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Immunological Study for the Role of Probiotic for Control on the *Leishmania donovani*

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ABSTRACT

Kefir is a fermented milk (drink) produced by the action of several microorganism mostly *lactic acid, acetic acid* bacteria and yeasts. This study included for using Kefir as immunomodulor to cell-mediate immune response against *Leishmania donovani* in the mice compared with pentostam by measured level of three cytokines (IL-12, IL-4, and IFN-γ) in serum. The infected mice administration Kefir (5ml/day) for three weeks the results showed significant($p \leq 0.05$) increase occur in levels of IL-12 were (518, 725 and 405.6 pg/ml) respectively compared to control (170 pg/ml) and IFN-γ were (388, 698and 421 pg/ml) compared with control (163.16 pg/ml), but the levels of IL-4 was no significant change after one week (42.83 pg/ml) compared to control (47.8±5 pg/ml) and then decreased in the second and third week, levels were (40.16 and 34.16 Pg/ml) respectively. Also the administrated only kefir to the mice lead to increase the level of IL-12 and IFN-γ in the second week compared with pentostam treatment mice (297.33 pg/ml)for IL-12 and (267.83 pg/ml) for IFN-γ but was no significant change occur in level of IL-4 between kefir and pentostam treatment after 7, 14 and 21 days. This investigation revealed that Kefir and pentostam approximately had the same efficacy to effect on parasite with safety advantage kefir as natural adjuvant product to enhance cellular immunity.

KEY WORDS: *Leishmania donovani*, kefir, pentostam, cytokines, IFN-γ

1. INTRODUCTION

Leishmania donovani is an obligate intracellular hemoflagellate parasite causes visceral Leishmaniasis or kala azar that occur about 500,000 cases every year in many part of the world[1] . Elimination *leishmania* parasite depend on the development of type I immune responses characterized by initial production of Interleukin-12(IL-12) by antigen-presenting cells (APCs) that induce Th1 T cell follow secretion interferon-γ (IFN-γ) to activation macrophage to killing intracellular amastigotes by production of nitric oxide (NO) and reactive oxygen species (ROS), while the disease progress occur in T helper-2 (Th2) response and cytokine produce such as IL-4 [2]. The existing drugs against visceral leishmaniasis are toxic and its vaccine absence so immunomodulation therapy that demonstrated in several studies by some immunomodulators that specifically boost the anti-microbial activities of the immune cells via enhanced pro-inflammatory cytokine(IL-12 and IFN-γ) production and host-protecting Th1 response mediated lead to resolution of pathology[3]. Kefir (a complex probiotic) is a fermented milk by action microorganisms considered as strong immunomodulator and kefir consumption activated immunity by stimulation macrophage to increase phagocytosis and augmenting nitric oxide (NO) and cytokine production particular pro-inflammatory cytokine resulting increased polarization of the immune response toward Th1type and decreased Th2 type response [4] .Therefore the present study was evaluated the role of kefir to enhance cell-mediated immunity either by cytokine production or cytokine inhibition in mice infected with visceral leishmaniasis.

2. MATERIAL AND METHOD

2.1 *Leishmania* antigen preparation

L. donovani isolated was obtained from the department of biology \ College of Sciences \ University of Thi-Qar. It was maintained *in vitro* by serial passage in semisolid medium (NNN) each 5 days. 1×10^8 promastigote/ml in a 0.2 ml volume of BPS for intraperitoneal (i.p) injection into mice [5].

2.2 kefir grains

Kefir grains were obtained from Los Angeles / California / United States. Cultivation grain in milk every day, fermented milk (kefir) was used in search [6]

2.3 Animals

Seventy eight male *albino* mice aged 8-12 weeks, weighing 20-28 gm were obtained from National Center for Drug Control and Research, housed under standard condition in animal house of biology department in College of science / AL-Mustansrya University.

