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Immunomodulatory effect of pleuran (β-glucan from *Pleurotus ostreatus*) in children with recurrent respiratory tract infections

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ABSTRACT

Objectives: Recurrent respiratory tract infections (RRTIs) represent a very important problem in daily clinical practice because of their significant contribution to morbidity in children. Several natural nutritional supplements have been used in the prevention of RRTIs, but the clinical efficacy of only a few preparations is supported by scientific evidence.
Materials and methods: In a double-blind, placebo-controlled, randomised, multicentre study, we have observed a group of 175 children (aged 5.65 ± 2.39 years) with more than 5 respiratory infections that occurred during the 12 months prior to the beginning of the study. Children were randomised into an active group, treated with Imunoglukan P4H® syrup (with pleuran-β-glucan from *Pleurotus ostreatus* and vitamin C), or a placebo group (vitamin C only). During the 3 visits, within a 12-month period, questionnaires were completed, and blood samples were examined for immune parameters.
Results: In the active group, 36% of the children did not suffer from any respiratory infections throughout the treatment, compared to 21% in the placebo group ($p<0.05$). Imunoglukan P4H® also significantly decreased the frequency of flu and flu-like disease and the number of lower respiratory tract infections. Imunoglukan P4H® treatment resulted in a statistically significant modulation of humoral and cellular immunity.
Conclusions: Results from this study demonstrate that Imunoglukan P4H® is effective in the prevention of RRTIs in children. Furthermore, our results also revealed complex immunomodulatory activity of this product. This is the first double-blind, placebo-controlled study in children with RRTIs that has addressed the preventive effects of pleuran on morbidity caused by respiratory infections.

1. Introduction

Physicians are challenged by the difficulty in diagnosing and treating recurrent respiratory tract infections (RRTIs). According to the epidemiological studies, approximately 6% of children younger than 6 years of age suffer from RRTIs. In developed countries, up to 25% of children less than 1 year of age and 18% of children between the ages of 1 to 4 years experience RRTIs [1]. These figures have increased the awareness of RRTIs among the medical community.

The definitions of RRTIs are too arbitrary, too generic, or too restrictive. However, at least one of the following criteria has to be present to diagnose an RRTI: ≥6 respiratory infections per year; ≥1 respiratory infection per month involving the upper airways from September to April; or ≥3 respiratory infections per year involving the lower airways [2]. Thus, physicians must determine whether the high morbidity caused by respiratory infections in normal children is related to the physiological immaturity of the immune system. Physicians must also assess whether increased exposure to environmental risk factors alters other underlying pathological conditions (immunological or not), thereby predisposing these children to respiratory infections [3,4]. The majority of children with RRTIs do not have recognised immunodeficiencies, but some children may have low levels of certain immunological parameters, such as reduced levels of immunoglobulin isotypes. However, a number of the observed immunological alterations are of questionable significance and may not be related to the increased susceptibility to respiratory infections [5].

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The management of children with RRTIs consists of excluding other pathological conditions that result in respiratory infections by administering the appropriate treatment. Children diagnosed with classical RRTIs are usually treated with immunomodulation. Many available preparations have a potential or confirmed immunomodulatory effects to prevent and treat respiratory infections in children. However, the therapeutic efficacy of only a few of these preparations has been evaluated in controlled clinical trials.

In our previous open label study, we investigated the effect of Imunoglukan P4H® (pleuran, insoluble β-glucan isolated from *Pleurotus ostreatus* combined with vitamin C) in a group of children suffering from RRTIs [6]. β-Glucans possess immunomodulatory activity in both non-specific and specific arms of immune response [7]. The use of pleuran resulted in increased NK-cell number [8,9], stimulated phagocytic activity and enhanced post-vaccination antibody production [10]. Vitamin C, which is also contained in the syrup, has confirmed several immunomodulatory effects, either on the stimulation of lymphocytes proliferation [11] and phagocytic activity [12], on the production of different cytokines (*e.g.* interferon gamma) [13,14] or through the stimulation of cellular immunity [15]. The combination of pleuran and vitamin C therefore could provide beneficial simultaneous immunomodulatory activity, resulting in a synergistic clinical effect. Our previously published open clinical trial confirmed the effect of Imunoglukan P4H® on the decline of respiratory morbidity in children. There were no side effects observed during this trial [6]. Based on these promising results, we have designed a randomised, multicentre, double blind, placebo-controlled study, in which we investigated the preventive clinical effect and immunomodulatory activity of Imunoglukan P4H® (pleuran, insoluble β-glucan isolated from *P. ostreatus* combined with vitamin C) in the group of children suffering from RRTIs. As we have observed a vulnerable population of children, mainly for ethical reasons, we have decided to use an active placebo in the form of vitamin C.

