Immunoenhancing Properties of Thymosine and Immunoferron on Leishmania donovani Pathogenicity In Experimental Animals

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ABSTRACT
Golden hamsters (Mesocricetus auratus) were treated intraperitoneally with 20 μg of semipurified thymosine and 10 μg of immunoferron for six successive days, then infected with 5x10^6 promastigotes of L. donovani. After 60 days post infection, the number's of total parasite burden, spleen and liver indices, delayed type hypersensitivity and histopathological changes in spleen and liver were investigated. The total parasites burden, liver and spleen weight were lower (P<0.01) than untreated, while the average of DTH was higher than (P<0.01) untreated one. Also, the histopathological change for spleen and liver less effect than untreated control. The result of investigation indicated that the semi purified thymosine and immunoferron activate the immune system against visceral Leishmaniasis.

INTRODUCTION
Visceral leishmaniasis is a serious systemic diseases caused by an
intracellular protozoan parasite of reticuloendothelial cells, called *Leishmania donovani* complex spp(1). The world health organization (WHO) has considered the Leishmaniasis to be the among six more important infections disease of human world wide(2). In the last decades, there was four to six fold rise in the number of cases of this disease in Iraq(3). The disease initiated by fever tends to be intermittent, chills, and sweating may be present. The spleen and liver are greatly enlarged due to increase of reticuloendothelial and kupffer cells which contain parasites. Thrombocytopenia, markedly leucopenia, and anemia develop together with the disease(4). The anemia is due to reduce red cells life span and mild degree of reduction of erythropoeisis.

Previously Reinner and Malemud(5) demonstrated that intracellular parasitism of *L. donovani* caused major increase in macrophage synthesis of prostoglandins (PGF$_{2\alpha}$), 5-Hydroeicosatetraenoic acid (12,15-HETE), such finding indicates that infection is associated with increased cycloxygenation and lipoxygenation of Arachidonic acid (AA). This result has been supported by Al-Dulami, 1992(6), indicating that Esculetin (an inhibitor of 5-lipoxygenase) reduce the parasite load *in vivo* and *in vitro*(7).

Immunity with the leishmanial infection is associated with the stimulation of protective T-cells, which produce lymphokines that are very important to activated the macrophages resulting in the elimination of parasites(8). In the recent years a considerable attention has been focused on the effect of thymic extract, which has immunological properties to induce both cellular and humoral immunity. In this study Thymosine and Immunoferron were attempted as Immunostimulator against *L. donovani* in experimental animals.

**MATERIALS & METHODS**

Extraction of Thymosine and semi purification from Thymus glands

The extraction of Thymosine and semi purification according to Goldstein, 1979(9).

**Animals:**
Sixty male of golden hamster *Mesocricetus auratus*, 7-8 weeks of age and 80gm body weight were used.

**Immunoferron:**
Glycophosphoptical (Arabian Company) prepared by dissolved 10mg in sterilized D.W. to obtained final concentration of 10µg/0.1 ml.

**Parasites and their Maintenance:**
Culture of *Leishmania donovani* (MOHM/IQ/BRC1 (AA3) was
obtained from the Department of Biology, college of science, University of Baghdad. *L. donovani* promastigotes were cultured at 26°C in semi sold media such promastigotes culture were normally ready for used within six days. When adequate numbers of cultured promastigotes were obtained they were harvested and used for infections in golden hamsters.

**Virulent Parasites:**
These were obtained by inoculating intrapaitonelly into BALB/C mice and the infection left to develop for one month animals were sacrificed and biopsies were taken from the liver and spleen inoculated at stationary phase and washed in sterile P.B.S by centrifugation. The parasites were counted in Neubauer chamber and parasites densities adjusted to give a concentration of $5 \times 10^8$ in 0.2ml volume of P.B.S for (I.P) injection into hamsters.

