Kafkas Univ Vet Fak Derg
19 (2): 183-190, 2013
DOI: 10.9775/kvfd.2012.6792

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Cytologic-Enzymologic Diagnosis of Experimental Pneumonia Induced by *Klebsiella pneumoniae* Serotype II in Rats and Its Treatment with Free and Liposomal Enrofloxacin [1]

Ahmet Levent BAS [1] Kamil UNEY [1] Hasan Huseyin HADIMLI [2] Ali Demir SEZER [3] Fatih HATIPOGLU [4] Mehmet MADEN [5] Julide AKBUGA [3]

[1] *This study was supported by The Scientific and Technological Research Council of Turkey (Project No: 106 O 099)*

1 Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Selcuk, TR-42031 Konya - TURKEY
2 Department of Microbiology, Faculty of Veterinary Medicine, University of Selcuk, TR-42031 Konya - TURKEY
3 Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, University of Marmara, TR-34668 Istanbul - TURKEY
4 Department of Pathology, Faculty of Veterinary Medicine, University of Selcuk, TR-42031 Konya - TURKEY
5 Department of Internal Medicine, Faculty of Veterinary Medicine, University of Selcuk, TR-42031 Konya - TURKEY

Makale Kodu (Article Code): KVFD-2012-6792

Summary

Enrofloxacin (ENR) rapidly localizes in eukaryotic cells *in vitro* but does not remain for prolonged periods, thereby reducing the ENR efficacy of defense against intracellular pathogens. Delivery of ENR in a liposome-encapsulated form may enhance its intracellular residence time. In this study, experimental pneumonia was induced in healthy and dexamethasone-treated rats using *Klebsiella pneumoniae* serotype II. Free and liposome-encapsulated ENR were injected intravenously into the infected animals at a dose of 7.5 mg/kg/day for 5 days. Samples of tissue, plasma and bronchoalveolar lavage (BAL) fluid were obtained at 1, 2, 3 and 4 days and 1, 2, 3 and 4 weeks after the first antibiotic treatment. All of the samples were evaluated cytologically, enzymologically, microbiologically and pathologically. It was determined that cytologic and enzymologic diagnoses of BAL fluid are not meaningful for evaluating the treatment of the experimental pneumonia in rats. However, it was established that the use of ENR in liposomal form at a dose of 7.5 mg/kg for 5 days is more effective than the free form both in the treatment of *K. pneumoniae* infections and in the prevention of recurrent infections. Liposome-encapsulated antimicrobial agents should provide another choice for antimicrobial therapy in the future, but further investigation must be completed before clinical use.

Keywords: *Enrofloxacin, Experimental pneumonia, Klebsiella pneumoniae, Liposome, Rat, Treatment*

Ratlarda *Klebsiella pneumoniae* Serotip II İle Oluşturulan Deneysel Pnömonilerin Sitolojik-Enzimolojik Teşhisi ve Serbest ve Lipozomlanmış Enrofloksasin İle Tedavisi

Özet

Enrofloksasin (ENR) *in vitro* ortamlarda ökaryotik hücrelerde hızla lokalize olur, ancak uzun süre kalamadığı için hücre içi patojenlere karşı onun etkinliği azalır. Lipozomla kapsüle edilmiş formda ENR uygulanması hücre içi ortamlarda kalış zamanını artırabilir. Bu çalışmada, sağlıklı ve deksametazon uygulanmış ratlarda *Klebsiella pneumoniae* serotip II kullanılarak deneysel pnömoni oluşturuldu. Serbest ve lipozomla kapsüle edilmiş ENR enfekte hayvanlara intravenöz olarak beş gün boyunca 7.5 mg/kg/gün dozda enjekte edildi. İlk antibiyotik uygulamasından sonraki 1, 2, 3, 4. gün ve 1, 2, 3, 4. haftalarda plazma, doku ve bronko-alveolar lavaj (BAL) sıvısı örnekleri alındı. Tüm örnekler sitolojik, enzimolojik, mikrobiyolojik ve patolojik olarak değerlendirildi. Ratlarda deneysel pnömoninin tedavisinin değerlendirilmesinde BAL sıvısının sitolojik ve enzimolojik teşhisinin anlamsız olduğu belirlendi. Ancak, enrofloksasinin beş gün boyunca 7.5 mg/kg dozda lipozomal formda kullanımının hem *K. pneumoniae* enfeksiyonlarının tedavisinde hem de tekrarlayan enfeksiyonlarının önlenmesinde serbest formdan daha etkili olduğu tespit edildi. Lipozomla kapsüle edilmiş antimikrobiyal ajanlar gelecekte antimikrobiyal tedavide diğer bir seçenek sağlayacaklardır, ancak klinik kullanımdan önce çok sayıda araştırma yapılmalıdır.

Anahtar sözcükler: *Enrofloksasin, Deneysel pnömoni, Klebsiella pneumoniae, Lipozom, Rat, Tedavi*

İletişim (Correspondence)
+90 332 2232690
alevent@selcuk.edu.tr

INTRODUCTION

The greatest problem of fluoroquinolone antibiotics in the treatment of infections caused by intracellular bacteria is that the antibiotics cannot become effectively concentrated in the cells [1], and they cannot sustain a sufficient intracellular level long enough to display the required antibacterial effect. The cellular/extracellular (C/E) concentration rate of enrofloxacin (ENR) was defined as 9 in polymorphic mononuclear leucocytes (PMNLs) and as 5 in alveolar macrophages (AMs). When the cells were exposed to the environment without ENR, 80% of the intracellular AMs and 90% of the PMNLs moved out of the cell within the first ten minutes. In addition, *Staphylococcus aureus* isolated from the alveolar macrophages was found to be less sensitive to the bactericidal effect of ENR compared to the bacteria outside of the cell [2]. Various studies have shown that liposomal forms of fluoroquinolones can significantly increase the effectiveness of the antibacterial action compared to free forms [3,4].

Klebsiella pneumoniae is an opportunistic, pathogenic microorganism that causes nosocomial infections in people with immune system deficiency. *K. pneumoniae* infections occur in a wide range of presentations, from mild urinary tract infections to severe bacteremia as well as pneumonia associated with high mortality and morbidity [5]. Pneumonia caused by *K. pneumoniae* is generally complicated and, depending on the presence of pulmonary abscesses and multilobular dispersion, should be treated quickly with antibiotics that have fast clinical results and efficiency [6].

