



## STUDYING THE EXPRESSION OF FOXP3 IN MICE LYMPHOID ORGANS TREATED WITH PREDNISOLONE AND PROBIOTIC BY IMMUNOHISTOCHEMICAL TECHNIQUE

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### ABSTRACT

The aim of this study was to investigate the side effects of immunosuppressive drug (Prednisolone) and the protective effects of probiotic on the expression of FOXP3 in spleen and lymph node of albino mice. Thirty adult male albino mice were included in this study; divided into 6 groups (five mice in each group) and treated daily for 14 days. Group T1 was administered orally with 0.4 ml of ( $1 \times 10^8$  CFU/ml) *S. boulardii* for 7 days then injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 7 days, control T1 was administration 0.4 ml of ( $1 \times 10^8$  CFU/ml) *S. boulardii* only for 14 days. Group T2 was injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 7 days then administered orally with 0.4 ml of ( $1 \times 10^8$  CFU/ml) *S. boulardii* for 7 days, control T2 was injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 14 days. Group T3 was administered orally with 0.4 ml of ( $1 \times 10^8$  CFU/ml) *S. boulardii* and injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 14 days, control T3 was administration normal saline only for 14 days. Group T4 was administered orally with 0.6 ml of ( $1 \times 10^8$  CFU/ml) *S. salivarius* for 7 days then injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 7 days, control T4 was administration 0.6 ml of ( $1 \times 10^8$  CFU/ml) *S. salivarius* for 14 days. Group T5 was injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 7 days then administered orally with 0.6 ml of ( $1 \times 10^8$  CFU/ml) *S. salivarius* for 7 days, control T5 was injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 14 days. Group T6 was administered orally with 0.6 ml of ( $1 \times 10^8$  CFU/ml) *S. salivarius* for 7 days and injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 14 days, control T6 was administration normal saline only for 14 days. The expression of FOXP3 was increased in spleen and lymph node of groups treated with probiotic (yeast and bacteria) and prednisolone drug by immunohistochemical technique.

**KEYWORDS:** Prednisolone, *Saccharomyces boulardii*, *Streptococcus salivarius*, FOXP3, spleen, lymph node.

### INTRODUCTION

Prednisolone is a synthetic glucocorticoid, a derivative of cortisol, which is used to treat a variety of inflammatory and auto-immune conditions. It is the active metabolite of the drug prednisone and it is used especially in patients with hepatic failure, as these individuals are unable to metabolize prednisone into prednisolone. It is a white, hygroscopic, crystalline powder, shows polymorphism, very slightly soluble in water, alcohol and in methyl alcohol, sparingly soluble in acetone, slightly soluble in dichloromethane and store in airtight storing containers to protect it from light. Prednisolone has multiple adverse effects including susceptibility to infection, impaired wound healing, growth suppression in children, osteoporosis, aseptic necrosis of bone, cataracts, glucose intolerance, fluid retention, hypertension, emotional lability, insomnia, manic and depressive psychosis, gastric ulcers, hyperlipidemia, polyphagia, obesity, and acne<sup>[1]</sup>. *Saccharomyces boulardii* (*Sb*) is a tropical strain of yeast first isolated from lychee and mangosteen fruit in 1923 by French scientist Henri Boulard. It is related to but distinct from *Saccharomyces cerevisiae* in several taxonomic, metabolic, and genetic properties, (*Sb*) was a thermophilic, nonpathogenic which administered in Western Europe for the prevention and treatment of a variety of diarrheal diseases. Preclinical and experimental

