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Original Article (Pages: 10731-10742)

Subcutaneous Immunotherapy and Synbiotic Combination Shift T-Helper 1 and Cytotoxic T Cells in Allergic Rhinitis

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Abstract

Background: Synbiotics have been used in the prevention and treatment of various immunological diseases. We aimed to investigate the synergistic clinical and immunologic effects of synbiotics and subcutaneous allergen immunotherapy (SCIT) combination in patients with allergic rhinitis.

Materials and Methods: Nineteen individuals with allergic rhinitis were enrolled in this single blind, placebo-controlled trial between 2015 and 2016 in Qaem Hospital, Mashhad, Iran. Patients were randomly divided into two groups: A) Immunotherapy plus one synbiotic capsule per day, and B) Immunotherapy plus placebo for two months. The Sino-nasal outcome test (SNOT-22), and mini rhinoconjunctivitis quality of life questionnaire (RQLQ) were filled by patients or their parents while intracellular expression of interlukin-4 (IL-4), interferon-gamma (IFN-gamma), and Forkhead Box P3 (FOXP3), and variations in the T helper 1 (Th1), T helper 2 (Th2) and T regulatory cells and cytotoxic T lymphocytes (CTL) frequency were examined by flow cytometry assay at baseline, after 2 and 6 months of intervention.

Results: Nineteen individuals with allergic rhinitis aged between 5 and 55 years participated in this study. No significant difference in the frequency of symptoms between the two groups was observed after 2 and 6 months of intervention ($p>0.05$). A significant increase in the percentage of Th1 cells was recorded in group A compared to group B ($p= 0.02$). CTL enhancement percentage was significantly increased in group A compared to group B after 2 months ($p=0.013$).

Conclusion: Subcutaneous Immunotherapy concomitant with synbiotics administration may have temporarily increased the percentage of Th1 cells, but no significant clinical differences were observed.

Key Words: Allergic rhinitis, Synbiotics, Probiotics, Subcutaneous allergen immunotherapy.

*Please cite this article as: Moazzen N, Ahanchian H, Jabbari azad F, Mohammadi M, Farid R, Nikpoor AR, et al. Subcutaneous Immunotherapy and Synbiotic Combination Shift T-Helper 1 and Cytotoxic T Cells in Allergic Rhinitis. Int J Pediatr 2020; 8(1): 10731-742. DOI: **10.22038/ijp.2019.14040**

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Received date: Feb.17, 2019; Accepted date: Dec.12, 2019

1- INTRODUCTION

Allergic rhinitis is a growing allergic disease; it affects 10-30% of the population worldwide. Its prevalence in the International Study of Asthma and Allergies in Childhood study (ISAAC) varied between 0.8 and 14.9% in 6 to 7-year-old children and between 1.4 and 39.7% in 13 to 14-year-old individuals (1). Today, various treatments are available for this disease including antihistamines, intranasal corticosteroids and immunotherapy (2). Subcutaneous immunotherapy is an effective treatment for allergic rhinitis. Its impact on the underlying immunologic mechanisms causes a balance in the immune system and consequently symptoms alleviation. Its clinical benefits such as decrease in symptoms score and need for medications persist even after cessation of immunotherapy. Successful allergen immunotherapy (AIT) is associated with suppression of inflammatory cells in target organs, T helper type 2 (Th2) down regulation and induction of T regulatory cells. These events are associated with antigen specific Th2 and delayed immune deviation in favor of Th1 response (3).

As taking any of these treatments can cause various side effects. Therefore, discovery of novel therapeutic modalities with minimum complications and better cost-effectiveness is of great importance. In this regard the use of synbiotics seems promising. Synbiotic is a nutritional supplement combining probiotics (bacteria) and prebiotics (sugars). Probiotics are live microorganisms that when administered in an adequate dose, may be beneficial to the host. In recent years, probiotics have been introduced as a novel therapeutic strategy in the treatment of infections and immune disorders (4). The highest documented effect of these agents is in the prevention and treatment of infectious and antibiotic induced diarrhea (5). The hypothesis that probiotics may be useful in the management of allergic disease and eczema is supported by evidence stating that the intestinal microbiota in allergic children is different from that of the normal population (6). Thus intestinal dysbiosis may cause or worsen allergic diseases and this supports the hypothesis that probiotic bacteria could be used in the prevention and treatment of allergic diseases and eczema (7). Several systematic reviews have investigated probiotics and/or synbiotics in the prevention and treatment of atopic dermatitis (AD) (8, 9). Other studies have reported that only probiotic products that contain Lactobacillus and Bifidobacterium can reduce the incidence of eczema (10).