**Experimental protocol:**
The animals were divided into five groups and each group contained (12) animals as the following:
1 – Group of animals treated with 20μg of semi purified thymosine for six successive days.
2 - Group of animals treated with 10μg of immunoferron for six successive days.
3 - Groups of animals injected with 10 μg of thymosine and 10 μg of immunoferron for six successive days.
4 - Group of animals injected with 0.5ml of 5% Glycogen and served as a positive control.
5 - Groups of animals injected with 0.5ml of P.B.S and served as a (negative control).
All animals were injected with 5x10^7 promastigotes at day seven after treated with immunodulaters compounds. Then the animals were sacrificed after (60) day's post- infection and a concern of the following parameters was made according to(10).

1 - Spleen and liver index.
2 - Total parasite burden (TPB) and prophylactic index.
3 - Delayed type hypersensitivity test to Leishmanial antigen.
4 - Histopathological changes in spleen and Liver's.

**Spleen and Liver Index:**
All animals were weighed at the end of experimental time. Then their spleen and liver aseptically harvested and weighed. Then organ index was calculated according to(11) as to the following equation.

Total parasites burden in liver and spleen:
A cut section of liver and spleen was blotted thoroughly on filter paper, and impression was made on glass slides. Then air dried smear's were fixed in methanol for 3-5 minutes, and stained with Geimsa for 20min. Slides were examined under Oil immersion and the ratio of amastigotes to organ cells nuclei was determined.

Liver or spleen parasites burdens were quantified according to Stauber 1958(12) as following equation formula.

\[
\text{Leishmania donovani} = \frac{\text{liver or spleen weight/mg}}{\text{ratio of amastigotes} \times 200-000 \text{ unit per liver or spleen.}}
\]

While the prophylactic index was calculated according to Riffaat et al., (1989)(11) as the formula.

\[
\text{No. of amastigotes/Spleen or liver in Infected and treated animal} \times 100
\]

\[
\text{No. of amastigotes/spleen or liver In infected and non-treated animal}
\]

Cellular immune response (Delayed type hypersensitivity)

Leishmanial skin test antigens were prepared by suspending washed promastigotes of \textit{Leishmania donovani} (5x10^7/ml) in a solution of 0.5\% phenol saline, hamster were injected intradermally with (0.2ml) of \textit{L. donovani} in left leg, the right legs was injected with (0.1ml) phenol saline (control). The footpad thickness was measured with averiner caliper after 24 hr after the injection of Leishmanin antigens, and the difference in the thickness between the control and Animal weight

\[
\text{Organ Index} = \frac{\text{Organ weigh}}{\text{Animal weigh} \times 1000}
\]

antigen injected foot was considered as the delayed type hypersensitivity.

Histopathological change in spleen and liver:

Tissues samples from spleen and liver were prepared for histopathological studies after sacrificing of hamster's fixed in Bouin's solution for one day, then processed and section were cut by a microtome 4-5 \(\mu\)m in thickness and stained with Haemtoxylin and Eosin according to Bancroft & Steven's 1982(13).

Statistical Analysis:

Analysis of variance (one way) ANOVA was used to compare the results.

**RESULTS**

Weight of spleen, liver and organ index:

Table (1) shows the changes in weight and organ index for spleen and liver in animals post 60 day's of infection after treated with
immunomodulator's for 6 day's the liver index was (38.52, 41.73, 33.44) in groups of animals treated with semipurified thymosine immunoferron, and groups treated with immunoferron and semipurified thymosine respectively, comparing with infected untreated control (59.39) and negative control (29.52) while the splenic index was (2.07, 1.99, 1.84) for treated group respectively comparing with positive control reached (3.80) and negative control reached (1.54). Quantified of total parasites burden (TPB) and prophylactic index in spleen:

Table (2) shows the changes in amastigotes loads, and prophylactic index in spleen post 60 days of infection in animals treated with semipurified thymosine and immunoferron for successive six days before infection. The total parasite burden in the groups treated with semipurified thymosine, immunoferron, semipurified thymosine and immunoferron reach (25.42, 24.97, 14.64) x 10⁶ respectively comparing with untreated control reached (67.85) x 10⁶ while the prophylactic index a raised significantly (P< 0.05) till reach to (62.53, 63.19, 78.40) for treated groups respectively.