2. Materials and methods

This study enrolled children (n = 175) between the ages of 2 and 10 years with a history of RRTIs. Children between the ages of 2 and 5 years and between the ages of 6 and 10 years were diagnosed with >5 and >3 respiratory infections each year, respectively. Patients with a history of serious internal disorders were not able to participate in the study and were excluded. Furthermore, patients who were treated with other immunomodulators or with antibiotics 14 days prior to enrolment were also excluded.

2.1. Study design

This clinical trial was a randomised, double-blind, placebo-controlled study conducted in 19 Paediatric Departments of Allergy and Immunology across Slovakia and the Czech Republic between February 2009 and October 2010. The study was approved by the Ethical Committees of Slovak Medical University and St. Elizabeth's Oncology Institute and was conducted under Good Clinical Practice regulations. The parents or legal guardians of the children received detailed information regarding the study protocol and subsequently signed consent forms.

Children were randomised into two different treatment groups, which received either Imunoglukan P4H® syrup (n = 81) or a placebo (n = 94). Demographic and basic characteristics of both therapeutic groups were completed using a standard questionnaire. Children that met the inclusion criteria were required to take 1 mL per 5 kg of Imunoglukan P4H® syrup (10 mg of pleuran and 10 mg of vitamin C in 1 mL of syrup) or placebo (10 mg of vitamin C in 1 mL of syrup) every morning on an empty stomach for 6 months. There were no visible differences between the placebo and Imunoglukan P4H® vials. The active substance of the administered natural product was extracted by unique and patented technology from the *P. ostreatus*. The active substance within this natural product was previously isolated, identified and chemically characterised by Karacsonyi and Kuniak [16]. This natural product is registered and distributed in 16 European and non-European countries and is endotoxin free. The testing for toxicity was performed by the Institute of Preventive and Clinical Medicine of Slovak Medical University (Final Report No. E-51/05) and the tests were performed in compliance with the criteria of the Directive of Good Laboratory Practice and Directive 2004/10/EC of the European Parliament and the Council of 11th February 2004.

Subjects were examined before the administration of Imunoglukan P4H® or placebo (V1), 6 months after the administration (V2), and after 6 months without treatment (V3). At the end of each period, venous blood samples (two vacutainer tubes) were collected. Patients were monitored using basic laboratory and immunological parameters, which included glycaemia and specific IgE antibodies against a standardised panel of inhalant and food allergens. The number and duration of infections and antibiotic therapies was also evaluated throughout the study. Self-reporting of RRTI symptoms, before and after the supplementation period, was used in this study. A validated health questionnaire, which addressed the overall health status and RRTI symptoms, was also administered and included questions about the potential side effects. All adverse events were documented throughout the study. Investigators' terms of adverse events were coded using the Medical Dictionary for Regulatory Activities.

Of the 175 children enrolled into the study, 10 children (5.7%) withdrew as a result of non-compliance and 7 children (4.0%) participated in only one of the three visits. Of the initial 175 children, 158 children (90.3%) successfully completed the study. In total, 17 children were excluded from the study because of missing or incomplete medical records, with a similar distribution between the active and placebo treatment groups.

2.2. Blood sampling

Venous blood samples were drawn from a peripheral arm vein, collected into an evacuated tube and treated with either EDTA or sodium heparin. White and red components of the blood count were examined by sampling with an 18-parameter haematological analyser Bayer Advia 60 (Siemens AG, Munich, Germany) using the company's reagents.