These data raise the possibility of prevention and treatment of AD by modulation of the microbiome (11, 12). Regardless of these findings further studies are yet required to determine the best therapeutic approach, formulation, dosage, timing and other environmental factors that may influence treatment, before being widely administered in the clinical setting (11, 12). To date, few relative studies are present on the administration of probiotics and/or synbiotics in the prevention and treatment of other allergic diseases such as allergic rhinitis and asthma (13, 14). As controlled clinical trials are the gold standard for assaying clinical efficacy, we aimed to investigate the possibility of improvement in allergic rhinitis with the use of synbiotics and their synergistic effect along with subcutaneous allergen specific immunotherapy (SCIT).

2- MATERIALS AND METHODS

2-1. Study design and population

This single blind, placebo controlled pilot study was performed between July 2015 and June 2016 in Qaem Hospital, Immunology Department, Mashhad University of Medical Sciences, Mashhad, Iran. All patients or their parents signed a written informed consent prior to

participation in the study. Patients were randomly divided into two groups: A) Immunotherapy plus one synbiotic capsule per day, and B) Immunotherapy plus placebo for two months.

2-2. Study subjects

Nineteen individuals aged 5-55 years who were diagnosed with allergic rhinitis were enrolled in this study. Allergic rhinitis was confirmed by allergist immunologist based on history taking, physical examination and skin prick test for common aeroallergens in the region. Two patients withdrew from the study within the first two months, due to personal reasons; one was in group A (Immunotherapy plus one synbiotic capsule per day), and one was in group B (Immunotherapy plus placebo). Seventeen patients completed the study and provided blood samples at each time point (**Figure.1**).

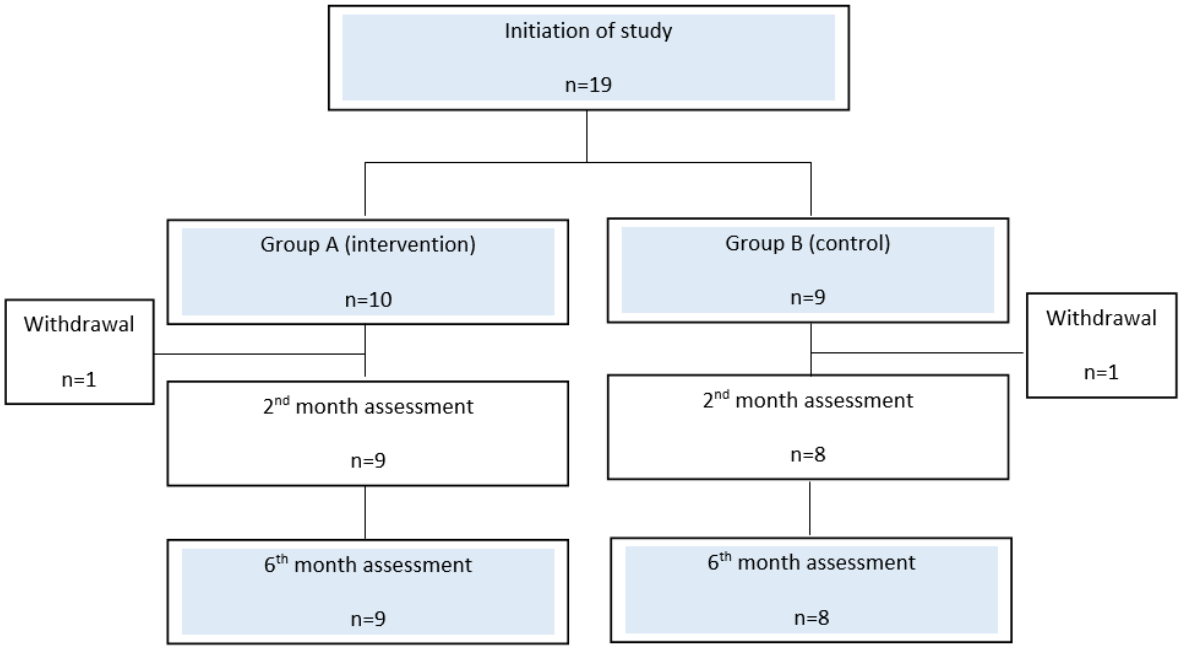

Fig.1: Flowchart of the study.

