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RESEARCH ARTICLE

Investacte the Role of *Weissella Confuse* on TNF-α in Serum and Liver Tissue of Mice Infected with *Leishmania Donovani*

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Abstract

Leishmaniasis is one,of the vector-borne diseases, caused by obligate protozoan parasites, they are.transmitted by sand, flies as extracellular. Flagellatedepromastigotes thatereplicate as an intracellulareparasite in.mononuclear cells of mammalian.hosts. Probiotics are microorganisms that provide health benefits when consumed and characterized by being of human origin, probiotics have benefits in health and diseases, there is some clinical applications using probiotic like: immunity function by increasing the number of IgA-producing plasma cells, improving phagocytosis as well as increasing the proportion of T lymphocytes and Natural Killer cells. The present study it is efficiency against *Leishmania donovani* compared with pentostam drug *in vivo* by measuring the levels of immune cytokines (TNF-α) in serum and liver tissue of infected mice and treatment with *Weissella confuse* bacteria and its supernatant for three weeks. The results of measuring the levels of TNF-α showed significant increase in mice serum and the concentration of TNF-α in liver tissue by immunohistochimsry showed increase in TNF-α secretion of treated mice with *Weissella confuse* bacteria and pentostam compared to non infected mice while mice treated with *Weissella confuse* supernatant showed less increase compared to the two previous groups. Probiotic bacteria and its supernatant have an affect on *Leishmania* donovani parasite through induce pro and anti-inflammatory cytokines *in vivo*.

Keyword: *Leishmania, Probiotic, TNF-α, Weissella confuse, Immunohistochimsry.*

Introduction

Leishmaniasis is one, of the vector-borne diseases, caused by obligate protozoan parasites of the genus. Leishmania, they are. Transmitted by various species of sand, flies belonging the. Genus of Phlebotomine aseextracellular. Flagellatedepromastigotes thatereplicate as an intracellulareparasite (aflagellate amastigotes) in.mononuclear cells of mammalian hosts [1]. Probiotics are microorganisms that provide health benefits and characterized by being of human origin, non-pathogenic and having high resistance to passing through the intestine [2].

Because it's effective in health and disease, some clinical applications using probiotic like :(LAB) may improve immune function by increasing the number of IgA-producing plasma cells, phagocytosis and the proportion of T lymphocytes and Natural Killer cells [3]. Probiotics may stimulate the host's immune response to invading pathogens and compete with enteric pathogens, increase the acidity of the intestinal environment, synthesize compounds that destroy pathogens [4]. Weissella spp. are gram-positive, catalase negative, asporogenous, non-motile produce bacteriocins, extracellular polysaccharides (EPS) and hydrolytic enzymes [5]. *Weissella confusa* presented good growth and survival under tough conditions. Also showed auto-aggregation, strong cell surface hydrophobicity, and moderate biofilm formation ability indicating the potential for adhesion on to the host cell as multiple aggregates.

Thermostability, cholesterol removal, ß-galactosidase production and proteolytic activity of the strain indicated potential functional ability[6].TNF- α is a potent pro-inflammatory cytokine ,one of the major biological roles of TNF- α is in the host defense against intracellular pathogens including parasitic infections. Trans membrane TNF- α expressed on glutaraldehy defixed pre-stimulated human T-cells induced monocytes to secrete IL-10 in a cell–cell contact manner .TNF-R1 and -R2 on the monocyte surface are stimulated by

transmembrane TNF-a on glutaraldehyde-fixed pre-stimulated human CD4+ T cells produce TNF-a[7].

It is thus considered that transmembrane TNF-a plays an important role in monocyte cytokine production in T-cell–monocyte cognate interaction. the host immune response is a key determinant of disease outcome following infection with the parasite [8].

Materials and Methods

Parasite Strain and Culture

Leishmania donovani was isolated from the bone marrow of an infected child, the strain was obtained from biotechnology center/ AL-Naharin University, it was cultured and maintained by serial passage in NNN media each 8 days and incubated at 26°C.

Leishmania Antigen Preparation

One milliliter of promastigote culture in stationary phase washed three times with phosphate buffered saline by centrifuge 4000 rpm in 15 minutes then adjusted to concentration 1×10^7 parasite\ml.

Preparation of Free Bacterial Cells and Supernatant

Weissella bacteria were inoculated into MRS broth at 37°C , 5% CO2 for 24 hour, then the culture was centrifuged at 6000 rpm for 15min, supernatant was extracted by pasteol pipette into Eppendrof tubes and bacterial cells were taken, washed and adjust in to 1×10^9 cell /ml [9].