Cytologic examination of the bronchoalveolar lavage (BAL) fluid is commonly used for the diagnosis of respiratory diseases. It is used to determine the cellular and humoral elements of the lower respiratory airways [7,8]. The BAL provides important information for the identification of cellular structures and the definition of the scope of the inflammation [9]. Direct examination and cultures of BAL can also be used to define the etiologic agent and aid in the selection of the appropriate antimicrobial drug [10]. In pulmonary diseases, the concentration of some cytoplasmic enzymes, such as lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP), which are found in the cell membrane, and some lysosomal enzymes, including beta-glucuronidase and beta-N-acetyl glucosaminidase, are increased. Microprotein (MP) and urea (UREA) concentrations in the BAL are regarded as indicators of changes in the respiratory membrane and in vascular permeability [11,12].

The purpose of this study was to evaluate the clinical effectiveness of free (F-ENR) and liposomal ENR (L-ENR) microbiologically, cytologically, enzymologically and pathologically in experimental respiratory tract infections induced by *K. pneumoniae* serotype II in rats.

MATERIAL and METHODS

Preparation and Purification of Liposomes: ENR loaded multilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs) were prepared using a modified dry film method [13,14]. The F- and L-ENR were separated by gel filtration on a Sephadex G-50 column at 25°C (1.0 cm in diameter, a gel bed height of 20 cm, Sigma Chemical Co., USA), and PBS (pH 7.4) was used as the medium [15].

Visualization and Transmission Electron Microscopy (TEM) Study: The final liposomes were visualized under an electron microscope using a negative staining technique (JEOL, Tokyo, Japan) at 10.000x magnification [13].

Determination of Liposome Size and Zeta Potential: Size and zeta potential measurements were performed at 25°C using a Malvern Zetasizer (Nano ZS, Malvern Instruments, Worcestershire, UK) and the results are expressed as the mean of three measurements [14,16].

Encapsulation Capacity: Triton X-100 solution (0.1 ml, 1% v/v) was added to 1 ml freshly prepared liposomal suspension and vortexed. Then, a 0.1 ml sample was adjusted to 10 ml with PBS (pH 7.4). The amount of drug present in the resulting solution was measured spectrophotometrically at 270 nm (BioSpec-1601, Shimadzu, Japan). Each measurement was performed in triplicate [17].

In Vitro Release Study: The *in vitro* drug release from the liposomal systems was determined using dialysis sacks (Sigma, 250-7U USA) and 5 ml liposomal suspension was poured into the sack. The dialysis sacks containing the liposomal preparations were suspended vertically in a beaker containing 200 ml PBS (pH 7.4), and the contents of the beaker were stirred using a magnetic stirrer at 37±1°C. The samples were withdrawn periodically and determined spectrophotometrically at 270 nm [17].

Animals: In this study, 280 Sprague-Dawley female rats (4-5 months of age, 250-300 g) were used (Selcuk University, Medicine Faculty, Kombassan Experimental Animals Unit). The experimental animals were maintained in polysulfone cages including five rats in each cage, and heat, light and humidity (24±2°C, 12/12 h light/darkness and 55±5% relative humidity) were controlled. Food and water were provided *ad libitum*. Before the experiment, approval of the Ethical Committee (No: 2005/011) was obtained from Selcuk University, Veterinary Faculty Ethical Committee.

Bacteria and Infection Model: *K. pneumoniae* serotype II (Dr. Sylvain Brisse, Unité Biodiversité des Bactéries Pathogènes Emergentes et Plate-forme Santé Publique Institut Pasteur, France) was used to infect the rats. We defined a $1x10^6$ bacteria/ml concentration as sufficient to cause experimental pulmonary infection. After the general health of the rats was examined, experimental pulmonary infection was induced in healthy rats and in rats that were given 0.5 mg/kg dexamethasone (0.4% dexamethasone with drinking water for 1 week; Richter Pharma AG) [18], using *K. pneumoniae* serotype II according to the method stated by Bakker-Woudenberg [19].

In Vivo Experimental Design and Sampling Procedure: Bolus doses of F-ENR and L-ENR were given intra-venously into the tail vein at a dosage of 7.5 mg/kg b.w. in rats infected with *K. pneumoniae* serotype II. Antibiotic applications started 24 h after bacterial inoculation into the trachea and continued at 24 h intervals for five days. The rats were divided into two groups as control and experimental groups. The control groups were divided into three groups with 8 rats in each group as follows: group I, positive control group with experimental infection but no antibiotic treatment (PC1); group II, positive control group with experimental infection after dexamethasone application but no antibiotic treatment (PC2); and group III, negative control group with healthy rats that did not receive any drug treatment (NC). The experimental groups were divided into the four following groups with 64 rats in each group: group I, F-ENR-treated group with experimental infection (FE); group II, F-ENR-treated group with experimental infection after receiving dexamethasone (FED); group III, L-ENR-treated group with experimental infection (LE); and group IV, L-ENR-treated group with experimental infection after receiving dexamethasone (LED). The PC1 and PC2 groups were euthanized by CO_2 inhalation on the fifth day following bacteria inoculation into the trachea. The experimental groups were euthanized by CO_2 inhalation following drug application on day 1, 2, 3 and 4 and week 1, 2, 3, and 4. The chest cavities were opened under sterile conditions, and the BAL fluid, lungs, liver, kidney and spleen were removed. Microbiologic, histopathologic and cytologic-enzymologic analyses of the samples were performed.

Microbiological Analysis: The quantitative bacteria numbers were defined for the BAL and lung samples by a microdilution method [20]. In addition, the susceptible colonies were identified using classic microbiology methods, and we determined whether they were *K. pneumoniae.* The samples of the other tissues (livers, kidney and spleen) were cultured on blood agar and MacConkey agar to isolate *K. pneumoniae* and other bacteria.

Analysis of BAL Fluid: The BAL fluid samples were evaluated cytologically and enzymologically [21,22]. The cytological differences and differential cell numbers were defined using two different staining techniques (Wright-Giemsa and Papanicolaou). LDH, AST, GGT, MP and urea concentrations were measured. These analyses were performed using a commercial kit with an autonalyzer (BioMerieux ILAB 300 Plus).

Pathological Analysis: After the BAL fluid was taken, the macroscopic findings were recorded. Tissue samples were collected and stored in 10% buffered formaldehyde. The tissues were prepared in paraffin blocks using an automatic tissue processor (Leica TP 1020, Nussloch, Germany) according to routine laboratory procedures, stained with hematoxylin and eosin (HE) [23] and examined using a light microscope (Olympus, Model BX51TF, Olympus, Tokyo, Japan).

Statistical Analysis: The values for bacteria numbers as defined micro-biologically were calculated. The results of the microbiology and the BAL fluid analysis were evaluated using one-way ANOVA. The differences between the groups were defined using *Duncan's* test. The results obtained in the microscopic examinations of the lung and liver tissues were evaluated using a *Mann-Whitney U* test. P<0.05 was accepted as statistically significant.

