Praca oryginalna *Original paper*

Comparison of immunomodulatory effects of free and liposomal levamisole administered intraperitoneally to rats

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Received 02.01.2024 *Accepted 20.02.2024*

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Summary

This study aimed to compare the effects of immunomodulatory treatment of rats with free and liposomal levamisole. Liposomes were freshly prepared and administered intraperitoneally to animals after characterization studies. The study was divided into Experiment 1 and Experiment 2. Experiment 1 involved daily administration at immunomodulating doses (2.5 mg/kg; IP) for three days, followed by a three-day break and further daily administration at immunomodulating doses (2.5 mg/kg; IP) for three days. Experiment 2 involved weekly administration at immunomodulating doses (2.5 mg/kg; IP) for four weeks. Immunomodulatory parameters, IL-5, IL-10, TNF-α, and IL-1β, were compared between free levamisole and liposomal levamisole formulations. No difference in TNF-α was found between the groups in Experiment 1 and Experiment 2. In Experiment 1, the free levamisole group showed higher values of IL-1β than did liposomal and control groups. IL-1β values in Experiment 2 were higher in the control group than in the free levamisole group. IL-5 values in Experiment 2 were higher in the control group than in the free levamisole group. In Experiment 1, IL-10 values were higher in the liposomal levamisole group and in the control group than they were in the free levamisole group. Thus it was determined that free and liposomal forms of levamisole, which have been found to have immunomodulatory effects in different animal species in many studies, also showed such effects in rats. It was concluded that studies on other animal species and other doses are needed to obtain more detailed information about the immunomodulatory effects of free and liposomal levamisole.

Keywords: Immunomodulatory, levamisole, liposome, rat

The immune system is the body's defensive mechanism. It uses both innate and adaptive immunity to recognize and eliminate harmful parasites, viruses, and bacteria in the body (21). Innate immunity, which is the primary defense against pathogens, relies on phagocytes, such as macrophages and dendritic cells (1). Adaptive immunity, involving humoral and cell-mediated immunity, is the body's second line of defense and is highly selective to potentially hazardous external antigens (28). The spleen, the center of humoral and cellular immunity, is packed with lymphocytes and macrophages, while the thymus secretes hormones that encourage the development and differentiation of T cells (23). Immunomodulatory drugs including cyclosporine, levamisole (LVM), and imiquimod were previously used to regulate the immune system. Furthermore, taking natural nutritional supplements may also help to promote immunological function (9).

Liposomes are nanoparticles that were first described in the 1960s. They typically consist of lipids and fatty acids arranged in a spherical bilayer membrane that encloses an aqueous compartment (5, 25). Liposomes provide a convenient platform for the encapsulation of hydrophilic and hydrophobic molecules of different sizes, including tiny compounds, big proteins, and nucleic acids (11, 15, 22). Drug molecules can be shielded from degradation by incorporation into liposomes, which may also result in regulated release and targeted delivery. The dosage can thus be changed to reduce potential toxicity while preserving the thera-

peutic concentration at the site of action. In addition, liposomes can overcome physiological barriers and lengthen the circulation time of the inserted molecules, both of which can enhance their pharmacokinetic properties (3, 39). Encapsulation in liposomes reduces the side and toxic effects of drugs. It even allows two drug formulations that are incompatible with each other to be used together (27).

LVM is a broad-spectrum anthelmintic that is used in humans and animals to treat pulmonary and gastrointestinal nematode infections (41). Its anthelmintic action consists in paralyzing susceptible parasites by stimulating their sympathetic and parasympathetic ganglia (51). LVM also enhances the immune systems of both humans and animals (48). There are conflicting opinions regarding LVM's effectiveness as an immunomodulator (43). It has been demonstrated that LVM has immunostimulant effects on non-specific immune responses in fish and shellfish. Additionally, it has been noted that LVM raised the white blood cell count and enhanced the survival rate of Persian sturgeon (*Acipenser persicus*) fry. It was demonstrated that feeding them LVM for 15 days affected stress reactions. LVM also lowered cortisol levels in pacus (*Piaractus mesopotamicus*) under stress and promoted defenses against *Aeromonas hydrophila* bacterial infection in (17, 18, 37, 40).