2.3. Serum studies

IgE antibodies specific to common aeroallergens were measured by performing a solid-phase immunoassay (Frima, Stat). The levels ≥0.35 IU/mL were considered positive and indicated the atopic status of the examined subject. Serum glycaemia and concentrations of the four immunoglobulin isotypes (IgG, IgA, IgM and IgE) were also examined.

2.4. Subpopulations of leukocytes and lymphocytes

Differential counts of leukocytes, as well as subpopulations of lymphocytes and NK cells, were assessed immediately after sampling. Cells were counted on the flow cytometer FC500 (Beckmann Coulter, Brea, CA, USA) after staining with monoclonal antibodies (Immunotech, Prague, Czech Republic) according to the manufacturer's instructions. A four-colour fluorescent protocol was used with the following composition: $CD4^+CD19$-FITC/$CD8^+CD16^+CD56$-PE/CD3-PC5/CD45-PC7. The absolute counts of all cell populations were calculated from the examined blood count. The following populations, based on the $CD45^+$ leukocyte gate, were quantified from the CD4 *vs.* side scatter (SS) cytogram: lymphocytes (low SS), T lymphocytes ($CD3^+$), T helpers ($CD3^+CD4^+$), T cytotoxic cells ($CD3^+8^+$), B lymphocytes ($CD19^+$) and NK cells ($CD3\text{-}8^+16^+56^+$).

Absolute and relative counts of NK cells were also examined on the cytometer using separate analysis parameters (CD8-FITC/CD16$^+$ CD56PE/CD3PC5/CD45PC7).

2.5. Statistical analysis

The results are presented as mean values ± standard deviation (SD). Data were analysed with the software package SPSS version 9.0 (SPSS Inc. Chicago, IL, USA). Student's two-tailed *t*-test, Student's paired test, and the chi-squared test were used for statistical comparisons of the parametric data. Non-parametric data were analysed using Mann–Whitney test and Fisher's exact test. *p* values lower than 0.05 were considered to be statistically significant.

3. Results

The demographic characteristics of the studied population are presented in Table 1. The two groups of the studied children did not differ in gender, age, mean weight, or number of upper respiratory tract infections (URTIs) in the previous 12 months or proportion of atopic children (Table 1). The number of children younger than 5 years of age was similar in both groups, with 52 (55.3%) and 47 (58.0%) in the active and placebo treatment groups, respectively ($p = 0.761$).

Active treatment resulted in a significant reduction in respiratory morbidity. Throughout the treatment period, 36% and 21% of the children in the active and placebo groups, respectively, did not suffer from any respiratory infections ($p<0.05$) (Fig. 1). Imunoglukan P4H® also significantly decreased the number of flu and flu-like diseases, as well as the frequency of lower respiratory tract infections in the active group compared to the placebo group (0.20 ± 0.55 per 12 months *vs.* 0.42 ± 0.78 per 12 months, $p<0.05$). In this double-blind trial, the paediatricians evaluated the effect of the treatment in the active group as very good (significant reduction of morbidity) in 66.0% of children compared to 38.3% in the placebo group ($p<0.001$, Table 2). The evaluation mark "good effect" of the treatment (mild reduction of respiratory infections) was more frequent in the placebo group (44.4% *vs.* 27.7%, $p = 0.021$, Table 2). However, total clinical efficacy, which was expressed by the summary of the evaluations "very good effect" and "good effect", clearly favoured the use of the active substance — pleuran (93.6% *vs.* 82.7%, $p = 0.024$, Table 2).

At the beginning of the study, results obtained from the various examined parameters did not differ between the children treated with either the active or the placebo substances. The active and placebo groups did not show any differences in specific humoral and cellular immunity parameters, as well as NK cells, at the beginning of the treatment (Table 3).

Imunoglukan P4H® showed a significant influence on the specific humoral immunity parameters (Table 4). In both the active and placebo groups, we observed a decline in the absolute number of CD19$^+$ B lymphocytes during the 12 months of the study. In the active group, the concentration of immunoglobulin G increased during the treatment period and remained heightened throughout the duration of the study. In the placebo group, we did not detect any significant changes in this parameter. In both treatment groups, we observed a gradual increase in the concentration of immunoglobulin A from the first to the third visit, but the differences were higher in the active group. During the treatment period, children treated with the active substance had an increase in the concentration of immunoglobulin M during the first 6 months, and the levels remained stable throughout the next 6 months of follow-up. In the placebo group, there were no changes in IgM concentration.

