

## Biochemical, Immunomodulatory and Antioxidant Properties of Levamisole at Different Storage Conditions and Administration Routes

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**Abstract:** Levamisole is a broad-spectrum nicotinic anthelmintic drug; widely used in veterinary medicine. Levamisole actions are variable depending on storage temperature and administration route. The present study was conducted to test levamisole effects on immune responses and antioxidant status of rats at different storage temperature and administration routes. The experimental rats were allocated into four experimental groups and two controls. Group 1 was given levamisole orally. Group 2 was injected levamisole intramuscularly. In group 1 and 2 levamisole was stored at 4°C for 72 h and given every two days for three weeks at a dose of 2.5 mg kg<sup>-1</sup> body weight. Group 3 and 4 were treated same as group 1 and 2 but levamisole was stored at 37°C. Serum levels of total IgG and IgA and plasma levels of glutathione reductase (GSSG-R), TAC concentrations in addition to GST and GSH-Px activities were measured. The results indicated that the storage of levamisole at 4°C significantly increased serum IgG and IgA levels in rats and improved rat's antioxidant status through significant increase in glutathione related enzymes (GSSG-R, GST and GSH-Px) and the TAC. Levamisole stored at 37°C increased measured antioxidant biomarkers but decreased the rats' non-specific humoral immunity. Moreover, the intramuscular administration induced better antioxidant properties than oral administration of levamisole. In conclusion, levamisole actions could be specifically directed towards certain types of immune responses due to changes in storage temperature with better response to injectable routes than oral routes with more antioxidant activities.

**Key words:** Antioxidant biomarkers, humoral immunity, immunoglobulines, levamisole

### INTRODUCTION

Levamisole (LMS), the L isomer of tetramisole (2, 3, 5, 6-tetrahydro-6-phenylimidazo [2, 1-b] thiazole) is a broad-spectrum nicotinic anthelmintic and therefore widely used in veterinary medicine (Renoux, 1980). Moreover Stogaus and King (1995) reported that levamisole has more useful physiological immune-stimulant effect. As known, treatment and prevention of infectious diseases are the most common reasons to use immunomodulators/immunostimulants in veterinary medicine (Blecha, 2001).

Following a long history of experimental and clinical uses in therapy of different diseases, LMS demonstrated significant immunomodulatory activities. It can stimulate formation of antibodies to various antigens, enhance T-cell responses by stimulating T cell activation, proliferation and maintenance (Cuesta *et al.*, 2004; Jin *et al.*, 2004; Bilandzic *et al.*, 2010), potentiate the functions of monocytes and macrophages and increase neutrophils mobility, adherence and chemotaxis (Anderson *et al.*, 1992; Blecha, 2001; Bozic and Mrljak,

2001; Chen *et al.*, 2008) and production of several cytokines including IFN- $\gamma$ , IL-6, IL-12, IL-18 and IL-1 (Johnkoski *et al.*, 1997; Szeto *et al.*, 2000; Chen *et al.*, 2008).

There are sufficient evidences concerning the antioxidant properties of levamisole, in particular, about its effects on the major cellular redox system glutathione (GSH/GSSG) (Ince *et al.*, 2010; Lake *et al.*, 2012). LMS exerts beneficial and protective effects on the SH-groups in plasma and liver (Kumar *et al.*, 1980; Chwiecko *et al.*, 1991), possibly through increasing intracellular glutathione (thiol) concentration (Obiri *et al.*, 1990; Chwiecko *et al.*, 1991). Hanson (1986) and Hanson *et al.* (1991) found that solutions of LMS stored at 4°C consistently enhanced the lymphocyte proliferation in response to concanavalin A (Con A) more than did freshly prepared solutions, while solutions of LMS stored at 37°C had an immunoinhibitory activity. They suggested that the opposing results reported might be due to the degradation of three products of LMS and their relative concentrations, which vary according to the used storage temperature (Hanson and Heidrick, 1991).