studies of *Sb* had demonstrated an anti-inflammatory, antimicrobial, enzymatic, metabolic and antitoxin activity investigation of *Sb* oral administration in healthy volunteers and demonstrates several cellular and humoral changes in peripheral blood. Among its effects are the increase of erythrocytes, leucocytes, polymorphs, neutrophils, complement components C3, C5, C3d, serum anticomplementary activity and leucocyte chemokinesis, especially when autologous serum and antigen have been added to the culture medium and decrease of complement haemolytic activity (CH50), classic and alternative pathways<sup>[2]</sup>. *Streptococcus salivarius* is a Gram-positive bacterial commensal which colonizes the human oral cavity throughout the host's life, and is generally associated with health, these characteristics made this species attractive to investigate as a potential oral probiotic. *S. salivarius* strains K12 was selected for further study based on its *in vitro* inhibitory activity against *Streptococcus pyogenes*, this strain encodes multiple bacteriocins; bacterially produced substances with the capacity to either inhibit other bacteria attempting to colonize the same niche, or to act as signaling molecules. *S. salivarius* K12 produces the bacteriocins salivaricin A2 and salivaricin B<sup>[3]</sup>. *S. salivarius* K12 has been shown in placebo controlled studies to prevent recurrent streptococcal induced pharyngitis in adults (20 patients

receiving the probiotic for 90 days), and children (45 patients receiving the probiotic for 90 days, then a 6 month follow-up period), as well as reduce halitosis by limiting the production of volatile sulfur compounds from anaerobic bacteria<sup>[4]</sup>. The aim of the study is to demonstrate the expression of FOXP3 in mice lymphoid organs after treated with prednisolone and probiotic.

## MATERIALS & METHODS

### Animals and experimental design

Group T1 was administrated orally with 0.4 ml of ( $1 \times 10^8$  CFU/ml) *Sacchromyces boulardii* for 7 days then injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 7 days, control T1 was administration 0.4 ml of ( $1 \times 10^8$  CFU/ml) *S. boulardii* only for 14 days. Group T2 was injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 7 days then administrated orally with 0.4 ml of ( $1 \times 10^8$  CFU/ml) *S. boulardii* for 7 days, control T2 was injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 14 days. Group T3 was administrated orally with 0.4 ml of ( $1 \times 10^8$  CFU/ml) *S. boulardii* and injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 14 days, control T3 was administration normal saline only for 14 days. Group T4 was administrated orally with 0.6 ml of ( $1 \times 10^8$  CFU/ml) *S. salivarius* for 7 days then injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 7 days, control T4 was administration 0.6 ml of ( $1 \times 10^8$  CFU/ml) *S. salivarius* for 14 days. Group T5 was injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone for 7 days then administrated orally with 0.6 ml of ( $1 \times 10^8$  CFU/ml) *S. salivarius* for 7 days, control T5 was injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 14 days. Group T6 was administrated orally with 0.6 ml of ( $1 \times 10^8$  CFU/ml) *S. salivarius* for 7 days and injected intraperitoneally with 0.1 ml of 2.5 mg/kg prednisolone drug for 14 days, control T6 was administration normal saline only for 14 days. After 14 days animals were sacrificed to obtained the spleen and lymph node for immunohistochemical study.

### Immunohistochemical study

**Kit components were used in the study was listed below:**

FOXP3 (2A11G9) mouse monoclonal IgG (200)µg, Peroxidase, Block Serum Block, Serum block (Goat serum), Biotinylated Antibody (Goat anti-mouse IgG), HRP-Avidin, Substrate buffer (10X concentrate), Peroxidase substrate (50Xconcentrate), DAB chromogen (50X concentrate), Negative control (normal mouse IgG) according to<sup>[5]</sup>. A vidin D-HRP complex for staining of formalin-fixed,paraffin-embedded tissue sections according to<sup>[6]</sup>.

### Procedure

1. Clean glass slides with 95% ethanol; treat with subbing solution and air dry.
2. Cut four to six micron thick tissue section using microtome and apply o slides. Deparaffinize as follows: 3x 80% xylene for 5 minutes each, 2x 100% ethanol for 10 minutes each and 2x 95% ethanol for 10 minutes each. Wash in deionized H<sub>2</sub>O for 1 minute on stir plate. Aspirate excess liquid from slides.

