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Clinical Pediatrics: Open Access

Research Article

Prebiotics and Probiotics: New Adjuvant Therapies for Children with Idiopathic Relapsing Nephrotic Syndrome

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ABSTRACT

Background: Idiopathic Nephrotic syndrome (INS) is an autoimmune disease characterized by repeated relapses with allergic conditions. The etiology remains unknown, new evidence correlates to the dysfunction of T Regulatory cells (T-Reg) which could be due to gut microbiota dysbiosis.

Aim: To investigate the effect of prebiotics and probiotics as adjuvant therapies for children with relapsing idiopathic nephrotic syndrome.

Methods: The study was designed as a prospective open label randomized clinical trial; involving 30 children diagnosed as relapsing NS. Children were randomly divided into two groups, group treated with prednisone only and group treated with prebiotics and probiotics in addition to prednisone. Fresh stool samples were collected from the children. *Lactobacillus* species were isolated and identified by conventional microbiological methods. Counting the total number of *Lactobacillus* species were also performed in each stool sample. The populations of T-reg cells in Peripheral Blood Mononuclear Cells (PBMC) were analyzed using the flow cytometry.

Results: Children treated with prebiotics and probiotics in addition to steroids showed a significant increase in ($CD4^+/CD25^+/FOXp3^+$) T-reg in peripheral blood (p-value<0.0001) and a higher count of *Lactobacilli* species in their stool (p-value<0.003) with a significant decrease in the rate of relapses in this group compared to group 1 (p-value<0.0001).

Conclusion: Treatment with prebiotics and probiotics increases T-reg cells and decreases the rate of relapses of INS significantly.

Keywords: Prebiotics; Probiotics; T-reg; Relapsing idiopathic nephrotic syndrome; Blood mononuclear cells

INTRODUCTION

Nephrotic Syndrome (NS) is one of the commonest pediatric renal diseases, characterized by leakage of protein from the blood into the urine through damaged glomeruli. It is a clinco-laboratory syndrome characterized by nephrotic-range proteinuria (≥ 40 mg/m^2/hour or urine protein/creatinine ratio ≥ 200 mg/mL or 3^+ protein on urine dipstick), hypoalbuminemia (<25 g/L) and generalized edema. The first-line treatment for INS is oral corticosteroids that can decrease mortality (down to 3%) and induce remission in approximately 80% of patients. It has been noticed that between 80% and 90% of children over 1 year of age presenting with NS respond to treatment with steroids within 4 weeks Steroid-Sensitive Nephrotic Syndrome (SSNS) [1]. In children with SSNS, the subsequent course of illness can be quite variable, with the majority of children having at least one episode of relapse and up to 50% having either Frequently Relapsing Nephrotic Syndrome (FRNS) (≥ 2 relapses in first 6 months or ≥ 4 relapses in any 1-year period) or Steroid-Dependent Nephrotic Syndrome (SDNS) (relapse, while on steroid therapy or within 2 weeks of steroid cessation).

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Received: 19-Mar-2024, Manuscript No. CPOA-24-3677; **Editor assigned:** 24-Mar-2024, PreQC No. CPOA-24-3677 (PQ); **Reviewed:** 07-Apr-2024, QC No. CPOA-24-3677; **Revised:** 01-Jun-2024, Manuscript No. CPOA-24-3677 (R); **Published:** 28-May-2024, DOI: 10.35248/2572-0775.24.09.269

Citation: Mohammed RA, Hussein SK, Gaber SN, Ahmed FA, Abdelfattah W, Said ES (2024) Prebiotics and Probiotics: New Adjuvant Therapies for Children with Idiopathic Relapsing Nephrotic Syndrome. Clin Pediatr. 09:269.

