Critical Appraisal of Air Pouch Infection Model in Rats

Zeynep Saribas¹, Hakan Ergun², Soner Mamuk², Özgen Köseoglu-Eser¹, and Mehmet Melli²

¹Hacettepe University, School of Medicine, Department of Medical Microbiology, Sihhiye 06100, Ankara, Turkey; ²Ankara University, School of Medicine, Department of Medical Pharmacology, Sihhiye 06100, Ankara, Turkey

Abstract. The aim of this study was to assess the efficacy and pharmacokinetic profiles of gentamicin, vancomycin, and levofloxacin in a rat air pouch model, in which Staphylococcus aureus (ATCC 25293) was used as the test organism. Antibiotic treatments (i.p.) were started 1 hour after bacterial inoculation and continued for 5 days. Bacterial counts and antibiotic concentrations were determined in pouch exudates that were obtained on the 5th day of antibiotic treatment. The following observations were made: 1) The concentrations of gentamicin or vancomycin in the exudate were found to be below the detection limit. 2) Levofloxacin and ciprofloxacin exhibited a dose-dependent effect on bacterial counts in the exudate. 3) The antibacterial efficacy of levofloxacin was found to be enhanced when the total daily dose of 10 mg was divided into smaller parts. The present study also showed that the air pouch infection model was a valuable tool to assess the pharmacokinetic and pharmacodynamic properties of antibiotics.

Key words: Staphylococcus aureus, air pouch infection model, gentamicin, vancomycin

Introduction

Several in vivo experimental models for the efficacy assessment of antibiotics are available [1, 2, 3]. Among these models, the air pouch infection model enables collection of the exudate that is formed in the air pouch. The exudate thus sampled can then be used to determine bacterial count and antibiotic concentrations simultaneously at the infection site. The latter property of the model allows for the evaluation of the pharmacodynamics and pharmacokinetics of the antibiotics simultaneously. This may be information essential in optimizing dosing regimens for antibiotics that target a particular microorganism [4].

Due to the fact that the antibiotic concentration at the site of infection is governed by complicated time-dependent processes in vivo, minimum inhibitory concentration (MIC), as an in vitro measure of antibiotic efficacy, should not provide sufficient information to anticipate the response in vivo. Therefore, different measures that combine all such information together have been used to predict efficiency of the antimicrobial therapy. Examples of such measures are: the ratio of area under the plasma concentration-time curve (AUC) to MIC (AUC/MIC); the ratio of peak concentration (C max) to MIC (C max/MIC); or the time period (T) in which the drug concentration (C) stays above the MIC value (T of C>MIC) [5, 6]. In clinical studies, pharmacokinetic data for antibiotics are generally obtained from blood sample analyses. However, time-dependent blood or plasma concentrations of antibiotics do not necessarily reflect their pharmacokinetics at the infection site.

The aim of the present study was to reevaluate the air pouch infection model to optimize the characteristics of air pouch formation and the maximum observable duration of antibiotic treatment, and to assess the efficacy and pharmacokinetic profile of three different groups of antibiotics, namely gentamicin, vancomycin, and levofloxacin or ciprofloxacin.

Materials and Methods

Animals. The study was performed on female Wistar albino rats weighting 200–250 g. The study was approved by Ankara University Animal Ethical
Committee and was conducted according to the European Community guidelines for the use of experimental animals. The animals were housed before and during the experiments in an air-conditioned room with an ambient temperature 21 ± 3°C and a 12-h light/dark cycle (light on at 7:00 AM) and were fed with their ordinary diet and allowed to drink water ad libitum.

**Formation of air pouches.** The air pouch was created by subcutaneous injection of 30 ml sterile air along with 1 ml of 1% suspension of croton oil (Sigma, St. Louis, MO, USA) in tricaprylin oil (Fluka, Taufkirchen, Germany). At the second day of air injection, the excess amount of air was withdrawn, as originally suggested by Brauner et al [12]. However, after having experienced several perforations which seemed due to the second puncture described above, the “air-suction” procedure was avoided in all the experiments described below.

**Inoculation of Staphylococcus aureus ATCC 25293.** One week after the initial injection of air, 1 ml (10^6 cfu) of *S. aureus* ATCC 25293 suspended in 0.1 ml of 5% gastric mucin (Sigma, St. Louis, MO, USA) was inoculated into the cavity [7]. The optimum amount of *S. aureus* ATCC 25293 was studied with each in 1 ml of 10^5, 10^6, and 10^7 cfu. The samples were diluted and spread, as previously mentioned. The optimum bacterial counts were obtained with 1 ml of 10^6 cfu of *S. aureus* ATCC 25293 inoculations. The remaining studies were all performed with the same amount.

After the inoculation of *S. aureus* ATCC 25293 and without any manipulation thereafter, animals were observed for nine days. At the 5th day of inoculation, prominent pouch skin coloration was observed. These color changes started to perforate mostly after the 5th day of inoculation period. Due to this limitation, the experiments were performed for only five days after *S. aureus* inoculation.

