Scientific Research

Ratanasampil (Tibetan Medicine, RNSP) Reduces β-Amyloid Protein (Aβ) and Pro-Inflammatory Factor Levels and Improves Cognitive Functions in Mild-to-Moderate Alzheimer's Disease (AD) Patients Living at High Altitude

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ABSTRACT

Ratanasampil (RNSP) is a traditional Tibetan medicine used for the treatment of stroke and cerebrovascular diseases. Previous discoveries that RNSP can reduce β-amyloid protein levels and increase learning and memory in Alzheimer's mouse models (Tg2576) led us to investigate whether RNSP can improve cognitive functions in Alzheimer's patients. In this study, 146 AD patients living in Qinghai province received either one gram or 0.33 gram daily of RNSP for 16 weeks. Placebo patients received Piracetam. Serum $A\beta_{40}$ and $A\beta_{42}$ levels were measured at the beginning of the study and after 4 and 16 weeks of treatment. Compared to the same group before treatment, MMSE scores, ADAS-cog scores and ADL scores were significantly improved ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively) in patients after 16-week treatment with high-dose RNSP. No significant differences were observed in either the low-dose RNSP or placebo groups ($p > 0.05$, $p > 0.05$). After 16-week treatment, serum TNF-α, IL-1β, IL-6 and $A\beta_{42}$ levels were significantly decreased ($p < 0.01$) in the high-dose RNSP group, whereas no significant differences were found in the low-dose and placebo groups. The $A\beta_{42}/A\beta_{40}$ ratio was significantly decreased after 4-week and 16-week treatment in the high-dose RNSP group ($p < 0.05$, $p < 0.01$). Furthermore, serum $A\beta_{42}$ concentrations had a strong positive correlation with TNF-α, IL-1β and IL-6 levels. There were no observable adverse effects in either treatment or control groups. We conclude that further clinical trials of RNSP in Alzheimer disease are warranted.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease commonly seen in the elderly population of developed countries [1,2] as well as in China [3]. The precise mechanisms that cause AD are still unknown. Multiple pathways are known to contribute to the cognitive deficits found in AD by disruption of neuronal signalling involved in learning and memory. These pathways are altered by β-amyloid peptide (Aβ) production and accumulation, aberrant tau phosphorylation, inflammation, oxidative damage, and dysregulation of neurontransmitters and receptors [4]. Many efforts have been made to develop pathway-specific interventions to block causal events in AD pathogenesis and to improve cognitive function in AD patients.

Various studies have shown that immune-mediated inflammatory responses are closely associated with AD pathogenesis [5]. Overproduction of pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α), inter-leukin-1β (IL-1β) and IL-6, triggers pro-inflammatory responses and promotes neuronal cell damage and excessive Aβ deposition. Both Aβ and pro-inflammatory factors are activators of neurotoxic pathways that lead to brain cell dysfunctions and death [6-8]. Therefore, anti-inflammatory drugs may help to improve brain function in patients with Alzheimer's disease. Several epidemiological studies have shown that patients on long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) have a lower risk of developing AD than others of the same age group [9].

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Traditional Chinese Medicine (TCM) have been used for the treatment of dementia and reportedly improve cognitive function and capabilities of daily living [10]. *Ratanasampil* (RNSP), which means *seventy-taste-pearl-balls* in the Tibetan language has been used for the treatment of stroke and cerebrovascular diseases. Our previous studies showed that RNSP reduced β-amyloid protein levels and increased learning and memory in Alzheimer's mouse models (Tg2576) [11,12]. Here, we report that RNSP improved cognitive functions in mild-to-moderate Alzheimer's patients living at high altitude. Furthermore, we found that RNSP also decreased serum $A\beta_{42}$ concentration and therefore the $A\beta_{42}/A\beta_{40}$ ratio, and reduced the level of several pro-inflammatory factors, including TNF-α, IL-1β and IL-6. These data suggest that RNSP could be a new therapeutic drug for the treatment and prevention of AD.

2. Subjects and Methods

2.1. Subjects

146 patients with mild-to-moderate AD living in Xining and its contiguous areas (at altitudes of 2000 - 3200 meters) in the Province of Qinghai, were recruited for this study during the period of January 2005 to December 2007. Out of 146 patients, 5 patients were lost to follow-up and 1 patient died of pneumonia during the treatment. Therefore 140 patients completed the 16-week of treatment.

The study took place at the Institution of Geriatric of Qinghai Provincial Hospital. These patients were divided into three groups: high-dose RNSP treatment (1 g/day), low-dose RNSP treatment (0.33 g/day) and controls. All three groups were carefully selected to be comparable with respect to age, gender, handedness, level of education, MMSE score, and baseline functioning. Enrolled patients were required to have stable medical conditions for at least 3 months prior to the study and to have an absence of systemic disorders that might confound a diagnosis of AD.