2-3. Intervention

Patients who met the eligibility criteria were randomized into two groups as follows: A: Immunotherapy plus synbiotics (n=10), and B: immunotherapy plus placebo (n=9). The patients were unaware of which was the placebo, and which the synbiotic was. They were told to use one capsule every day and if any other drug was needed during the first two months, they had to inform the researcher. Patients did not pay any money for the symbiotic capsule and after the first month they were asked about any side effect of the drug and they were given a new package of the drug for the second month. The cluster protocol was employed for immunotherapy in the buildup phase and then monthly injections were performed. The cluster protocol was adapted from the Middleton's text book (15). Allergen extracts used for test and treatment were manufactured by the Greer Company (Mail: GREER®, PO Box 800 Lenoir, NC 28645). Eighteen patients were administered with weed mix while in one patient tree mix extract was used for immunotherapy. Patients in the synbiotic group (with and without immunotherapy) received capsules daily for two months.

Lactocare® is a synbiotic that contains 1 billion CFU/Capsules of *Lactobacillus casei, Lactobacillus rhamnosus, Streptococcus thermophilus, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium infantis, Lactobacillus bulgaricus*, and *Fructooligosaccharide* (Zist Takhmir, Tehran, Iran). Patients in the placebo plus immunotherapy group received placebo capsules without synbiotics which were matched for size, shape, and color and manufactured by the same company. All patients were highly advised to cease treatment in case of any need for antibiotic consumption and start again after the completion of the course of the treatment. Non-sedative antihistamine (Loratadine) was administered at the dose of 10mg orally during the course of immunotherapy till reaching the maintenance dose at 8th week of treatment, and 10mg orally only one day before immunotherapy afterwards. Intranasal corticosteroids and other treatments were individually administered based on the patient's symptoms and severity of allergic rhinitis.

2-4. Measuring tools

Clinical and laboratory assessment was performed for each participant once after 2 months and once again at the end of the 6-month intervention course. Mini Rhinoconjectivitis Quality of Life Questionnaire (mini RQLQ) (16), and Sino-nasal outcome test-22 (SNOT-22) (17) scores, and also changes in skin reactivity by skin prick test, changes in intracellular expression of interlukin-4 (IL-4), interferin-gamma (IFN-gamma) and Forkhead Box P3 (FOXP3) and variation in the frequency of T helper 1 (Th1) and Thelper 2 (Th2), T regulatory and cytotoxic T lymphocytes (CTL) were measured for each participant by flow cytometery. Minimum and maximum score in the mini RQLQ Questionnaire are 1 and 6 in each item (total score 0-84), and in SNOT 22 Questionnaires are 1 and 5 in each score (total score 110), respectively. Higher scores show more functional impairment due to rhinoconjuctivitis. A 5mL blood sample was taken from all individuals and the buffy coat was separated. For measurement of intracellular expression of IL-4, IFN-gamma and FOXP3, frequency of CD4+ Th1 and Th2, CD4+, CD25+, CD127-, FOXP3+ T-regs and CD8+ cytotoxic T-lymphocytes, were analyzed by flow cytometry assay. The flow cytometry kits were purchased from the Biolegend Company (9727 Pacific Heights Blvd. San Diego, CA 92121). A skin prick test was performed at each time point and the results were compared. No side effects were reported during the treatment course.

2-5. Ethical consideration

The study protocol was approved by the Ethics Committee of Mashhad University of Medical Sciences and an informed consent was obtained from each participant prior to study entrance (Code: IR.MUMS.fm.REC.1394.154). Also, the study was registered in Iranian Clinical Trial studies: IRCT2015120123235N4.

2-6. Inclusion and exclusion criteria

Individuals aged between 5-55 years old who were diagnosed with allergic rhinitis were enrolled in this study. Patients were excluded from the study in case of simultaneous administration of antibiotics, systemic corticosteroids or immunosuppressive drugs, or being diagnosed with other major medical conditions especially autoimmune disease or immunodeficiency, malnutrition and pregnancy.

2-7. Statistical analysis

Statistical analysis was performed by the statistical package for social sciences (SPSS) software version 16.0 (IBM Inc, Chicago, Il, USA). Continuous data were checked for normality using the Shapiro-Wilk test. Normally distributed data were

described using mean and standard deviation (SD). Categorical data were presented as frequency and percentage. Distribution pattern of the continuous data was compared using Chi-square test. Continuous data were compared between groups using the student t-test. Repeated measures analysis of variance (ANOVA) was used to compare data between and within groups during the study. The significance level was set at P<0.05 in all analyses.

