0.001
Joint tenderness[a,b]	46.88	23.53	0.001	41.74	23.76	0.001	16.29	38.82	0.001
Vexation[a]	38.50	21.25	0.001	36.10	21.33	0.024	20.57	38.79	0.011
Cold joint[a]	38.05	13.21	0.001	34.88	12.01	0.002	20.10	39.30	0.038
Fatigue[a]	38.29	13.34	0.001	35.52	11.65	0.007	20.59	36.69	0.001
Intolerant to cold[a,b]	38.38	13.27	0.001	35.77	11.51	0.013	21.42	35.92	0.009
Cold limbs[a,b]	38.22	13.3	0.001	35.34	31.75	0.003	21.07	36.25	0.003
Nocturia[b]	39.08	13.2	0.001	37.66	10.45	0.226	20.19	35.21	0.039
Numb limbs[b]	39.68	13.18	0.001	39.26	19.56	0.892	20.43	34.99	0.048
Heavy limbs[b]	39.48	13.35	0.001	38.71	39.87	0.621	22.39	36.87	0.001
Sore waist[b]	39.8	13.19	0.001	39.59	19.38	0.928	20.28	35.13	0.047
Yellow[b] and turbid urine[c]	39.8	13.15	0.001	39.59	19.38	0.915	20.85	34.60	0.067
Thirst[b]	39.66	13.17	0.001	39.22	19.58	0.872	20.60	34.84	0.078
Dizziness[b]	39.14	12.91	0.001	37.80	10.38	0.225	23.68	31.98	0.460
Fever[b]	38.87	13.12	0.001	37.08	10.78	0.110	21.10	34.37	0.172
Hot joint[b]	39.36	13.03	0.001	38.40	10.04	0.295	22.22	33.33	0.416

The data are mean rank, and higher mean rank represents more improvement on the symptom.
[a]Symptoms improved both in all groups.
[b]Symptoms showed tendency of improvement in ETABJ group ($P < 0.05$).

ETA group compared with a group with blueberry. No severe adverse event was found in ETABJ group.

Symptom improvement in ACR20 responders in a blueberry group

Table 3 showed the improvement for joint-related symptoms such as pain, tenderness, swollen and stiffness, and non-joint-related symptoms such as vexation, cold joint, fatigue and intolerance with cold in ETABJ group compared with other groups ($P < 0.05$). Meanwhile, more improvement was also found for the non-joint-related symptoms such as numb limbs, heavy limbs, sore waist, and nocturia in ETABJ group compared with other groups ($P < 0.05$).

Blueberry consumption reduces the side effects caused by etanercept

We proposed that blueberry consumption reduced the side effects caused by etanercept with its anti-oxidant and anti-inflammation activities (Coban et al. 2014; Esposito et al. 2014). Thus, the side effects were investigated with the combination therapy of etanercept and blueberry. The results showed that blueberry could reduce most side effects (mental diseases, retrobulbar optic neuritis, gaining weight, infection, cutaneous vasculitis, diarrhea, uveitis, pancytopenia, Asthenia, anorexia, thoracic pain and Chronic cough) caused by etanercept while most of the side effects could not be observed before the therapy (Table 4).

mRNA levels of inflammation-related genes in JIA patients

To keep RNA integrity, the time between RNA isolation and qRT-PCR was about 30 min. No significant ($P > 0.05$) difference was observed the expression levels of IL1 alpha, IL1 beta and IL1RA mRNAs in all groups before the study (Fig. 1). No significant ($P > 0.05$) difference was observed in these mRNA levels in ETA group before and after a 6-month follow-up (Fig. 1). Blueberry diet consumption had a significant effect on the mRNA expression

Table 4. Main side effects in patients with juvenile idiopathic arthritis (JIA).