2.2. Eligibility Requirements for Participants

The diagnosis of mild-to-moderate AD was made by the employing "Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)"criteria and the "National Institute of Neurological Disorders and Stroke/ Alzheimer's Disease and Related Disorders Association (NINDS/ADRDA)" criteria [13]. Briefly, a mini-mental state examination (MMSE) score between 10 and 24 points; a Hachinski ischemia score $\leq$ 4 points; and a Hamilton depression scale score (HAMD) < 7 points was used to diagnose mild-to-moderate AD. Brain CT scans and MRIs showed temporal lobe atrophy, a smaller hippocampus, ventricular dilatation, cortical atrophy and white matter lesions.

2.2.1. Exclusion Criteria for This Study Included

1) Vascular dementia or other physical or chemical factors causing dementia such as intracranial lesions.

2) Serious heart, lung, liver, kidney, blood diseases, or other serious mental illness.

3) Any critical condition, because it is difficult to evaluate accurately drug efficacy and safety.

4) Taking mental performance enhancement drugs (smart drugs, central nervous system stimulants), chronic non-steroidal anti-inflammatory drugs or hormone supplements within 4 weeks of initial treatment.

Patients or their guardians agreed to participate in this study and signed informed consent forms. The protocol was approved by the Qinghai Medical Ethics Committee.

2.3. Study Design

2.3.1. A Randomized, Double-Blind, Controlled Trial of Drug Design

The high-dose treatment group was given a single oral dose of 1 g of (RNSP every day Qinghai Jinke Tibetan Medicine Pharmaceutical Co. LTD, Zhunzi Z63020062). The low-dose treatment group received a single oral dose of 0.33 g/day of RNSP. The control group was treated with a single 24 mg dose of piracetam per day (WuXi KaiXi Pharmaceutical Co., Ltd.).

RNSP and the control drug were re-packed at the Center into the same size and color capsules. The center randomly assigned numbers to the drugs and distributed them to the patients.

All patients did not take any mental enhancement drugs or central nervous system stimulants during the whole procedure (16 weeks). However, some complications such as hypertension and diabetes were treated accordingly and any drug adverse reactions were recorded.

2.3.2. Efficacy Assessment

All patients were assessed 3 times (before treatment, after 4 weeks of treatment, and at the end of treatment (16 weeks) by accredited psychologists using MMSE, ADAS-cog and the ADL measurements.

1) The Alzheimer's Disease Assessment Scale cognitive subscale (ADAS-cog) was used to assess the patient's cognitive function, including memory, attention, language, orientation, visuo spatial, skill and ability to understand and operate 12 tasks. The lower the score, the better was the cognitive function [14].

2) The Mini-Mental State Examination (MMSE) was also used to assess the patient's cognitive function. This measures 5 factors: orientation in time and space, instantaneous recall and short term memory, attention and calculation ability, language, and constructional capacities. The higher the MMSE score, the better was the cognitive function [15].

3) The activity of daily living scale (ADL) was used to determine a person's ability to perform tasks of daily

living. This includes 6 items of physical assessment screenings (self-care) and 8 items of functional assessment screening (tool-use ability). The lower the score means the better the ability of daily living [16].

2.3.3. Tolerability and Safety Assessment

Physical examination included vital signs (blood pressure, heart rate, and body temperature), 12-lead electrocardiograph (ECG), and laboratory tests (including serum chemistry, hematology, and urinalysis). They were conducted before drug administration, after 4 weeks of treatment and at the end of the study period after 16 weeks of treatment.

All laboratory tests were performed at an accredited central laboratory of the Qinghai Provincial Hospital. Adverse events were assessed throughout the study period based on direct questioning and spontaneous reports, and were recorded regardless of their suspected relations to the study drugs. All data obtained from the study were monitored by qualified investigators.

2.3.4. Aβ and Pro-Inflammatory Cytokines Measurements

Blood samples (10 ml) were collected from all patients before drug administration, after 4 weeks of treatment and at the end of the study period after 16 weeks of treatment. The samples were centrifuged to separate the serum which was stored in $-70°C$ until testing

$A\beta_{40}$ and $A\beta_{42}$ levels were measured using an enzyme linked immunosorbent assay, (ELISA, Adlitteram Diagnostic. Laboratories Co. (USA) Corporation) according to the manufacturer's protocol.

IL-1β, IL-2, IL-6, IL-8 and TNF-α concentrations were measured by radioimmunoassay (Tianjin Jiuding Medical and Biological Co., Ltd.), following the manufacturer's recommendations.