The mechanism of action for the immunomodulatory effects of Levamisole are not well understood. It is believed that it restores cell-mediated immune function in peripheral T-lymphocytes and stimulates phagocytosis by monocytes. Its immune stimulating effects appear to be more pronounced in animals that are immune-compromised (38).

Levamisole helps to normalize the CD4/CD8 ratio in autoimmune disorders and enhances cellular immunity. It also enhances the synthesis of interleukin 1 by macrophages and the synthesis of interferons by lymphocytes, as well as the proliferative response of lymphocytes. The dentritic cell (DC) will begin to mature upon exposure to extracellular stimuli. The primary function of DCs, or antigen-presenting cells, is to collect, prepare, and deliver these antigens to unprimed T cells. Levamisole promotes DC maturation, which raises the expression of costimulatory molecules, such as CD80, CD83, and CD86, as well as major histocompatibility complex (MHC) molecules. T cell activation occurs via antigen and MHC complexes. Activated T cells release interferon gamma and thus strengthen immunity (27, 43).

Levamisole has little effect on B lymphocytes. It stimulates T lymphocytes more. It has been reported by researchers that levamisole directs the immune balance to type 1 response by initiating Th1 activation and upregulation of IL5 and TNF-α (30). Debrabander et al. (13) identified various cytokines that could be used as monitoring parameters for the potential immunomodulatory mechanism of action of levamisole. These are TNF-α, IL-1β, IL-2 or IL-6. Another important cytokine that regulates the immune system response is interleukin IL-10 (36). One of the pro-inflammatory cytokines of the immune system's initial response is interleukin-1β. Interleukin (IL-1β) and tumor necrosis factor (TNF-α) are implicated in apoptosis, inflammation, and cell division. Through a counteracting network, cytokines, such as transforming growth factor β (TGF-β) and interleukin-10 (IL-10), control the activity of TNF-α and IL-1β (42, 53).

This study aimed to compare the effects of immunomodulatory treatment of rats with free and liposomal levamisole. Since IL-5, IL-10, TNF-α, and IL-1β values had previously been analyzed for immunomodulatory effects, they were analyzed for the immunomodulatory effect of levamisole in our study.

Material and methods

Materials. The chemicals used in this study, lecithin (L-α-Lecithin, Soybean), chloroform, and methanol, were purchased from Sigma-Aldrich. Cholesterol was obtained from Acros Organics (Belgium).

The devices used in the study included a rotary evaporator (Isolab), centrifuge (Isolab), zeta-sizer (Malvern Zetasizer Nano-ZS ZEN3600), refrigerator (Arçelik 270530EB), ultrasonic bath (MEDISSON), vortex (IKA® MS 3), UV-VIS spectrophotometer (Shimadzu UV-1900i), precision scales (Denver Instrument), scanning electron microscope (JEOL Neoscope JCM-5000), and a gold-plating device (Quorum).

Preparation and characterization studies of liposomal LVM. Liposomal LVM was prepared by the method of Susar et al. (47), which is a modification of a method used by Bangham et al. (6). For this purpose, lecithin, LVM and cholesterol were weighed in a ratio of 3 : 1 : 1. The substances were dissolved in chloroform and methanol solvents in a 1 : 1 ratio. The lipid film was obtained by rotation with a rotary evaporator at 250 rpm for 30 min. To ensure complete volatilization of solvents from the lipid film, it was kept open at 4°C overnight. To remove the lipid film, 10 ml of distilled water was cycled in a rotary evaporator for 10 min. It was kept in an ultrasonic bath for 5 min to reduce the particle size. The liposomal LVM was centrifuged at 27,000 g for 1 h at 4°C to remove free LVM. The liposomal LVM obtained was stored at 4°C for administration to animals.

The particle size, polydispersity index, zeta potential, and encapsulation efficiency of the liposomes were determined by methods described by Çoban et al. (12).

The encapsulation efficiency of liposomal LVM was calculated by an indirect method. After liposome formation, the dispersion was centrifuged for one hour at 27,000 g. The amount of supernatant was used to calculate the encapsulation efficiency (EE%) by an indirect method. Every measurement was made three times. The procedure was set up at a wavelength of 213 nm. For particle size and polydispersity index measurement, 120 µl of the sample and 1880 µl of distilled water were taken. Three measurements were made at 25 ± 0.1°C and averaged. For zeta potential,

Fig. 1. Liposome preparation steps: 1 – dissolving substances, 2 – lipid film production in a rotary evaporator, 3 – lipid film obtained, 4 – lipid film retrieval, 5 – LVM

1 ml of the sample and 1 ml of distilled water were used at the same temperature.