3. All subsequent steps are carried out at room temperature in a humifide chamber. Tissue section should not be allowed to dry out at any time during the procedure.
4. Incubate specimens for 20 minutes in 1-3 drops of serum block. Aspirate serum for slides.
5. Dilute primary antibody to 0.5-5 µg/ml as determined by titration antibody should be diluted in serum block (provided).
6. Incubate for 2 hours, rinse with PBS, and wash in PBS twice for 2 minutes each on stir plate. Aspirate excess liquid from slides.
7. Incubate specimens for 30 minutes in 1-3 drops of biotinylated secondary antibody. Rinse with PBS, and wash in PBS twice for 2 minutes each on stir plate. Aspirate liquid from slides.
8. Incubate specimens for 30 minutes in 1-3 drops of Avidin D-HRP complex. Rinse with PBS, and wash in PBS twice for 2minutes each on stire plate. Aspirate liquid from slides.
9. During the above incubation step, prepare HRP substrate in the substrate mixing bottle as follows (sufficient for 15-20 slides): remove tip from mixing bottle and combine 1.6 ml deionized H<sub>2</sub>O 5 drops 10x substrate buffer, 1drop 50x DAB chromogen and 1 drop 50x peroxidase substrate.
10. Add 1-3 drops of HRP substrate to each slide. Develop until light brown staining is visible, usually 30 seconds - 10 minutes, although up to 20 minutes may be required. The section may be checked for staining by rinsing with deionized H<sub>2</sub>O and viewing under a microscope. If necessary, add additional HRP substrate and continue to incubate. Rinse with deionized H<sub>2</sub>O and transfer to deionized H<sub>2</sub>O wash for 2 minutes on stire plate.
11. Dehydrate sections as follows: 2x 95% ethanol for 10 seconds each, 2x 100% ethanol for 10 seconds each and 3x 80% xylene for 10 seconds each. Wipe off excess xylene from edges of slide.
12. Immediately add 1-2 drops of permanent mounting medium and cover with glass cover slipe. Observe by light microscopy.

### Scoring Analysis

The slides were examined by light microscope 100X, 200X, 400X and after comparing the sections slides with positive and negative control slides, the scores were determined. Lymphocyte infiltrating cells were scored according to<sup>[7]</sup> and <sup>[8]</sup>. Cytoplasmic intensity was scored to negative staining , low ,moderate and high according to deep of brown color of the marker.

### FOXP3 expression

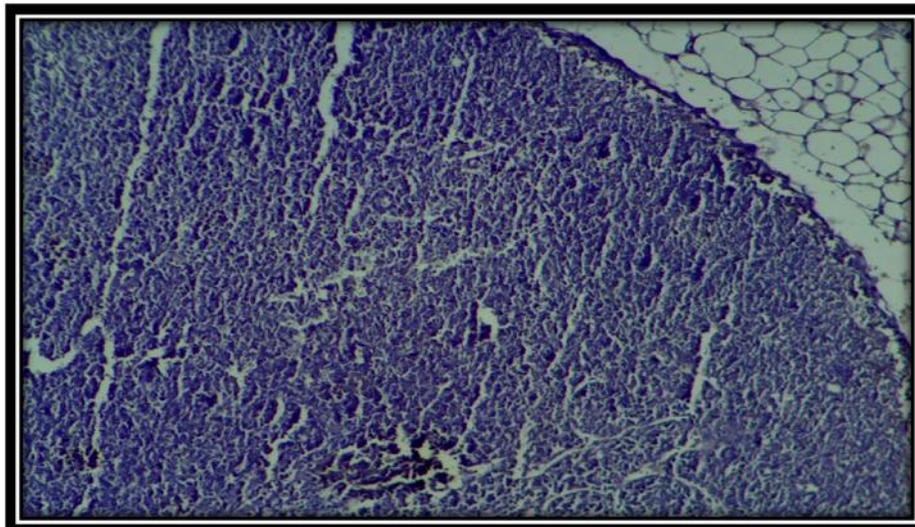
- No staining cells	0
- (1-25%) cells	+1
- (26-50%) cells	+2
-(51-100%)	cells+3