3- RESULTS

Nineteen patients were recruited, whereas 17 completed the trial, baseline characteristics of participants are shown in **Table.1**; there were no significant differences between the two groups in terms of age, gender, history of atopy, type and grade of allergic rhinitis and other baseline characteristics.

Table-1: Baseline characteristics of the studied patients

Variables		Group A	Group B
Number		9	8
Mean age (years)		22	24.33
Gender (Male/Female)		7/3	6/3
Type of AR	Seasonal	3 (30%)	3 (33.3%)
	Perennial	7 (70%)	6 (66.7%)
Activity	Normal	4 (40%)	4 (44.4%)
	Impaired	6 (60%)	5 (55.6%)

AR= Allergic rhinitis.

There was no significant difference in the frequency of symptoms between the two groups after 2 and 6 months of treatment (P= 0.10 and P=0.47, respectively). The patients completed the SNOT-22 and mini RQLQ questionnaires before the intervention, after 2 months and finally after 6 months of treatment. **Table.2** shows the mean of SNOT score in each point time. **Table.3** shows the mean of RQLQ score in each point time. The mean SNOT scores were 40.66±22.57, 15.11±10.34, and 11.13±9.36 for group A, and 52±24.06, 18.62±11.84, and 14±18.36 for group B at the mentioned time points, respectively. The same mean mini RQLQ values were 42.55±13.18, 11.22±6.09, and 10.88±5.51 for group A, and 43.75±12.93, 16.87±12.43, and 10.50±8.05 for group B, respectively. According to the repeated measures test, no significant difference in SNOT score (P= 0.32) or mini RQLQ score (P= 0.064) was obtained between the two groups.

Table-2: SNOT score mean and standard deviation in group A (Immunotherapy+ synbiotic), and B (Immunotherapy+ placebo).

SNOT-22 score	Group	Mean	Std. deviation	Number
SNOT score before treatment	Group A	40.66	22.57	10
	Group B	52	24.06	9
SNOT score after 2 months	Group A	15.11	10.34	10
	Group B	18.62	11.84	9
SNOT score after 6 months	Group A	11.33	9.36	9
	Group B	14	11.36	8

SNOT: Sino-nasal outcome test; according repeated measure analysis, there was no significant difference between two groups (P=0.32).

Table-3: Mean of difference between RQLQ score before and after 2 months in groups A (Immunotherapy + synbiotic), and C (synbiotic alone).

Mini RQLQ score	Mean	Standard deviation	Number
Immunotherapy + synbiotic	31.33	12.29	10
Just symbiotic	17.85	14.57	7

RQLQ: Quality of Life Questionnaire.

CD4+, CD25+, CD127-, Foxp3+ T regulatory cells percentage and intracellular Foxp3 expression at baseline were measured after 2 and 6 months of treatment. With repeated measures analysis, no significant difference in T regulatory cells percentage was found between the two groups after 6 months of intervention (P= 0.66). The decrease in the number of cells was statistically insignificant in both groups (P=0.88 in group A, and P=0.46 in group B). Based on repeated measures analysis no significant difference was achieved in intracellular Foxp3 expression between the two groups (P= 0.80), whereas a significant difference was observed within groups after 6 months of intervention (P= 0.048) (**Figure.2**).