Symptoms (% of all cases of JIA)	ETA (57 cases)	ETAPJ (63 cases)	ETABJ (65 cases)	P values
Mental diseases	6 (10.53)	7 (11.11)	1 (1.54)	$P < 0.05$
Retrobulbar optic neuritis	5 (8.77)	6 (9.52)	1 (1.54)	$P < 0.05$
Weight gain	4 (7.02)	4 (6.35)	0 (0)	$P < 0.05$
Infection	8 (14.04)	9 (14.29)	0 (0)	$P < 0.05$
Cutaneous vasculitis	10 (17.54)	12 (19.05)	1 (1.54)	$P < 0.05$
Diarrhea	6 (10.53)	6 (9.52)	1 (1.54)	$P < 0.05$
Uveitis	6 (10.53)	7 (11.11)	1 (1.54)	$P < 0.05$
Pancytopenia	3 (5.26)	4 (6.35)	1 (1.54)	$P < 0.05$
Asthenia, anorexia	3 (5.26)	4 (6.35)	1 (1.54)	$P < 0.05$
Thoracic pain	5 (8.77)	6 (9.52)	0 (0)	$P < 0.05$
Chronic cough	1 (1.75)	2 (3.17)	0 (0)	$P < 0.05$

There are significant differences via ETABJ group if $P < 0.05$.

of IL1 alpha, IL1 beta and IL1RA compared with those from other groups. A significant increase in the mRNA levels of IL1RA could be observed in a blueberry consumption group ($P < 0.05$) (Fig. 1C). The mRNA levels of IL1RA reached the highest level in ETABJ group compared with other groups (Fig. 1C). In contrast, the mRNA of IL1 alpha and beta reached the lowest level in ETABJ group (Fig. 1A, B). All the results suggest that certain concentrations of blueberry can promote the secretion of IL1RA and also inhibit the levels of IL1 alpha and IL1 beta.

Protein levels of inflammation-related cytokines in JIA patients

Just as mRNA level of the cytokines, the serum protein levels of three cytokines showed the same changing trend after adding blueberry juice. No significant ($P > 0.05$) difference was observed for serum protein levels of all cytokines in all groups before the study (Fig. 2). No significant ($P > 0.05$) difference was observed for protein levels of all cytokines in ETA group before and after a 6-month follow-up either (Fig. 2). Blueberry diet consumption had a significant effect on the protein expression of IL1 alpha, beta and IL1RA compared with those from other groups. A significant increase in the protein levels of IL1RA could be observed in a blueberry consumption group ($P < 0.05$) (Fig. 2C). The protein levels of IL1RA reached the highest level in ETABJ group compared with other groups (Fig. 2C). In contrast, the protein levels of IL1 alpha and IL1 beta reached the lowest level in ETABJ group (Fig. 2A, B). All the results suggest that certain concentrations of blueberry can promote the secretion of IL1RA while the blueberry can also inhibit the levels of IL1 alpha and IL1 beta.

Discussion

Since the introduction of biologic therapies, the pharmacological treatment approaches for RA have changed substantially. The efficacy of combination therapy with a biological product is superior to the monotherapy depend on a kind of medicine (Kuriya et al. 2010). A combination therapy has a greater initial effect on clinical remission than monotherapy. A combination therapy has been widely reported for JIA treatment (Tynjala et al. 2011; Record et al. 2011; Ramanan et al. 2014), but no combination therapy with a biological product has been reported for the therapy of JIA yet. Oxidative stress was increased in JIA patients (Breda et al. 2013; Lipinska et al. 2015). On the other hand, JIA is the most common disease in pediatric rheumatology and characterized by chronically joint destruction and inflammation (Koos et al. 2013; Abramowicz et al. 2013). Fortunately, anti-oxidant and anti-inflammation properties of blueberry have been widely reported with fewer side effects (Kraujalyte et al. 2015; Chiabrando and Giacalone 2015; Diaconeasa et al. 2015; Kang et al. 2015). Thus, blueberry therapy was chosen a kind of functional food to be combined with etanercept for JIA treatment in the study.