Gut dysbiosis may cause a variety of disorders which are characterized by immune alterations and chronic inflammation that affects the gastrointestinal tract and spread to the systemic circulation. Dysbiosis of gut microbiota has been reported in chronic kidney diseases and there is a strong connection between dysbiosis of gut microbiota and INS. The distinct pathogenesis and reasons for relapse of INS stay unknown exactly, though it has been proposed that impaired T-cell function is involved since it was first described by Shalhoub in 1974. Probiotics are live microorganisms that give a health benefit to the host when administered in an adequate amount that was used for treating different pathologies in animal models [2].

MATERIALS AND METHODS

Lactobacillus acidophilus, one of the probiotics, is a natural resident of human gastrointestinal systems, is of significant industrial and medical attention because these species are supposed to show an important role in human health by its impact on the intestinal flora. Prebiotics are "non-digestible food ingredients" that beneficially influence the host by selectively stimulating the growth and/or activity of one or a few numbers of bacteria that enhance the host health. The beneficial effects of probiotics achieved by modulation of the immune system through modification of the gut microbiota, competitive adhesion to both epithelium and mucosa, enhancing of the gut epithelial barrier. For instance, a decreased percentage of ($CD4^+/CD25^+/FoxP3^+$) T lymphocytes, classically known as T regulatory cells (T-regs), were reported in patients with autoimmune diseases [3].

This study was designed for the first time, up to our knowledge, to investigate the effect of prebiotics and probiotics as adjuvant therapies to decrease the rate of relapse in children with relapsing INS. The research ethics committee of Fayoum medical university, Egypt approved the study protocol N: R81. Written informed consents were obtained from the parents. The study was conducted from March 2018 to October 2019. Our study is prospective open label randomized clinical trial. Thirty children with relapsing, steroid-responsive INS with normal kidney function were included in the study from the clinic of the pediatric department at Fayoum university hospital. INS was diagnosed based on edema, a urine protein to creatinine ratio ≥ 300 mg/dL or ≥ 3^+ protein on a urine dipstick and hypoalbuminemia ≤ 2.5 g/dL. Children less than 2 years and more than 12 years old, those with congenital or secondary NS, steroid-resistant or steroid-dependent, children with abnormal kidney function tests, children taking previous antibiotic treatment within the last 2 months of study enrollment, children on anti-inflammatory treatment or immune regulating medications, those taking probiotics prior to enrollment to the study, or children with history of Crohn's disease, inflammatory bowel disease, celiac disease or other malabsorption disorder were excluded from the study. All the patients were successfully treated with prednisolone according to the KDIGO (Kidney Disease: Improving Global Outcomes) guidelines, pediatric nephrology (60 mg/per m^2 or 2 mg/kg day for 4 weeks, gradually reducing the dose through a period of 2-3 months). Remission was defined as normal serum albumin (>3.0 g/dL) and normal urinary protein excretion (dipstick trace or negative for at least 3 consecutive days) [4].

The patients were randomly divided into 2 groups: Group 1 (control group): Given steroid therapy as explained above (n=15), group 2 (treated group): Given prebiotics and probiotics as adjuvant for steroid therapy for 2 months (n=15) during alternate day therapy of steroids and this the 1st time that is used in INS. Drug used: Lactonorm® sachet 5 mg (contain 30% probiotics (each sachet contain 5 billion freeze-dried *Lactobacilli*) and 65% prebiotics). The children were given one sachet during alternate day therapy of steroids. At the end of the treatment course (8 weeks), urine, stool and blood samples were collected from the two groups for analysis. Patients are followed for 18 months for detecting the rate of relapses by routine urine analysis and detection of albuminuria.

Detection of the microbial load of *Lactobacilli* in the stool: Fresh stool samples were collected from the children in tight plastic boxes and kept refrigerated up until referred to the research lab of medical microbiology and immunology department at faculty of medicine, Fayoum university. Within 2-hours, feces were homogenized and 1.0 g was extracted and diluted 1:5 in saline solution (1.0 g of feces+4.0 ml 0.9% saline). The saline diluted samples were then homogenized and diluted again (1:100). From the 1:10 dilution, successive dilutions were performed up to 1. Then one hundred microliters of each the diluted samples were spread onto the surface of de Man Rogosa and Sharpe (MRS) (Oxoid, France) agar plates. Plates were incubated anaerobically at 37°C for 48 and 72 hours. Any rod, aerotolerant, gram-positive, catalase-negative, non-spore-forming, non-motile isolates were regarded as *Lactobacilli*.