LMS therapeutic efficacy depends mainly on several factors, such as dose timing and the use of different cell types and experimental models (Renoux, 1980). Renoux's group has early suggested the importance of route of LMS administration in affecting graft rejection. The change in LMS administration from oral route to intramuscular injection was found to affect significantly the drug bioavailability in plasma (Fernandez *et al.*, 1998). To our knowledge, the possible relationship between methods of LMS administration and its storage efficacy on the immune responses is not yet examined and data are not clear and scarce. The current study investigated the effects of LMS stored at two different temperatures and administrated orally and intramuscularly on immune and antioxidant status of Sprague-Dawley rats.

## MATERIALS AND METHODS

**Experimental animals:** Specific pathogen free, immunocompetent, male Sprague-Dawley rats weighting 300-400 g were purchased (National Research Centre, Cairo) and housed for 2 weeks acclimation period. All animals were clinically normal. Rats were fed *ad libitum* by fair laboratory chow and potable fresh and clean water. Rats were kept at temperature of  $23\pm 2^{\circ}\text{C}$ .

**Formulation of LMS solutions:** Levamisole solutions were prepared with LMS.HCL pure powder (KAHIRA Pharmaceutical and chemical Industries Company, Cairo, Egypt). The purity was determined by the manufacture to be greater than 99.0% with spectrophotometry. The powder was irrigated by sterile deionized distilled water for oral solutions and water for injection (pyrogen free) for intramuscular solutions. Solutions then were transferred in 4 dark amber glass sterile (autoclaved) prescription bottles. One bottle of each formulation (for oral and intramuscular usage) was stored in methanol at  $4^{\circ}\text{C}$  refrigerator. The other two bottles were stored in incubator at  $37^{\circ}\text{C}$  from light. The storage period for all was 72 h and the apparent pH of each solution was measured using a digital pH meter after preparation and was found to range between (7.25-7.5).

**Experimental design:** Rats were randomly allocated into four experimental and two control groups each group with 10 rats. Group I (G1): rats given LMS.HCL stored at  $4^{\circ}\text{C}$  orally, Group II (G2): rats given LMS.HCL stored at  $4^{\circ}\text{C}$  i.m., Group III (G3): rats given LMS.HCL stored at  $37^{\circ}\text{C}$  orally, Group IV (G4): rats given LMS.HCL stored at  $37^{\circ}\text{C}$  i.m.. Control 1 (C1): rats received normal saline orally as a placebo and Control 2 (C2): rats received normal saline i.m. as a placebo.

LMS.HCL solutions were administrated to the four experimental groups at the standard and well tolerated dosage ( $2.5\text{ mg kg}^{-1}\text{ b.wt.}$ ) orally and intramuscularly based on Stogaus and King (1995) and Lake *et al.* (2012). Both LMS.HCL solutions and normal saline were given at same time with one day intervals (day after day) for three weeks (21 days). Blood sampling was done on days 2, 12, 22 after starting. Serum and plasma were harvested after centrifugation at 3,000 rpm for 15 min and stored at  $-70^{\circ}\text{C}$  until analysis. Erythrocytes lysate was prepared according to Paglia and Valentine (1967) method. Red blood cells were collected by centrifugation (4000 rpm x 10 min at  $4^{\circ}\text{C}$ ) and the plasma was removed. Then cells were washed once with 10 volumes of cold saline. The red cell pellets were lysed by adding 4 volumes of cold deionized water to the estimated pellet volume. Red cell stroma was removed by centrifuging and the resulting clarified supernatant was collected and kept in freezer at  $-70^{\circ}\text{C}$  until analysis.

**Biochemical analysis:** Total serum immunoglobulins IgG and IgA concentrations were evaluated according to the methods of Zilva and Pannall (1984) and Chan *et al.* (1995), which based on nephelometric assessment of immunoglobulin/specific antiserum insoluble complexes. Plasma glutathione reductase (GSSG-R) activity was measured spectrophotometrically (Goldberg and Spooner, 1983) and plasma total glutathione S-transferase (GST) activity (cytosolic and microsomal) was measured spectrophotometrically (Habig *et al.* 1974). Total Antioxidant Capacity (TAC) in plasma was assessed colorimetrically based on method of Koracevic *et al.* (2001). Cellular glutathione peroxidase (c-GSH-Px) in erythrocytes was measured using an indirect coupled enzymes method (Paglia and Valentine, 1967). Erythrocytes hemoglobin levels were determined as described by Zilva and Pannall (1984).