According to TCM theory, the therapy of RA is often performed based on syndrome differentiation of cases and individual therapy is more likely to be considered. The therapeutic procedures used in this study were based on the recommendations by professional Chinese medical doctors. In our study, ACR20, ACR50 and ACR70 response were higher in a group in combination with blueberry compared with other groups after a 6-month follow up, suggesting there was significantly clinical improvement for JIA. Meanwhile, more improvement on the non-joint-related symptoms (Table 3) and fewer adverse events were observed in a therapy combined with blueberry (Table 4).

Here, a total of 18 symptoms in RA were classified into four kinds of combined factors (symptom combinations). The classification of symptom combinations was similar to the categories differentiated with theory of TCM in RA patients. RA was divided into four patterns: cold, hot, mixed cold and hot, and deficient RAs (Liu 1986). The results suggest that the clinical experience based on TCM has the identical classification as those from clinical statistic data. Furthermore, this suggests that all symptoms in RA are taken into consideration during the diagnostic clas-

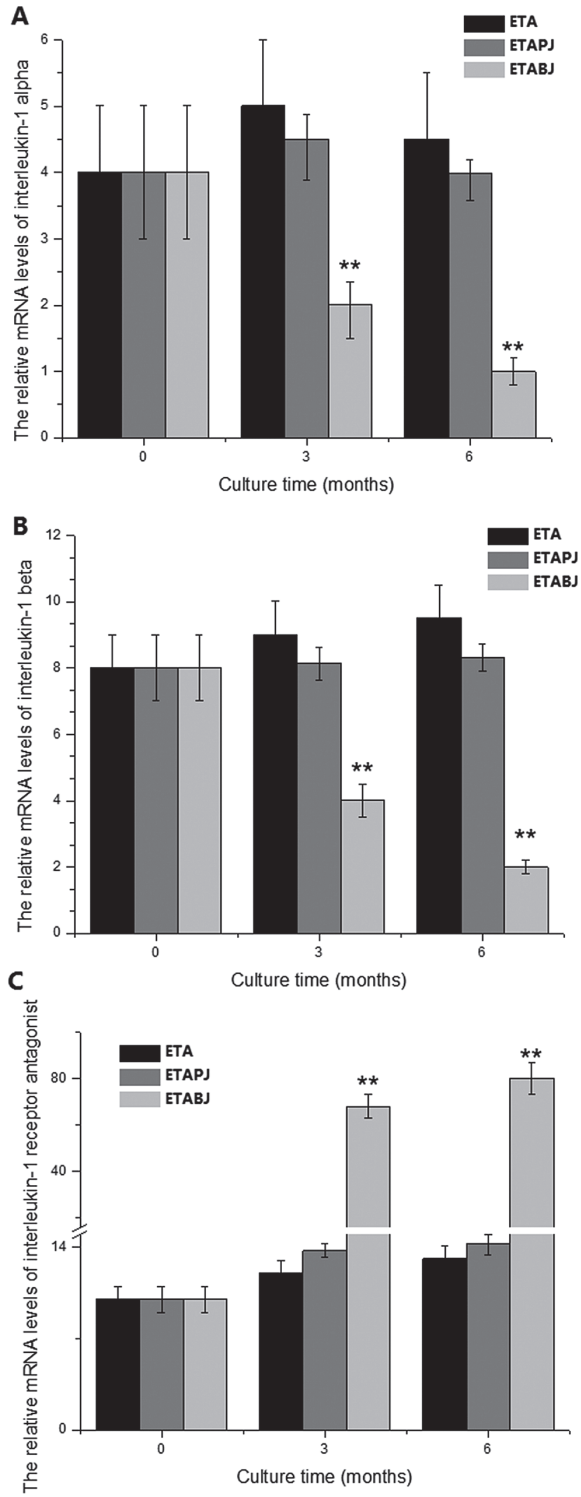

Fig. 1. mRNA levels of inflammation-related genes in JIA patients.
A. The relative mRNA levels of IL1 alpha in ETA, ETAPJ and ETABJ groups. B. The relative mRNA levels of IL1 beta in ETA, ETAPJ and ETABJ groups. C. The relative mRNA levels of IL1RA in ETA, ETAPJ and ETABJ groups. The mRNA levels of IL1 alpha, IL1 beta and IL1RA were normalized to GAPDH expression and expressed as relative concentrations in arbitrary units. Values were presented as means ± standard error (S.E.). **P < 0.01 vs. ETA group.