Animals

Ninety six Male *albino* mice aged between 8-12 weeks, weighing 20-28 gm was obtained from National Center for Drug Control and Research, housed under standard condition in animal house in the biology department in College of Science/AL-Mustansrya university.

Ninety mice were infected with 1×10^7 parasite/ml *L. donovani* promastigotes by injection intraperitonial [10]. After fourteen days the parasite infection six mice were sacrificed, and prepared impression smears on a slide from the liver,then stained by Gamisa stain , examined under a microscope to confirm the presence of parasites. Then the remaining infected mice were divided into five groups, each group contain 18 mice . The last group (fifth) of the uninfected was considered as a negative control. Then the groups were inoculated as a follow:

- Group 1: inoculated orally by stomach tube (0. 1ml/day) normal saline considers as control positive group.
- Group 2: inoculated with *Weissella confuse* bacteria cell (0.1ml/day) for 21 days and considers as bacteria group
- Group 3: inoculated bacterial supernatant (0. 1 ml/day) for 21 days considers as suspention group.
- Group 4: injected with (0.01ml/day) from Pentostam drug by intramuscular for 21 days considers as a Pentostam group.
- Group 5: (non infected: inoculated orally by stomach tube (0. 1ml/day) normal saline considers as negative control.

Determination of Cytokine Levels

The serum levels of TNF- α were analyzed by ELISA (human Systems, Germany). The cytokines were quantified using manufacturer's protocol.

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to effect of different factors in study parameters. Least significant difference-LSD test (ANOVA) was used to significant compare between means in this study, (Snedcor and Cochran, 1980).

Immunohistochemistry Study

Livers were removed from the sacrificed mice at the same time collecting blood , fixed in 10% formalin, processed and staining with hematoxylin and eosin then examined under the light microscope to study the score of TNF-α in liver tissue

Results

Concentration of TNF-α

Concentration of TNF-α in Serum

After mice are infected with *Leishmania* parasite the serum levels of TNF-α was gradually increased through three weeks in control positive group (73.77 pg/ml) compared with control group, table (3-5) with significant differences ($P<0.01$) also shown pentostam and bacterial supernatant groups were continuous elevation reached in third week (90.23 pg/ml and 85.41 pg/ml) respectively. While In *Weissella confuse*

group the level of TNF-α has increased in first weeks (89.88pg/ml) and decrease in the second week but in the last week increased again and reached to (86.62pg/ml

Table 1: The serum Concentration of TNF-α in the experimental mice groups after three weeks(Mean ± SE)

Groups	Serum concentration of TNF-α (pg/ml) Mean ± SE		
	7 days	14 days	21 days
Control -	25.08 ± 0.19 e	26.07 ± 0.21 d	25.70 ± 0.25 c
Control +	66.83 ± 1.52 d	69.88 ± 3.52 c	73.77 ± 2.17 b
Pentostam	83.15 ± 1.67 b	85.41 ± 0.68 b	90.23 ± 2.03 a
Bacteria	89.88 ± 1.78 a	91.63 ± 2.18 a	86.62 ± 1.95 a
supernatant	77.99 ± 1.40 c	81.83 ± 1.02 b	85.41 ± 0.68 a
LSD value	4.331 **	5.831 **	5.468 **

** ($P<0.01$).
Means having with the different letters in same column differed significantly

Concentration of TNF-α in Liver Tissue by Immunohistochemistry Assay

After mice are infected with *Leishmania* parasite then inoculated with *Weissella confuse* bacteria and its supernatant and pentostam . TNF-α concentration determined in the liver tissue by using Immunohistochemistry assay. The result showed that the infected mice after three weeks showed a high concentration compaired to non infected mice with (+++ score) this strong intensity was the same in pentostam and bacterial cell group while *Weissella confuse* supernatant group showed less intensity with only (++score). Table 2

Table 2: Concentration of TNF-α in liver tissue for experimental groups after three weeks

Groups	Concentration TNF-α (score)
Control (-)	+
Control (+)	+++
pentostam	+++
Bacterial cell	+++
Bacterial supernatant	++