Identification of species of *Lactobacilli* was confirmed by biochemical reactions. The number of Colony-Forming Units for each gram (CFU/g) was determined. The number of *Lactobacillus* and total colonies ($\log_{10}$ CFU g^{-1}) developed was calculated for each stool sample [5].

Measurement of T. Regulatory cells (T-regs) in peripheral blood: Peripheral blood T-regs were measured by (FlowXTM human regulatory T cell from R and D systems (bio-techne), catalog number: FMC021). Peripheral blood was obtained in EDTA containing sterile vacutainers. Peripheral Blood Mononuclear Cells (PBMCs) were isolated using lymphocyte separation medium (Ficol Hypaque solution) according to the manufacturer's instructions. PBMCs are kept in RPMI medium overnight then prepared for staining for flow cytometry. The following day, PBMCs (1×10^6 per sample) were washed with 2.0 mL of flow cytometry staining buffer (R and D systems R, Catalog #FC001), by spinning at 300 × g for 5 minutes, using 5.0 mL flow cytometry tubes. 4 µL of CD4-FITC (fluorescein isothiocyanate) and 4 µL of CD25-APC (allophycocyanin) antibodies were added then the mixture incubated for 30-45 minutes at 2°C-8°C in the dark. Cells were washed two times with cold 1X Phosphate-Buffered Saline (PBS), resuspended in fresh 1X FoxP3/transcription factor fixation buffer using 0.5 mL/tube then incubated at 2°C-8°C for 30 minutes. Wash two times was done with fresh, cold, 1X FoxP3 permeabilization and wash buffer. Seven µL of FoxP3 antibody was added to the cells and incubated for 30 minutes at 2°C-8°C. Cells were washed one time with cold 1X FoxP3 permeabilization and wash buffer then resuspended in flow cytometry staining buffer and run on

a flow cytometer. Cells were acquired on FACSCanto 10 (BD biosciences). Data were analyzed using FACS DIVA software [6].

Statistical analysis

The collected data were organized, tabulated and statistically analyzed using SPSS software statistical computer package version 18 (SPSS Inc, USA). For quantitative data, the median and Interquartile Range (IQR) were calculated. Mann-Whitney-U test was used as a test of significance to compare study groups. Regarding clinical data, an independent t-test was used to test the differences between the two groups of patients. A paired t-test was used in comparing the different readings of study variables. For qualitative data: The number and percent distribution was calculated. Spearman correlation was run to identify the relation between the studied parameters. For interpretation of the results of tests of significance, significance was adopted at $P \leq 0.05$.

RESULTS AND DISCUSSION

There was no significant change between the two groups of the study regarding age, mode of delivery and nutritional history in the 1st year of life (Table 1). At the end of the treatment (3 months) with prednisolone in group 1, there was a significant increase in serum albumin, decrease in body weight and serum cholesterol with a significant decrease in urinary Albumin: Creatinine (A/C) ratio and increase in systemic blood pressure [7]. And at the end of treatment with prednisolone (3 months) in addition to probiotics and prebiotics (2 months with prednisolone) in group 2, there was a significant increase in serum albumin, decrease in body weight and serum cholesterol with a significant decrease in urinary (A/C) ratio and increase in systemic blood pressure (p-value<0.0001) (Table 2).

Table 1: Comparing mean age, nutritional history and mode of delivery between the two groups of the study.