**Antibiotic treatment.** Antibiotic treatments were started 1 hour after the inoculation of bacteria. All antibiotics were given by intraperitoneal (i.p.) injection. Dose response experiments for each antibiotic were performed to determine the optimum dose. Control group for gentamicin studies (n=15), gentamicin 2.5 mg/kg/day (n=10), 5 mg/kg/day (n=12) and 10 mg/kg/day (n=10); control for vancomycin studies (n=11), vancomycin 5 mg/kg/day (n=10), 10 mg/kg/day (n=9) and 20 mg/kg/day (n=10); control group for ciprofloxacin studies (n=9), ciprofloxacin 5 mg/kg/day (n=11), 20 mg/kg/day (n=9) and 40 mg/kg/day (n=12) mg/kg/day, and control group for levofloxacin studies (n=10), levofloxacin 5 mg/kg/day (n=8), 20 mg/kg/day (n=7) and 40 mg/kg/day (n=8) dose response studies were performed. Only ciprofloxacin and levofloxacin were found to be effective in these experiments. Therefore, we further investigated only the properties of levofloxacin as a representative of the fluoroquinolone group. In this second group of experiments, the

---

**Table 1.** Dose-response data of gentamicin and vancomycin against *S. aureus* ATCC 25293 with once a day i.p. injection for 5 days in the rat air pouch. Data is given as mean (± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Gentamicin 2.5 mg/kg/day</th>
<th>Gentamicin 5 mg/kg/day</th>
<th>Gentamicin 10 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus (5x10^5 cfu/ml)</td>
<td>224 (±57)</td>
<td>255 (±90)</td>
<td>232 (±92)</td>
<td>186 (±78)</td>
</tr>
<tr>
<td>Control</td>
<td>Vancomycin 5 mg/kg/day</td>
<td>Vancomycin 10 mg/kg/day</td>
<td>Vancomycin 20 mg/kg/day</td>
<td></td>
</tr>
<tr>
<td>S. aureus (5x10^5 cfu/ml)</td>
<td>258 (±130)</td>
<td>247 (±34)</td>
<td>287 (±84)</td>
<td>220 (±55)</td>
</tr>
</tbody>
</table>
total daily dose of levofloxacin (10 mg/kg/day) was administered once, twice, or four times a day.

Pharmacokinetic studies. A separate group of animals was used to determine blood-concentration vs. time. Bloods samples, before and after drug administration, were collected under general anesthesia at the following time points: right before, and 15, 30, 45, 60, 90, 120, 180, 240 and 360 minutes after drug administration. General anesthesia was induced by 80 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride (intramuscular), and maintained by 40 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride, administered every 45 minutes during the blood collection period. The temperatures of the surgical table and the room were kept constant at 37°C and 22°C, respectively. Blood samples were collected from the right common carotid artery, which was cannulated with a 0.76 mm (inside diameter) polyethylene catheter (Durect Corp., Cupertino, CA, USA).

Samples of pouch exudates were drawn from the inoculation-treatment group on the fifth day of antibiotic treatment (see below).

Bacterial counts. Bacterial counts and antibiotic concentrations were determined in the samples of pouch exudates. Exudate samples (1 ml/animal) were obtained on the 5th day of treatment (steady state phase). Samples were diluted (1:10) in phosphate buffered saline (PBS). Diluted samples (20 μl in triplicates) were plated on blood agar, and incubated for 24 hours at 37°C. Results are expressed as cfu/ml.

Antibiotic assays. Blood and pouch exudate samples for gentamicin and vancomycin measurements were centrifuged at 10,000 rpm at 4°C for 15 minutes and stored at -80°C. Gentamicin and vancomycin were determined in the central laboratory of Ibn-i Sina Hospital of Ankara University by a method of
“Fluorescence Polarization Immunoassay” using the Abbott Axym® system without prior extraction.

Blood and pouch exudate samples for levofloxacin measurement were extracted as follows: After the addition of 20 µl internal standard (0.1 mg/ml gatifloxacin) and 1200 µl acetonitrile to the 200 µl sample, the mixture was vortexed for 5 minutes and centrifuged at 4500 rpm at 4°C for 5 minutes. The organic phase was evaporated by vacuum centrifugation (MaxiDry Plus, Heto Holten A/S, Allerod, Denmark). The residue was diluted by 200 µl mobile phase and stored at -80°C [8]. Levofloxacin was analyzed with Agilent Technologies 1200 series high pressure liquid chromatography (HPLC) system (Agilent Technologies Inc., USA). Thirty µl of the sample solution was injected into the HPLC system. The diode-array detector wavelength was set to 290 nm. Separation was performed with an Agilent Eclipse XDB C18 (5 µm, 4.6x150mm) column (Agilent Technologies Inc., USA). The column temperature and autosampler temperature were set to 40°C and 20°C, respectively. The flow rate was 1.2 ml/min. The mobile phase consisted of acetonitrile/methanol/citric acid (0.2 M) (10/10/80, v/v/v) [9].