RESULTS

Liposome Characterization: The ENR–loaded liposomes were obtained using the dry lipid film method [19]. The characterization of the liposome shape and surface was evaluated by TEM, and spherical liposomes were obtained, as observed in *Fig. 1*. The particle size of the liposomes, with surface charges of +50.3±2.6 mV and +55.3±1.7 mV, were 2.58±0.29 mm and 4.65±0.37 mm. The encapsulation capacities of the liposomes were 83.7±0.6% and 95.3±0.1% *(Table 1)*. The slowest ENR release and highest encapsulation drug amounts can be obtained by using the LUVs rather than the MLVs.

Microbiological Findings: When the number of bacteria isolated from the BAL samples of infected rats was compared between the groups, we observed significant differences between the results obtained on different days. No bacterial isolation was performed on the BAL samples in the 4th week *(Table 2)*. We determined that the

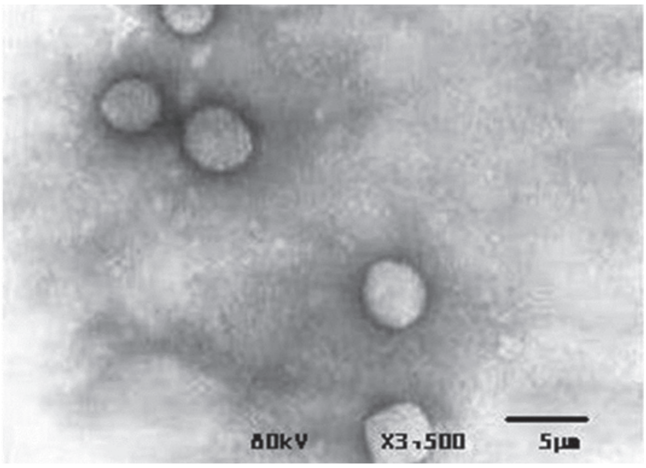

Fig 1. TEM photograph of LUV formulation (The molar ratio of DPPC: cholesterol:ENR:SA is 4.7:17.3:1.0:2.0)

Şekil 1. LUV formülasyonunun TEM fotoğrafı (DPPC:cholesterol:ENR:SA molar oranları 4.7:17.3:1.0:2.0'dir) number of re-isolated bacteria was higher in the lung samples of the infected rats than in the BAL samples *(Table 2)*. When the days and groups were considered, the highest morbidity was found on the 3rd day in the LED group and on the 2nd day in the FED group *(Table 3)*.

Findings of BAL Cytology and Enzymology: Results of cytologic analysis between the 6 groups of rats with experimental respiratory tract infection are given in *Table 4*. The comparisons of the measured enzyme activities in the BAL fluid (LDH, GGT, and AST) with the urea and MP concentrations are presented in *Table 5* and *Table 6*.

Pathological Findings: Although the pathological findings depended on infection development were found in the control and experiment groups after histo-pathological examination of the tissue samples, there were no statistically significant differences among the groups. Some of the macroscopic findings are shown in *Fig. 2*, and the histopathological findings are shown in *Fig. 3*.

DISCUSSION

In this study, ENR-loaded MLVs and LUVs, which were prepared using the optimum formulations of our previous studies [13,14], were given to rats with experimental pneumonia, and we evaluated the efficiency of the liposomal treatment. The characterization of the liposomes' shape and surface was evaluated using a transmission electron microscope (TEM). The liposomes were spherical in shape and were found to be multilamellar and large unilamellar forms. The MLVs were smaller and had lower encapsulation capacity than the LUVs (P<0.05) *(Table 1)*. The ENR release from the LUVs was much slower than that observed from the MLVs (data not given). This might be due to the particle size and encapsulation capacity of the liposomes. When we

Table 1. *Codes and formulations of ENR-loaded MLVs and LUVs*
Tablo 1. *ENR yüklenen MLV ve LUV'ların kodları ve formülasyonları*

Codes	Molar Ratios of DPPC: Cholesterol:ENR:SA	α-tocopherol (mg)	Internal Phase [Chloroform (ml)]	External Phase [PBS, pH 7.4 (ml)]	Mean Particle Size (mm ± SD)	mV (± SD)	Encapsulation Capacity (% ± SD)
MLV	7:26.0:1.5:3.0	15	5	5	2.58±0.29	55.3±1.7	83.7±0.6
LUV	4.7:17.3:1.0:2.0	15	5	5	4.65±0.37	50.3±2.6	95.3±0.1

Table 2. *The comparisons of re-isolation of K. pneumoniae in the BAL and lung samples of the experimental groups*
Tablo 2. *Deney gruplarının BAL ve akciğer örneklerinde K. pneumoniae'nın yeniden izolasyonlarının karşılaştırılması*

Sample	Group	Day 1	Day 2	Day 3	Day 4	Day 5	Week 2	Week 3	Week 4
BAL	FE	$2.69\pm1.10^{a,y}$	$1.84\pm0.96^{a,xy}$	$0.66\pm0.44^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$	$0.87\pm0.87^{a,xy}$	$0.30\pm0.30^{a,x}$	$0.00\pm0.00^{a,x}$
	FED	$3.03\pm0.74^{a,y}$	$0.64\pm0.45^{a,x}$	$2.53\pm0.80^{a,y}$	$2.56\pm0.48^{c,y}$	$0.00\pm0.00^{a,x}$	$0.23\pm0.23^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$
	LE	$2.68\pm0.81^{a,y}$	$0.97\pm0.68^{a,x}$	$1.30\pm0.70^{a,xy}$	$0.60\pm0.41^{ab,x}$	$0.32\pm0.32^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$
	LED	$7.48\pm0.47^{b,z}$	$2.27\pm0.88^{a,y}$	$6.50\pm0.73^{b,z}$	$1.29\pm0.53^{b,xy}$	$0.59\pm0.39^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$
Lung	FE	$4.65\pm0.85^{a,x}$	$3.05\pm0.93^{a,wx}$	$2.47\pm1.08^{a,vwx}$	$2.08\pm0.95^{a,vw}$	$0.78\pm0.52^{a,vw}$	$0.37\pm0.37^{a,v}$	$0.36\pm0.36^{a,v}$	$1.66\pm0.87^{a,vw}$
	FED	$7.73\pm0.60^{b,x}$	$6.70\pm0.29^{b,x}$	$7.33\pm0.51^{c,x}$	$5.76\pm0.83^{b,wx}$	$0.37\pm0.37^{a,v}$	$1.66\pm0.71^{a,v}$	$2.19\pm0.75^{b,v}$	$4.71\pm0.91^{b,w}$
	LE	$4.62\pm0.35^{a,x}$	$1.75\pm0.73^{a,w}$	$1.24\pm0.63^{a,vw}$	$0.73\pm0.48^{a,vw}$	$0.00\pm0.00^{a,v}$	$1.62\pm0.81^{a,w}$	$0.00\pm0.00^{a,v}$	$0.00\pm0.00^{a,v}$
	LED	$6.60\pm0.45^{b,z}$	$3.37\pm0.82^{a,xy}$	$5.13\pm0.79^{b,yz}$	$2.21\pm0.95^{a,wx}$	$0.96\pm0.64^{a,vw}$	$0.50\pm0.50^{a,vw}$	$0.00\pm0.00^{a,v}$	$0.00\pm0.00^{a,v}$