Change in cellular immune response (Delayed type hypersensitivity):
This parameter's, investigated the potential role of semipurified thymosine and immunoferron to induce a protective immune response against infection with *L. donovani*. As shown in Table (3) the delayed type hypersensitivity showed increased (P < 0.05) in footpad swelling till reach (0.35, 0.62, 0.74) mm in group of animal treated with semipurified thymosine, immunoferron and treated with semipurified thymosine and immunoferron respectively comparing with positive control which reached (0.15) mm.

**Histopathological Study**
Histological pictures of spleen in infected and non-treated animals showed follicular hyperplasia and widening of the white pulp, hyperplasia of macrophages and lymphocytes (Figure-1). Also, the picture of liver in these animals, showed, slight congestion, hyperplasia of Kupffer cells, infiltration of lymphocyte and macrophages leads to granuloma formation enclosed to blood vessel, with amyloidosis and fatty change Fig. (2) while the histological pictures of spleen and liver Fig. (3,4) in infected and treated with semipurified thymosine and treated with immunoferron showed decreasing in infiltration of lymphocyte and macrophages the while pulp looked normally. The number and size of granuloma was lower then infected and non treated groups.

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DISCUSSION

The activation of macrophages by thymosine and immunoferron leads to change in the surface receptors, which used by leishmanial promastigotes for entering macrophages. However, it is possible that immigrant monocytes represent the responding effector phagocyte(14). The results were consistent with Mahmoud and Tuwajrni (1991)(15) when demonstrated a suppressed proliferation of *L. donovani* amastigotes in both spleen and liver of glucon pre-treatment animals. Also, the reduced total parasite burden observed when infected mice with treated interferon-α as also was observed by Groft *et al.*, (1992)(16).

The results of this study demonstrated that immunopotentiation with thymosine and immunoferron induced a significant protection in hamster against Leishmaniasis. Also, spleen and liver weight reduced to nearly normal weight when treated the infected animals with visceral Leishmaniasis by immunoferron and semipurified thymosine for successive six days consistent with Al-Dulami (1992)(6) when preinfection mice treated with Esculetin daily for six day's. The increased weight of liver and spleen in infected and non-treated animals in this study also observed by Afrin & Ali(17) when reported that liver and spleen weight had increased by 1.5 and 4.7 fold, correlating with visceral proliferation of amastigotes in these organs, the same result reported by Riffaat *et al.*, (1989)(11) when treated visceral Leishmaniasis with ivermectin, when examined immunological change in animals infected with *L. donovani*, the low level of parasite-specific delayed type hypersensitivity in (DTH) response correlated with disease progression in hamester's while the groups treated with semipurified thymosine and immunoferron expressed strong delayed type hypersensitivity. Cellular immune response play an important role in the pathogenesis and healing of Leishmaniasis. This indicated that semipurified thymosine and immunoferron have the ability to activate macrophages and increase proliferation of these cells with lymphocyte and induce the secretion of lymphokines and resulted into granulomatous lesion. The results were consistent with (18,19) when treated visceral Leishmaniasis with polysaccharides daily for six day the DTH were increased.

The histopathological change of infected liver reported in this study were consistent with Sequire *et al.*, (1989)(20) when described granuloma change in liver for 24 weeks. The increase in mature granuloma were correlated with cell immune response as shown in our results. When DTH were decreased during experimental periods, also
the increase of granuloma were correlated with liver weight which increased along infected periods. The histopathological change in liver of treated groups of immunoferron and semipurified thymosine showed a little congestion, hyperplasia of macrophages, granuloma like forming groups and also, correlated with increase in liver weight and reduction in parasite load. These results were consistent with Squires et al., (1989)(20) when treated visceral Leishmaniasis with interferon-γ. The histopathological change in infected spleen were consistent with Gutierrezes et al., (1984)(21) the cause of necrosis depends on immune reaction the decrease in lymphocyte will lead to inhibition of immune cells response. The decrease B and T cells leads to decrease in cells mediated response, which determine weather the infection remain localized or become generalized. The histological changes and increase in spleen weight are consistent with (22,23). Reported the same change after 8 weeks of infected golden hamster's with L. major. The aggregation of macrophages and lymphocytes depends on activation effect of Thymosine and Immunoferron, which a raise the cellular immune response.