### RESULT

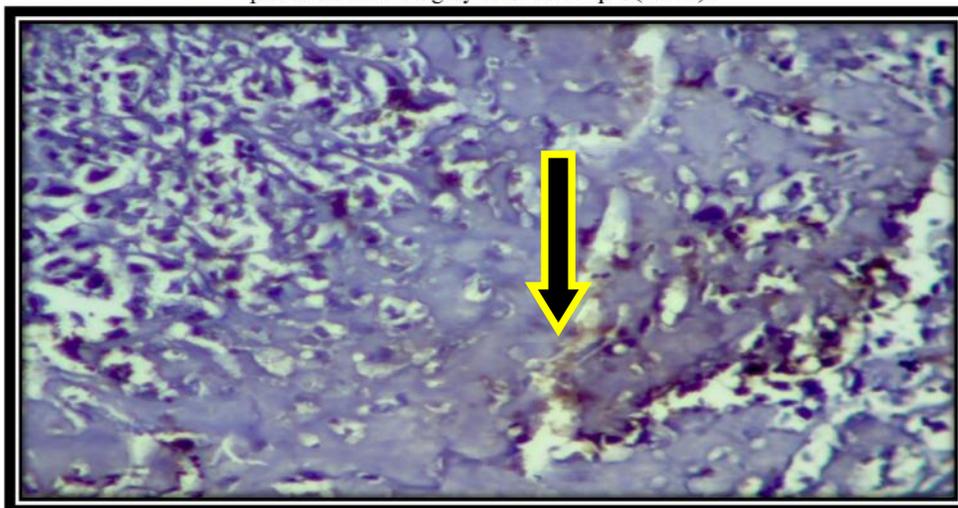
The result showed that the expression of FOXP3 in mice treated by (0.4) ml of ( $1 \times 10^8$ ) CFU/ml *Sacchromyces boulardii* then followed by 0.1ml of (2.5) mg/kg prednisolone drug (groupT1), is higher in spleen when compared with spleen control (*Sb* only) (+2,+3) (+1,+2) respectively Figure (2,3,4) and lymph node of (groupT1)

showing the same expression of FOXP3 as compared with control group (+1,+2) Figure (6,7). Mice when treated by 0.1 ml of (2.5) mg/kg prednisolone drug then followed by (0.4)ml of ( $1 \times 10^8$ )CFU/ml *Sacchromyces boulardii* (group T2), showing decrease the expression of FOXP3 in spleen as compared with control group(drug only)(-ve,+1) (+2,+3) respectively Figure (1,2,3,4) and lymph node of (groupT2) showing decrease expression of FOXP3 as compared with control group (-ve,+1)(+1,+2) respectively Figure(5,6,7). Mice when injected by 0.1 ml of (2.5)mg/kg prednisolone drug and administrated by (0.4)ml of ( $1 \times 10^8$ )CFU/ml *Sacchromyces boulardii* together (group T3), showing increase the expression of FOXP3 in spleen as compared with control group (Normal saline only) (+1,+2) (-ve,+1) respectively Figure(1,2,3) and lymph node of (group T3), showing increase expression of FOXP3 as compared with control group (+1,+2) (-ve,+1) respectively Figure (5,6,7). Mice when administrated by (0.6) ml of ( $1 \times 10^8$ ) CFU/ml *Streptococcus salivarius* then injected by 0.1 ml of (2.5) mg/kg prednisolone drug (group T4),showing the same expression of FOXP3 in