2.4. Statistical Analyses

The statistical package SPSS for Windows Ver. 11.5 was used to do statistical analyses. Pearson's correlation was used for linear correlation analysis. All data are shown as mean ± standard deviation (±SD). Multiple group comparisons were made using analysis of variance (ANOVA). Comparisons between the groups were made by t test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. The Demographic Characteristics of the Study Population Are Not Significantly Different between the High-Dose RNSP (Single Dose, 1 g/day), Low-Dose RNSP (Single Dose, 0.33 g/day) and Control Groups

The demographic characteristics of the study population are presented in **Table 1**. All patients were ethnic Han Chinese. Low-dose, high-dose, and placebo groups comprised respectively 40 patients (19 males and 21 females), 50 patients (23 males and 27 females), and 50 patients (25 males and 25 females). The patients ranged in age from 60 to 85 years old (mean age 74.5 ± 8.1 years old) in the low-dose group, from 59 to 84 years old (mean age 75.2 ± 6.9 years old) in the high-dose group and from 61 to 85 years old (mean age 74.7 ± 6.9 years old) in the control group. All three groups had similar education levels. Psychological tests showed that all the patients were in the mild to moderate range of AD. Based upon a review of psychological testing records, the average length that the participants had been diagnosed with AD was 2 years for all 3 groups. The basal MMSE scores prior to our treatment were 17.80 ± 4.60 in the low-dose treatment group, 17.89 ± 5.29 in the high-dose treatment group, and 17.57 ± 5.68 in the control group. Statistical analyzes showed that the demographic characteristics (gender, age, ethnicity, height and weight), education levels, average length of patients diagnosed as AD and basal MMSE scores of all three groups were not significantly different.

Figure 1 displays a flow diagram that describes the

Table1. Demographic characteristics of the study population by group at baseline.

	RNSP (1 g/day) (n = 50)	RNSP (0.33 g/day) (n = 40)	Placebo (2.4 g/day) (n=50)	*p*-value
Gender				
Male/Female	23/27	19/21	25/25	0.524
Age (years)	75.2 ± 6.9	74.5 ± 8.1	74.7 ± 6.9	0.551
Height (cm)	169 ± 9.1	167 ± 10.2	168 ± 11.3	0.298
Weight (Kg)	71 ± 20.1	72 ± 22.3	72 ± 68.9	0.687
BMI (kg/m^2)	26 ± 5.2	25 ± 7.1	26 ± 8.9	0.913
Education (years)	6.1 ± 2.0	6.4 ± 2.1	6.2 ± 1.8	0.876
Duration of dementia (years)	2.1 ± 2.6	2.3 ± 3.0	2.5 ± 2.3	0.639
MMSE baseline score	17.89 ± 5.29	17.80 ± 4.56	17.57 ± 5.68	0.240

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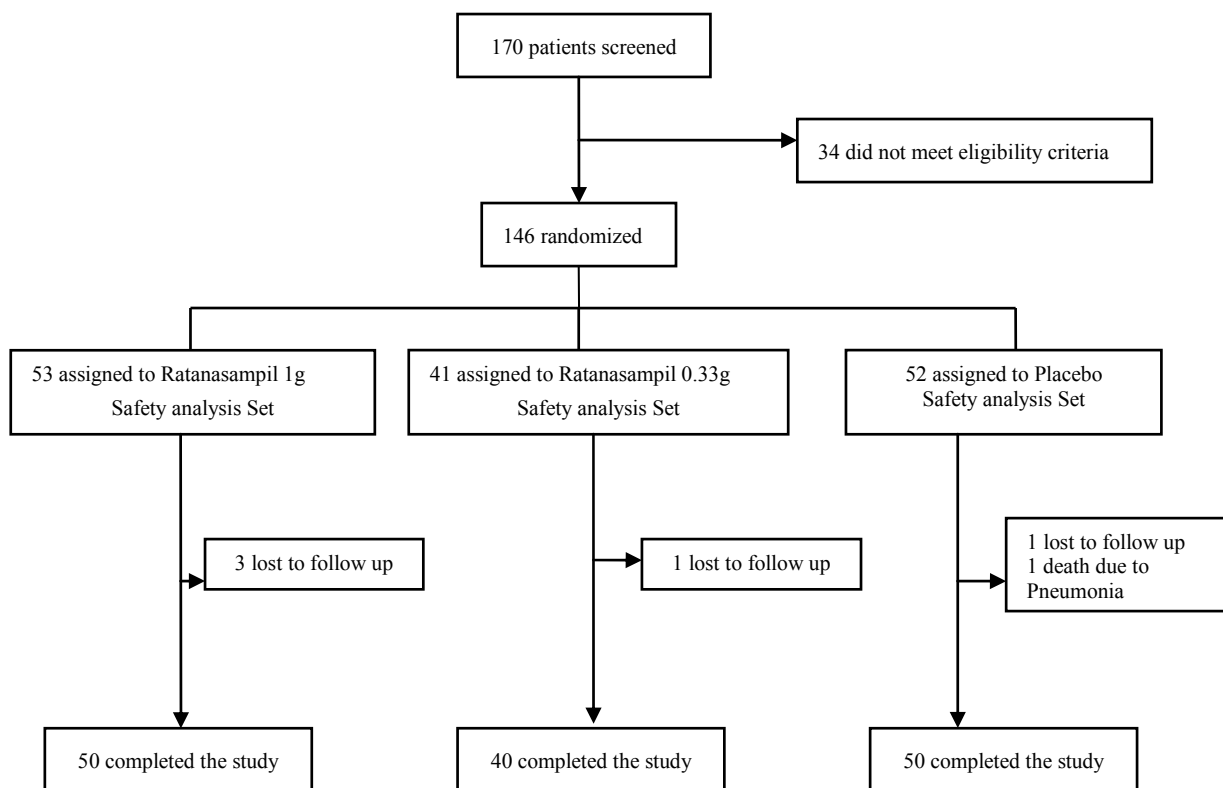
Figure 1. The figure displayed a flow diagram that describes the trial from screening until the end of the study. 170 patients were screened and 34 failed meet the eligibility criteria. 146 patients were enrolled in this study.