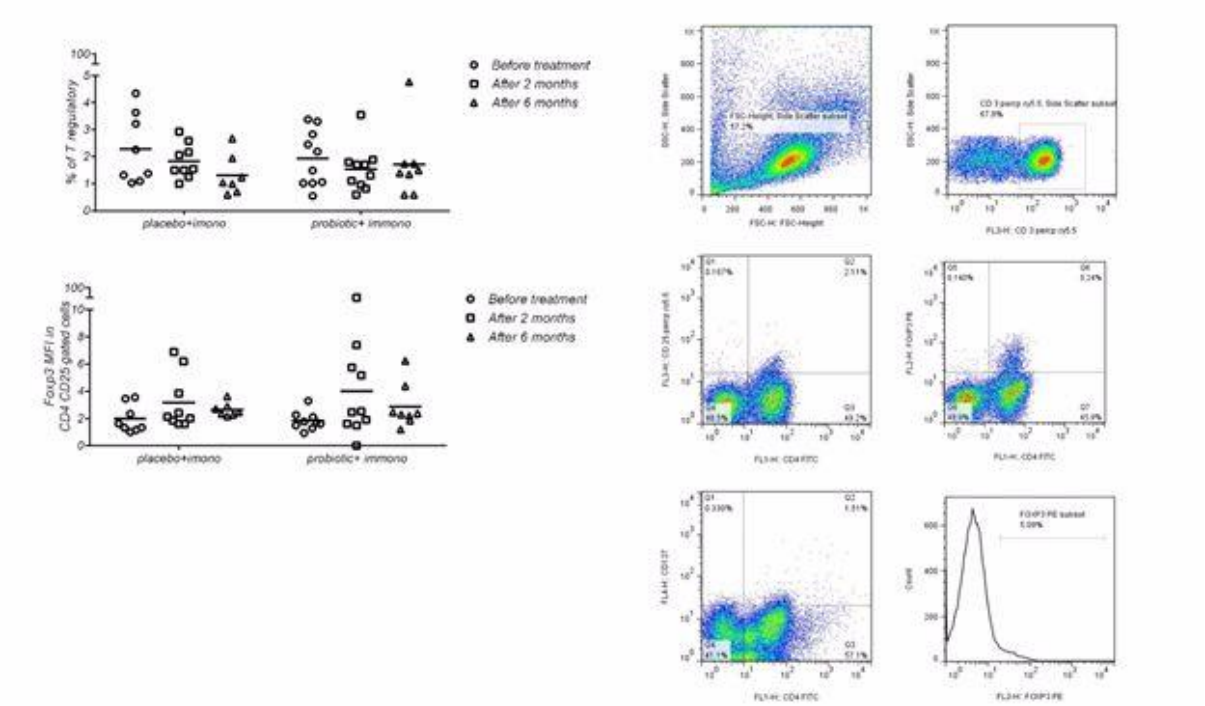

Fig.2: Density plots of CD25, FOXP3, CD127 and flow cytometry diagram of FOXP3. Isolated PBMCs were stained as described in the methods section. Frequency of stained cells is shown in the upper right quadrant for CD25, CD127 and FOXP3.

CD4+ Th2 cells and the intracellular expression of IL-4 were measured during treatment. Repeated measures analysis revealed no significant difference between the two groups after 6 months of intervention (P=0.44). However, repeated measure analysis showed a significant difference within each group (P= 0.04). With sample analysis for intracellular IL-4 expression in CD3+ CD4+ T cells, no significant difference was detected between the two groups (P=0.16) and within each group (P=0.40). CD4+Th1 cells and their intracellular

expression of IFN-gamma were also measured by the flow cytometry technique during treatment. With repeated measures ANOVA, no statistically significant difference was seen between the two groups after 6 months (P=0.11). However, after 2 months, a significant difference was recorded (P=0.012). Moreover, a statistically significant decrease was observed within each group after 6 months (P=0.005). Regarding the intracellular expression of INF-gamma, there was no significant difference between (P=0.11), and within (P= 0.61) the two groups (**Figure.3**). CD8+T cells (CTL cells), and their intracellular IFN-gamma expression were also measured in defined intervals. Repeated measures ANOVA revealed no significant difference in CTL percentage within the two groups during follow up (P=0.16) (**Figure.4**).