Liposome formulation structures were evaluated by scanning electron microscopy (SEM) (JEOL Neoscope JCM-5000). The samples were gold-plated for 5 minutes and then images were taken. Liposomal LVM samples were examined at a voltage range of 10-15 kV and suitable images were recorded.

Experimental design. A total of 32 male Wistar albino rats (weighing 250-350 g, 6-7 weeks old) were obtained from the Balıkesir University Experimental Animal Production, Care, Application, and Research Center. The animals were divided into six groups. The study was conducted as Experiment 1 and Experiment 2. Experiment 1 involved daily administration at immunomodulating doses (2.5 mg/kg; IP) for three days, followed by a three-day break and daily administration at immunomodulating doses (2.5 mg/kg; IP) for another three days. Experiment 2 involved weekly administration at immunomodulating doses (2.5 mg/kg; IP) for four weeks.

Experiment 1

The rats were divided into three groups: a control group, a free LVM group, and a liposomal LVM group. The procedure was as follows:

Group A: physiological saline was administered daily at immunomodulating doses (2.5 ml/kg; IP) for three days and then for another three days after a three-day break (n = 4);

Group B: free LVM hydrochloride was administered daily at immunomodulating doses (2.5 mg/kg; IP) for three days and then for another three days after a three-day break (n = 4); (n = 6);

Group C: liposomal LVM hydrochloride was administered daily at immunomodulating doses (2.5 mg/kg; IP) for three days and then for another three days after a three-day break (n = 4); (n = 6);

Experiment 2

The rats were divided into three groups: a control group, a free LVM group, and a liposomal LVM group. The procedure was as follows:

Group D: physiological saline was administered weekly at immunomodulating doses (2.5 ml/kg; IP) for four weeks (n = 4);

Group E: free LVM hydrochloride was administered weekly at immunomodulating doses (2.5 mg/kg; IP) for four weeks (n = 6);

Group F: liposomal LVM hydrochloride was administered weekly at immunomodulating doses (2.5 mg/kg; IP) for four weeks (n = 6).

The rats were kept under conventional laboratory conditions (12 hours light/12 hours dark, 22°C, 40-60% relative humidity, ad libitum feeding and watering) for the duration of the experiment. This study was carried out with the permission of the Animal Experiments Local Ethics Committee of Balikesir University (BAUN-HADYEK) (permission no. 2023/2-4 of March 30, 2023). The study was financed and supported as a project by the Balikesir University Scientific Research Coordinatorship (BAP Project No: 2022/124).

Sample collection. Blood samples were collected 24 hours after the last drug administration. The rats were euthanized by cervical dislocation without anesthesia. The blood samples were centrifuged at 3500 × g for 15 minutes to separate the serums and stored at –80°C until further analysis.

Biochemical analysis. The levels of TNF-α (Bioassay Technology Laboratory, BT Lab, E0764Ra, Zhejiang, China), IL-1 β (Bioassay Technology Laboratory, BT Lab, E0119Ra, Zhejiang, China), IL5 (Bioassay Technology

Laboratory, BT Lab, E0134Ra, Zhejiang, China), and IL10 (Bioassay Technology Laboratory, BT Lab, E0108Ra, Zhejiang, China) were measured using commercially available ELISA kits according to the manufacturer's protocol and an ELISA reader (SPECTROstar Nano, BMG LABTECH GmbH, Ortenberg, Germany).

Statistical evaluation. Statistical analysis of the data was performed with the SPSS 26 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and GraphPad Prism (Prism 9 for Windows, version 9). The statistical significance level of differences between groups was accepted as $p = 0.05$.

Results and discussion

The average liposomal size was 1536 nm, with an LVM encapsulation efficiency of 91.31%, a polydispersity index of 0.97, and a zeta potential of –0.173. Encapsulation efficiency is a crucial metric that indicates how well nanocarriers can shield the loaded actives (16). The zeta-potential, a surface charge characteristic of liposomes, is an essential parameter for determining the stability, *in vivo* efficacy, and biological destiny of colloidal systems. Dynamic Light Scattering (DLS) is frequently used to estimate the zeta potential because the charge on the surface of liposomes controls their movement, which in turn affects the intensity of the scattered light. A charge of more than +30 mV or less than –30 mV indicates good stability against fusion and the absence of an aggregating tendency in particles (46). PDI, a measure of the size homogeneity of dispersed particles, normally has a range of 0 to 1 (32). The data obtained in our study are consistent with. Encapsulation efficiency was high, and polydispersity index and zeta potential results were similar to those in the literature.