trial from screening until the end of the study. 170 patients were screened and 34 failed meet the eligibility criteria. 146 patients were enrolled in this study. 53 patients began the high-dose RNSP treatment (1 g/day), 41 patients began the low-dose RNSP (0.33 g/day) and 52 patients began placebo. In the end, 50 patients (attrition rate 5.7%) in the high-dose RNSP treatment group, 40 patients (attrition rate 2.4%) in the low-dose RNSP treatment group and 50 patients (attrition rate 3.8%) in the placebo group remained in the study after 16-weeks. The reasons for attrition included lost to follow up and death.

3.2. High-Dose RNSP (1 g/day) Significantly Improved Cognitive Functions in Mild-to-Moderate Alzheimer's Patients after 16-Week Treatment

All 3 groups (high-dose, low-dose, and control) were evaluated at the beginning of treatment, at 4 weeks and at the 16 week termination. MMSE scores, ADAS-cog scores and ADL scores for each group are presented in **Table 2** and **3** and **Figure 2**. The basal MMSE, ADAS-cog and ADL scores were similar among the 3 groups ($p > 0.05$). After 4 weeks of treatment, the high-dose RNSP group showed significant improvement in MMSE scores (3 out of 5 sub-scores including orientation in time and space, instantaneous recall and short term memory were

significantly improved) and ADAS-cog scores. However, the ADL score was only slightly changed after 4 weeks of treatment ($p > 0.05$). Interestingly, after treatment for 16 weeks, the high-dose RNSP group showed a further significantly improved MMSE score (all 5 sub-scores were significantly improved), much decreased ADAS-cog scores (better cognitive functions), and significantly improved ADL scores（both physical and functional assessment screenings were significantly improved). Although there was a slight improvement in the low-dose RNSP treatment group, the difference was not statistically significant ($p > 0.05$).

3.3. High-Dose RNSP Significantly Decreased the Levels of Aβ42 and Pro-Inflammatory Factors Associated with Alzheimer's Disease

Blood samples from all 3 groups (high-dose, low-dose and control) were collected 3 times during the 16-week treatment (at the beginning of treatment, at 4 weeks and 16 weeks after treatment) to examine the levels of Aβ and several AD-related pro-inflammatory factors. The results are shown in **Table 4**. The basal levels of Aβ, TNF-α, IL-1β, IL-6, IL-8 were similar among all 3 groups ($p > 0.05$). After 4 weeks of treatment, TNF-α, IL-1β, IL-6 and Aβ_{42} levels were significantly reduced in the high-dose RNSP treatment group compared to the control group,

Table2. MMSE scores (including all 5 sub-scores) before and after treatment (4-weeks and 16-weeks of treatment) in the 3 groups (mean ± SD points).

Groups	Time points	MMSE Total scores	Ⅰ Orientaton in time and space	II instantaneous recall and short term memory	III attention and calculation ability	IV constructional capacities	V language
High-dose RNSP group	Before treatment	17.89 ± 5.23	6.22 ± 2.84	2.84 ± 0.65	1.43 ± 1.44	0.36 ± 0.72	6.98 ± 1.96
	4-week	20.70 ± 5.33^{ac}	7.18 ± 2.45^a	2.95 ± 0.30^{ce}	2.00 ± 1.84	0.89 ± 0.97^a	7.68 ± 1.48
	16-week	23.70 ± 4.48^{bdf}	8.36 ± 1.95^{bdf}	2.98 ± 0.15^{ce}	2.64 ± 1.82^{bdf}	1.57 ± 1.02^{bdf}	8.14 ± 1.07^{be}
Low-dose RNSP group	Before treatment	17.80 ± 4.56	6.10 ± 2.66	2.73 ± 0.64	1.47 ± 1.76	0.43 ± 0.73	7.17 ± 1.64
	4-week	18.17 ± 5.09	6.30 ± 2.42	2.53 ± 0.94	1.37 ± 1.72	0.50 ± 0.78	7.47 ± 1.33
	16-week	18.90 ± 5.46	6.50 ± 2.32	2.80 ± 0.97	1.66 ± 1.45	0.73 ± 1.01	7.63 ± 1.60
Control group	Before treatment	17.57 ± 5.35	5.93 ± 2.88	2.70 ± 0.75	1.43 ± 1.77	0.44 ± 0.63	7.00 ± 1.98
	4-week	17.83 ± 5.66	6.17 ± 2.55	2.88 ± 1.01	1.33 ± 1.73	0.49 ± 0.78	7.33 ± 1.65
	16-week	18.23 ± 6.00	6.59 ± 3.44	2.81 ± 1.04	1.39 ± 1.47	0.62 ± 1.02	7.57 ± 1.88
F-Value		6.039	3.739	2.520	2.821	7.531	2.050
p-Value		0.000	0.000	0.011	0.005	0.000	0.041