	Group 1 (15 patients) (control group)	Group 2 (15 patients) (treated group)	P-value
Age (mean ± SD)	5.9 ± 2	5.4 ± 1.7	0.535
Nutritional history	33% breast feeding, 33% artificial milk, 33% mixed breast and bottle	33% breast feeding, 33% artificial milk, 33% mixed breast and bottle	1
Mode of delivery	77.8% Caesarian Section (CS), 22% normal vaginal deliver	73.8% Caesarian Section (CS), 22% normal vaginal deliver	

Table 2: Clinical and laboratory results of group 1 and group 2 before and after prednisone therapy.

	Group 1			Group 2		
	Before prednisone treatment	After prednisone treatment	P-value	Before prednisone treatment	After prednisone treatment	P-value
Weight (kg)	19.7 ± 3.8	17.7 ± 3.8	<0.0001*	19.5 ± 3.2	17.4 ± 3.1	<0.0001*
Systolic blood pressure (mmHg)	94.4 ± 10.1	111.1 ± 9.3	<0.0001*	95 ± 11.2	111.7 ± 9.9	<0.0001*
Diastolic blood pressure (mmHg)	61.7 ± 9.4	75 ± 7.1	<0.0001*	60.7 ± 9.6	72.7 ± 7.2	<0.0001*
Serum albumin (mg/dl)	2.1 ± 0.4	4.6 ± 0.3	<0.0001*	2.2 ± 0.4	4.6 ± 0.3	<0.0001*
Serum cholesterol	329 ± 37.7	157 ± 15.7	<0.0001*	323.4 ± 27.7	167.5 ± 18.3	<0.0001*
Urinary [a]A/C ratio	713.4 ± 143.8	206.1 ± 25.1	<0.0001*	685.1 ± 123.7	206.6 ± 23.5	<0.0001*

By comparing the two groups of the study at the end of treatment (3 months), there was no significant change between group 1 and group 2 in the body weight, the blood pressure, the kidney functions, serum albumin, serum cholesterol and A/C ratio. While by comparing the two groups as regard the rate of relapses during the follow up for 18 months, there was a significant decrease in the number and frequency of relapses in group 2 which was treated by prebiotics and probiotics in addition to steroids compared to group1 which was treated by steroids only (p-value<0.0001) (Table 3) [8].

Table 3: Comparison of clinical and lab results between the two groups at the end of the treatment.

	Group 1 prednisone therapy only	Group 2 prednisone therapy in-addition to probiotics and prebiotics	P-value
Weight (kg)	17.7 ± 3.8	17.4 ± 3.1	0.853
Systolic blood pressure (mmHg)	111.1 ± 9.3	111.7 ± 9.9	0.893
Diastolic blood pressure (mmHg)	75 ± 7.1	72.7 ± 7.2	0.461
Serum albumin (mg/dl)	4.6 ± 0.3	4.6 ± 0.3	0.971
Serum cholesterol	157 ± 15.7	167.5 ± 18.3	0.165
Urea (mg/dl)	26 ± 4.4	25.4 ± 3.9	0.730
Creatinine (mg/dl)	0.5 ± 0.1	0.5 ± 0.2	0.377
Urinary [a]A/C ratio	206.1 ± 25.1	206.6 ± 23.5	0.962
Number of relapse during follow up for 18 month	3.8 ± 0.8	0.4 ± 0.5	<0.0001*

The microbial load of *Lactobacillus* in the stool: There was a significant increase in the number of *Lactobacilli acidophilus* in stool of patients treated with probiotics and prebiotics with steroids (group 2) compared to patients treated with steroids only (group1) (p-value 0.003) (Table 4).

Level of T-regulatory cells (T-reg) in the peripheral blood: Analysis of T-reg by flow cytometry. There was a significant increase in the number of total lymphocytes in the peripheral blood in (group 2) compared to (group 1), (p-value=0.008). There was a significant increase in the number of CD4 (helper T cells) in group 2 compared to group 1 (p-value=0.001) [9]. There was a significant increase in the percentage of T-reg ($CD4^+/CD25^+/FOXP3^+$) of not from total lymphocytes and of not from total $CD4^+$ cells (both p-value<0.0001). FOXP3 MFI and percentage of not from $CD4^+$ were significantly increased in group 2 compared to group 1, (p-value 0.048 and 0.035 respectively) (Table 4).