Then 54 mice were infected with 1×10^8 promastigote per ml in a 0.2 ml volume of BPS by injected intraperitoneal. After one day, all mice were divided into 5 groups each group contain 18 mice then each group inoculated as a follow:

1. Group1 (none infected): inoculated orally by stomach tube (0.1ml/day) normal saline consider as control.
2. Group2 (infected): inoculated orally by stomach tube (0.1ml/day) normal saline consider as infected mice.
3. Group3 (none infected): inoculated orally by stomach tube kefir (5ml/day) for 21 days consider as kefir group.
4. Group4 (infected): inoculated orally by stomach tube kefir (5ml/day) for 21 days consider as kefir treatment group.
5. Group5 (infected): injected with (0.01 ml/day) from pentostam drug by intraperitoneal each day for 21 days consider as pentostam treatment group.

2.4 Collection of Blood

After 7, 14, 21 days post-infection and treatment, from each six mice about 2 ml of blood were collected and obtain serum.

2.5 Serum Level of Cytokines

Serum level of three cytokines (IL-12, IL-4, and IFN-γ) was determined by using KOMA Cytokine ELISA Kits (Korea), which were designed for the quantitative measurement of cytokine in mice.

2.6 Statistical Analysis

The data for various parameters were subjected to statistical analysis SPSS, software program using analysis of variance (ANOVA).

3. RESULT

3.1 Serum level cytokine

To examine the role of Kefir to induce immune response in a murine experimental *Leishmania* model, were determined by measured the levels of three cytokines (IL-12, IL-4, and IFN-γ) in serum for all experimental groups (kefir, pentostam and control groups) after 7,14 and 21 days.

1. Serum level of IL-12

Serum level of IL-12 was increased in all experimental groups in all weeks compared to the control was (170±10.14pg/ml) (figure 4-1). The results showed gradual increasing occur in level of Il-12 in three weeks after infection of positive control group

(259.33±15.5, 322.33±12.2 and 508.1±14.1pg/ml) respectively. While in kefir group mice (GK) was observed high level at first week (604.6±7.9 pg/ml) then gradually decrease occur in the high levels at second and third weeks (500.66±15.71 and 274.83±16 pg/ml) respectively, but remained higher than control group. While after treatment infected mice with kefir (TK) the level of IL-12 was begin increased after first and second weeks (518±23.71, 725±8.71 pg/ml) respectively, but return decrease at third week (405.6±7.23 pg/ml) although remained more than(GK) group with significant difference (P≤0.05) between them in the same week. In treatment pentostam groups the results have been volatile, high level occurred in the first week (524±7.21 pg/ml) and then got low in the second week (297.33±14.12) then in the third week returned to rise again 502.8 ±13 pg/ml). There was significant difference (P≤0.05) found within the same groups and with control.