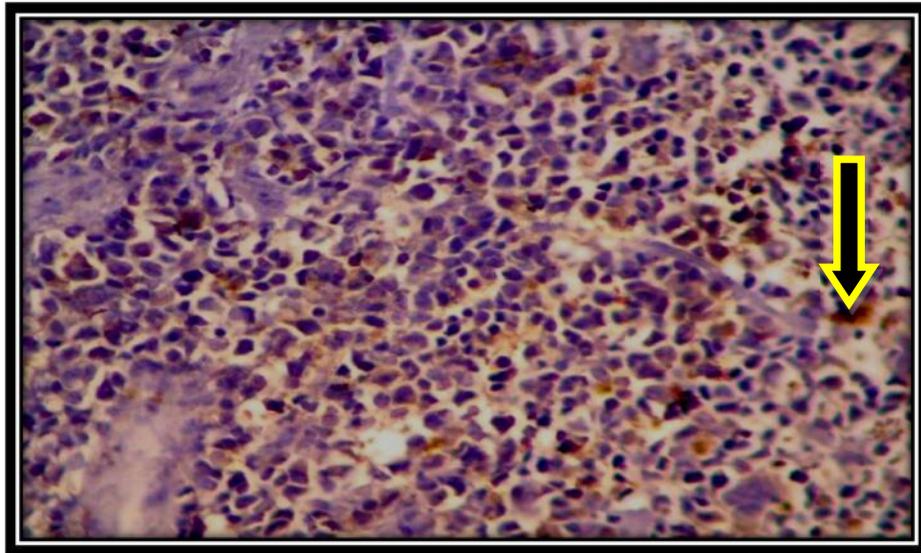
spleen as compared with control group (*Ss* only) (+1,+2) Figure (2,3) and lymph node of (groupT4) showing decrease expression of FOXP3 as compared with control group (-ve,+1)(+1,+2) respectively Figure(5,6,7). Mice when injected by 0.1 ml of (2.5)mg/kg prednisolone drug then followed by (0.6) ml of( $1 \times 10^8$ )CFU/ml *Streptococcus salivarius* (groupT5),showing decrease the expression of FOXP3 in spleen as compared with control group(drug only)(+1,+2)(+2,+3) respectively Figure(2,3,4) and lymph node of (groupT5), showing the same expression of FOXP3 as compared with control group (+1,+2) Figure(6,7).Mice when injected by 0.1 ml of (2.5)mg/kg prednisolone drug and administrated by(0.6) ml of ( $1 \times 10^8$ )CFU/ml *Streptococcus salivarius* together (group T6), showing the expression of FOXP3 in spleen is the same as compared with control group(Normal saline only)(-ve,+1) Figure (1,2) and lymph node of (groupT6) showing increase the expression of FOXP3 as compared with control group (+1,+2)(-ve,+1) respectively Figure (5,6,7).



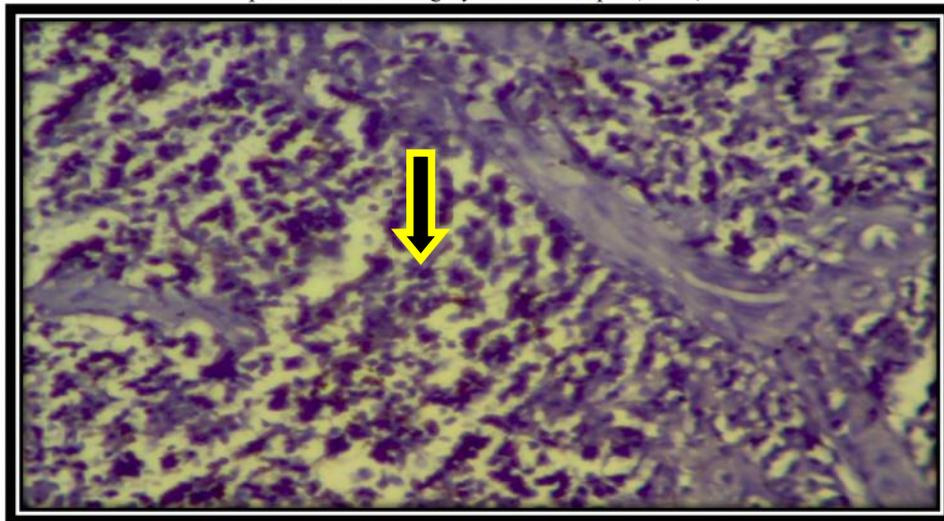
**FIGURE 1:** showing the negative scoring (-ve) of FOXP3 expression in spleen of mice treated with probiotic and prednisolone drug by IHC technique (100X).