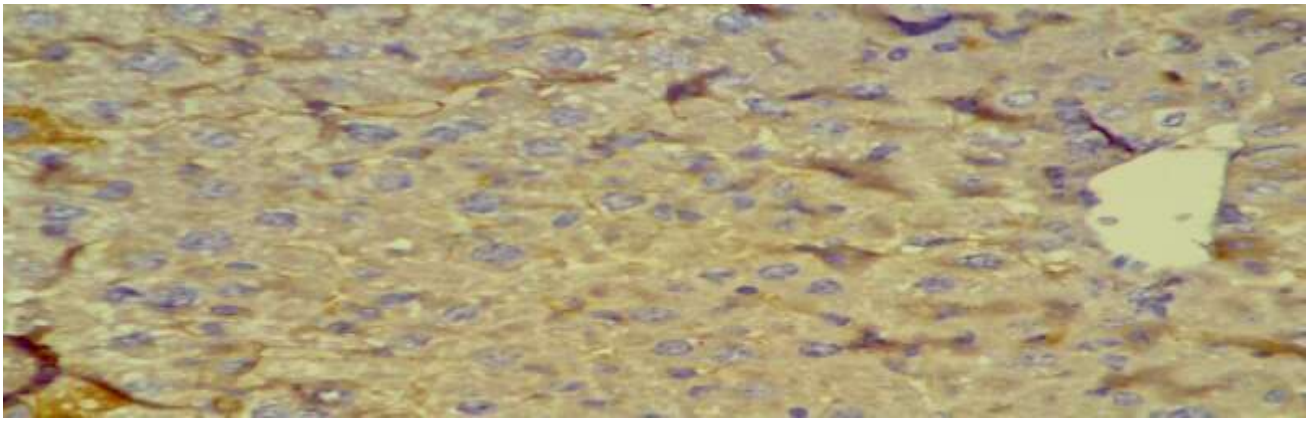

Figure 2: A section in non-infected mice liver shows a low concentration of TNF-α (+) with dark-brown pigmented in lymphocytes cell and hepatic cells in light brown. (40X)

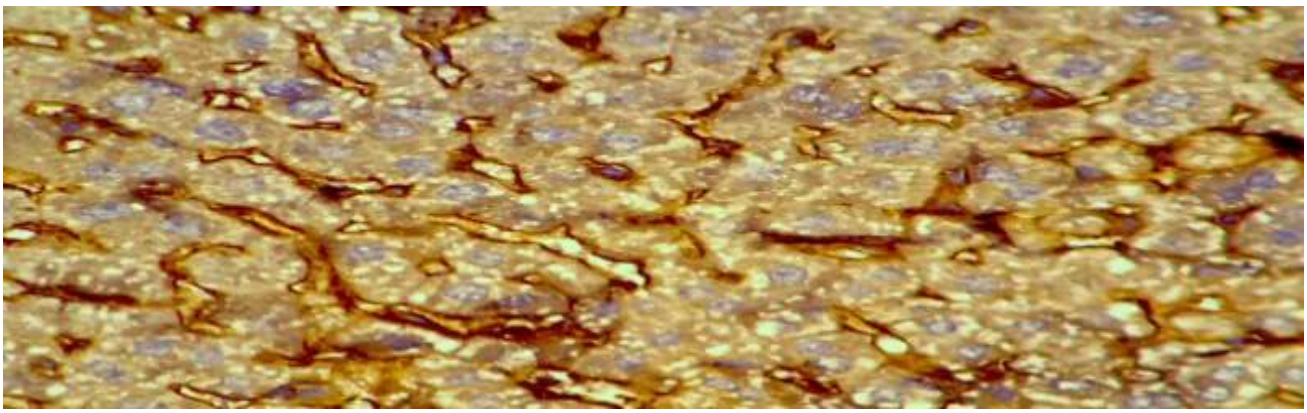

Figure 3: A section in infected with *Leishmania donovani* after three weeks shows shows high concentration of TNF-α (+++)with dark-brown pigmented in lymphocytes cell and hepatic cells in light brown. (40X)

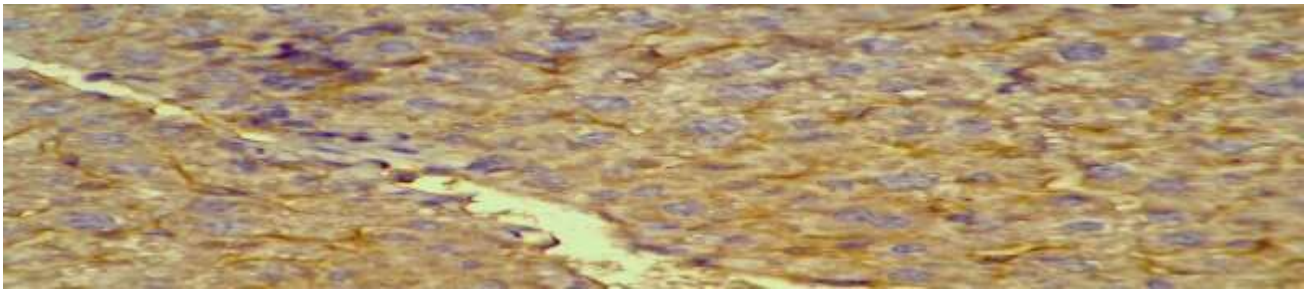

Figure 4: A section in treated with *Weissella confuse* after three weeks shows moderate intensity of TNF-α (++)with dark-brown pigmented in lymphocytes cell and hepatic cells in light brown. (40X)

