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 Lipozolamlanmış Enrofloksasin İle Tedavisi

Özet

Enrofloksasin (ENR) in vitro ortamlarda ökaryotik hücrelerde hızla lokalize olur, ancak uzun süre kalamadığı için hücre içi patojenlere karşı onun etkiliği azalır. Lipozomla kapsül edilmiş formda ENR uygulanması hücre içi ortamlarda kalış zamanını artıracabilir. Bu çalışmada, sağlıklı ve deksametazon-uğurlananuşturulan ratlarda Klebsiella pneumoniae serotip II kullananlar deneySEL pnömoni oluşturdu. Serbest ve lipozomla kapsül edilmiş ENR enfekte hayvanlara intravenoz olarak başın tüm boyunca 7.5 mg/kg/gün dozda enjekte edildi. İlk antibiyotik uygulanmasından sonra 1, 2, 3, 4, gün ve 1, 2, 3, 4, haftalarda plazma, doku ve bronkoalveolar 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 önemini anlamsız olduğu belirlendi. Ancak, enrofloksasinin başın tüm boyunca 7.5 mg/kg dozda lipozomal formda kullanılmının hem K. pneumoniae enfeksiyonlarının tedavisinde hem de tekrarlanan enfeksiyonların önlenmesinde serbest formdan daha etkili olduğu tespit edildi. Lipozomla kapsül edilmiş antimikrobiyal ajanlar gelecekte antimikrobiyal tedavide diğer bir seçeneken sağlayacaklardır, ancak klinik kullanmanın önce çok sayıda araştırma yapılmalıdır.

Anahtar sözcükler: Enrofloksasin, Deneysel pnömoni, Klebsiella pneumoniae, Lipozom, Rat, Tedavi
**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, 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 polymorphonuclear 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. Various studies have shown that liposomal forms of fluoroquinolones can significantly increase the effectiveness of the antibacterial action compared to free forms.

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. 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.

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. The BAL provides important information for the identification of cellular structures and the definition of the scope of the inflammation. 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. 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.

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. 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.

**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.

**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.

**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.

**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.

**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 1x10⁶ 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 intravenously 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 CO2 inhalation on the fifth day following drug application into the trachea. The experimental groups were euthanized by CO2 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
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 histopathological 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, were given to rats with experimental pneumonia, and we evaluated the efficiency of the liposomal treatment. The characteristic 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 4. Cytological evaluation of the BAL fluids of the control and experimental groups (n=8)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>PC</th>
<th>FE</th>
<th>LE</th>
<th>FED</th>
<th>LED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil %</td>
<td>76.97±1.57a</td>
<td>38.16±2.45b</td>
<td>40.02±2.30c</td>
<td>34.43±3.32d</td>
<td>56.49±3.40e</td>
<td>61.40±3.02f</td>
</tr>
<tr>
<td>Macrophage %</td>
<td>3.70±1.67a</td>
<td>9.39±2.80b</td>
<td>6.22±0.98cd</td>
<td>6.65±0.88de</td>
<td>7.09±1.31ef</td>
<td>3.34±0.55g</td>
</tr>
<tr>
<td>Eosinophil %</td>
<td>9.37±1.88a</td>
<td>46.31±2.12b</td>
<td>46.12±2.32c</td>
<td>50.92±3.32d</td>
<td>28.11±3.51e</td>
<td>25.68±3.22f</td>
</tr>
<tr>
<td>Lymphocyte %</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.49±0.04</td>
<td>0.66±0.36</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

Different letters (a, b, c, d, e) in the same line are significantly different (P<0.05). Data is presented as mean±SD.
liposomal gentamicin rather than multiple doses of free gentamicin. 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 3rd 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 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

### Table 6. Urea and microprotein concentrations in the BAL fluids of the control and experimental groups (n=8)

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

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
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. The increase in macrophage number in the FED and LED groups compared to the PC is explained as a classical leukocyte response (neutrophilia, lymphopenia, monocytosis and eosinopenia) to the corticosteroid application. 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. 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. 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. Thus, the increase in enzyme activities of LDH, a cytoplasmic enzyme and GGT, located on the brush-like edges of cell membranes, 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. Dere and Polat, 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 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 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