Table 4: Median and IQR for the T-regulatory cells in blood and the microbial load of *Lactobacillus* in the stool.

	Group 1			Group 2			P-value
	Median	IQR		Median	IQR		
Lymphocytes %	59.4	50.1	68.3	78	67.4	89	0.008*
CD4%	32	28.2	35.5	44.1	43.2	46.2	0.001*
T-regs% of CD4 (CD4+/CD25+/FOXP3+)	6.1	5.2	8	19	14	22	<0.0001*
T-regs% of whole lympocytes (CD25+/FOXP+-CD4+)	4	1.8	4	8.3	8	9	<0.0001*
FOXP3+/CD4	11.5	7.8	14.7	15.2	13.5	17	0.035*
FOXP3 MFI	890	775	2009	2745	980	18997	0.048*
Lactobacilli in stool	5000	4500	6000	3000000	166000	5000000	0.003*

DISCUSSION

Relapsing nephrotic syndrome in children is a major health problem that needs long term steroid therapy which has multiple side effects such as slowing of growth, weight gain, hypertension and dyslipidemia. The pathogenesis of INS rests unknown. It seems to be due to the imbalance between T helper 1 and T helper 2 cells. Also, Th17 were highly expressed and Th1 exhibited lower expressions. Th2 cytokines (Interleukin (IL)-13, IL-4, IL-5) are augmented in patients with nephrotic syndrome, and changes in vascular permeability have been defined, which is tangled in the pathogenesis of proteinuria. Concluded that T-regs number and serum Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) were significantly suppressed at the onset of INS and glucocorticoid therapy [10]. The suppression of (Treg) leads to massive proteinuria in INS. Treg cells are adaptable inhibitory control of immune responses. Numerous mechanisms of Treg-mediated immune suppression have been proposed like secretion of immunosuppressive cytokines such as Interleukin 10 (IL-10) or transforming growth factor beta, and functional modification or killing of Antigen Presenting Cells (APC). Also, Kaneko et al. suggested that the etiology of INS, in which Minimal Change Nephrotic Syndrome (MCNS) accounts for the majority of cases, has been attributed to dysregulated T cells. It has been proved that microbiome composition is strongly associated with the development of allergic and immunological diseases which are more frequently observed in children who have a less diverse gut microbial flora in their early life. Studies hypothesized that one cause of relapse in INS patients is related to dysbiosis of gut microbiota; in particular, the butyric acid-producing bacteria leads to decreased butyric acid levels and abnormalities in T-regs differentiation in the lamina propria mucosae of the large intestine. The presence of gut dysbiosis in patients with relapsing INS remains unclear. This work, for the first time, used the prebiotics and probiotics in combination with prednisone in a trial to improve gut dysbiosis and to enhance the production of T-regs to prevent relapses in children with INS.

History taking of the patient in our study showed that two-third of patients delivered by Caesarean Section (CS) and fed artificial milk during infancy which affects the composition of gut microbiota. Dramatic changes occur in the human gut microbiota immediately after birth. By approximately 3 years of age, the gut microbiota is similar to that found in adults. Factors that prevent the establishment of normal gut microbiota during this critical period include CS delivery, formula milk and the administration of antibiotics during infancy [11].