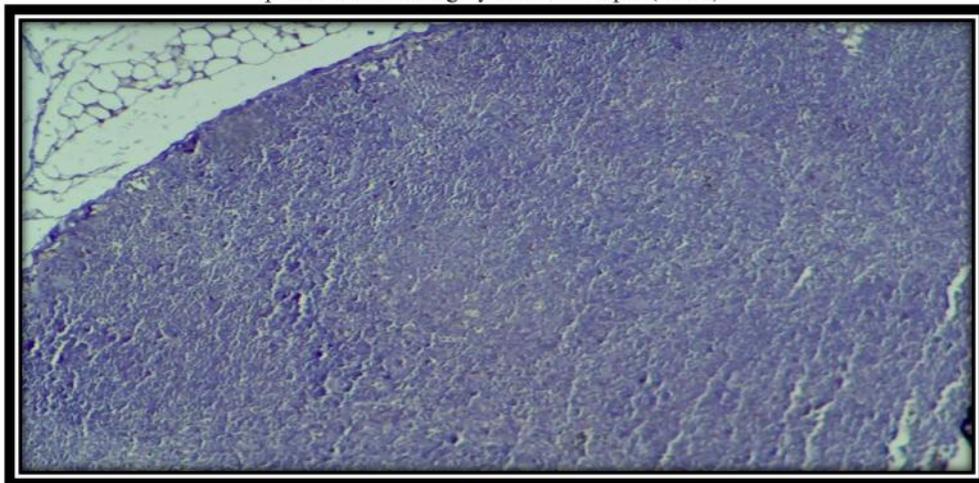
**FIGURE 2:** (arrow) showing the positive scoring (+1) of FOXP3 expression in spleen of mice treated with probiotic and prednisolone drug by IHC technique(400X).



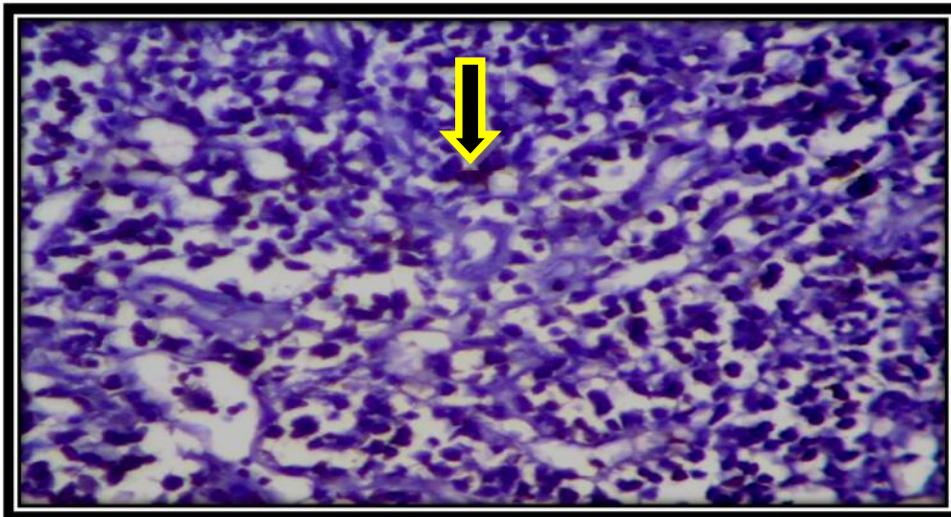
**FIGURE 3:** (arrow) showing the positive scoring (+2) of FOXP3 expression in spleen of mice treated with probiotic and prednisolone drug by IHC technique (200X).



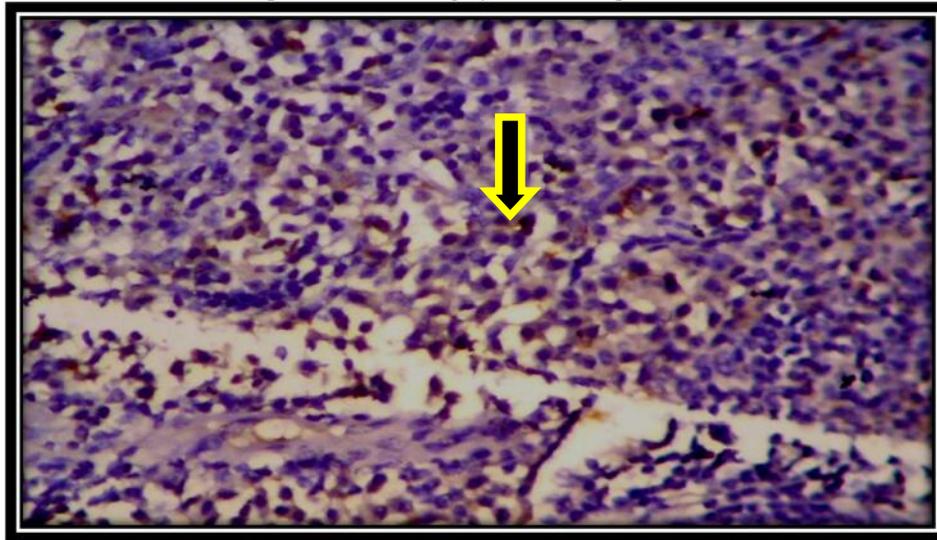
**FIGURE 4:** (arrow) showing the positive scoring (+3) of FOXP3 expression in spleen of mice treated with probiotic and prednisolone drug by IHC technique (200X).