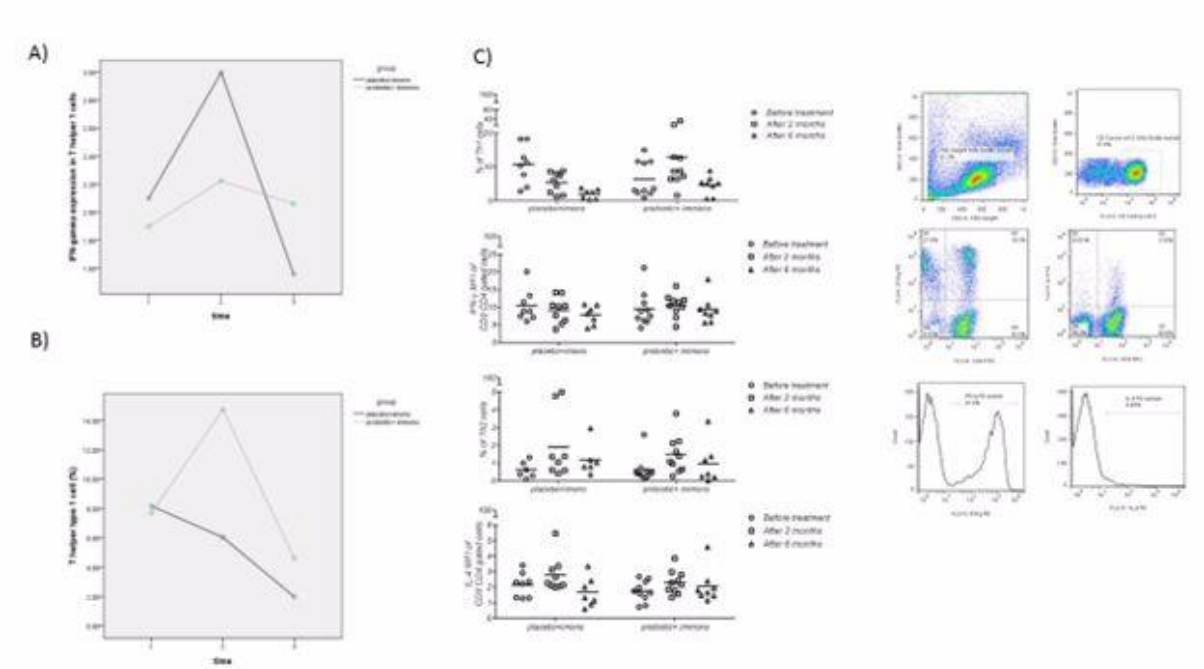

Fig.3: A) IFN-gamma gene expression in T helper 1 cells during intervention, B) T helper 1 cell percentage during intervention, C) Density plots of CD3, IL-4, and IFN-gamma.

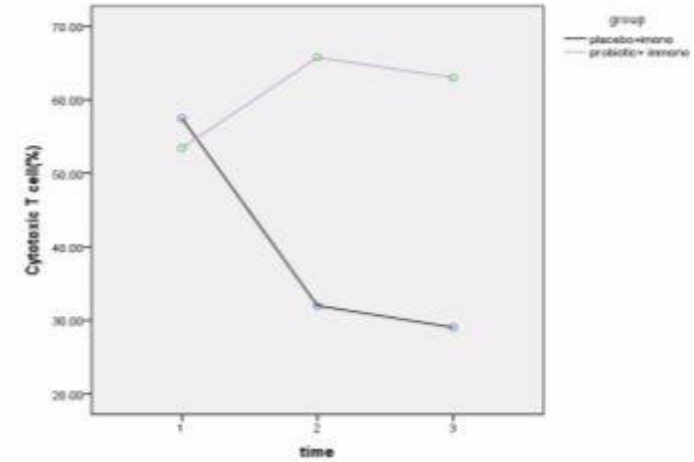

Fig.4: Cytotoxic T cells percentage in defined intervals. 1: at beginning, 2: after 2 months, 3: after 6 months.

Regarding the CTL percentage during treatment, the samples were analyzed after 2 and 6 months of treatment with T-test. A significant difference was recorded between the two groups accordingly (P=0.013, and P=0.094 in group A and B, respectively). IFN-gamma expression in cytotoxic T cells was also measured by flow cytometry during treatment. No significant difference was revealed between (P=0.12), and within (P=0.90) the two groups (**Figure.5**).