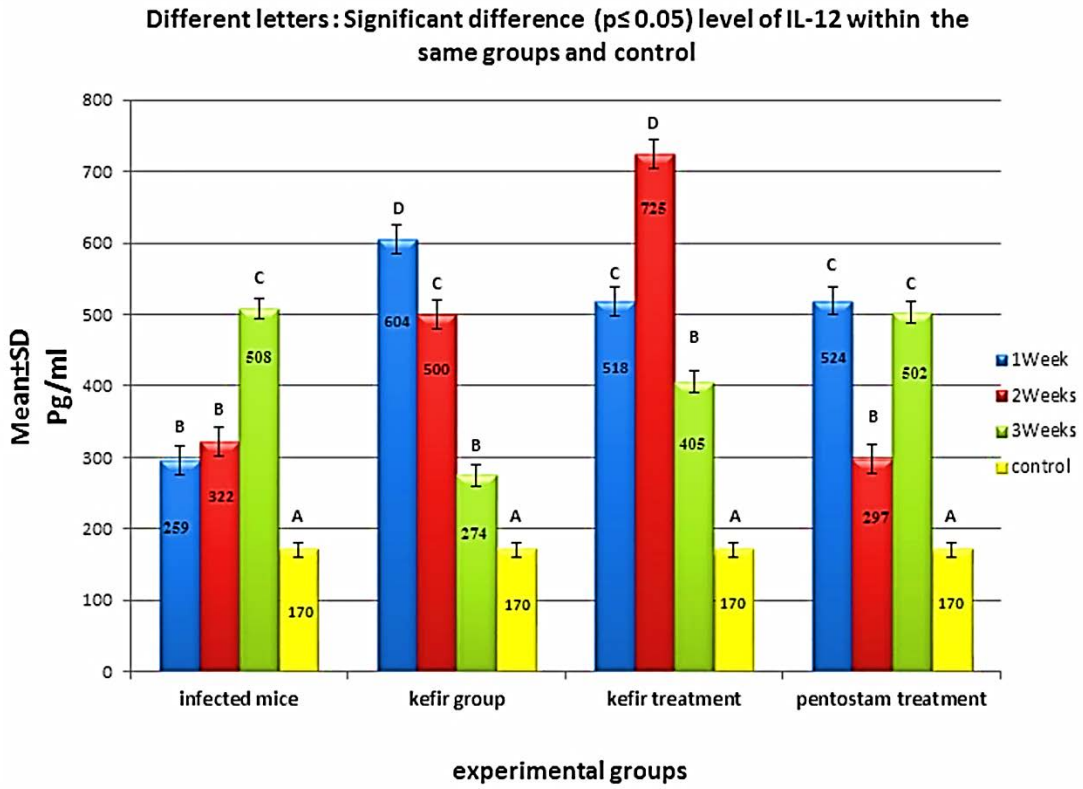

Figure 3-1: IL-12 level in Serum of experimental group and control.

2. Serum level of IL-4

Most of experimental groups was significant (P ≤ 0.05) recorded decrease serum level of IL-4 compared to control (47.8±5 pg/ml) (figure 4-2). In positive control(infected mice) group the lowest level was in the first week (29.66±3.82 pg/ml) then increased to reached nearly the normal value in the second and third week(38.66±1.6 and 41.5±4.66 pg/ml) respectively. Whereas the results of kefir group was observed no change in first week compared to control (51.1±4.4 pg/ml) then decreased through the second and third week (39±4.93 and 37.16±3.8 pg/ml) respectively. While in treatment kefir group no changes occur in IL-4 value after first and second week (42.83±4.9 Pg/ml and 41.16±3 Pg/ml) respectively, but in the third week significant decrease was evidence (P ≤ 0.05) (34.16±2.99 Pg/ml). No importance changes happen in the level of cytokine during the first week (43.5±4.1 pg/ml)in treatment Pentostam

group, then began decreased after second and third week (31.5±3.1 and 29.33±2.16 Pg/ml) compared to control , on the other hand there was no significant different ($P \geq 0.05$) found between treatment pentostam group and treatment kefir group in third week.