No local or systemic adverse effects were observed in the rats after intraperitoneal administration of free and liposomal LVM at a dose of 2.5 mg/kg. The groups in Experiment 1 were compared with each other. No significant statistical difference was found between the groups in TNF-α ($p > 0.682$) and IL-5 ($p > 0.563$). Statistically significant differences in IL-10 were found between Groups B and C ($p < 0.002$) and between Groups A and C ($p < 0.003$). Groups B and C ($p < 0.006$) were found to differ. Statistically significant differences in IL-1 β were found between Groups B and C ($p < 0.001$) and between Groups A and C ($p < 0.008$). It was found that Groups A and B ($p < 0.001$) showed a difference.

The groups in Experiment 2 were compared with each other. A statistically significant difference was determined between the groups in IL-5 ($p < 0.014$). Groups D and E showed a significant difference ($p < 0.010$). There was no significant difference between groups E and F ($p > 0.310$). A statistically significant difference in IL-1 β was determined between the groups ($p < 0.032$). Groups D and E showed a significant difference ($p < 0.011$), but there was no significant difference between Groups E and F ($p > 0.584$). In the experimental design, which was carried out as Experiments 1 and 2, no difference was found in terms of TNF-α and IL-5 when the two application methods were compared with each other. Statistically significant differences were observed in IL-1 β and IL-10. In terms of IL-10, Group B showed better results than Group D ($p < 0.0001$), Group E ($p < 0.0003$), or Group F ($p < 0.0001$). In terms of IL-1 β, Group B showed better results than Group D ($p < 0.0057$), Group E ($p < 0.0001$), or Group F ($p < 0.0001$).

Liposomal LVM under SEM is shown in Figure 2. TNF-α and IL-1 β levels of the rat groups are shown in Figure 3, whereas IL-5 and IL-10 levels are presented in Figure 4.

Cytokines are signaling molecules in protein or glycoprotein structures that are produced and secreted by more than one cell, especially immune system cells. They are involved in the development and differentiation of T, B, and hematopoietic cells, and,