**Statistical analysis:** The obtained data were statistically analyzed by one-way Analysis of Variance (ANOVA) followed by the Duncan multiple test. All analyses were performed using the statistical package for social science (SPSS 13.0 software). Values of  $p < 0.05$  were considered to be significant.

## RESULTS

**Effect of LMS storage and rout of administration on IgG and IgA concentration in rats:** As shown in Table 1, serum IgG concentrations varied significantly in all experimental groups at 12 and 22 days ( $p < 0.001$ ). Serum levels of IgG in rats given LMS.HCL solutions stored at  $4^{\circ}\text{C}$ , orally and intramuscularly were higher in G1 and G2

than in G3 and G4. IgG levels were at day 12 and 22 were 236±5.27 and 256±11.93 respectively in group 1, and were 239±7.09 and 263.5±5.77 in day 12 and 22, respectively in group 2 and they are higher than respective control groups (C1, 210.13±9.2 and C2, 212.67±9.07). Moreover, it is worth noting that IgG concentrations in G3 and G4 groups were significantly low among all groups without difference between the two administration routes (Table 1). Regarding serum IgA concentrations, there are differences in days 2, 12 and 22 among experimental groups (p<0.05). Serum IgA concentrations in G1 (40.33±3.51 at day 22) and G2 (43.33±2.52 at day 22) groups considered significantly the highest amongst all the examined groups. While serum levels of IgA in G3 (20.67±2.08 at day 22) and G4 (18.27±2.53 at day 22) groups recorded the lowest values without difference between the two administration routes. Moreover, there are time dependent increase and decrease in serum levels of IgG and IgA in LMS used at 4 and 37°C, respectively relative to each respective control and day 2 in all examined groups.

**Effect of LMS storage and rout of administration on GST and GSS-R activity in rats:** As seen in Table 2, plasma

GST and GSSG-R activities were higher at days 12 and 22 in rats' given LMS.HCL solutions stored at 4°C (456.5±17.37 in G1 and 476.5±12.5 in G2) than G3 and G4 groups given LMS.HCL solutions stored at 37°C (415±19.58 in G3 and 418.25±13.87 in G4). However, the activity of GST enzyme was significantly higher in all examined groups, compared to their respective control groups. The intramuscular delivery of LMS solution stored at 4°C achieved the best level of significance at day 22 (87.79±4.86 in G2 and 61.7±6.52 in G4) (Table 2). The overall response regardless rout of administration is time dependent pattern.

**Effect of LMS storage and rout of administration on plasma total antioxidant (TAC) and erythrocytes glutathione peroxidase activities in rats:** Plasma TAC activities were significantly higher in G2 (1.89±0.1), G3 (1.83±0.18) and G4 (2±0.07) groups than G1 (1.81±0.12) group (Table 3). Moreover, there were no significant differences between G1 and G3 in response to TAC status. While on day 22, the four experimental groups exhibited equal marked improvement in their antioxidant status compared to control groups (Table 3). On other hand, GSH-Px enzymatic activity at all examined groups

Table 1: Changes in serum IgG and IgA levels after administration of levamisole in rats

Groups	Serum IgG levels (mg dL <sup>-1</sup> )			Serum IgA levels (mg dL <sup>-1</sup> )		
	2nd day	12th day	22nd day	2nd day	12th day	22nd day
G1	201.33±11.02	236.50±5.27 <sup>c</sup>	256.33±11.93 <sup>c</sup>	28.33±3.51	33.33±2.52 <sup>cd</sup>	40.33±3.51 <sup>e</sup>
G2	211.33±8.96	239.33±7.09 <sup>c</sup>	263.50±5.77 <sup>c</sup>	29.67±0.58	36.33±4.04 <sup>d</sup>	43.33±2.52 <sup>e</sup>
G3	198.33±2.89	192.33±4.04 <sup>a</sup>	186.67±5.86 <sup>a</sup>	28.33±1.53	25.50±1.32 <sup>ab</sup>	20.67±2.08 <sup>a</sup>
G4	208.33±8.74	194.17±7.01 <sup>a</sup>	178.00±7.55 <sup>a</sup>	29.17±3.4	23.10±2.59 <sup>a</sup>	18.27±2.53 <sup>a</sup>
C1	208.33±10.69	210.13±9.2 <sup>b</sup>	217.00±3.61 <sup>b</sup>	25.77±4.14	30.00±1.73 <sup>bc</sup>	29.90±1.15 <sup>b</sup>
C2	206.00±7.94	212.67±9.07 <sup>b</sup>	211.33±8.5 <sup>b</sup>	29.00±2.65	28.87±3.59 <sup>bc</sup>	32.00±3.00 <sup>b</sup>