The Mini-Mental State Examination (MMSE) was used to assess the patient's cognitive function of three groups in 3 times. This measures 5 factors: orientation in time and space, instantaneous recall and short term memory, attention and calculation ability, language, and constructional capacities. After 4 weeks of treatment, the high-dose RNSP group showed significant improvement in MMSE scores (3 out of 5 sub-scores including orientation in time and space, instantaneous recall and short term memory were significantly improved). Results were expressed with the means ± S.E.M. $^a p < 0.05$, $^b p < 0.01$ versus control group; $^c p < 0.05$, $^d p < 0.01$ versus the same group before treatment; $^e p < 0.05$, $^f p < 0.01$ versus low-dose RNSP treatment group

Table 3. ADAS-cog scores and ADL scores (including 2 aspects of screenings) before and after treatment (4-weeks and 16-weeks of treatment) in the 3 groups (mean ± SD points).

Group	Time Points	ADAS-Cog	ADL Total Scores	Self-Care Ability	Tool-Use Ability
High-dose RNSP group	Before treatment	25.27 ± 5.11	39.98 ± 14.41	21.84 ± 8.78	17.82 ± 6.54
	4-week	23.11 ± 4.08^b	36.20 ± 12.91	19.61 ± 7.16	16.86 ± 6.91
	16-week	19.93 ± 4.40^{bdf}	31.64 ± 12.00^{ae}	17.93 ± 6.15^{ae}	14.16 ± 6.72^{ae}
Low-dose RNSP group	Before treatment	25.47 ± 4.33	39.47 ± 17.45	22.57 ± 10.05	17.23 ± 8.40
	4-week	24.10 ± 4.62	38.63 ± 17.30	22.03 ± 9.80	16.80 ± 8.42
	16-week	23.37 ± 4.99	37.80 ± 15.73	21.07 ± 8.45	16.60 ± 8.38
Control group	Before treatment	25.83 ± 3.70	40.47 ± 18.04	22.97 ± 10.38	17.83 ± 8.51
	4-week	25.43 ± 5.22	39.40 ± 17.86	22.30 ± 10.04	17.30 ± 8.63
	16-week	24.10 ± 5.11	39.70 ± 18.11	22.60 ± 10.37	17.07 ± 8.64
F-value		6.233	1.901	1.36	2.429
p-value		0.000	0.056	0.214	0.033

All 3 groups (high-dose, low-dose, and control) were evaluated at the beginning of treatment, at 4 weeks and at the 16 week termination. ADAS-cog scores and ADL scores for each group are presented. The resulted showed high-dose RNSP significantly improved ADL scores (both physical and functional assessment screenings). Although there was a slight improvement in the low-dose RNSP treatment group, the difference was not statistically significant ($p > 0.05$). There was no significant difference observed in placebo group ($p > 0.05$). Results were expressed with the means ± S.E.M. $^a p < 0.05$, $^b p < 0.01$ versus control group; $^c p < 0.05$, $^d p < 0.01$ versus the same group before treatment; $^e p < 0.05$, $^f p < 0.01$ versus low-dose RNSP treatment group.

($p < 0.05$). Interestingly, after treatment for 16 weeks, the concentrations of TNF-α, IL-1β, IL-6 and Aβ_{42} were further decreased compared to the control group ($p < 0.01$). The Aβ_{42}/Aβ_{40} ratio was significantly reduced after both 4-weeks and 16-weeks of treatment ($p < 0.05$, $p < 0.01$). However, there were no significant differences in IL-8 and Aβ_{40} levels between the high-dose RNSP treatment and control groups.

Compared to the control group, the levels of IL-6 in the low-dose RNSP treatment group were also decreased after 16 weeks of treatment, while no significant differences were observed in the levels of other pro-inflammatory factors. There was also no difference in the control group before and after treatment.

Linear correlation analyses showed that serum $A\beta_{42}$ levels had a positive correlation with the levels of TNF-α (r = 0.564), IL-6 (r = 0.271) and IL-1β (r = 0.205) ($p < 0.01$, $p < 0.05$ and $p < 0.05$). However, we found no correlation between the $A\beta_{42}$ levels and IL-8 levels ($p > 0.05$).