Different letters in the same column (a, b, c) and line (v, w, x, y, z) are significantly different (P<0.05), Data is presented as mean±SD ***FE,*** *free enrofloxacin;* ***FED,*** *free enrofloxacin+dexamethasone;* ***LE,*** *liposomal enrofloxacin;* ***LED,*** *liposomal enrofloxacin + dexamethasone, Isolated bacteria numbers were calculated by transforming ln*

Table 3. *Morbidity[a] and mortality[b] levels in the experimental groups*
Tablo 3. *Deney gruplarında morbidite[a] ve mortalite[b] düzeyleri*

Group	Parameter	Day 1	Day 2	Day 3	Day 4	Day 5	Week 2	Week 3	Week 4
FE	Morbidity	8/8	5/8	2/8	2/8	1/8	0/8	0/8	3/8[c]
	Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
FED	Morbidity	8/8	6/8	7/8	6/8	1/8	2/8	2/8	4/8
	Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
LE	Morbidity	8/8	3/8	2/8	2/8	0/8	0/8	0/8	0/8
	Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
LED	Morbidity	8/8	5/8	8/8	4/8	2/8	1/8	1/8	1/8
	Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

[a] *The number of infected animals after bacteria was introduced into the trachea and the treatment was given;* [b] *the number of animals that died after bacteria was introduced into the trachea and the treatment was given; and* [c] *the number of dead and infected animals,* ***FE,*** *free enrofloxacin;* ***FED,*** *free enrofloxacin+dexamethasone;* ***LE,*** *liposomal enrofloxacin;* ***LED,*** *liposomal enrofloxacin+dexamethasone*

***Table 4.** Cytological evaluation of the BAL fluids of the control and experimental groups (n=8)*
***Tablo 4.** Kontrol ve deney gruplarının BAL sıvılarının sitolojik değerlendirilmesi (n=8)*

Parameter	NC	PC	FE	LE	FED	LED
Macrophage %	76.97±1.57^{a}	38.16±2.45^{c}	40.02±2.30^{c}	34.43±3.02^{c}	56.49±3.49^{b}	61.40±3.02^{b}
Lymphocyte %	3.70±1.67^{bc}	9.39±2.80^{a}	6.22±0.98^{abc}	6.65±0.88^{abc}	7.09±1.31^{ab}	3.34±0.55^{a}
Neutrophil %	9.37±1.88^{c}	46.31±2.12^{a}	46.12±2.32^{a}	50.92±3.32^{a}	28.11±3.51^{b}	25.68±3.22^{b}
Eosinophil %	0.00±0.00	0.00±0.00	0.00±0.00	0.49±0.34	0.66±0.36	0.00±0.00

*Different letters (a, b, c) in the same line are significantly different (P<0.05), Data is presented as mean±SD **NC,** negative control; **PC:** positive control; **FE,** free enrofloxacin; **FED,** free enrofloxacin+dexamethasone; **LE,** liposomal enrofloxacin; **LED,** liposomal enrofloxacin+dexamethasone*

***Table 5.** Enzyme levels in the bronchoalveolar lavage fluids of the control and experimental groups (n=8)*
***Tablo 5**. Kontrol ve deney gruplarının bronko-alveolar lavaj sıvılarında enzim düzeyleri (n=8)*

Parameter	Group	Day 1	Day 2	Day 3	Day 4	Day 5	Week 2	Week 3	Week 4
LDH (U/l)	NC	10.44±2.51^{c}	10.44±2.51^{c}	10.44±2.51^{cd}	10.44±2.51^{de}	10.44±2.51^{e}	10.44±2.51^{b}	10.44±2.51^{cd}	10.44±2.51^{c}
	PC1	12.40±3.03^{c}	12.40±3.03^{c}	12.40±3.03^{cd}	12.40±3.03^{de}	12.40±3.03^{de}	12.40±3.03^{b}	12.40±3.03^{cd}	12.40±3.03^{c}
	PC2	4.90±0.77^{c}	4.90±0.77^{c}	4.90±0.77^{d}	4.90±0.77^{e}	4.90±0.77^{e}	4.90±0.77^{b}	4.90±0.77^{d}	4.90±0.77^{c}
	FE	5.33±0.94^{c}	8.00±2.74^{c}	19.40±2.25^{c}	14.50±1.12^{cd}	22.70±2.07^{bc}	19.40±1.49^{b}	17.60±1.61^{bc}	34.10±21.02^{bc}
	FED	52.90±9.64^{b}	48.50±7.68^{a}	42.40±4.90^{a}	43.40±5.56^{a}	35.64±4.96^{a}	53.80±15.97^{a}	37.50±7.00^{a}	83.80±27.25^{ab}
	LE	68.70±7.79^{a}	23.50±2.25^{b}	28.80±2.90^{b}	22.90±2.43^{bc}	21.22±2.05b^{cd}	25.36±2.36^{b}	28.00±4.71^{ab}	25.20±2.15^{bc}
	LED	45.67±2.82^{b}	39.20±1.93^{a}	30.10±3.28^{b}	29.10±3.28^{b}	29.40±3.06^{ab}	67.45±10.65^{a}	32.27±3.61^{a}	115.91±41.9^{a}
GGT (U/l)	NC	3.33±0.44^{c}	3.33±0.44^{d}	3.33±0.44^{b}	3.33±0.44^{c}	3.33±0.44^{d}	3.33±0.44^{c}	3.33±0.44^{bc}	3.33±0.44^{c}
	PC1	4.40±0.58^{c}	4.40±0.58^{bcd}	4.40±0.58^{d}	4.40±0.58^{bc}	4.40±0.58^{d}	4.40±0.58^{c}	4.40±0.58^{abc}	4.40±0.58^{bc}
	PC2	5.30±0.86^{c}	5.30±0.86^{bcd}	5.30±0.86^{bcd}	5.30±0.86^{abc}	5.30±0.86c^{d}	5.30±0.86^{abc}	5.30±0.86^{ab}	5.30±0.86^{abc}
	FE	5.11±0.77^{c}	7.10±1.44^{b}	5.60±0.64^{bcd}	6.90±0.81^{abc}	7.50±1.27^{abc}	6.00±0.94^{abc}	4.70±0.76^{abc}	4.60±0.54^{bc}
	FED	8.70±1.94^{ab}	11.20±0.99^{a}	14.10±3.17^{a}	9.00±2.77^{a}	9.82±0.92^{ab}	7.80±1.29^{a}	6.40±1.54^{a}	6.70±1.15^{a}
	LE	10.10±1.30^{a}	7.00±2.11^{bc}	9.10±1.04^{b}	7.70±1.36^{ab}	10.22±1.22^{a}	7.45±0.99^{ab}	4.40±0.79^{abc}	5.40±0.56^{ab}
	LED	5.56±1.04^{bc}	5.20±0.81^{bcd}	9.10±1.04^{bc}	7.20±1.33^{abc}	5.00±0.92^{cd}	5.18±1.09^{abc}	2.60±0.45^{c}	3.27±0.38^{c}
AST (U/l)	NC	13.11±1.01^{c}	13.11±1.01^{bcd}	13.11±1.01^{c}	13.11±1.01^{bc}	13.11±1.01^{bc}	13.11±1.01^{b}	13.11±1.01^{bc}	13.11±1.01^{cd}
	PC1	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}
	PC2	18.20±1.37^{ab}	18.20±1.37^{ab}	18.20±1.37^{ab}	18.20±1.37^{ab}	18.20±1.37^{ab}	18.20±1.37^{a}	18.20±1.37^{ab}	18.20±1.37^{abc}
	FE	14.33±1.39^{bc}	13.00±1.62^{bcde}	14.00±1.45^{bc}	13.20±0.61^{bc}	17.70±1.33^{abc}	14.20±0.36^{b}	11.90±2.23^{c}	4.20±2.20^{e}
	FED	4.30±1.22^{d}	7.60±2.18^{e}	6.20±1.10^{d}	13.60±2.29^{bc}	5.73±1.34^{c}	16.50±3.27^{a}	14.30±3.46^{bc}	21.30±4.69^{ab}
	LE	13.20±1.69^{c}	14.60±2.65^{bc}	11.70±2.45^{c}	15.20±2.90^{bc}	15.56±4.18^{bc}	18.91±3.02^{a}	9.20±1.88^{cd}	9.50±1.70d^{e}
	LED	14.56±2.64b^{c}	8.50±1.27d^{e}	10.40±0.97^{c}	11.60±1.81^{c}	6.90±1.28^{c}	18.00±3.95^{a}	4.91±0.76^{d}	10.73±2.91^{cde}