Data are presented as Mean±SE. SE: Standard error for 10 rats per each group. Mean values with different superscript letters in the same column are significantly different at (p<0.05)

Table 2: Changes in plasma GST and GSSG-R activities after administration of levamisole in rats

Groups	Plasma GST activity (U L <sup>-1</sup> )			Plasma GSSG-R activity (U L <sup>-1</sup> )		
	2nd day	12th day	22nd day	2nd day	12th day	22nd day
G1	335.50±33.75	456.50±17.37 <sup>c</sup>	335.50±33.75	41.97±2.85	64.68±5.77 <sup>c</sup>	82.44±4.32 <sup>c</sup>
G2	340.25±38.34	476.50±12.26 <sup>c</sup>	340.25±38.34	43.46±2.94	66.03±3.5 <sup>c</sup>	87.79±4.86 <sup>c</sup>
G3	324.25±27.94	415.00±19.58 <sup>b</sup>	324.25±27.94	43.86±3.07	55.50±3.97 <sup>b</sup>	62.55±4.84 <sup>b</sup>
G4	331.00±15.64	418.25±13.87 <sup>b</sup>	331.00±15.64	41.70±2.01	49.12±8.85 <sup>ab</sup>	61.70±6.52 <sup>b</sup>
C1	344.25±29.65	360.75±24.24 <sup>a</sup>	344.25±29.65	39.56±3.19	46.32±3.15 <sup>a</sup>	44.71±4.61 <sup>a</sup>
C2	336.25±21.76	364.50±28.22 <sup>a</sup>	336.25±21.76	42.98±4.25	44.89±5.41 <sup>a</sup>	47.33±3.14 <sup>a</sup>

Data are presented as Mean±SE. SE: Standard error for 10 rats per group. Mean values with different superscript letters in the same column are significantly different at (p<0.05)

Table 3: Changes in plasma TAC and erythrocytes GSH-Px activity after administration of levamisole in rats

Groups	Plasma TAC status (mM L <sup>-1</sup> )			Erythrocytes' GSH-Px activity (mU gHb <sup>-1</sup> )		
	2nd day	12th day	22nd day	2nd day	12th day	22nd day
G1	1.00±0.28	1.45±0.28 <sup>b</sup>	1.81±0.12 <sup>b</sup>	442.19±37.94	544.64±10.55 <sup>b</sup>	713.5±28.01 <sup>bc</sup>
G2	0.99±0.24	1.53±0.11 <sup>bc</sup>	1.89±0.1 <sup>b</sup>	416.48±49.81	543.09±14.71 <sup>b</sup>	708.42±35.77 <sup>bc</sup>
G3	1.00±0.26	1.52±0.11 <sup>bc</sup>	1.83±0.18 <sup>b</sup>	441.58±22.81	540.70±14.02 <sup>b</sup>	700.02±31.54 <sup>b</sup>
G4	1.04±0.18	1.79±0.17 <sup>c</sup>	2.00±0.07 <sup>b</sup>	404.63±21.36	552.21±20.76 <sup>b</sup>	743.77±11.72 <sup>c</sup>
C1	1.08±0.23	0.94±0.25 <sup>a</sup>	1.01±0.1 <sup>a</sup>	416.38±13.94	416.25±39.23 <sup>a</sup>	411.79±24.78 <sup>a</sup>
C2	1.02±0.13	0.99±0.16 <sup>a</sup>	1.04±0.21 <sup>a</sup>	415.20±44.4	424.48±20.89 <sup>a</sup>	422.02±16.64 <sup>a</sup>