| Table (1): Spleen and liver index of golden hamster infected with L. donovani after treatment with Semi purified Thymosine and Immunoferron. |
| Liver Indices | Spleen Indices |
| +ve control 59.39 3.80 | SD |
| Extract 20 μg/gm 38.52 2.07 | Immunoferon 10μg/gm 41.73 1.99 |
| SD | EXT+Immuno 10+5 μg/gm 33.4 1.84 |
| -ve Control 29.52 1.54 | SD |
| N=6 | P<0.05 |
| LSD of liver = 1.02 | LSD of spleen = 0.028 |

Table (2): Total parasite burden and prophylactic index in groups of golden hamster's treated with semipurified thymosine and...
immunoferon and infected with *L. donovani*.

<table>
<thead>
<tr>
<th>Spleen weight (mg)</th>
<th>No. of <em>Leishman donovani</em></th>
<th>Body / cell Total No. of parasite/spleen</th>
<th>% Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>+veControl 295 1.15 67.85</td>
<td>--</td>
<td>6</td>
<td>6.67 0.0931 4.921</td>
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<tr>
<td>SD 6.67 0.0931 4.921</td>
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<tr>
<td>Extract 20μg/gm 163 0.78 25.42** 62.53</td>
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<tr>
<td>SD 8.21 0.0994 3.552</td>
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<tr>
<td>Immunoferon 10μg/gm 181 0.69 24.97** 63.19</td>
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<tr>
<td>SD 7.19 0.0931 2.110</td>
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<tr>
<td>Ext + Immuno 10+5 μg/gm 183 0.40 14.64** 78.42</td>
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<tr>
<td>SD 8.28 0.0886 2.699</td>
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<tr>
<td><strong>P&lt;0.001 LSD=18.25</strong></td>
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Table (3): The delayed hypersensitivity index in hamster's treated with semipurified thymosine and immunoferon and infected with *L. donovani*.

<table>
<thead>
<tr>
<th>DTH Index</th>
<th>+ve control 0.71</th>
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<tbody>
<tr>
<td>SD 0.231</td>
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<tr>
<td>Extract 20μg/gm 1.21</td>
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<tr>
<td>SD 0.155</td>
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<tr>
<td>Immunoferon 10μg/gm 1.48</td>
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<tr>
<td>SD 0.362</td>
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<tr>
<td>Immuno + EXT 5+10 μg/gm 1.60</td>
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<tr>
<td>SD 0.86</td>
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<tr>
<td>-ve control 0.86</td>
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<td>SD 0.198</td>
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Figure (1): Cross section in the liver of hamster infected with *Leishmania donovani* after (60) day's post infection with *L. donovani* showed the infiltration of lymphocyte and macrophages leads to formation of typical Granulomia. Stained with (H&E) under magnification power of (125X).

Figure (2): Cross section in the liver of hamster treated with immunoferron and semipurified thymosine showed little infiltration of lymphocyte and macrophage stained with (H&E) under magnification power of (125X).

Figure (3): Cross section in the spleen of hamster after 60 day's post infection with *L. donovani* showed expansion in white pulp area due to increase in percentage of lymphocyte and macrophage stained with (H&E) under magnification power (125X).

Figure (4): Cross section in the spleen of hamster treated with semipurified thymosine and immunoferron and infected with *L. donovani* showed normal sizes of white pulp with the rare infiltration of lymphocyte and macrophage stained with (H&E) under magnification power of (125X).

REFERENCES