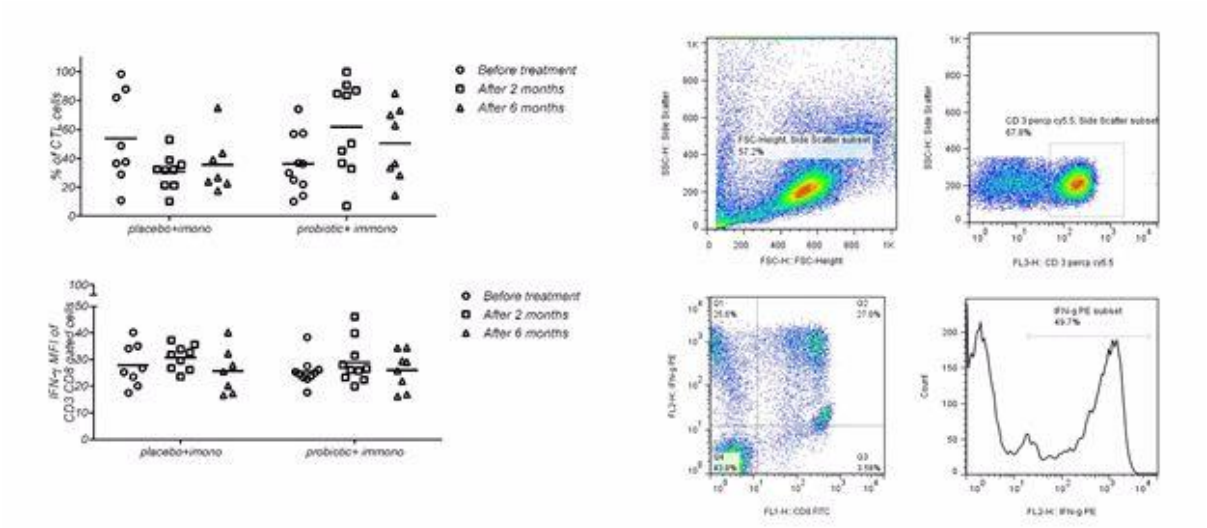

Fig.5: Intracellular expression of INF-gamma in cytotoxic T cells during the intervention course.

All patients in group A and B underwent skin prick test before and 6 months after the intervention. Since all patients received weed mix allergen extract for immunotherapy, skin prick test was repeated for Russian thistle (Salsola kali), Lamb's quarter and Common Pigweed. The student t-test revealed no significant differences in wheal size before and 6 months after treatment for Russian thistle (P= 0.99), and Common Pigweed (P=0.65). However, a statistically significant decrease in the wheal size was recorded for lamb's quarter (P<0.001).

4- DISCUSSION

Due to the increasing prevalence of allergic diseases and their impact on human life, especially on the quality of life, the discovery of novel therapeutic approaches with higher efficacy and minimal side effects are highly anticipated. In the current study we investigated the possibility of improvement in allergic rhinitis with the use of synbiotics and its synergistic effect with subcutaneous allergen specific immunotherapy. A significant difference was achieved for Th1 cell percentage between the two groups (group A, Immunotherapy+ synbiotic), and (group B, Immunotherapy+ placebo). Therefore, it could be suggested that probiotics plus immunothearpy can induce faster enhancement in Th1 cells. This finding was in agreement with Van Overtvelt et al.'s study in 2010 which investigated the immunomedulatory properties of 11 strains of lactic acid bacteria and their capacity to enhance sublingual immunotherapy efficacy in a murine asthma model. *L. helventicus* skewed CD4+ T cells toward IFN-gamma and IL-10 gene expression (a Th1 and possibly T reg profile) whereas *L. casei* only elicited Th1 differentiation (18). In another study in 2012 on murine models of sublingual immunotherapy, *B. bifidum* was found to be able to establish Th2 responses

toward the Th1/regulatory T cell profile and can be a valid adjuvant candidate for allergen specific immunotherapy (19). Our synbiotic product contained *L. casei* which may be the cause for the similar results in both Th1 enhancement and potentiating tolerance induction in immunotherapy. Considering that all the patients enrolled in this study were administered antihistamines and intranasal corticosteroid spray and were also recommended to avoid allergens; as we expected, a significant improvement was seen in symptom scores and quality of life scores before and after treatment in both groups. These results were in agreement with the findings of the clinical trial conducted by Rossi et al., in Italy (2016); they investigated the safety of co-treatment of SLIT and probiotics and their positive effects on allergic symptoms (24); 30 patients with allergic rhinitis undergoing SLIT, randomly received probiotic or placebo and were evaluated after 2 and 4 months. The intervention group received an industrial combination of *Lactobacillus rhamnosus* LR05, *Bifidobacterium lactis* BS01 and FOS.

This combination was administered 14 days before the first ITS sublingual administration and then continued for 4 months thereafter. In this study improvement in symptoms score occurred in both groups with a greater decrease in the SLIT + probiotic group, although it did not reach statistical significance. There was also a significant reduction in the medication score and a greater number in well days. In a similar study, Irmawati et al., investigated the role of SLIT and probiotics or probiotics alone on clinical parameters of childhood asthma such as symptoms score, medication score and FEV1 reversibility (20). This clinical trial was performed on 6 to 17-year-old asthmatic children. Forced expiratory volume during 1st second (FEV1) reversibility, symptoms score, and medication score improved in the three groups, although not statistically significant the most marked improvements were FEV1 reversibility and decrease in symptoms and medication scores in the probiotic groups (20). In both the above mentioned studies (19, 24), and in our study, improvement in symptoms score was observed, yet not statistically significant. This might be explained by the fact that medical treatment in allergic rhinitis is very successful and a large sample size is needed to confirm the efficacy level of a new intervention. This may also be due to the administered probiotic strains, dose and duration of intervention. In 2016, Jerzynska et al., investigated the clinical and immunologic efficacy of SLIT with and without probiotics or vitamin D supplementation. They observed a significant diminution in symptoms score and medication score in study groups that received SLT compared to the control group (21).