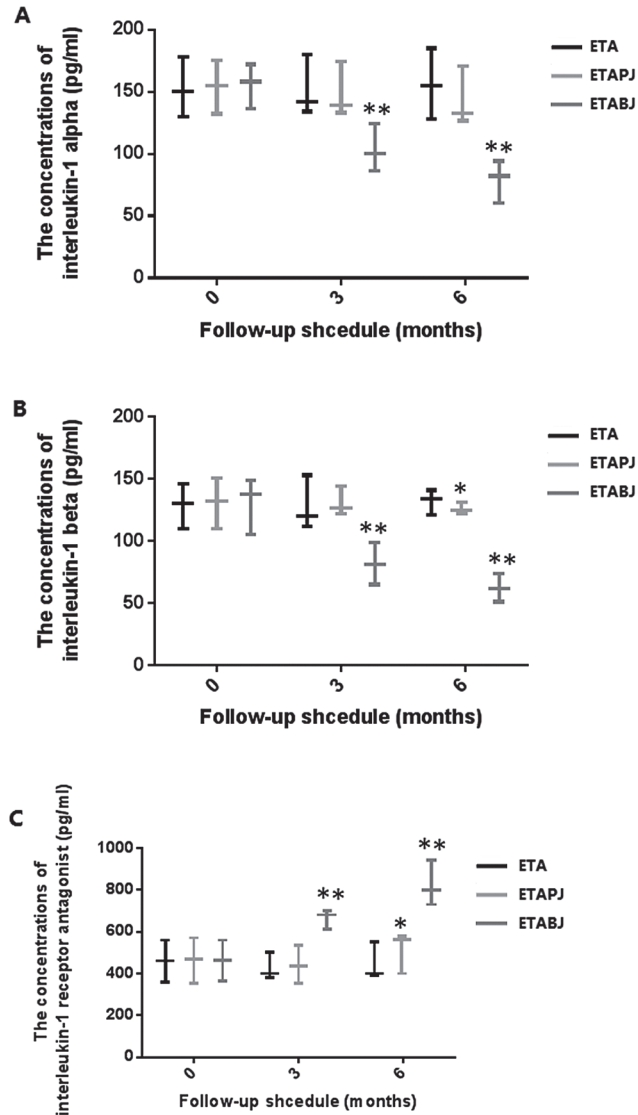

Fig. 2. Serum levels of inflammation-related genes in JIA patients.
A. The relative protein levels of IL1 alpha in ETA, ETAPJ and ETABJ groups. B. The relative protein levels of IL1 beta in ETA, ETAPJ and ETABJ groups. C. The relative protein levels of IL1RA in ETA, ETAPJ and ETABJ groups. Values were presented as three bars. The highest values were shown as an up bar. The lowest values were shown as a down bar. The average values were shown as a middle bar. *P < 0.05 vs. ETA group, **P < 0.01 vs. ETA group.

sification and more clinical investigations will be needed.

Though more non-joint related symptoms improved in a blueberry group, it was still worthy of analyzing the relation between ACR20 response and all the combinations of symptoms. Our results indicated that the loading values of the factors in effective cases (ACR20) had the tendency to be lower than those from the factors in non-responders. The loading values of symptom combination one in responders were much lower than those in non-responders. The results suggested that the ACR20 response evaluation

was used mainly to judge the improvement on the symptom combination one, which was related with all joint symptoms.