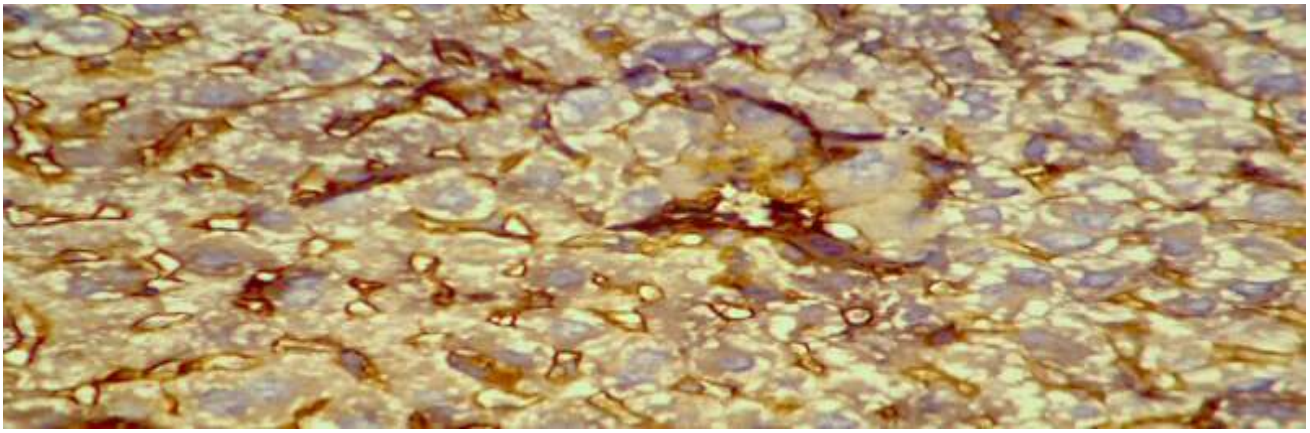

Figure 5: A section in treated with treated with *Weissella confuse* cell after three weeks shows high intensity of TNF-α (+++)with dark-brown pigmented in lymphocytes cell and hepatic cells in light brown. (40X)

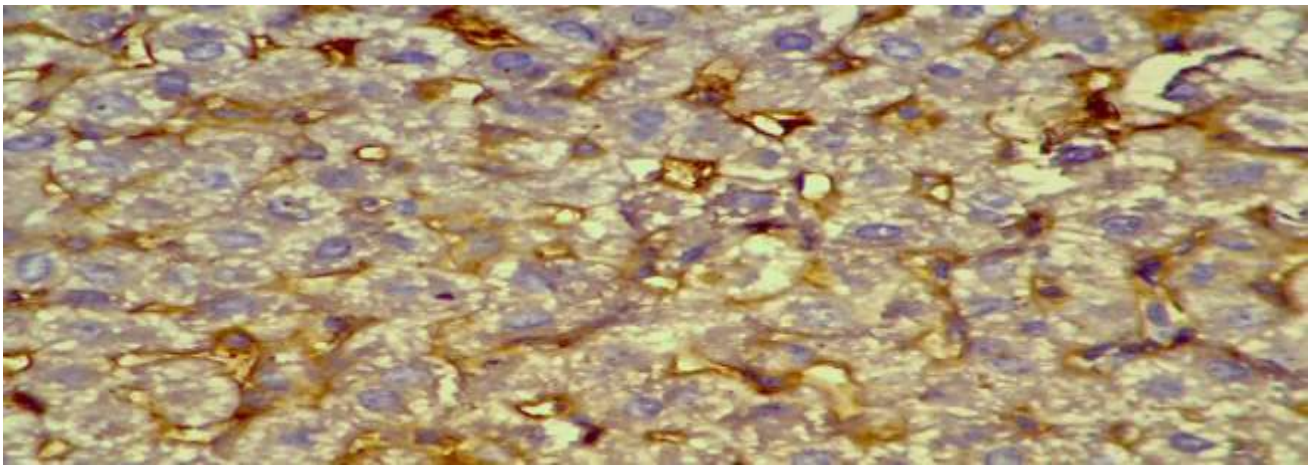

Figure 6: A section in treated with treated with pentostam after three weeks shows high intensity of TNF-α (+++)with dark-brown pigmented in lymphocytecell and hepatic cells in light brown. (40X)

Discussion

The results of this study show an increase occurs in serum level of TNF-α during three weeks post-infection as showed by DE F Michelin *et al* [11]. Who reported a significantly increased serum level of TNF-α in visceral leishmaniasis patients compared to healthy controls, also confirmed by [12]. Pro-inflammatory TNF-α cytokine is important in the defense against many infections, being involved in macrophage activation and granuloma formation [13], and up regulation of chemokine production and expression of cell adhesion molecules required for cellular recruitment [14]. Wilhelm *et al* [15].