In the current study, patients with relapsing INS have gut dysbiosis in the form of a very low number of beneficial *Lactobacillus acidophilus* in the stool. At the end of the course of the prebiotics and probiotic therapy in addition to steroids, there was a significant increase in the number of *Lactobacilli acidophilus* in the stool of the patient received prebiotics and probiotics (group 2) was found compared to group 1. This result proves the ability of the probiotics to resist the gastric juice and bile salt, persist passage through the upper GIT, multiply, colonize and function in the gut. Probiotics modify the composition of gut microbial species by preserving the balance and suppressing the growth of potential pathogenic bacteria in the gut. Although the time of permanence of the probiotic bacteria in the intestinal lumen (72 h) is enough to induce changes in the gut immune cells, increasing the number of macrophages and Dendritic Cells (DCs) of the lamina propria and improving their functionality, but children, in our trial, who stopped lactonorm sachets after short period 3-4 weeks showed rapid relapse so, we encourage parents to give the new treatment for 8 weeks to establish the improvement of immunity and to build high level of T-reg. Our results agreed by Tsuji et al. concluded that pediatric relapsing INS patients show gut microbiota dysbiosis, characterized by a decreased proportion of butyric acid-producing bacteria and lower fecal butyric acid quantities, concomitant with reduced circulatory T-regs due to reduction in their induction and differentiation. Natural T-reg cells play a key role in maintaining peripheral tolerance *in vivo* through the active suppression of self-reactive T-cell activation and expansion. Deficiency or dysfunction of Tregs has been described in several autoimmune diseases. Recently, the use of Tregs for therapeutic purpose in the treatment of autoimmune diseases is gaining significance. In 2009, Hashimura, et al. first reported that a patient with a mutated FOXP3 gene resulted in a small number of T-regs in INS. Since then, T-regs have drawn particular attention as key players in the pathogenesis of INS. Our results showed that the percentage of T-reg ($CD4^+/CD25^+/FOXP3^+$) cells of total lymphocytes and the total CD^{4+} cells, FOXP3 MFI and percentage of $CD4^+$ were significantly increased in group 2 compared to group 1. Kaneko et al. reported that INS is a renal manifestation of a T-cell dysregulation, in which cytokines act as modifiers of podocyte ultrastructure. Our results are also in agreement with Kawamoto et al. who studied the interaction between the microbiome and immune homeostasis and found that the regulatory T cell mediates the balance of microbiota by the production of immunoglobulin A. Konstantinov, et al. reported that probiotics produced enlarged levels of IL-10. There is due to their ability to bind to the lectin Dendritic Cell (DC)-Specific Intercellular adhesion molecule 3-Grabbing Nonintegrin (DC-SIGN) DC specific receptors and extracellular protein such as S-layer protein A (SlpA) secreted by *L. acidophilus* NCFM has been shown to bind DC-SIGN and encourage IL-10 production in DCs which induce T-regs. Another study also showed up-regulation of surface MHC class II and B7-2 (CD86), by *Lactobacilli* spp. which is suggestive of DC maturation. The probiotics reduced the inflammation severity due to the production of IL-10 and the generation of greater numbers of Treg cells expressing TGF-B. Shah, et al. proved that *Lactobacillius* L-92 induced $CD4^+/CD25^+/FOXP3^+$ regulatory T cell in mice. Thus, L-92 might affect the composition of microbiota through modulation of the mucosal immune system. The beneficial effect of probiotics in allergy processes is well described. The IgE increase is one of the most relevant signs that characterize this process. Probiotics are efficient in decreasing this immunoglobulin, as well as in alleviating symptoms. However, the mechanisms mediated for the alleviation of allergy have not been described. The relationship between gut microbiota and the development of allergic diseases is still unclear. Dwivedi, et al., reported that the probiotic *Lactobacillus acidophilus* and *Bifidobacterium longum* increased $FoxP3^+$ Tregs