3.4. Toxicological Measurements

Clinical adverse events

Patients from all 3 groups reported occasional nausea, anorexia, dizziness, mild diarrhea and dry mouth (See **Table 5**). These symptoms usually eased or disappeared without any special treatment. One patient from the control group died of pneumonia.

Laboratory Tests

The heart rate, PR interval, QRS wave and QTC of ECG showed no significant changes before and after treat- ment. Serum chemistry, hematology, and urinalysis (liver and kidney function, glucose, lipids, electrolytes) before and after treatment showed no significant changes in all 3 groups.
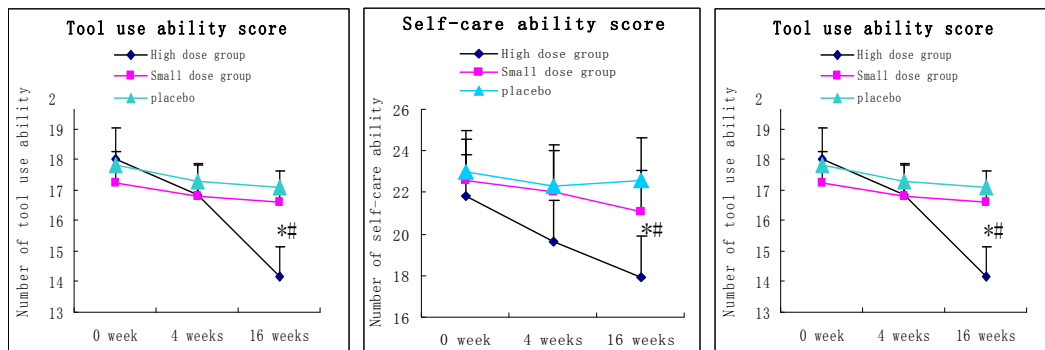
The basal ADL scores were similar among the 3 groups ($p > 0.05$). However, the ADL score was only slightly changed after 4 weeks of treatment ($p > 0.05$). After treatment for 16 weeks, the high-dose RNSP group showed a further significantly improved ADL scores (both physical and functional assessment screenings were significantly improved). Results were expressed with the means ± S.E.M. [*]$p < 0.05$ versus control group; [#]$p < 0.05$ versus the same group before treatment.

Figure 2. ADL total scores and its two sub-scores before and after treatment (4-weeks and 16-weeks treatment) in the 3 groups (mean ± SD points).

Table 4. Serum levels of TNF-α, IL-1β, IL-6, IL-8, $A\beta_{40}$ and $A\beta_{42}$ before and after treatment (4-weeks and 16-weeks of treatment) in the 3 groups (mean ± SD).

group	patient number	Time (week)	TNF-α (ug/l)	IL-1β (ug/l)	IL-6 (ug/l)	IL-8 (ug/l)	$A\beta_{40}$ (ng/ml)	$A\beta_{42}$ (ng/ml)	$A\beta_{42/40}$
RNSP (1 g/day)	53	0	1.79 ± 0.39	0.98 ± 0.45	156.84 ± 69.05	0.26 ± 0.21	17.38 ± 8.75	27.71 ± 9.25	1.96 ± 0.69
	50	4	1.56 ± 0.34[ae]	0.72 ± 0.37[ac]	118.00 ± 37.14[ae]	0.27 ± 0.19	14.01 ± 3.84	20.26 ± 5.92[bce]	1.52 ± 0.48[a]
	50	16	1.27 ± 0.24[bdf]	0.59 ± 0.32[bdf]	95.43 ± 32.45[bde]	0.25 ± 0.11	13.82 ± 3.77	17.76 ± 6.42[bdf]	1.33 ± 0.60[bce]
RNSP (0.33 g/day)	41	0	1.76 ± 0.44	0.99 ± 0.80	160.02 ± 18.73	0.25 ± 0.99	16.97 ± 9.18	28.04 ± 12.44	1.92 ± 0.96
	40	4	1.75 ± 0.98	0.93 ± 0.68	156.32 ± 29.44	0.24 ± 0.39	15.88 ± 7.16	26.08 ± 14.39	1.80 ± 1.44
	40	16	1.64 ± 0.65	0.84 ± 0.55	121.11 ± 40.37[ac]	0.22 ± 0.74	14.66 ± 8.62	22.47 ± 11.21	1.67 ± 0.91
Placebo (2.4 g/day)	52	0	1.72 ± 0.55	0.95 ± 0.22	160.02 ± 18.73	0.27 ± 0.42	17.04 ± 5.52	27.10 ± 11.45	1.92 ± 0.96
	50	4	1.70 ± 0.74	0.90 ± 0.41	155.32 ± 29.44	0.24 ± 0.39	16.70 ± 6.87	26.42 ± 14.67	1.87 ± 1.12
	50	16	1.68 ± 0.21	0.91 ± 0.62	150.11 ± 38.52	0.26 ± 0.62	16.25 ± 5.85	25.90 ± 3.26	1.79 ± 0.56
F-valve			9.539	10.920	4.283	1.430	1.383	4.321	4.594
p-valve			<0.001	<0.001	<0.01	>0.05	>0.05	<0.01	<0.01