Data are presented as Mean±SE. SE: Standard error for 10 rats per group. Mean values with different superscript letters in the same column are significantly different at (p<0.05)

were significantly higher relative to control groups ( $p < 0.05$ ) in time dependent manner. The levels were  $713.5 \pm 28.01$ ,  $708.42 \pm 35.77$ ,  $700.02 \pm 31.54$ ,  $743.77 \pm 11.72$  in G1, G2, G3 and G4 compared to C1 and C2 ( $411.79 \pm 24.78$  in C1 and  $422.02 \pm 16.64$  in C2).

## DISCUSSION

LMS is a compound that possesses a wide variety of immunological effects both *in vivo* and *in vitro* and acts as an immunopotentiator at lower dosages and on intermittent administration (Wright *et al.*, 1977). In the present study, total immunoglobulins of gamma and alpha classes were increased in all examined groups. IgG is the most abundant antibody class found in plasma and extracellular spaces of the internal tissues. In vertebrates, IgG constitutes approximately three fourths of the total immunoglobulins, while IgA, secreted primarily by mucosal lymphoid tissues to neutralize injurious agents in mucosal affection, is the next to IgG (Painter, 1998). Hence administration of LMS solutions stored at 4°C orally and intramuscularly to immunocompetent rats increased significantly both total serum IgG and IgA. The same immunopotentiating effects were observed by Soppi *et al.* (1979), Krakowski *et al.* (1999) and Pekmezci and Cakiroglu (2009). Generally, a significant difference in  $\gamma$ -globulin levels after LMS administration to immunocompetent species was recorded (Mohri *et al.*, 2005; Sadigh-Eteghad *et al.*, 2010). Collectively our findings and others suggest that LMS administration induced antibody production in the primed hosts and potentiate migration of immunoglobulins toward gamma globulin zone. Other explanation for the decrease in IgG and IgA in groups given LMS solutions stored at 37°C is the reduction in lymphocytes count (data not shown).

In this study, LMS showed an increase in antioxidant activity through its positive effects on GSH and other antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) as reported by ours and studies of Chwiecko *et al.* (1991) and Ince *et al.* (2010). However, scarce literatures are available about the possible effects of LMS on glutathione related enzymes, in particular, GST, GSSG-R and GSH-Px and its accompanied total antioxidant capacity. Glutathione transferases (GSTs) are ubiquitous scavengers of toxic compounds classified as a family of Phase II detoxification enzymes that have classically been described as catalyzing the conjugation of glutathione (GSH) to electrophilic compounds through thio-ether linkages (Ketterer, 2001). GSSG-R is an important antioxidant enzyme in maintaining an intracellular

reducing environment, which is critical to the cell against oxidative stress by catalyzing the reduction of glutathione disulfide (GSSG) to glutathione (GSH) (Argyrou and Blanchard, 2004). Glutathione peroxidases are a major family of functionally important selenoproteins which catalyze the inactivation of reactive oxygen and nitrogen species. These enzymes create a disulfide bond between two GSH molecules and form oxidized glutathione (GSSG) (Maiorino, 2004). So, the marked improvement in TAC status is referred to the enhancement in glutathione related enzymes GST, GSSG-R and GSH-Px. The same assumption was stated by Filomeni *et al.* (2002).

Total and differential leukocyte counts indicated that LMS solutions stored at 37°C tend to enhance the immune response towards more cellular responses, while solutions stored at 4°C tend to more humoral actions (data not shown). In addition both types of solutions were able to improve the antioxidant status of rats, possibly through keeping high GSH/GSSG ratio. Also, obtained results indicate that the intramuscular injection compared with oral route of LMS solutions stored either at 4°C or 37°C might be the most suitable route to express actions levamisole. In conclusion, LMS modulates immunoglobulins and antioxidant activity of rats with high response to intramuscular than oral route. Moreover, LMS stored at low temperature highly potentiates immune status of rats.

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