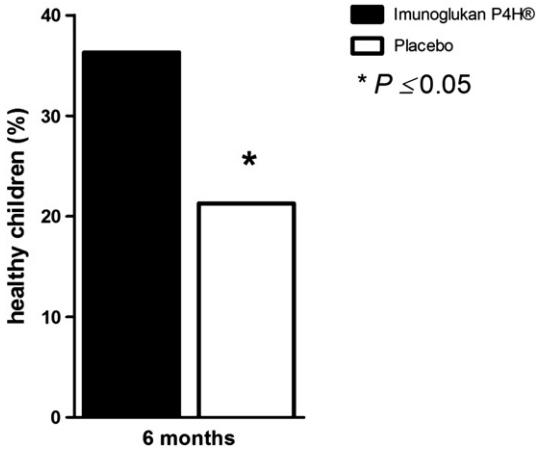

Fig. 1. The proportion of the subjects without any respiratory infections throughout the treatment period in active and placebo groups.

Treatment with Imunoglukan P4H® also caused changes in specific cellular immunity and in NK cells (Table 5). The absolute number of CD3$^+$, CD4$^+$ and CD8$^+$ T lymphocytes gradually declined throughout the study in both groups. However, treatment with Imunoglukan P4H® prevented the decline in CD8$^+$ T cytotoxic lymphocytes, and the numbers remained stable throughout the study without any over-stimulation of this cellular subpopulation. During the treatment period, in the active group, we observed an increase of absolute number of NK cells, which returned to the initial value by the end of the study. The placebo treatment did not cause any changes in the NK cell number.

The treatment was well tolerated, and none of the participants withdrew from the trial as a result of side effects or intolerability. The tolerability of Imunoglukan P4H® was the same as the placebo. We did not find any significant adverse effects related to the treatment.

4. Discussion

In our double blind, placebo-controlled clinical trial, we investigated the clinical efficacy and immunomodulatory effect of a natural immunomodulator, Imunoglukan P4H®. Our results showed a significant decline in respiratory morbidity, including a decrease in the number

Table 1
Demographic characteristics of the studied populations.

Parameter	Whole group	Active group	Placebo group	*p*
Number	175	94	81	–
Males	97 (55.4%)	54 (57.4%)	43 (53.1%)	n.s.
Females	78 (44.6%)	40 (42.6%)	38 (46.9%)	n.s.
Age [years]	5.65 ± 2.39	5.78 ± 2.53	5.51 ± 2.22	n.s.
Weight [kg]	27.08 ± 25.19	27.52 ± 27.24	26.54 ± 22.61	n.s.
Atopy	76 (43.4%)	44 (46.8%)	32 (39.5%)	n.s.
Number of RTIs/12 months before the study	6.53 ± 2.34	6.27 ± 2.10	6.86 ± 2.58	n.s.

n.s. — non-significant, RTIs — respiratory tract infections.

Table 2
Evaluation of the treatment effects, according to the paediatricians, in a double-blind study design.

Evaluation of the treatment effect	Active group	Placebo group	*p*
Very good effect (significant reduction of respiratory morbidity)	62 (66.0%)	31 (38.3%)	<0.001
Good effect (mild reduction of respiratory morbidity)	26 (27.7%)	36 (44.4%)	0.021
Very good + good effect (general reduction of respiratory morbidity)	88 (93.6%)	67 (82.7%)	0.024
None effect (without changes in respiratory morbidity)	6 (9.3%)	14 (17.3%)	0.024

Table 3
The inter-group comparison of selected immune parameters at the beginning of the study.

