



## CAN LEVAMISOLE ALONE MAINTAIN THE IMMUNITY?

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## ABSTRACT

The objective of the present study is to check the ability of levamisole to maintain the immunity when given as pretreatment for chronic use. Mainly medicinal plants are used to maintain immunity, but due to lack of scientific evidence they are not given as main therapy. The animals were given pretreatment of levamisole for 15, 30 and 45 days before being immunosuppressed by cyclophosphamide and evaluated for heamagglutination titre, delayed type hyper sensitivity reaction, cyclophosphamide reaction and neutrophil adhesion test. It was found that at least 30 days pretreatment can prevent immunosuppression.

**Keywords:** Sheep red blood cells (SRBC), Heamagglutination titre (HA titre), Delayed hypersensitivity sensitivity reaction (DTH reaction), Cyclophosphamide induced myelosuppression, neutrophil adhesion test

## INTRODUCTION

Lack of immunity is the main cause of many diseases like cancer, AIDS, autoimmune disorder and many infections. Lymph node plays an important role in maintenance of immunity.<sup>1</sup> There are many researches going on development of vaccines to prevent immunosuppression for particular diseases, but they are specific. Newer vaccines include highly purified subunit antigens that are weakly immunogenic. Vaccine formulations often require adjuvants for increased immunological efficiency and better vaccination schedules.<sup>2</sup> It has been observed that many plants are used for non-specific activation of immune system. A number of plants used in traditional medicines for rejuvenation therapy and chronic ailments have been shown to stimulate immune responses and several active substances have been isolated. Plants like ashwagandha, shankpushpi, amala, indian ginseng, etc. are proved to be immunostimulant in immunosuppressed condition.<sup>3</sup> But it has been observed that the active content in plants vary from place to place and seasonal to seasonal with a huge controversy of heavy metal contents. Even the immunostimulant effect can be due to some synergism. The use of medicinal plants in modern medicine suffers from the fact that though hundreds of plants are used in the world to prevent or to cure diseases, scientific evidence in terms of modern medicine is lacking in most cases. However today it is necessary to provide scientific proof as whether to justify the use of plant or its active principles.<sup>4</sup> Therefore there is a strong need of synthesized drug, which can have a constant known therapeutic effect. Levamisole is one of the ideal candidates for this purpose because of its immunomodulatory effect.

Levamisole is mainly used as anthelmintic agent in veterinary purpose.<sup>5</sup> But in some countries its use is limited to immunomodulatory agent in humans in some cancers.<sup>6</sup> It is having immunostimulating effect in immunosuppressed condition.<sup>7</sup> It is used as a standard drug in immunosuppressed condition. But it has been found that it is also having useful effect in autoimmune diseases like nephrotic syndrome and rheumatoid arthritis. It helps to make steroid free period for up to 6 months to 1 year in nephrotic syndrome.<sup>8,9</sup>

The objective of the present research work is to check effect of chronic pretreatment with levamisole for a particular period on prevention of immunosuppression, when given immunosuppression.

## MATERIALS AND METHODS

**Animals:** Unused Male *Wistar* rats (150-200 g) were purchased from Hafkine institute, Mumbai, India. The rats were housed in polypropylene cages under standard conditions of temperature,

humidity and light at animal house of School of Pharmacy & Technology Management, Mumbai. Acclimation period of 7 days was given prior to experimentation. Water and feed were given ad libitum. The animal experiments were approved by institutional animal ethics committee (IAEC) of School of Pharmacy & Technology Management, SVKM's NMIMS University, Mumbai in accordance Committee for the purpose of control and supervision on experiments on animals (CPCSEA).

**Chemicals:** Levamisole hydrochloride was gift sample from Ashish Life Science (Mumbai, India). Endoxan (Baxter, USA) - a marketed formulation of cyclophosphamide was purchased. The other materials were of pharmaceutical and analytical grade and were procured from S. D. Fine Chem Ltd (Mumbai, India).

**Antigen:** Fresh blood was collected in Alsvet's solution in 1/1 proportion from local slaughter house where sheep are sacrificed. Sheep red blood cells (SRBCs) were separated by centrifugation and were washed three times in normal saline and adjusted to a concentration of  $1 \times 10^9$  cells/ml for immunization and challenge.