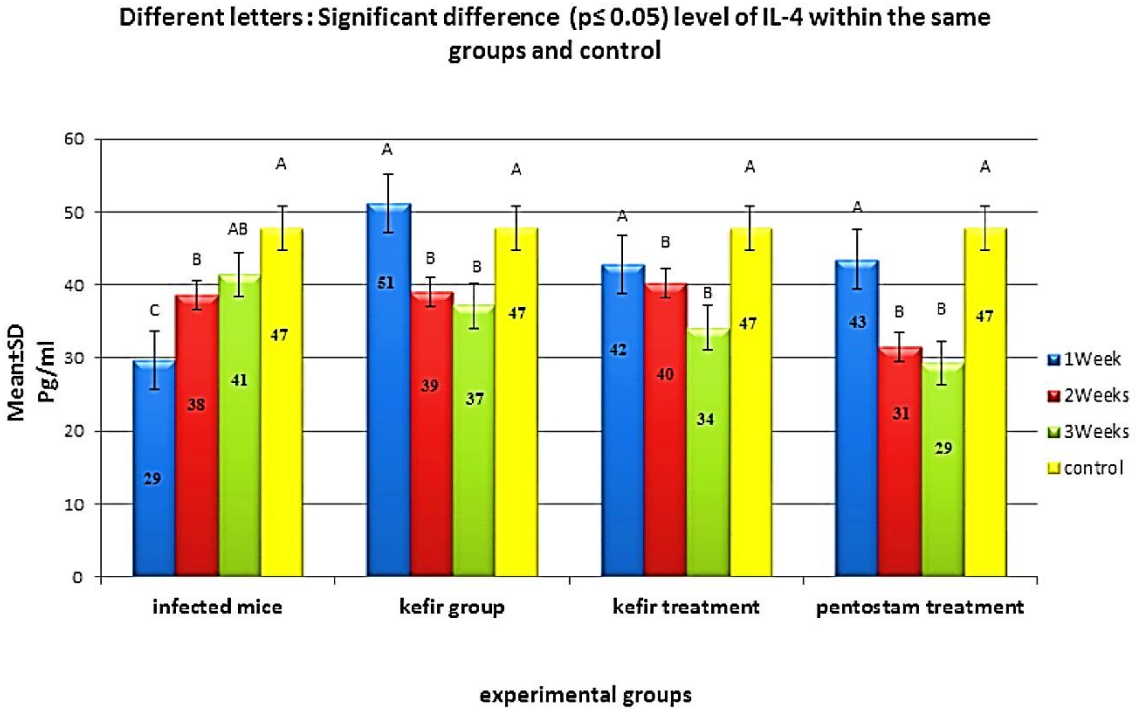

Figure 3-2: IL-4 level in Serum of experimental group and control .

3. Serum level of IFN-γ

The IFN-γ appeared significantly ($P \leq 0.05$) increased in all groups compared to control (163.16±8.14 pg/ml) (Figure 4-3). In the control group the positive increase has been gradual since the first week was (396.83±7.40), in the second week became (402±16 pg/ml) and reached to (488.5±10.5 pg/ml) in third week but in the kefir group it was observed the highest level after one week of administration kefir (639.8±12.79 pg/ml) then the risen value gradually lowered (500±23.94 pg/ml) in the second week to reach nearly the normal value in the third week (201.66±16.12 pg/ml). Irregular increasing was showed in the treatment kefir group in all weeks, whereas the high level after one week was scored (388.33±10.75 pg/ml), while the level in the second week was more increasing (698.83±12.41 pg/ml) then returned decreased in the third week (421.16±5.56 pg/ml). In the pentostam treatment group IFN-γ level increased in the first week (357.33±18.92 pg/ml) , then drop back in second week (267.83±13.12 pg/ml) and risen significantly (528.5±20.72pg/ml) in third weeks.