Parameter	Active group	Placebo group	*p*
Immunoglobulin G [g/L]	9.12 ± 2.39	8.70 ± 2.44	0.262
Immunoglobulin A [g/L]	1.04 ± 0.57	1.03 ± 0.53	0.905
Immunoglobulin M [g/L]	0.98 ± 0.42	1.00 ± 0.45	0.777
Immunoglobulin E [IU/L]	84.05 ± 109.74	62.99 ± 101.47	0.214
$CD3^+$ T-lymphocytes [%]	71.15 ± 7.42	69.97 ± 6.15	0.263
$CD3^+$ T-lymphocytes [10^9/L]	2.39 ± 0.86	2.41 ± 0.79	0.956
$CD4^+$ T-lymphocytes [%]	39.52 ± 7.27	38.77 ± 7.20	0.498
$CD4^+$ T-lymphocytes [10^9/L]	1.34 ± 0.52	1.36 ± 0.49	0.823
$CD8^+$ T-lymphocytes [%]	25.93 ± 6.48	24.79 ± 5.66	0.225
$CD8^+$ T-lymphocytes [10^9/L]	0.89 ± 0.37	0.87 ± 0.36	0.710
$CD19^+$ B-lymphocytes [%]	16.29 ± 5.14	17.47 ± 5.12	0.128
$CD19^+$ B-lymphocytes [10^9/L]	0.57 ± 0.31	0.62 ± 0.29	0.297
$CD15CD56^+$ NK cells [%]	11.19 ± 8.79	10.26 ± 5.98	0.428
$CD15CD56^+$ NK cells [10^9/L]	0.33 ± 0.21	0.34 ± 0.20	0.733

of RRTIs in the active group. Imunoglukan P4H® treatment also resulted in complex immunomodulatory activity on innate and adaptive immunity. The increase in all three immunoglobulin isotypes demonstrated that Imunoglukan P4H® treatment supported the physiological maturation of the humoral immune response. Moreover, Imunoglukan P4H® slowed down the decline of T-cytotoxic lymphocytes, and also caused the increase in the NK cell number. During the study, we did not detect any relevant adverse effects (clinical or laboratory) due to the active treatment with Imunoglukan P4H®. To the best of our knowledge, our study is the first randomised, placebo-controlled clinical trial that studied the effects of pleuran (β-glucan from *P. ostreatus*) on respiratory morbidity and on selected immune parameters in children with RRTIs.

Children with RRTIs represent a great challenge for physicians, from both a therapeutic and preventive point of view. In recent years, with the increased incidence of antibiotic resistance, the interest in preventive treatment has intensified. Several immunomodulators are available for the prevention of RRTIs. Metabolites and components of medicinal mushrooms have been used in medicine for many centuries. β-Glucans, a heterogeneous group of glucose polymers, are biologically active polysaccharides that are responsible for the observed clinical efficacy of mushroom extracts. β-Glucans appear to be an important, interesting group of natural immunomodulating substances, which are associated with a low risk of side effects. Furthermore, our results also demonstrate that Imunoglukan P4H® is also effective and safe in children. There are only a few studies that address the potential preventive effects of β-glucans on RRTIs. In another multicentre, open clinical trial, Imunoglukan P4H® had a similar effect on the course and frequency of RRTIs [6]. A positive response to the treatment (≥50% reduction of the frequency of RRTIs) was observed in 71.2% of the children enrolled in this study. The average annual incidence of respiratory infections in children with the positive response to the treatment was 3.6, which was significantly lower compared to unresponsive patients (3.6 *vs.* 8.9, $p<0.001$) [6]. The effect of β-glucans on the frequency of respiratory infections was also studied in several trials in groups of athletes. Pleuran resulted in a similar clinical efficacy, as respiratory infections were decreased during and after intensive training [8]. Talbott and Talbott (2009) conducted a study in marathon runners treated with insoluble yeast β-glucan and observed a significant reduction in the incidences of upper respiratory tract infections (URTIs) in the active group [17]. In a recent clinical trial, treatment with yeast β-glucan decreased the total number of days with URTI symptoms [18]. Another study evaluated the efficacy of a topical medical device, based on colloidal silver and carbossimetyl β-glucan, in a group of 100 children with viral rhinitis. Treatment with topical β-glucan resulted in a significant improvement of the global symptomatic score compared to children treated with a saline solution [19]. Conversely, in the study by Nieman et al. (2008), application of a soluble oat β-glucan prevented neither the incidence of infections nor the exercise-induced changes in the immune system. However, in this study, the treatment period was shorter and they used soluble cereal β-glucan [20]. It has been suggested that the full clinical immunomodulation effect of β-glucans can be observed after several weeks of application. The effect is more evident with insoluble β-glucan treatment, which acts on the immune cells located within the Peyer's patches of the gut. In the literature, no other clinical trials have investigated the effect of other β-glucans on the incidence of respiratory tract infections.