*Different letters (a, b, c, d, e) in the same column are significantly different (P<0.05), Data is presented as mean±SD **LDH,** lactate dehydrogenase; **GGT,** gamma-glutamyl transferase; **AST,** Aspartate aminotransferase; **NC,** negative control; **PC1:** positive control for FE and LE; **PC2,** positive control for FED and LED; **FE,** free enrofloxacin; **FED,** free enrofloxacin+dexamethasone; **LE,** liposomal enrofloxacin; **LED,** liposomal enrofloxacin+dexamethasone*

compared the MLVs and LUVs, we found that the LUVs were approximately two times bigger than the MLVs. This particle size difference affects the drug encapsulation capacity. The particle size of the LUVs was approximately 4.65±0.37 mm, and they had the highest encapsulation capacity *(Table 1).* We suggest that increasing the drug encapsulation capacity produced the slow release of ENR. This result was similar to the results found in the literature [3,13,14]. The optimized LUVs with the appropriate particle size and distribution, high surface charge and drug release properties used in the *in vitro* studies were chosen for use in the *in vivo* experiments, and the treatment efficacy on rats with experimental pneumonia was partially investigated.

In a previous study, in rats in which pneumonia was induced by *K. pneumoniae*, the clinical therapeutic effect of liposome-encapsulated gentamicin was found to be higher than the free form [24]. In this study, while clear clinical findings were not observed in infected rats with normal immune systems and those with immune systems that were depressed by dexamethasone after L-ENR treatment in the 2nd, 3rd, and 4th weeks, the fact that clinical symptoms arose in the 4th week in rats given F-ENR suggested that experimental infection reoccurred. For that reason, liposome encapsulation increased the effectiveness of ENR. Studies showed that liposome-encapsulated CPR provided full protection after aerosol inhalation when compared to free CPR in mice in which respiratory infection was induced by *F. tularensis* [25].

In rats with weakened immune systems, the therapeutic effectiveness was found to be higher with one dose of

Table 6. *Urea and microprotein concentrations in the BAL fluids of the control and experimental groups (n=8)*
Tablo 6. *Kontrol ve deney gruplarının BAL sıvılarındaki üre ve mikroprotein konsantrasyonları (n=8)*

Parameter	Group	Day 1	Day 2	Day 3	Day 4	Day 5	Week 2	Week 3	Week 4
UREA (mg/dl)	NC	3.11±0.42[b]	3.11±0.42[b]	3.11±0.42[b]	3.11±0.42[b]	3.11±0.42[c]	3.11±0.42[b]	3.11±0.42[b]	3.11±0.42[c]
	PC1	3.10±0.36[b]	3.10±0.35[b]	3.10±0.35[b]	3.10±0.35[b]	3.10±0.35[c]	3.10±0.35[b]	3.10±0.35[b]	3.10±0.35[c]
	PC2	3.20±0.36[b]	3.20±0.36[b]	3.20±0.36[b]	3.20±0.36[b]	3.20±0.36[c]	3.20±0.36[b]	3.20±0.36[b]	3.20±0.36[c]
	FE	3.22±0.32[b]	3.20±0.29[b]	4.40±0.37[b]	3.80±0.39[b]	3.50±0.37[bc]	3.80±0.39[b]	4.40±0.43[b]	3.50±0.34[c]
	FED	3.30±0.37[a]	3.60±0.31[b]	3.20±0.36[b]	3.60±0.52[b]	3.55±0.41[bc]	3.80±0.42[b]	3.90±0.53[b]	4.20±0.29[bc]
	LE	3.20±0.57[b]	4.30±0.52[a]	4.40±0.43[b]	4.50±0.37[b]	4.56±0.24[b]	5.00±0.40[b]	4.50±0.60[b]	5.70±0.93[ab]
	LED	4.56±0.56[a]	5.30±0.82[a]	8.10±1.19[a]	7.20±1.01[a]	7.00±0.65[a]	13.36±5.30[a]	6.73±1.45[a]	6.09±0.69[a]
MP (mg/dl)	NC	179±98[b]	179±98[b]	179±98[b]	179±98[b]	179±98[cd]	179±98[b]	179±98[bc]	179±98[bc]
	PC1	225±27[b]	225±27[b]	225±27[b]	225±27[b]	225±27[bcd]	225±27[b]	225±27[b]	225±27[bc]
	PC2	242±34[b]	242±34[b]	242±34[b]	242±34[b]	242±34[bcd]	242±34[b]	242±34[b]	242±34[bc]
	FE	136±18[b]	202±26[b]	171±27[b]	179±26[b]	141±18[d]	162±23[b]	69±14[c]	80±20[c]
	FED	202±35[b]	201±33[b]	176±26[b]	165±49[b]	316±45[abc]	516±123[a]	415±54[a]	370±55[ab]
	LE	374±48[a]	496±53[a]	404±81[a]	455±44[a]	337±48[ab]	259±41[b]	248±45[b]	348±51[ab]
	LED	517±71[a]	419±25[a]	477±38[a]	393±48[a]	426±49[a]	316±61[b]	217±29[b]	483±124[a]