**FIGURE 5:** showing the negative scoring (-ve) of FOXP3 expression in lymph node of mice treated with probiotic and prednisolone drug by IHC technique (100X).



**FIGURE 6:** (arrow) showing the positive scoring (+1) of FOXP3 expression in lymph node of mice treated with probiotic and prednisolone drug by IHC technique (200X).



**FIGURE 7:** (arrow) showing the positive scoring (+2) of FOXP3 expression in lymph node of mice treated with probiotic and prednisolone drug by IHC technique (200X).

## DISCUSSION

The result of the recent study showed that the expression of FOXP3 was increased in spleen and lymph node of mice treated with probiotic and prednisolone drug, treated of mice with prednisolone drug cause enhance expression of the FOXP3 gene as a result of suppression T-cells by prednisolone drug. This result agreed with many studies<sup>[9]</sup> who mentioned that the percentages of CD4+FOXP3+ and CD25+FOXP3+ were significantly increased in newly diagnosed patients with diabetes type 1,<sup>[10]</sup> who mentioned that children with atopic dermatitis had significantly greater increases in FOXP3 expression after stimulation with both food allergen and inhalant allergen and<sup>[11]</sup> who mentioned that FOXP3 was significantly more elevated in asthmatics on corticosteroids than steroid naive asthmatics. Treg cells defined by the expression of CD4 + CD25+ and the transcription factor FOXP3 which play a critical role in the maintenance of peripheral tolerance and in the control of autoimmunity<sup>[12]</sup> and<sup>[13]</sup>. Impairment of Treg development and/or function can precipitate a variety

of autoimmune diseases, whereas a higher frequency of Tregs can render the immune system hypo responsive to pathogens. The result of the group treated with probiotic then prednisolone drug show a lower expression of FOXP3 as compared with the group treated with prednisolone drug then probiotic and group treated with probiotic and drug together, and these results agreed with<sup>[14]</sup> who reported that oral administration of a certain probiotic strain could increase FOXP3+ Tregs and<sup>[15]</sup> who mentioned that probiotics have ability to modulate immune system and induced both Tcell and B-cell hyporesponsiveness and down-regulated T helper (Th) 1, Th2, and Th17 cytokines without apoptosis induction. Systemic corticosteroids therapy like prednisolone drug lead to several side effects<sup>[16]</sup>. So that the group treated with prednisolone drug show several immunosuppressive effects on of this the alteration of expression of FOXP3 by immunohistochemical technique. In this study when the immunosuppressed mice treated with probiotic (yeast and bacteria) cause protection and immunomodulation effect

leading to increased FOXP3 expression by immunohistochemical study. These effect of probiotic returned to their ability to improve the host health one of them stimulation of host immunity [17] and [18] suggested that selective probiotics may induce the expression of FOXP3 and development of Tregs in atopic individuals. Several studies demonstrated that probiotic might induce a protective effect in modulating both innate and adaptive host immunity to respond against pathogen infection. Probiotic may work in several different ways: including helping to control intestinal homeostasis, by preventing pathogens from colonizing, by promoting beneficial enzyme production, by improving the gastrointestinal lining permeability, or by improving immune responses [19].

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