The recognition and response to β-glucans is mediated primarily by cell surface receptors. To date, four receptors have been identified, which include scavenger, complement receptor 3, lactosylceramide, and more recently, dectin-1 receptors [21]. The structure of β-glucans consists of non-cellulosic polymers of β-glucose with glycosidic bonds in the β(1–3) position and with a certain proportion of β(1–6) bound glucose molecules. This unique branched structure is responsible for its biological activity [16,22]. Other studies have demonstrated that the isolation and purification procedures influenced the activity of β-glucans. Thus, different studies, using various sources and forms of β-glucans, yielded different results [16]. Large molecular weight β-glucans (*e.g.*, pleuran) appear to activate leucocytes directly. Their phagocytic, cytotoxic and anti-microbial activities are mediated by production of reactive intermediates, pro-inflammatory mediators, cytokines, and chemokines [23]. Regardless of the parenteral or peroral

Table 4
The changes in the specific humoral immunity parameters during the study.

Parameter	Visit 1	Visit 2	Visit 3
Active Imunoglukan group			
$CD19^+$ B-lymphocytes [%]	16.29 ± 5.14	16.16 ± 4.96	15.96 ± 4.61
$CD19^+$ B-lymphocytes [10^9/L]	0.57 ± 0.31¶¶¶	0.56 ± 0.27†††	0.48 ± 0.22
Immunoglobulin G [g/L]	9.12 ± 2.39$^{***, ¶¶}$	9.44 ± 2.48	9.36 ± 2.42
Immunoglobulin A [g/L]	1.04 ± 0.57$^{**, ¶¶¶}$	1.17 ± 0.66	1.23 ± 0.64
Immunoglobulin M [g/L]	0.98 ± 0.42**	1.04 ± 0.42	1.00 ± 0.37
Placebo group			
$CD19^+$ B-lymphocytes [%]	17.47 ± 5.12	16.99 ± 5.05	15.69 ± 4.80
$CD19^+$ B-lymphocytes [10^9/L]	0.62 ± 0.29	0.67 ± 0.56†††	0.49 ± 0.21
Immunoglobulin G [g/L]	8.70 ± 2.44	9.05 ± 2.21	8.83 ± 2.14
Immunoglobulin A [g/L]	1.03 ± 0.53¶¶¶	1.09 ± 0.49†	1.17 ± 0.59
Immunoglobulin M [g/L]	1.00 ± 0.45	1.06 ± 0.44	1.01 ± 0.35

Comparison among the three visits:
Visit 1 *vs.* Visit 2: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.
Visit 1 *vs.* Visit 3: $^{¶}p<0.05$, $^{¶¶}p<0.01$, $^{¶¶¶}p<0.001$.
Visit 2 *vs.* Visit 3: $^{†}p<0.05$, $^{††}p<0.01$, $^{†††}p<0.001$.

Table 5
The changes in the specific humoral immunity and NK cell parameters during the study.