Etanercept has been approved very effective for the therapy of JIA (Horneff et al. 2015). However, etanercept therapy is often associated with significant side effects. Uveitis is the main extra-articular manifestation affecting the life quality of JIA patients. The patients, with a history of uveitis, will have higher uveitis risk after etanercept treatment (Foeldvari et al. 2015). The association between etanercept therapy and increased rate of infection has also been reported in JIA patients (Davies et al. 2015). Due to its multiple immune regulatory functions, etanercept can induce inflammation in many organs, such as eye, skin, and gastrointestinal tract (Kakkassery et al. 2010). All these side effects were reduced or avoided after consuming blueberry. Blueberries prevent urinary tract infections with the following reasons: specific compounds, proanthocyanidins, were found in blueberries (Rodriguez-Mateos et al. 2012). Proanthocyanidins prevent bacteria from sticking to the walls of the gastrointestinal tract. Blueberries can inhibit the growth of bacteria with large amounts of vitamin C (Sanchez-Moreno et al. 2008). Anthocyanins, extracted from blueberries, can inhibit trinitrobenzene-sulfonic-acid-induced colitis in mice. Furthermore, dietary blueberry consumption can lower the risk of inflammatory bowel disease (Wu et al. 2011). Blueberries are edible fruits and have been widely reported for their potential health benefits. Vaccinium corymbosum, isolated from blueberries, shows anti-inflammatory and antinociceptive activities. Thus, its consumption will be beneficial for the therapy of inflammatory diseases (Torri et al. 2007).

To understand the molecular mechanism for the functions of blueberries on JIA, the levels of pro-inflammatory cytokines IL1 alpha, IL1 beta and IL1RA were measured. The result showed that Blueberry improved the therapeutic effect of etanercept on the patients with juvenile idiopathic arthritis by reducing the levels of IL1 alpha and beta, and reducing the levels of IL1RA. However, the molecular mechanism is not only limited to the cytokines interleukins. For an example, nuclear factor-kappaB (NF-kappaB), an important the transcription factor, can be activated by oxidative stress and pro-inflammatory stimuli. NF-kappaB controls the expression of many genes related with the inflammatory responses. A previous job shows that the anthocyanins isolated from bilberries and black currants, can prevent and treat chronic inflammatory diseases by inhibiting NF-kappaB transactivation and decreasing the concentrations of pro-inflammatory chemokines and cytokines (Karlsen et al. 2007). On the other hand, blueberries have been already successfully used (combination therapy) in other chronic inflammatory diseases, such as Peyronie's disease (PD) (Paulis et al. 2012, 2013): a long-term combination therapy with blueberries is the best treatment for PD.

Certainly, one important issue should be considered here. The present text suggests that RNA was prepared from serum samples. If so, the rationale for the use of serum RNA is still unknown. Although RNA has not been a strong biomarker candidate because of its instability, RNA in serum is an emerging field for noninvasive diagnosis. The discoveries of tumor-derived RNA in the serum of patients have opened a whole novel field for investigating gene expression as a noninvasive way (Tsui et al. 2002). Real-time quantitative reverse transcription-PCR (qRT-PCR) makes serum RNA detection assays more sensitive. The serum RNA is a remarkable finding because it is more labile than DNA in blood. Presently, the exact mechanisms that protect circulating RNA remain unclear. Theoretically, to keep RNA integrity, the time between RNA isolation and qRT-PCR should be reduced to the minimum as soon as possible. Some methods, such as snap-freezing of serum or adding a stabilizing reagent, should be considered to reduce the chances of RNA degradation.

Certainly, there are some limitations for present study. Firstly, JIA was considered in a small population. Secondly, the blueberry juice can be absorbed slowly via digestive system, shows a better therapeutic result and produces fewer side effects. However, the detail molecular mechanism for its functions remains unclear; Finally, double blind study was not performed here because of two different ways used (injection taken for etanercept and oral taken for blueberry juice).

In sum, more ACR20, ACR50 and ACR70 responsiveness, and more improvement on symptoms and fewer adverse events are observed in blueberry treatment combined with ETA. Blueberry can ameliorate the symptoms of JIA patients by reducing the levels of inflammation-related genes, IL1 alpha and beta, and increasing the level of IL1RA. Meanwhile, blueberry can reduce the side effects caused by etanercept. Thus, the combination therapy of etanercept and blueberry is a potential method for the therapy of JIA.

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Conflict of Interest

The authors declare no conflict of interest.

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