**Experimental design:** The animals were divided into five groups.

- Control group
- 15 days pretreatment
- 30 days pretreatment
- 45 days pretreatment
- Negative control group (For cyclophosphamide induced myelosuppression only)

All the above groups are given cyclophosphamide (30 mg/kg, i.p.) for last 10 days during their pretreatment with levamisole (2.5 mg/kg, p.o.). The parameters evaluated after chronic treatment are as follow:

1. Heamagglutination titre (HA titre)
2. Delayed hypersensitivity reaction (DTH reaction)
3. Cyclophosphamide induced myelosuppression
4. Neutrophil adhesion test

The detail method for each evaluation parameter is given below:

**1. Heamagglutination titre (HA titre)<sup>10,11,12,13,15,16,17:</sup>**

Each group is immunized by i.p. administration of 0.2 ml of  $1 \times 10^9$  SRBC/ml suspension after their pretreatment. Blood sample is collected and serum is separated from the blood sample for primary antibody determination after one week of immunization with SRBC. After collection of blood sample, each group is challenged by 0.2 ml of  $1 \times 10^9$  SRBC/ml. Again, blood samples are collected after a week and secondary antibody determination is done.

Antibody levels were determined by hemagglutination technique. Briefly, equal volume of individual serum samples of each group was pooled. Two fold dilutions of pooled serum samples were made in 25 µl volumes of normal saline in 96 well plate microtitration plates and to it 25 µl of 1% suspension of  $1 \times 10^9$  SRBC in saline is added. After mixing, the plates were incubated at 37°C for 2 h. and examined for agglutination. The highest no. of dilution of serum showing hemagglutination has been expressed as HA titre.

## 2. Delayed type hypersensitivity (DTH) response<sup>3,14,17</sup>

In this method, six animals per group are immunized by i.p. administration of  $1 \times 10^9$  SRBCs/rat after their chronic pretreatment and challenged by s.c. administration of  $0.25 \times 10^9$  SRBCs/rat into right hind foot pad. The volume for the inflammation was measured after challenge at 24 and 48 h by Plethysmometer (IITC Life Science, USA). The differences obtained for pre- and post-challenge foot volumes were taken for the measurement of DTH and were expressed in % inflammation.

## 3. Cyclophosphamide induced myelosuppression<sup>10, 11, 13, 14</sup>

Group I serves as control group receiving saline solution. During pretreatment, all the groups receive cyclophosphamide for last 10 days, while group V is given cyclophosphamide alone at 30 mg/kg i.p. After the pretreatment and 10 days of cyclophosphamide treatment, blood samples were collected from the retro-orbital plexus of individual animals and analyzed for hematological and serological parameters by automated hematology analyzer (Sysmex, Japan).

## 4. Neutrophil adhesion test<sup>11,13</sup>

Group I was served as control group and received saline solution. After the treatment, blood samples are collected by puncturing retro orbital plexus into vials having EDTA as an anti-coagulant and analyzed for total leukocyte cell (TLC) and differential leukocyte cell (DLC) counts using automated hematology analyzer (Sysmex, Japan). After initial counts, blood samples were incubated with 80 mg/ml of nylon fiber for 15 min. at 37°C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index of blood sample. The percent neutrophil adhesion was calculated as shown below:

$$\text{Neutrophil adhesion \%} = \frac{(NI_u - NI_t)}{NI_u} \times 100$$

Where  $NI_u$  is neutrophil index of untreated blood samples and  $NI_t$  is the neutrophil index of treated blood samples.

**Statistical analysis:** Data were expressed as mean  $\pm$  standard deviation (n=6), and statistical analysis was carried out by one way ANOVA followed by unpaired student's t-test, \*p<0.05 was considered as statistically significant.

## RESULTS

### 1. Hemagglutination titre (HA titre)

The HA titre was used to assess humoral immune response. It was observed that as the number of the pretreatment day's increases, the primary and secondary antibody increases in rats. The augmentation of the humoral immune response to SRBC by levamisole solution is evidenced by increase in the antibody titres in the blood of rat.