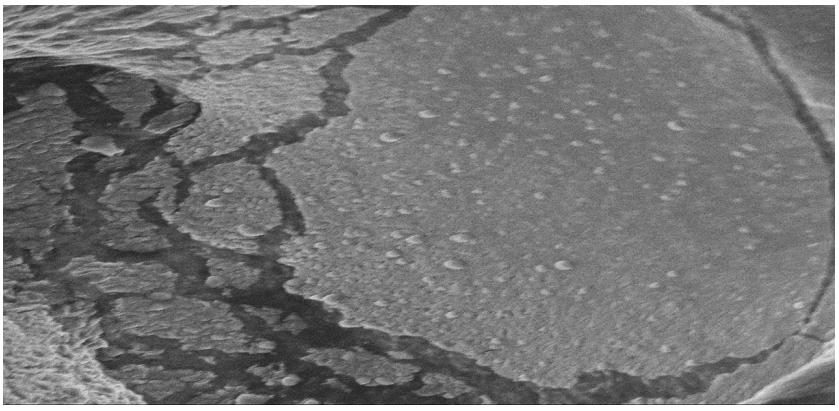

Fig. 2. Liposomal LVM under scanning electron microscopy

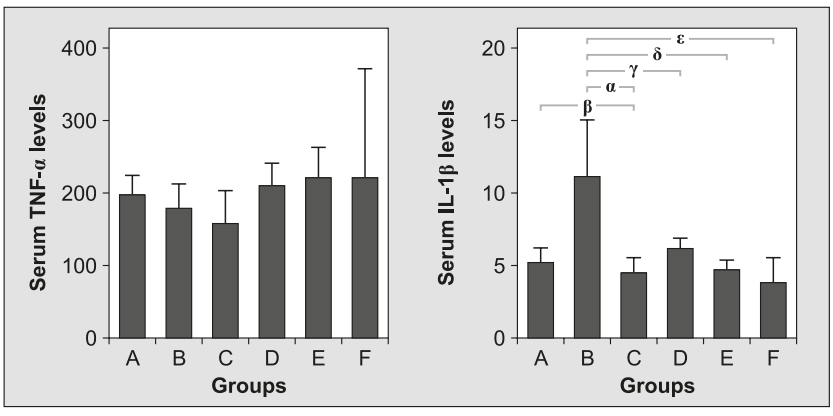

Fig. 3. TNF-α and IL-1 β levels of rat groups
Explanations: α – Group C compared to Group B; β – Group C compared to Group A; γ – Group D compared to Group B; δ – Group E compared to Group B; ε – Group F compared to Group B

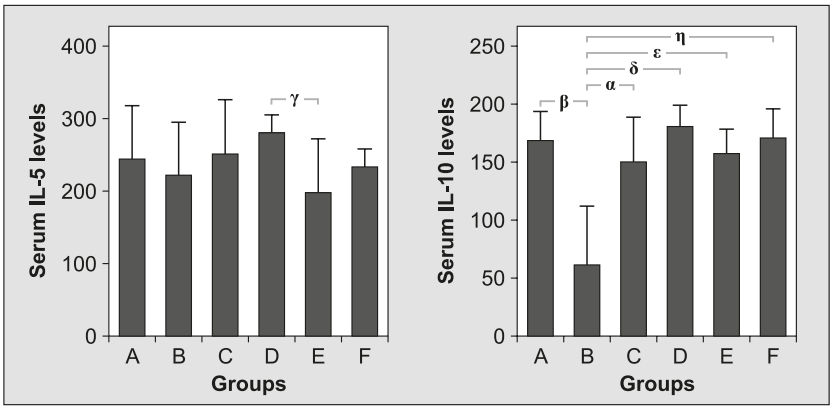

Fig. 4. IL-5 and IL-10 levels of rat groups
Explanations: γ – Group E compared to Group D; α – Group C compared to Group B; β – Group B compared to Group A; δ – Group D compared to Group B; ε – Group E compared to Group B; η – Group F compared to Group B

accordingly, provide intercellular communication by creating hormone-like effects at every stage of the immune response. They also regulate biological activities, such as inflammation, the proliferation, development and activation of cells, and morphogenesis (44). The cytokine function depends on several variables, such as the tissue levels of cytokines, activation signal, exposure time and duration, target cell characteristics, and the experimental model under investigation (8). Although research suggests that IL-10 can also be pro-inflammatory in some situations, it is generally considered to be an anti-inflammatory cytokine (26, 29, 31). Research on mice has demonstrated that a lack of IL-10 causes inflammation (20, 33, 52). It functions as an anti-inflammatory and guards against illness and tissue damage by reducing or eliminating the unwarranted or excessive reaction of the innate immune system to microbial antigens. IL-10 often reduces the production of pro-inflammatory cytokines by targeting them (36). In our study, liposomal LVM produced higher IL 10 levels than free LVM in the groups which were administered the drugs at immunomodulating doses (2.5 mg/kg; IP) for three days and then for another three days after a three-day break. The IL 10 level in the control group was found to be higher than in the group that was administered free LVM.

TNF-α is a cytokine that exhibits pleiotropic effects on different types of cells. It is known to play a role in the etiology of some inflammatory and autoimmune disorders and has been identified as a key regulator of inflammatory responses (7). TNF-α plays a critical role in the physiological process of an aberrant immune response. While TNF-α can help control the immune system, producing too much or too little of it can be dangerous and may even cause disease. Though its exact function in illnesses is unknown, TNF-α is widely known to accelerate the course of disease when it activates and accumulates fibroblasts, leading to joint erosion, fibrosis, and stricture formation (19). In our study, there was no change in TNF-α levels either in the groups which were administered the drugs at immunomodulating doses (2.5 mg/kg; IP) for three days and then for another three days after a three-day break or in the groups which received them weekly at immunomodulating doses (2.5 mg/kg; IP) for four weeks. This fact suggests that, although free and liposomal formulations of LVM do not have immunomodulatory effects, they do not have harmful effects either.