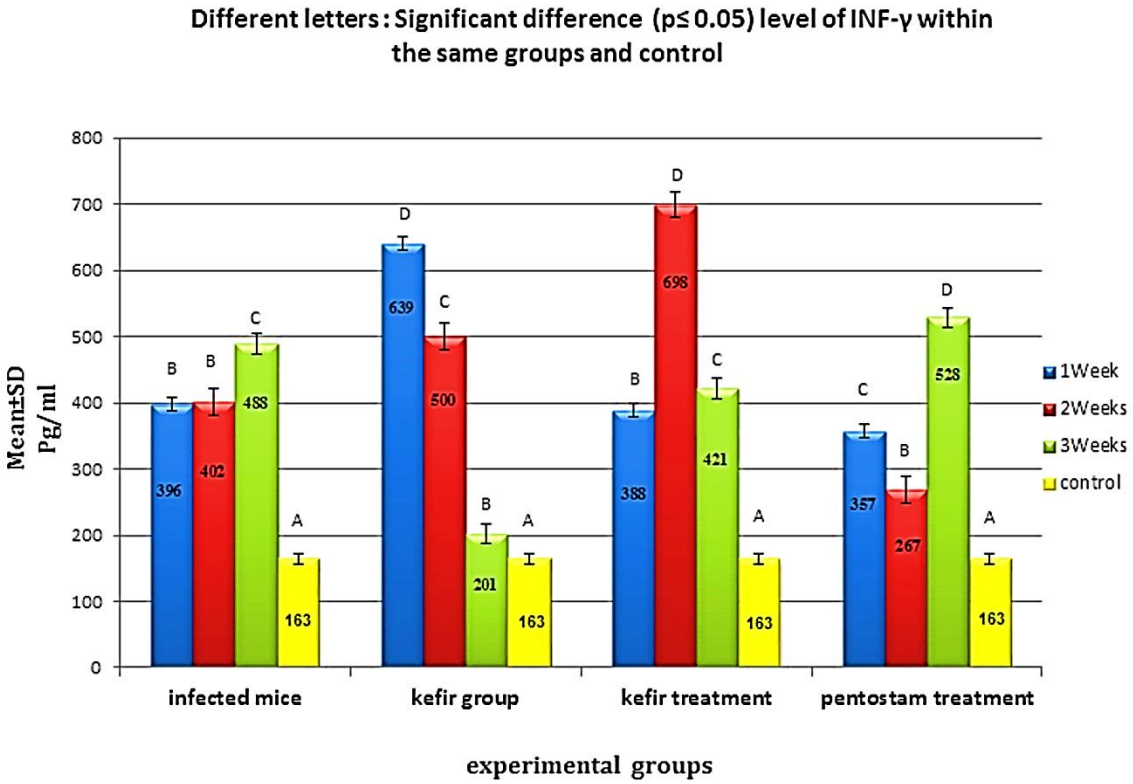

Figure 3-3: Serum level of IFN-γ in experimental group and control.

4. DISCUSION

The main observation in this study (as first study) was evidence restore and enhance host immune response against *L. donovani* parasite by kefir. Some studies on human infection and murine model leishmaniasis have demonstrated that immunity and healing occur with induction of predominant Th1 response and IFN-γ induced by macrophage activation, in contrast induction of the Th2 and producing IL-4 have been showed to correlated with disease progress [7]. In Results the study was observed the early host immunity against *Leishmania donovani* in the first week post-infection when showed increased occur in serum level of IL-12 and IFN-γ versus decrease level of IL-4. After that the parasite began the fighting with immune system in the next weeks leading to rising of IL-12 and IFN-γ with returning level IL-4 to normal range in the third week .This statue agreed with Caldas *et al.* [8] confirmed that circulating levels of IL-12 and IFN-γ were elevated in VL patients, Kip *et al*. [9] mentioned in his research that IL-12 and IFN-γ level were found to be significantly elevated in patient with active visceral leishmaniasis. While Ansari *et al*. [10] found that in VL the level of IL-4 was decrease or not different from control group. Murine models of *leishmania* have demonstrated that host defense mechanisms include IL-12 induced Th1 cytokine such as IFN-γ which by activating macrophages ultimately eliminate the parasite through enhanced release of nitric oxide (NO) therefore during *leishmania* infection established that critical skewing from a pro-inflammatory to anti-inflammatory response result in sustained intracellular survival of parasite [11]. The disrupt the host cell signaling events by generation of effector molecules to evade killing by the host and involved every aspects of the manipulation/inhibition of host cell signaling , ranging from preventing the production of microbicidal molecules and defect cytokine to interfering with effective antigen

presentation [12]. The lypophosphpglycan and Glycoprotienase GP63 are important survival factors of *leishmania* that defect macrophage activation and targeted the IL-12, The Toll-like receptors (TLRs) signaling and major histocompatibility complex (MHC) Class II presentation that associated with inflammation, also they suggested that the blockage of Th1 cytokine receptor leading to defective response to IFN-γ signaling in infected macrophage [13] and Ray *et al.*[14] indicated that the infection occur although the presence of high level of IFN-γ due to fault in downstream signaling and negative regulation of the IFN-γ receptor that contributes to JAK2/STAT1 pathway inactivation in *Leishmania donovani*-infection cell.