### 2. Delayed type hypersensitivity (DTH) response

The cell mediated immune response was assessed by DTH reaction, i.e. foot pad reaction. The pre-treatment showed period dependent increase in DTH reactivity. Increase in DTH reaction in rat in response to T cell dependent antigen revealed the stimulatory effect of pre-treatment of levamisole on T cells. Thus, as the pre-treatment increases, the % edema is decreased in rats.

### 3. Cyclophosphamide induced myelosuppression

Cyclophosphamide at a dose of 30 mg/kg, i.p. caused a significant reduction in the hemoglobin (HGB), RBCs, WBCs and platelets count. Pretreatment with levamisole was able to restore the bone marrow activity as compared with cyclophosphamide treatment alone. Though, there was not much immunostimulant action seen with 45 days pretreatment as compared with 15 and 30 days pretreatment.

### 4. Neutrophil adhesion test

Pretreatment of drug evoked a significant increase in the in vitro adhesion to nylon fibres, which correlates the increase in % neutrophils. As percentage neutrophil increases, the antigen engulfment also increases.

**Table 1: Effect with pre-treatment of levamisole on antibody titres to antigenically challenged rat**

| Group            | No. of the pre -treatment days | Mean hemagglutination antibody (HA) titre |                    |
|------------------|--------------------------------|---|--------------------|
|                  |                                | Primary HA titre                          | Secondary HA titre |
| I- Control group | -                              | 6 $\pm$ 3.464                             | 10.67 $\pm$ 4.62   |
| II               | 15                             | 10.67 $\pm$ 4.62                          | 13.33 $\pm$ 4.62   |
| III              | 30                             | 18.67 $\pm$ 12.22                         | 21.33 $\pm$ 9.23   |
| IV               | 45                             | 26.67 $\pm$ 9.24                          | 32.0 $\pm$ 0.00    |

Values are in mean $\pm$ S.D, n=6. p<0.5 when compared with control group in all cases (statistics; one way ANOVA followed by unpaired t-test)

**Table 2: Effect of pre-treatment of levamisole on SRBC induced DTH in rat**

| Group | No. of pre-treatment days | Mean % edema at 24 hrs. | % change in DTH reaction at 24 hrs. | Mean % edema at 48 hrs. | % change in DTH reaction at 48 hrs. |
|-------|---------------------------|-------------------------|-------------------------------------|-------------------------|-------------------------------------|
| I     | -                         | 51.78 $\pm$ 4.55        | -                                   | 48.25 $\pm$ 6.75        | -                                   |
| II    | 15                        | 37.32 $\pm$ 14.92       | 27.93                               | 35.07 $\pm$ 4.79        | 27.32                               |
| III   | 30                        | 27.91 $\pm$ 6.77        | 46.10                               | 23.49 $\pm$ 4.45        | 51.32                               |
| IV    | 45                        | 27.97 $\pm$ 7.28        | 45.98                               | 23.64 $\pm$ 7.26        | 51.00                               |

Values are in mean $\pm$ S.D, n=6. p<0.5 when compared with control group in all cases (statistics; one way ANOVA followed by unpaired t-test)

**Table 3: Effect of pretreatment of levamisole on blood cells of rat treated with cyclophosphamide for 10 days.**

| Group | Hemoglobin concentration in gm. | RBC count in (%) million (cmm) | WBC count in thousands (cmm) | Platelet counts in thousands (cmm) |
|-------|---------------------------------|--------------------------------|------------------------------|------------------------------------|
| I     | 14.7±3.66                       | 8.62±1.65                      | 11.6±4.293                   | 840.5±82.73                        |
| II    | 12.15±2.62                      | 7.15±0.551                     | 3.8±0.99                     | 638.0±352.14                       |
| III   | 12.6±0.565                      | 7.47±0.59                      | 6.96±0.874                   | 761.33±179.89                      |
| IV    | 12.77±1.27                      | 7.35±0.662                     | 8.9±0.781                    | 804.66±145.22                      |
| V     | 11.25±0.07                      | 6.45±0.304                     | 0.6±0.141                    | 567.0±257/38                       |

**Group I-** control (without any drug treatment), **Group II-** 15 days pretreatment with levamisole, **Group III-** 30 days pretreatment with levamisole, **Group IV-** 45 days pre-treatment with levamisole, **Group V-** Cyclophosphamide treated group.