Blood samples from all 3 groups (high-dose, low-dose and control) were collected 3 times during the 16-week treatment (at the beginning of treatment, at 4 weeks and 16 weeks after treatment) to examine the levels of $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$ ratio of and several AD-related pro-inflammatory factors in serum. After 4 weeks and 16 weeks of treatment, TNF-α, IL-1β, IL-6 and $A\beta_{42}$ levels were significantly reduced in the high-dose RNSP treatment group compared to the control group, ($p < 0.05$, $p < 0.01$). The $A\beta_{42}/A\beta_{40}$ ratio was significantly reduced after both 4-weeks and 16-weeks of treatment ($p < 0.05$, $p < 0.01$). However, there were no significant differences in IL-8 and $A\beta_{40}$ levels between the high-dose RNSP treatment and control groups. The results are expressed with the means ± S.E.M. [a]$p < 0.05$, [b]$p < 0.01$ versus control group; [c]$p < 0.05$, [d]$p < 0.01$ versus the same group before treatment; [e]$p < 0.05$, [f]$p < 0.01$ versus low-dose RNSP treatment group.

Table 5. Adverse events reports for three groups.

Adverse events	RNSP 1 g/day (n = 50)	RNSP 0.33 g/day (n = 40)	Placebo (n = 50)
Nausea	5 (10.0%))	3 (7.5%)	4 (8.0%)
Anorexia	5 (10.0%)	4 (10.0%)	5 (10.0%
Dizziness	3 (6.0%)	1 (2.5%)	2 (4.0%)
Mild diarrhea	1 (2.0%)	1 (2.5%)	1 (2.0%)
Dry mouth	1 (2.0%)	0 (0.0%)	3 (6.0%)

Patients from all 3 groups reported occasional nausea, anorexia, dizziness, mild diarrhea and dry mouth. These symptoms usually eased or disappeared without any special treatment.

4. Discussion

Patients with AD suffer progressive memory loss, cognitive dysfunction, impairment of language skills, impairment in activities of daily living and behavioral and psychological abnormalities. AD ranks fourth among the leading causes of death, after cardiovascular diseases, cancer and stroke. In 2004, there were an estimated 18 million people suffering from AD worldwide according to the World Health Organization (WHO), with approximately 5 million AD patients in China. The prevalence rate for the population aged 60 years and over was about 1.26% [17]. According to the WHO, the AD population was expected to double to 34 million around 2025 [18]. While the rate of dementia is expected to double between 2001 and 2040 in developed nations, it is predicted it will increase by more than 300 percent in India, China and South Asia [19].

Qinghai is located on the Qinghai-Tibet Plateau. The climate in Qinghai is that of a typical continental plateau, which is dry, windy, and cold, with huge temperature differences between each region, day and night. People living in these areas experience many environmental stresses, including chronic hypoxia, strong ultraviolet radiation, high altitude and relative isolation from the outside world. Besides the relatively backward economic and medical situations, a high number of the elderly population suffer declines in memory and cognitive function [20-22]. Exploring methods of treatment of AD has thus become a significant focus for current medical research in this area.

Traditional Chinese medicine (TCM) has a time-honored history of several thousand years and there is a very rich record of Chinese herbal medicine used to treat dementia, a significant feature of AD. However, Tibetan medicine has not yet been systematically tested in AD clinical research. About 1450, two contradictory schools of thought (northern and southern) arose in Tibet. Each school had its own views concerning prescription methods and the Four Medical Classics. Sukar, a representative of the southern school, and his disciples conducted research on diseases in southern Tibet and developed *seventy-taste-pearl-balls* (RNSP)*,* which has been used as an anti-aging and anti-hypoxic drug ever since. It is

regarded as one of the most important Tibetan medicine treasures [23]. In the eighteenth century, RNSP was included in the well-known "One-thousand prescriptions", in which RNSP is described as able to treat various acute and chronic cerebrovascular diseases such as cerebral hemorrhage, cerebral infarction, epilepsy, and brain concussion. RNSP is composed of more than seventy components, including Zuotai, natural pearls, antelope horn, saffron, natural bezoar, benzoin, incense, and nine-eye stone. Through generations of the continuous practice of Tibetan medicine and recent clinical observations, it has been found that RNSP has sedative and anticonvulsant effects, improved learning and memory, improved circulation, reduced platelet aggregation, and antithrombotic properties [24,25].