Different letters (a, b, c) in the same column are significantly different (P<0.05), Data is presented as mean±SD ***MP,*** *microprotein;* ***NC,*** *negative control;* ***PC1:*** *positive control for FE and LE;* ***PC2,*** *positive control for FED and LED;* ***FE,*** *free enrofloxacin;* ***FED,*** *free enrofloxacin+dexamethasone;* ***LE,*** *liposomal enrofloxacin;* ***LED,*** *liposomal enrofloxacin+dexamethasone*

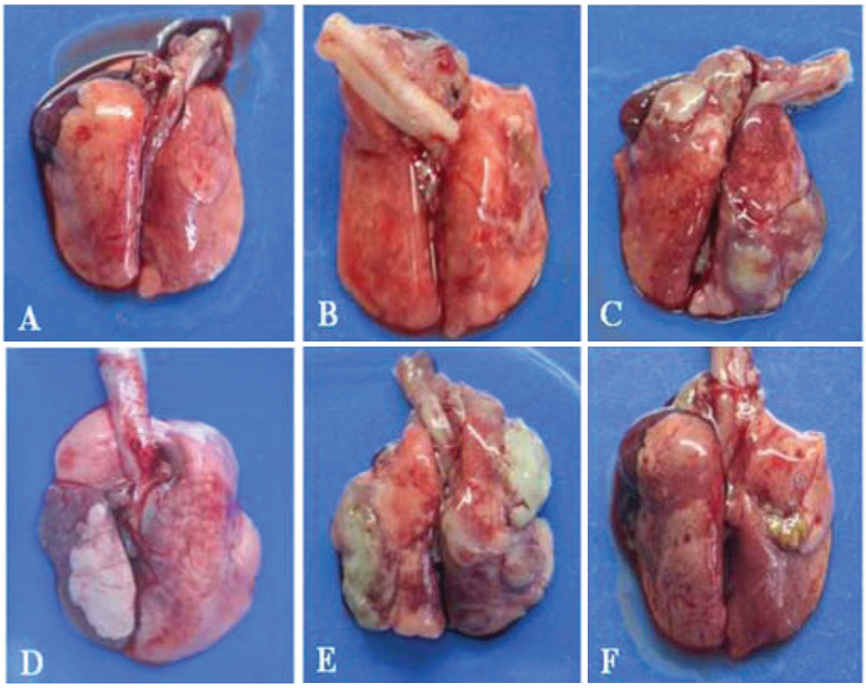

Fig 2. A- Mild congestion, NC, **B**- Pleuritis, PC1, **C**- Abscess containing yellow-green pus, LED-3rd day, **D**- Band shape hepatization in the left caudal lobe, SE-4th day, **E**- Pleuritis and abscess containing yellow-green pus, SED-4th day, **F**- Abscess containing yellow-green pus in the right cranial lobe, SED-2nd week

Şekil 2. A- Hafif konjesyon, NC, **B**-Pleuritis, PC1, **C**- Sarı-yeşil irin içeren abse, LED-3. Gün, **D**- Sol arka lobta kenarları keskin hepatizasyon, SE-4. Gün, **E**- Pleuritis ve sarı-yeşil irin içeren abse, SED-4. Gün, **F**- Sağ ön lobta sarı-yeşil irin içeren abse, SED-2. hafta

liposomal gentamicin rather than multiple doses of free gentamicin [26]. After F-ENR treatment in this study, in rats with a suppressed immune system, re-isolation of the bacteria was found in lung samples collected up to the 4th week, but in the BAL samples, re-isolation was found only on the 5th day. In the 3rd and 4th weeks, re-isolation could not be performed. However, after treatment with L-ENR, bacteria isolation was found in the lung samples, except during the 3rd and 4th weeks, and no bacterial reproduction was observed in the BAL samples in the 2nd, 3rd and 4th weeks. Nevertheless, there was less bacterial production in the BAL and lung samples of the LED group according to the FED group. However, in rats with depressed immune systems, the therapeutic effectiveness of L-ENR was found to be limited, even though L-ENR has better therapeutic effectiveness than F-ENR. Many studies [27,28] have stated that even with the improved therapeutic effectiveness of drugs encapsulated with liposomes, 100% of the bacteria could not be eliminated from the body. In addition to, the fact that death was not observed in the rats in all of the experimental groups is important in terms of showing the bactericidal effect of the drug. The fact that the morbidity rate was lower in the LE group than in the other groups can be explained by the fact that the