Parameter	Visit 1	Visit 2	Visit 3
Active Imunoglukan group			
$CD3^+$ T-lymphocytes [%]	71.15 ± 7.42	70.19 ± 7.70	70.97 ± 7.31
$CD3^+$ T-lymphocytes [10^9/L]	2.39 ± 0.86¶¶	2.38 ± 0.88†	2.18 ± 0.72
$CD4^+$ T-lymphocytes [%]	39.52 ± 7.27	38.86 ± 7.24	39.13 ± 8.10
$CD4^+$ T-lymphocytes [10^9/L]	1.34 ± 0.52¶¶¶	1.33 ± 0.51††	1.19 ± 0.42
$CD8^+$ T-lymphocytes [%]	25.93 ± 6.48	26.26 ± 5.82	26.91 ± 6.62
$CD8^+$ T-lymphocytes [10^9/L]	0.89 ± 0.37	0.89 ± 0.37	0.85 ± 0.39
$CD15CD56^+$ NK cells [%]	11.19 ± 8.79	11.62 ± 7.22	10.42 ± 5.91
$CD15CD56^+$ NK cells [10^9/L]	0.33 ± 0.21*	0.39 ± 0.31††	0.33 ± 0.23
Placebo group			
$CD3^+$ T-lymphocytes [%]	69.97 ± 6.15	69.58 ± 6.10	70.48 ± 5.93
$CD3^+$ T-lymphocytes [10^9/L]	2.41 ± 0.79¶	2.38 ± 0.64†	2.17 ± 0.59
$CD4^+$ T-lymphocytes [%]	38.77 ± 7.20	38.73 ± 6.75	39.60 ± 6.63
$CD4^+$ T-lymphocytes [10^9/L]	1.36 ± 0.49¶	1.32 ± 0.42†	1.21 ± 0.35
$CD8^+$ T-lymphocytes [%]	24.79 ± 5.66	24.73 ± 5.42	24.69 ± 5.79
$CD8^+$ T-lymphocytes [10^9/L]	0.87 ± 0.36¶¶	0.83 ± 0.26†	0.75 ± 0.28
$CD15CD56^+$ NK cells [%]	10.26 ± 5.98	11.11 ± 4.86	11.57 ± 5.26
$CD15CD56^+$ NK cells [10^9/L]	0.34 ± 0.20	0.37 ± 0.21	0.41 ± 0.36

Comparison among the three visits:
Visit 1 *vs.* Visit 2: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.
Visit 1 *vs.* Visit 3: $^{¶}p<0.05$, $^{¶¶}p<0.01$, $^{¶¶¶}p<0.001$.
Visit 2 *vs.* Visit 3: $^{†}p<0.05$, $^{††}p<0.01$, $^{†††}p<0.001$.

administration route, β-glucans influenced the immune system. Furthermore, β-glucans are also a useful tool to prime the host immune system, thereby increasing resistance against invading pathogens [24]. According to the published studies, β-glucans influence both the nonspecific and specific arms of the immune response, but these effects were mostly observed *in vitro*. Although the observed increase of B-lymphocytes was also confirmed in another study [25], our finding that all three immunoglobulin isotypes were increased is novel and has not been previously reported. The effect of pleuran on the function and markers of innate immunity was studied in several recent studies. After three months of the supplementation of pleuran in regularly training elite athletes, no reduction of the phagocytic function was found within the pleuran group, whereas a significant reduction of phagocytosis was observed in the placebo group ($p = 0.01$). Moreover, we have confirmed a significant increase in the pleuran group after three months of supplementation and additional 3 months of non-supplementation compared to the baseline value. No changes in the number of NK cells were measured in the placebo group [8]. The influence of pleuran on NK cells was also noted by other investigators [9,20] and our study confirmed the significant effect of pleuran on the NK cell numbers in children with RRTIs. Another animal study with pleuran showed the significant influence of this β-glucan on the functions and metabolic activities of phagocytic cells [10]. In addition, a recent clinical trial demonstrated that β-glucans have the ability to induce specific cellular immune responses in HIV-infected patients [26]. In our study, we tested the efficacy of vitamin C (used as active placebo) and of the combination of pleuran with vitamin C on the respiratory morbidity and immune parameters in the children with recurrent respiratory tract infections. Vitamin C has some confirmed immunomodulatory activities, either on the stimulation of lymphocyte proliferation [11] and phagocytic activity [12], on the production of different cytokines (*e.g.* interferon gamma) [13,14], or through the stimulation of cellular immunity [15]. However, most randomised placebo-controlled studies have been unable to clearly demonstrate that vitamin C by itself has the capacity to prevent respiratory tract infections [27]. The combination of pleuran and vitamin C may have a beneficial effect on the prevention of respiratory tract infections and on the immunomodulatory effect. This possible synergic effect merits further investigation.

The results provide new insight into the complex immunomodulatory effect of pleuran in children. Furthermore, our study supports the use of pleuran in the prevention of respiratory infections. The complex immunomodulatory activity of pleuran promotes the physiological maturation of the immune system in preschool children without any risk of over-stimulation of the maturating immune response and without the development of immunopathological conditions.

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