As well as some researchers added that the patient with active VL not depress production of these cytokine but there appears to be unresponsiveness that correlated with increased production of immunosuppressive cytokine such as IL-10 that secreted with IL-4 from Th2 [7]. IL-10 is down-regulation of IL-12 signals of macrophage activation via blocking Th1 activation [15, 16]. While the IL-4 skewing of Th1 to Th2 immune response during infection and if both IL-12 and IL-4 are present at the time of T- cell activation, the effect of the IL-4 dominates and the T lymphocytes polarize to become Th2 effectors [17]. Mondal *et al.* [18] reported that in visceral leishmaniasis the level of IFN- γ in serum remains high and suggesting that their source could be lymphoid organ where the parasite proliferation.

After treatment infected mice with kefir and compared with pentostam drug for three weeks, showed decrease occur level in of IL-4 with increased more levels of IL-12 and IFN-γ as marker to enhance cellular immune response of mice against infection when treatment with kefir and pentostam drug. These cases agreed with study of Thakur *et al.*, [19] when reported that important estimation the IFN-γ: IL-4 ratio to determining the drug response profile and when level of IFN-γ high and level of IL-4 is low that is indicated successful treatment in infected individual. While Mutiso *et al.*, [20] mentioned that the elevation level of IL-4 particularly associated with treatment failure. In infected mice The kefir appeared rapidity syntheses much more IL-12 and IFN-γ after two week followed clearly lowered these cytokine compared untreated mice and pentostam drug in the third week. Ansari *et al.* [21] suggested that the T-cell response may be began in kefir before chemical drug treatment. It is known during host defense in VL the efficacy of anti-leishmanial therapy is systematically associated with restored expression of IL-12 and INF-γ consequence leshmania-specific T-cell response [22].

Impaired IFN-γ signaling was detected in *Leishmania*-infected macrophages and was associated with activation of Tyrosine-protein phosphatase **(**SHP-1) by the parasite Therefore, it could be postulated that sodium stibogluconate (pentostam) may augment IFN-γ signaling in macrophages via inhibiting SHP-1 (and other Protein tyrosine phosphatases PTPases) and contribute to the clearance of intracellular *Leishmania*. Thus anti-*Leishmania* activity of sodium stibogluconate may derive both from augmenting cell signaling and from parasite killing inside cells [23]. By cytokine level scanning, increase polarization of the immune response towards Th1 type and decrease Th2 type response after kefir feeding healthy mice for 21 days only. It was scored highest level of IL-12 and IFN-γ and no significant change in level of IL-4 in first week then backed this high level but no less than normal range and decrease IL-4 less than control in the third week. In study by Vinderola *et al.* [24] established that the capacity of kefir in a murine model after administration through determined some cytokine level in the gut, intestinal fluid and blood serum were found elevation of IL-12 and IFN-γ, also Hong *et al.* [25] suggested that immunomodulation capacity of kefir return to lactic acid bacteria which most present microorganism in kefir that act by cytokine profile through a toll-like receptor pathway thereby induced the production of pro-inflammatory cytokine

which would potentially have effect on promotion of cell-mediate immune response against intracellular pathogen infection. The Toll-like receptors (TLRs) are crucial regulators of immune response against the pathogen, and after attachment with specific ligand they involved variety phenomenon like maturation phagocytosis and microbicidal activity of phagosome as well as production of inflammatory cytokine such as IL-12 and IFN- γ thus several studies confirm important of TLR signaling in the onset of the leishmanial pathogensis, susceptibility and resistance [26]. Previous study reported that immunomodulation properties of kefir related with exopolysaccharide and a lot of bioactive peptides which form during the fermentation process additionally they observed was linked to a cell-mediated response. Ebner *et al*. [27] established that kefir using for oral immunization it is ability to express biologically active molecules that can modulated immune response or function as adjuvant, in addition to the all derivation of this study there was added perception for strong connected small intestine immune system to the systemic immune system by the lymphatic and blood circulation because the small intestine harbors the Payer's patch that constitute the principal inductive site of the immune response after oral administration [24].

5. RECOMENDATION

Studying the effect of kefir combined with pentostam against *leishmania* parasite in addition the effect of kefir on the other immunological parameters. And investigation of the role of kefir as a preventive therapy against leishmaniasis and other parasites.

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