Kardani et al., performed a clinical trial in Indonesia in 2013 to evaluate the therapeutic efficacy of immunotherapy combined with probiotics and Nigella sativa on the number of Th17 cells and the improvement of clinical symptoms of asthma in 31 children with mild asthma. In the 14-week follow up, asthma control test (ACT) score showed a significant improvement in groups that received combination of immunotherapy with either or both Nigella sativa or probiotics (22). The discrepancies in the findings of the above mentioned studies and our study may be due to the duration of probiotic administration, different strains used and/or different routes of allergen immunotherapy. Activated T cells and their products play an important role in the pathogenesis of allergic diseases. There are two types of T CD4+ cells; those that preferentially secrete IL-4 (Th2), and those that preferentially secrete IFN- gamma (Th1). Among the very early to immediate

effects of allergen specific immunotherapy is the production of allergen specific T regulatory cells and the suppression of allergen specific Th1 and Th2 cells (8). In current study, the percentage of CD4+, CD25+, CD127- , FOXP3 +, T regulatory cells before and 2 and 6 months after treatment were measured by flow cytometry. A significant difference was observed neither within nor between the two groups. However, FOXP3 expression in T regulatory cells was enhanced in our study and its enhancement within groups was significant; whereas there was no significant difference between the two groups. In addition, these cells decreased during treatment which is in contrast to other studies stating that the induction of T regulatory cells occur within the first days of specific allergen immunotherapy (23).

Moussu et al., evaluated the enhancement of clinical efficacy of sublingual immunotherapy by three strains of Bifidobacterium as an adjuvant candid. They showed that *B. bifidium* can induce CD4+, $CD25^{high}$ FOXP3+, and T cells. The results of this study were similar to a previous clinical trials which demonstrated that *Lactobacillus plantarum* and *Lactobacillus helventicus* can induce the production of high levels of IFN-gamma and IL-10 in asthmatic mice under SLIT (24). The diversities in the results of different studies can be described by the various strains of bacteria administered or the different routes of allergen specific immunotherapy. As a hypothesis, the synergistic effects of probiotics in enhancing immunotherapy efficacy may occur only when both of them are administered orally to the gut associated lymphoid tissue. These results were similar to the study by Jerzynska et al. which showed that the enhancement in T regulatory cells percentage was significantly greater in the SLIT + probiotic group compared to the SLIT group alone (21).

One of the limitations of this study was the small sample size and short duration of the study due to financial retrains. Regarding the promising results observed in the current study clinical trials with a larger sample size and/or longer treatment course with probiotics are thus highly recommended. Regarding the enhancement of Th1 cells percentage during probiotic consumption, it seems that this agent can be administered as an adjuvant in allergic rhinitis patients undergoing subcutaneous allergen specific immunotherapy, thus improving treatment efficacy although its impact on symptoms score and quality of life was not remarkable in our clinical trial.

4-1. Study Limitations

This study had limitations as follows:

- Patients' reluctance to participate in this project
- Discontinuation of patient collaboration throughout project
- Drug interactions
- Low number of samples and
- Dangerous side effects occur throughout project.

5- CONCLUSION

Based on the results, adding synbiotic to subcutaneous allergen immunotherapy increases the percentage of T helper type 1 cells. Regarding enhancement in T helper type 1 cell percentage during probiotic consumption, it seems that this agent can be administered as adjuvant in allergic rhinitis patients undergoing subcutaneous allergen specific immunotherapy and thus improve treatment efficacy.

6- ABBREVIATIONS

ISAAC: International Study of Asthma and Allergies in Childhood study
AIT: Allergen Immunotherapy
Th: T helper
AD: Atopic Dermatitis
SCIT: Subcutaneous Allergen Specific Immunotherapy

Mini RQLQ: Mini Rhinoconjectivitis Quality of Life Questionnaire
SNOT-22: Sino-Nasal Outcome Test
IL-4: Interlukin-4
IFN-gamma: Interferon-gamma
FOXP3: Forkhead Box P3
CTL: Cytotoxic T lymphocyte
CD: Cluster of Differentiation
SD: Standard Deviation
ANOVA: Analysis Of Variance
FEV1: Forced Expiratory Volume during 1st second.

7- ACKNOWLEDGEMENTS

The authors would like to thank the research Council of Mashhad University of Medical Sciences for supporting the study financially.

8- CONFLICT OF INTEREST

The authors certify that they have No affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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