It has long been known that IL-1β, a member of the interleukin (IL)-1 family, is a strong inflammatory mediator. In autoinflammatory illnesses, IL-1β is a crucial cytokine that mediates inflammation. The primary role of IL-1β is to promote leukocyte activation, which causes fever and high concentrations of acute-phase proteins, which in their turn set off systemic inflammatory reactions. An intracellular sensor initiates IL-1β activation by triggering the inflammasome, which then uses caspase-1 to cleave pro-IL-1β into its active form (14, 45). Akpınar et al. (2) looked at the effects of ozone on local and systemic IL-1β and IL-10 levels in an experimental periodontitis model in rats. They revealed that ozone reduced alveolar bone resorption in rat periodontitis models. In our study, free LVM produced higher IL-1β levels than liposomal LVM in the groups which were administered these drugs at immunomodulating doses (2.5 mg/kg; IP) for three days and then for another three days after a three-day break.

IL-5 has multiple target cells, including basophils, eosinophils, and B cells. However, recombinant IL-5 exhibits pleiotropic effects on these cells in addition to supporting the proliferation and terminal differentiation of mouse B cells into antibody-secreting cells *in vitro*. Hematopoietic and non-hematopoietic cells, such as T cells, granulocytes, and natural helper cells, produce IL-5. Receptors made up of a common and IL-5-specific O-subunit allow IL-5 to drive proliferation and differentiation (49). Mohamed et al. (30) investigated the modulatory effects of levamisole and garlic oil on the immune response of Wistar rats. They found upregulation of IL-5 immune response. Nag et al. (34) investigated the effects of IL-5 on airway physiology and inflammation in rats. They found that IL-5 increased lung resistance 20 hours after antigen challenge. Wei et al. (50) investigated the effect of IL-5 on immune response and lung injury in rats with sepsis. They concluded that IL-5 can alleviate lung damage by regulating the immune response and inhibiting the systemic inflammatory response caused by sepsis. The data on IL-5 obtained in our study are compatible with the results of other studies.

Mohamed et al. (30) concluded that in rats given 2.5 mg/kg LVM every two days for four weeks the drug initiated Th1 activation and directed the immune balance to type 1 response through upregulation of IL2,

IL4, IL5, IFN, and TNFα. Nageshwari and Merugu (35) showed that LVM inhibited CD138 expression and affected IL6 levels in a dose-dependent manner. In a study on the effects of LVM on immunity and cancer treatment, Chen et al. (10) observed that LVM treatment increased the activation of T cells against Type 1 T helper immune response by stimulating INF-γ secretion. Kimball et al. (24) found that a single oral dose of LVM given to mice one to four days before macrophage detection caused a two-fold increase in IL1 production of macrophages, as well as a decrease in IL6 and TNF production of the same macrophages. They concluded that LVM affects cytokine production. Baca-Estrada et al. (4) conducted a study on the effect of IL-4 and IL-12 on liposomes. The researchers found that modification of the immune response can be achieved with much lower doses of IL-4 and IL-12 than previously thought and that such a therapeutic regime can preclude many of the complications that can result from repeated injections of high doses of cytokines. The immunomodulatory parameters evaluated in various other studies are both similar to and different from those in our study. Since many cytokines are used in the evaluation of immune response, it was thought that there might be parameter differences. Although the results of our study are different from those obtained by Mohamed et al. (30) in terms of TNF-α, they are similar in terms of IL-5. Although our results are different from those reported by Kimball et al. (24) in terms of TNF-α, they are similar in terms of IL-1β. It is thought that the cause of these differences may be the different frequency of the drug administration.

In summary, we successfully prepared liposomes consisting of LVM and investigated the microscopic morphology, encapsulation efficiency, polydispersity index, zeta potential, and pH of the liposomes. LVM is rapidly absorbed and shows its effect on the body. It is rapidly excreted from the body. Therefore, it should be transported through nanocarriers to prolong the duration of the drug's effect. Our study demonstrated that free and liposomal formulations of LVM have an immunomodulatory effect. Levamisole is a water-soluble drug. When liposomes are prepared, phospholipids used in the formulation increase the oil/water distribution coefficient of the drugs. This makes the drug more effective. While bioavailability increases, toxic effects decrease. The encapsulation of levamisole in liposomes is aimed at maximizing its efficacy without harming the body. Our study shows that levamisole may have immunomodulatory effect on healthy animals. However, in order to better understand the effects of liposomal levamisole, studies should be carried out on different animal species and different routes of administration and doses.

This study sheds light on the research to be carried out in this context.

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