Values are in mean±S.D, n=6. p<0.5 when compared with control group in all cases (statistics; one way ANOVA followed by unpaired t-test)

**Table 4: Effect of pretreatment of levamisole on neutrophil adhesion test**

| Group | TLC (10 <sup>3</sup> /ml) [A] |            | % Neutrophil [B] |            | Neutrophil index [A×B] |              | Neutrophil adhesion (%) |
|-------|-------------------------------|------------|------------------|------------|------------------------|--------------|-------------------------|
|       | UTB                           | FTB        | UTB              | FTB        | UTB                    | FTB          |                         |
| I     | 11.4±2.29                     | 11.07±2.04 | 21.8±4.98        | 21.77±4.77 | 245.02±50.26           | 238.04±49.25 | 2.76±3.73               |
| II    | 10.85±8.41                    | 9.9±6.79   | 38.35±20.01      | 38±20.78   | 331.9±105.58           | 305.64±52.14 | 10.02±8.11              |
| III   | 7.94±0.87                     | 7.93±1.72  | 19.67±7.97       | 18.47±7.31 | 152.27±47.71           | 138.44±21.75 | 11.48±9.37              |
| IV    | 6.85±0.64                     | 6.1±0.71   | 25.9±6.36        | 24.65±4.74 | 175.39±27.11           | 148.69±11.47 | 14.71±6.64              |

UB: untreated blood; FTB: fiber treated blood.

Values are in mean±S.D, n=6. p<0.5 when compared with control group in all cases (statistics; one way ANOVA followed by unpaired t-test)

## DISCUSSION

Antibody molecules, a product of B lymphocytes and plasma cells, are central to humoral immune responses, IgG and IgM are the major immunoglobulin which are involved in the complement activation, opsonization, neutralization of toxins, etc. The augmentation of the humoral immune responses to SRBCs by pretreatment of levamisole, as evidenced by increase in the antibody titre in rats (Table 1) indicated the enhanced responsiveness of T and B lymphocyte subsets, involved in the antibody synthesis. The value of haemagglutinating antibody titre increase as the no. of pretreatment day's increases indicates that immunostimulation was achieved through humoral immunity.

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defense against infectious organisms, infection of foreign grafts, tumor immunity and delayed-type hypersensitivity reactions. Therefore, increase in DTH reaction in rat in response to T cell dependent antigen revealed the stimulatory effect by pretreatment of levamisole on T cells (Table 2).

Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a significant reduction in the WBCs, RBCs, hemoglobin and platelets count. Combined treatment of cyclophosphamide and levamisole resulted in a restoration of bone marrow activity according to pretreatment as compared to cyclophosphamide treatment alone. Treatment with levamisole indicated that cyclophosphamide-induced immunosuppression was not restored to normal, but the animals treated with levamisole showed more normal activity as compared to cyclophosphamide-treated group. As shown in table 3, the levamisole treated group normalizes all the parameters of blood like RBCs, hemoglobin and platelet count level to normal level according to no. of pretreatment days.

The neutrophil, an end cell unable to divide and with limited capacity for protein synthesis is, nevertheless, capable of a wide range of responses, in particular chemotaxis, phagocytosis, exocytosis and both intracellular and extracellular killing. In the present study, pretreatment with levamisole evoked a significant increase in percent neutrophils. This is indicating that when any immunosuppression is given, the drug will try to prevent this immunosuppression and this may help in increasing immunity of body against microbial infections.

## CONCLUSION

The present investigation suggests that chronic pretreatment with levamisole may stimulate both cellular and humoral immune responses and thus prevent the immunosuppression in normal rat suggesting its therapeutic usefulness in disorders of immunological origin. Further studies using in vivo and in vitro models of immunomodulation are needed to elucidate the exact immunomaintenance property of levamisole and its mechanism of action.

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