Reported that in a visceral leishmania model TNF-α was found to be critical for resistance and resolution visceral leishmania infection and act in concert with IFN-γ through induction of nitric oxide (NO) production by activating macrophages to kill intracellular *Leishmania* parasites . Singh *et al* [16]. Suggested that interleukin 10 is required to control the inflammatory and potentially dangerous effects of extra, production of TNF-αe. Engwerda *et al* [17].

Showed that bloc k of TNF-α can result in increased susceptibility to leishmaniasis infection, or reactivation of disease following therapeutic cure and required for the control of *L. donovani* infection and for the formation of granulomas. This indicates that TNF-α plays an important role in the control of intracellular pathogens, especially those that infect macrophages, also Stanley *et al* [18]. Found that TNF-α critical for Th1 cell-dependentgranuloma formation in an experimental model of visceral leishmaniasis. Goff *et al* [19]. Who reported that the stimulation of the innate immune system ascribed to the early appearance of IL-12 and IFN- γtranscripts in the spleen.

The *lactobacilli* when administered 3 days before or on the same day of parasite infection versus 7 days before, demonstrated better protective respons. Mukhopadhyay and Ganguly [20] reported that lactic acide bacteria strain *Lactobacillus* has demonstrated a protective effect through increasing nonspecific resistance to the malarial parasite *Plasmodium chabaudi* as infection in NIH mice followed by lesser parasitemia and the viability of parasites extracted from the spleen of treated mice, serum of treated mice with lactic acid bacteria has shown more nitrice oxide concentratione which provides a protective effect upon parasite infection.

Also Galdeano and Perdigon [21] said that the stimulation of different subsets of immune system cells by the probiotics can help with production of cytokines, which is followed by induction and regulation of the immune response and remote effect through a nonspecific immune stimulation. Many studes mesuered the TNF- α in infected organs tissue, Taylor and Murray [22] noticed that challenging normal BALB/c mice with *Leishmania donovani* induced TNF-α in infected liver, and increasing tissue TNF levels reflected both initial control over parasite replication and subsequent near resolution of visceral infection, a study presented by Lima *et al* [23].

Indicate that the involvement of TNF-α in the development of lesions occurring at the acute phase of mice infection with other parasite like *Trypanosoma cruzi*, the presence of TNF-α emphasizes the important role of this cytokine in the destruction of parasitized macrophages and intracellular parasites, probably as part of the innate immune response to control the parasite multiplication, in the initial phase of infection with *Trypanosoma cruzi*. Anther study presented by Dheeb *et al* [24].

Who noticed that TNF-α in liver expression was moderate staining at 7 and 14 day post infection while there is intense staining at 21 post infection and suggested that aTNF- α expression levels was time-dependent increased and related with the progress of the infection. In the study done by [25] so the results of immunohistochemistry showed that the score and intensity of TNF in tissue staining increased after infection in animal organs, return to stimulate Th1 immune responses and provide the antigens to stimulate B and T cells to produce TNF- α. A study presented by (Hong *et al* ., 2009) [26] aimed to investigate the *in vitro* immuno-modulating capacity and mechanisms of lactic acid bacteria (LAB) and their supernatants.

The results demonstrated that supernatants, induced the production of pro-inflammatory cytokineTNF-α in murine peritoneal macrophages. These findings indicated that lactic acid bacteria (LAB) influenced the secretion of pro-inflammatory cytokines TNF-α which would potentially have beneficial effects on promotion of cell-mediated immune responses against tumours and also against intracellular pathogenic infections.

The pleiotropic cytokine TNF appears to be a prominent component of a diverse spectrum of both beneficial and harmful inflammatory responses [27].Among the beneficial effects of endogenous TNF is complex role in inducing macrophage activation and enhancing host antimicrobial defence, particularly against intracellular pathogens [28].The critical role of endogenous TNF in the multicytokine-mediated host defence response which controls experimental visceral leishmaniasis, a spread protozoal infection in macrophages of the liver [29].

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