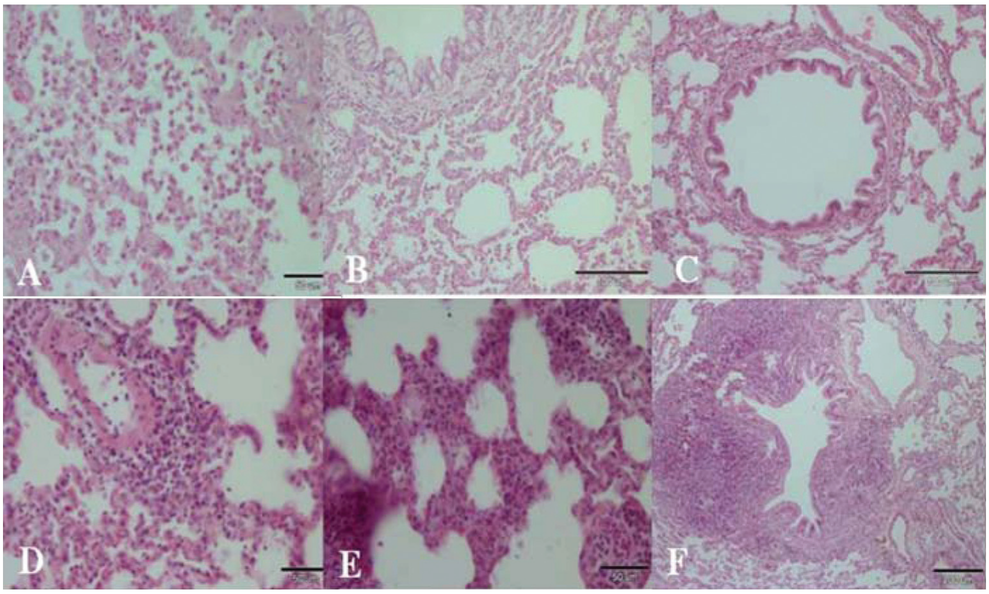

Fig 3. A- Alveolar macrophages and neutrophils in the alveolar lumen, SED-3rd day, H&E, Bar: 50 µm, **B-** Increases in the number of goblet cells, alveolar macrophages and neutrophils in the alveolar lumen, SED-3rd day, **C-** Mild perivascular mononuclear cell infiltrations, LE-4th day, H&E, Bar: 100 µm, **D-** Perivascular mononuclear cells and eosinophil infiltrations, LE-1st week, H&E, Bar: 50 µm, **E-** Thickness of interalveolar septa, LE-2nd week, H&E, Bar: 50 µm, **F-** Perivascular and peribronchiolar mononuclear cell infiltrations and lymphoid hyperplasia, SED-3rd week, H&E, Bar: 200 µm

Şekil 3. A- Alveol lumeninde alveoler makrofajlar ve nötrofiller, SED-3. gün, H&E, Bar: 50 µm, **B-** Alveol lumeninde goblet hücre, alveoler makrofaj ve nötrofil sayılarında artış, SED-3. Gün, **C-** Hafif perivasküler mononükleer hücre infiltrasyonları, LE-4. gün, H&E, Bar: 100 µm, **D-** Perivasküler mononükleer hücre ve eozinofil infiltrasyonları, LE-1. hafta, H&E, Bar: 50 µm, **E-** Alveoller arası septumda kalınlaşma, LE-2. hafta, H&E, Bar: 50 µm, **F-** Perivasküler ve peribronşiyal mononükleer hücre infiltrasyonları ve lenfoid hiperplazi, SED-3. hafta, H&E, Bar: 200 µm

drug was carried into the cell, increasing its therapeutic effectiveness.

In this study, when the cellular composition of the BAL fluid was evaluated compared to the control groups, the macrophage number decreased significantly compared to healthy rats (NC), and an increase in the neutrophil number was observed. These changes were evaluated as an indicator of experimental bacterial infection [9]. The increase in macrophage number in the FED and LED groups compared to the PC is explained as a classical leukocyte response (neutrophillia, lymphopenia, monocytosis and eosinopenia) to the corticosteroid application [29]. When the cellular composition of the BAL fluid was evaluated according to the differences in drug formations without taking the control groups into consideration, there was no difference between the FE and LE groups with the FED and LED groups, showing that free and liposomal forms of the drug did not affect cellular formation. Similarly, corticosteroid action was not effective in these two different drug treatments.

The increase in the enzyme activities in the BAL fluid (LDH, AST and GGT) reflects cell injury [11,21,22]. In this study, there were significant differences in the LDH and GGT enzyme activities of the BAL fluid between groups *(Table 5)*. It has been determined that the LDH enzyme in the BAL fluid can only be found extracellularly in the case of cell membrane injury, and can be a useful parameter for the diagnosis of injury in lung tissue [30]. Similarly, it has been reported that the increase in GGT enzyme activity in the BAL fluid can be an indicator of pulmonary endothelial cell injury [31]. Thus, the increase in enzyme activities of LDH, a cytoplasmic enzyme [32] and GGT, located on the brush-like edges of cell membranes [31] were evaluated as indicators of pulmonary injury. In this study, there were also significant decreases in AST enzyme activity of the BAL fluid between groups *(Table 5)*. AST is a cytoplasmic enzyme similar to LDH, yet AST tissue levels are lower than those of LDH [33]. Dere and Polat [34], in their study on paraquat toxicity in rats, found a significant increase in LDH enzyme activity and a significant decrease in AST enzyme activity at 2 and 16 h. In this study, the reasons for the differences in the AST enzyme level compared to the GGT and LDH enzyme levels can be attributed to low tissue levels and excessive variations within the groups.

Many studies [12,22,31] have shown that the BAL fluid protein and urea measurements in lung diseases can show vascular and respiratory permeability defects. In this study, when the increases in the enzyme activities were considered, the changes found in the MP and urea levels confirm the existence of respiratory and vascular permeability deformities and pulmonary damage. The high MP and urea concentrations in the LE and LED groups versus the FE and FED groups *(Table 6)* indicate that liposomal drug formations are more effective on vascular and respiratory membrane permeability.

In conclusion, ENR was successfully encapsulated into the LUVs and MLVs. The molar ratios of the DPPC, cholesterol and ENF in the liposome formulation are important for the drug encapsulation, vesicle size, electrophoretic mobility and drug release from the liposomes. The highest encapsulation capacity was obtained in LUVs. In the *in vivo* study, the data show that cytologic and enzymologic diagnoses of the BAL fluid are not meaningful in evaluating the treatment of the experimental pneumonia in rats. We concluded that the use of ENR in liposomal form at a 7.5 mg/kg dosage for five days was more effective than the free form both in the treatment of *K. pneumoniae* infections and in the prevention of recurrent infections. The pharmaceutical form of the antimicrobial agent, in addition to the choice of the agent, drastically affects the prognosis of infection. The liposome-encapsulated antimicrobial agents should provide another choice in antimicrobial therapy in the future, and further investigation is necessary before clinical use.

REFERENCES

1. Lizondo M, Pons M, Gallardo M, Estelrich J: Physicochemical properties of enrofloxacin. *J Pharm Biomed Anal*, 15, 1845-1849, 1997.

2. Schovers EJ, Van Leengoed AMG, Verheijden JHM, Nie Wold TA: Effects of enrofloxacin on porcine phagocytosis function. *Antimicrob Agents Chemother*, 43, 2138-2143, 1999.

3. Magallanes M, Dijkstra J, Fierer J: Liposome-incorporated ciprofloxacin in the treatment of murine salmonellosis. *Antimicrob Agents Chemother*, 37, 2293-2297, 1993.

4. Di Ninno VL, Cherwonogrodzlky JW, Wong JP: Liposome-encapsulated ciprofloxacin is effective in protection and treatment of BALB/C mice against *F. tularensis*. *J Infect Dis,* 168, 793-794, 1993.

5. Jarvis WR, Munn VP, Highsmith AK, Culver DH, Hughes JM: The epidemiology of nosocomial infections caused by *Klebsiella pneumoniae*. *Infect Control*, 6, 68-74, 1985.