In this study, we used comprehensive clinical and neurological rating systems to evaluate whether the Tibetan medicine RNSP could improve the cognitive function and activities of daily living in AD patients living at high altitude. We chose piracetam as the control medicine because it is a mild anti-inflammatory agent which has been tested and reported in numerous studies [26]. After high-dose RNSP (1 g/day) treatment for 4 weeks, the patients showed a significant improvement in the MMSE total score and the ADAS-cog score. Although compared to the control group there was an improvement in the ADL score as well, the difference was not statistically significant. After treatment for 16 weeks, the MMSE total scores (and its 5 sub-scores) and the ADAS-cog scores were further improved compared to the control group. ADL scores (both self-care ability and tool-use ability sub-scores) also improved significantly. However, there were no significant differences between the low-dose RNSP treatment group (0.33 g/day) and the control group in MMSE, ADAS-cog and ADL scores. These data suggested that high-dose RNSP treatment effectively improved clinical measures of cognitive functions in mild-to-moderate Alzheimer's patients living at high altitude. Cognitive processes including perception, attention, memory, calculating, thinking, language and understanding, are one of the basic psychological processes. Cognitive functions not only affect emotional, behavioral and other psychological processes, but also have direct

impact on the ability to perform activities of daily living. Therefore RNSP had positive effects on the quality of daily life in AD patients.

Neuroinflammation is now recognized as a crucial player influencing and linking $A\beta$ deposition and neuronal damage in Alzheimer's pathogenesis. Anti-inflammatory treatments are considered beneficial to AD patients. The possible mechanisms that inflammatory responses are involved in AD pathogenesis include the pro-inflammatory cytokine IL-1β as a key regulator of acute inflammatory processes in the CNS. Over-expression of IL-1β stimulates and accelerates the deposition of $A\beta$, which is an early event in AD pathogenesis [27]. IL-6 over-expression begins before the formation of senile plaques, suggesting that IL-6 plays an important role in their early development [28]. The IL-6 $-174°C$ allele in the promoter region of the IL-6 gene was over-represented in AD patients. Increased brain and serum IL-6 levels were associated with a higher risk of AD [29]. It has been found that there is a large amount of TNF-α around the senile plaques in the brains of AD patients. $A\beta$ and other proteins found in the senile plaques are potent activators that induce inflammatory responses, accompanied by leukocyte activation and inflammatory cytokine production. The activation of microglia increases the expression of TNF-α, suggesting that abnormal expression of high levels of TNF-α could be closely related to the pathogenesis of AD [30,31]. Genetic studies suggest a significant anti-inflammatory role of IL-4, IL-8, IL-10 and IL-13 in AD pathogenesis, although the mechanisms are not known [32].

Recent studies revealed that non-steroidal anti-inflammatory drugs reduced $A\beta$ production and deposition in AD animal models [33,34]. $A\beta$ is generated by the consecutive cleavage of the amyloid precursor protein (APP) by two proteases, β-secretase and γ-secretase [35]. $A\beta$ is a protein consisting of two major forms, $A\beta_{40}$ and $A\beta_{42}$. $A\beta_{42}$ is thought to be the major form that drives $A\beta$ aggregation and plaque formation. Elevated levels of $A\beta_{42}$ and an increased $A\beta_{42}/A\beta_{40}$ ratio are early events in AD pathology and the ratio may predict preclinical AD in cognitively normal patients [36]. Reports also showed that elevated serum $A\beta_{42}$ levels increased the risk of AD [37]. These data suggested that $A\beta_{42}$ could be a biomarker for AD diagnosis and the evaluation of treatment efficacy. The link between $A\beta$ deposition, $A\beta$ induced inflammation and AD provides a new therapeutic strategy for the treatment of patients suffering from the disease [38, 39].

Here we showed that high-dose RNSP reduced serum TNF-α, IL-1β, IL-6 and $A\beta42$ levels after treatment for 16 weeks. The $A\beta_{42}/A\beta_{40}$ ratio was decreased after 4 weeks of high-dose RNSP treatment and further decreased after 16 weeks of treatment. Statistical analyses showed a positive correlation between serum $A\beta_{42}$ levels and TNF-α, IL-1β and IL-6 levels. These results suggest that RNSP could inhibit inflammatory responses, reduce the serum levels of $A\beta_{42}$, decrease the $A\beta_{42}/A\beta_{40}$ ratio and slow down the progression of AD. Furthermore, there was no significant adverse medication reaction observed in either RNSP treatment or control groups during the whole procedure, which suggested that RNSP (even at a high dose) is safe and well tolerated. All these results indicated that Tibetan Medicine RNSP could have great potentials for the treatment of AD. In this study, we found a positive relationship between the levels of pro-inflammatory cytokines and $A\beta_{42}$ peptides and cognitive function scores. We aim to investigate further whether the changed levels of these cytokines and $A\beta_{42}$ predict the progression of AD on long-term follow-up. At the same time, we plan to increase our sample size and observe whether RNSP can further improve AD patients' cognitive function with an even longer period of treatment time, such as 6 months or 12 months. Additionally, we will investigate whether low-dose RNSP treatment for a longer period of time (6-12 months) can enhance AD patients' cognitive scores. Our future experiments will provide more information on the efficacy and safety of RNSP. The further clinical trials of RNSP in AD will study at different altitudes.

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