and IL-10, decreased TNF-α, decreased IL-4 and Treg-associated TGF-ß production so, prevent allergy. Guimaraes, et al., showed a significant increase in the percentage of circulating T-reg ($CD4^+$ $FoxP3^+$) cells in both Steroid-Sensitive (SS) and Steroid-Resistant (SR) nephrotic group if compared to healthy controls, indicating a potential compensatory mechanism in response to the INS. The effect of administration of probiotic proved also by Galdeano, et al. who reported that probiotic bacteria primes a Th1 profile response and increases levels of IL-10, IFN-γ playing an important role in the immunomodulation. Also, probiotic bacteria are emerging as a safe and natural approach for allergy prevention and treatment. Different mechanisms such as the generation of cytokines from activated pro-T-helper type 1, which favor the production of IgG production rather than IgE, have been suggested. Immunomodulatory mechanisms employed by probiotic bacteria in the gut mucosa by adherence to intestinal epithelial cells and activate them through the pattern recognition receptors. Intestinal epithelial cells release cytokines and chemokines that create a microenvironment in the gut lamina propria, allowing the clonal expansion of B cells to produce IgA. After probiotic stimulation, macrophages distant from the GT such as peritoneum and spleen, increase their functionality (cytokines production, phagocytic and microbicidal activity) emphasizing the innate immune response [12].

The rate of relapses during the follow- up, for one and half years, in group 2 that was treated by prebiotics and probiotics in addition to steroids was markedly reduced compared to group1 that was treated by steroids only which proves the effectiveness of prebiotics and probiotics therapy. Relapsed cases after probiotic course showed relapse after one year of stoppage of treatment. compared to steroid treated children who showed relapse after one month of stoppage of treatment, which proves the effectiveness of prebiotics and probiotics therapy.

Unfortunately, there were several limitations to the current study. First, there is no fecal analysis for the microbial load of lactobacilli and blood analysis for T-reg from the patient before prebiotic and probiotic therapy. Second, there are no healthy subjects included in the study. Third, fecal and blood butyric acid couldn't be measured to prove the mechanism by which pro and prebiotic can increase T.reg cells. Also the genomic analysis of gut microbes in pediatric nephrotic syndrome to prove dysbiosis can't be done [13].

CONCLUSION

Our study has the strengths that patients given prebiotics and probiotics in addition to steroids showed a significant decrease in the rate of relapses which proves that prebiotics and probiotics can be used as adjuvant therapies for the decreasing the rate of relapses in idiopathic nephrotic syndrome, improving the outcome and decreasing the need for steroid therapy with its many side effects. These beneficial effects for probiotics and prebiotics may be through increasing the level of T-reg cells.

RECOMMENDATIONS

We encourage pediatric nephrologist to give prebiotics and probiotic courses adjuvant therapies to steroid treatment for all children with idiopathic nephrotic syndrome to prevent relapses, to increase gut immunity and to decrease the need for further steroid therapy. Further researches are needed to clarify the possible underling mechanisms through which prebiotics and probiotic decrease the frequency of relapses in INS and to investigate the effects of different types of probiotics given for longer periods for relapses in INS.

ACKNOWLEDGEMENT

All authors acknowledge help from all paramedical personals to accomplish and finalize the clinical research work.

FUNDING

The research was funded personally, by microbiology and immunology department, faculty of medicine. Fayoum university and clinical and chemical pathology department, faculty of medicine, Cairo university.

CONFLICT OF INTEREST

(No financial or nonfinancial benefits have been received or will be received from any one related directly or indirectly to the subject of this article).

ETHICAL APPROVAL

The research ethics committee of Fayoum medical university, Egypt approved the study protocol N: R81. Written informed consents were obtained from the parents.

AUTHOR CONTRIBUTIONS

Rehab A. Mohammed: Invent the idea, searching the internet data-base, and the writing of the manuscript.

Sherin K. Hussein: Collection of patients' data, searching the internet data base.

Walaa Abdel Fattah: Share in laboratory tests, and final editing of the manuscript.

Sylvana N. Gaber: Share in microbiological laboratory tests, searching the internet data-base and the writing of the manuscript.

Fatma A. Ahmed: Share in microbiological laboratory tests and searching the internet data base.

Eman S. Said: Statistical analysis and searching the internet data base.

Amy F. Boushra: Invent the idea, searching the internet data base and final editing of the manuscript.

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