6. Cortes G, Alvarez D, Saus C, Alberti S: Role of lung epithelial cells in defense against *Klebsiella pneumoniae* pneumonia. *Infect Immun*, 70, 1075-1080, 2002.

7. Larkin HA: Veterinary cytology-cytological diagnosis of diseases of the respiratory tract in animals. *Ir Vet J*, 47, 304-312, 1994.

8. Lecuyer M, Dube PG, Difrussia R, Desnoyers M, Lagace A: Bronchoalveolar lavage in normal cats. *Can Vet J*, 36, 771-773, 1995.

9. Daniele RP, Elias JA, Epstein PE, Rossman MD: Bronchoalveolar lavage: Role in the pathogenesis, diagnosis, and management of interstitial lung disease. *Ann Intern Med*, 102, 93-108, 1985.

10. Mayer P, Laber G, Walzl H: Bronchoalveolar lavage in dogs; analysis of proteins and respiratory cells. *Zentralbl Veterinarmed A*, 37, 392-399, 1990.

11. Lopez A, Prior M, Yong S, Albassam M, Lillie LE: Biochemical and cytologic alterations in the respiratory tract of rats exposed for 4 hours to hydrogen sulfide. *Fundam Appl Toxicol*, 9, 753–762, 1987.

12. Van de Graaf EA, Jansen HM, Weber JA, Koolen MG, Out TA: Influx of urea during bronchoalveolar lavage depends on the permeability of the respiratory membrane. *Clin Chim Acta*, 196, 27-39, 1991.

13. Sezer AD, Baş AL, Akbuğa J: Encapsulation of enrofloxacin in liposome preparation and *in vitro* characterization of LUV. *J Liposome Res*, 14, 77-86, 2004.

14. Sezer AD, Akbuğa J, Baş AL: *In vitro* evaluation of enrofloxacin-loaded MLV liposomes. *Drug Deliv*, 14, 47-53, 2007.

15. Montero MT, Carrera I, Hernandez-Borrell J: Encapsulation of a quinolone in liposomes. Location and effect on lipid bilayers. *J Microencapsul*, 11, 423-430, 1994.

16. Berger N, Sachse A, Bender J, Schubert R, Brandl M: Filter extrusion of liposomes using different devices: Comparison of liposome size, encapsulation efficiency, and process characteristics. *Int J Pharm,* 223, 55-68, 2001.

17. Vyas SP, Goswami SK, Singh R: Liposomes based nasal delivery system of nifedipine: Development and characterization. *Int J Pharm*, 118, 23-30, 1995.

18. Martinez A, Regadera J, Jimenez E, Santos I, Gargallo-Viola D: Antifungal efficacy of GM237354, a sordarin derivative, in experimental oral candidiasis in immunosuppressed rats. *Antimicrob Agents Chemother*, 45, 1008-1013, 2001.

19. Bakker-Woudenberg IAJM: Experimental models of pulmonary infection. *J Microbiol Methods*, 54, 295-313, 2003.

20. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC: Color Atlas and Textbook of Diagnostic Microbiology. 4th ed., JB Lippincott Company, Philadelphia, 1992.

21. Maden M, Birdane FM, Alkan F, Hadimli HH, Şen I, Aslan V: Clinical, cytologic, bacteriologic and radiographic analysis of respiratory diseases in dogs. *Eurasian J Vet Sci*, 16, 43-50, 2000.

22. Maden M, Birdane FM, Alkan F, Hadimli HH, Şen İ, Aslan V: Specific enzyme activities in bronchoalveolar lavage fluid as an aid to diagnosis of tracheobronchitis and bronchopneumonia in dogs. *Res Vet Sci*, 71, 141-145, 2001.

23. Luna LG: Routine staining procedures. **In,** Luna LG (Ed): Manuals of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed., pp. 32-46, McGraw-Hill Book Company, New York, USA, 1968.

24. Schiffelers RM, Storm G, Bakker-Woundenberg IAJM: Therapeutic efficacy of liposomal gentamicin in clinically relevant rat models. *Int J Pharm*, 214, 103-105, 2001.

25. Wong JP, Yang H, Blasetti KL, Schnell G, Conley J, Schofield LN: Liposome delivery of ciprofloxacin against intracellular *Francisella tularensis* infection. *J Control Release*, 92, 265-273, 2003.

26. Schiffelers R, Storm G, Bakker-Woundenberg IAJM: Liposome-encapsulated aminoglycosides in pre-cinical and clinical studies. *J Antimicrob Chemother*, 48, 333-344, 2001.

27. Bakker-Woundenberg IAJM, Ten Kate MT, Guo L, Working P, Mouton JW: ciprofloxacin in polyethylene glycol-coated liposomes: Efficacy in rat models of acute or chronic *Pseudomonas aeruginosa* infection. *Antimicrob Agents Chemother*, 46, 2575-2581, 2002.

28. Bakker-Woundenberg IAJM, Ten Kate MT, Guo L, Working P, Mouton JW: Improved efficacy of ciprofloxacin administered in polyethylene glycol-coated liposomes for treatment of *Klebsiella pneumoniae* pneumonia in rats. *Antimicrob Agents Chemother*, 45, 1487-1492, 2001.

29. Meyer DJ, Coles EH, Rich LJ: Leukocytic tests and disorders. **In,** Mills L (Ed): Veterinary Laboratory Medicine-Interpretation and Diagnosis. 1th ed. 27-41, WB Saunders Company, Philadelphia, 1992.

30. Henderson RF, Driscoll KE, Harkema JR, Lindenschmidt RC, Chang IY, Maples KR, Barr EB: A comparison of the inflammatory response of the lung to inhaled versus instilled particles in F344 rats. *Fundam Appl Toxicol*, 24, 183-197, 1995.

31. Day BJ, Carlson GP, Denicola DB: Gamma-glutamyl transpeptidase in rat bronchoalveolar lavage fluid as a probe of 4-ipomeanol and alpha-naphthalene-induced pneumotoxicity. *J Pharmacol Methods*, 24, 1-8, 1990.

32. Beck BD, Gerson B, Feldman HA: Lactate dehydrogenase isoenzymes in hamster lung lavage fluid after lung injury. *Toxicol Appl Pharm*, 71, 59-71, 1983.

33. Vroon DH, Israili Z: Aminotransferases. **In,** Walker HK, Hall WD, Hurst JW (Eds): Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd ed., Chapter 99, Butterworth Publishers, Boston, 1990.

34. Dere E, Polat F: The effect of paraquat on the activity of some enzymes in different tissues of mice (*Mus musculus*-Swiss albino). *Turk J Biol*, 25, 323-332, 2001.