Standard curves were analyzed in the range from 10 to 1000 ng/ml for levofloxacin. The lower limit of quantification was 5 ng/ml. The coefficient of variation was less than 6% for the 50 and 500 ng/ml concentrations. Data acquisition was performed on ChemStation for LC-3D systems (Rev.B,03.01.317, 2007, Agilent Technologies Inc., USA).

Assessment of bacterial resistance. All the exudate samples were tested for the development of resistance to gentamicin, vancomycin, and levofloxacin. The E-test method was used to test the susceptibility of the test organism to gentamicin or vancomycin. The test was done according to the manufacturer’s instructions (AB-Biodisk, Solna, Sweden). Briefly, S. aureus colonies grown on blood agar were subcultured. The subcultures were adjusted to 0.5 McFarland turbidity standard. The bacterial suspension was homogenously spread on Mueller-Hinton agar and E-test strips were placed on the medium. After 24-hour incubation at 35°C, the MICs for either gentamicin or vancomycin were determined. In the case of levofloxacin, broth microdilution method was used according to Clinical Laboratory Standards Institute (CLSI) guidelines [10].

For gentamicin, MIC≤4 µg/ml, MIC=8 µg/ml or MIC≥16 µg/ml were considered “susceptible”, “intermediate”, or “resistant”, respectively. For vancomycin, the following thresholds for MIC values were used (as recommended by CLSI): MIC≤2 µg/ml (susceptible), MICs in [4-8] µg/ml (intermediate) or MIC≥16 µg/ml (resistant) [11].

Statistical methods. The effects of individual antibiotics doses on bacterial counts and the concentration of levofloxacin in the exudates were compared with one-way analysis of variance (ANOVA) followed by the Dunnet or Tukey test (levofloxacin exudates concentration). p< 0.05 was considered statistically significant.

Results

Antibiotic treatments

Gentamicin and vancomycin. Neither of the two antibiotics was found to be effective when compared to the relevant control groups (Table 1). Consistent with the latter observation, neither of the two antibiotics was found to be reaching a measurable concentration in the exudate. Nevertheless, analysis of blood concentrations indicated that these antibiotics were well absorbed when given i.p. (data not shown).

Levofloxacin and ciprofloxacin. Both antibiotics presented a dose dependent effect on bacterial counts (Figures 1 and 2). At the second part of the experiment, daily doses of 10 mg levofloxacin were given once (every 24 h), twice (every 12 h) or 4 times (every 6 h) a day. The results indicate that the antibacterial efficacy of levofloxacin is more pronounced when given in divided doses (Figure 3).

Pharmacokinetic results. Vancomycin and gentamicin were well absorbed after i.p. administration. However, the concentrations of either drug in exudate were below the detection limits (data not shown). Plasma levofloxacin concentrations were evaluated after the administration of 2.5, 5, and 10 mg/kg doses. The time-plasma levofloxacin
concentration curve is given in Figure 4. Exudates samples for levofloxacin were obtained at the end of the efficacy experiments. The levofloxacin levels in the exudates were well correlated with its effect on the bacterial count (Figure 4).

Detection of Antimicrobial Resistance. All the samples were susceptible to gentamicin, vancomycin, and levofloxacin. No resistance was observed in any of the isolates. MIC ranges for vancomycin, gentamicin and levofloxacin were 0.19-1, 0.25-2, 0.5-1 μg/ml respectively.

Discussion

In vivo experimental models have been widely used in pharmacology, especially in the preclinical phase of drug discovery studies. The thigh infection model has been broadly used to assess antibacterial efficacy. However, this model does not easily allow the measurement of the level of antibiotics at the infection site. The air pouch infection model, on the other hand, enables to the simultaneous evaluation of the effect and concentration of antibiotics at the infection site. In this study, the air pouch infection model was reevaluated critically. Details of the procedure are given. Pharmacokinetic and pharmacodynamic evaluations are reported in terms of bacterial counts and antibiotic concentration in pouch exudates.

There were several critical steps in the procedure of the air pouch infection model. One of them was the air withdrawal on the second day of air pouch initiation. This step was skipped in the present study, since a relatively high rate of pouch perforation was observed (in 5 days) when the latter step was performed. In addition, the viscosities of the exudates were found to be relatively low when the air withdrawal step was omitted.
These points, which may be critical only for relatively long treatment/observation periods, might have escaped attention in those studies where the antibiotic treatments were limited to 2-3 days [13, 14]. Therefore, we suggest not removing air when the observation period is longer than 3 days.

The maximum possible observation period in this model was also evaluated. We observed that almost half of the rats had a perforation on the 9th day of *S. aureus* inoculation, independently of whether the air was withdrawn or not, whereas no perforation was observed until the 5th day when the air withdrawal step was omitted. Therefore, we set the maximum observation period to 5 days.

We intended to investigate the development of resistance as well. However, during the period of the present observations, we did not observe any resistance to the antibiotics tested here. This was obviously not surprising, as the observation period in the present study was limited to 5 days. Hence, we should admit that the present model is not suitable for investigating resistance, at least not to Gram-positive microorganisms like *S. aureus*, for which the resistance develops in more than five days.

The antibiotics used in this study were selected based on their known effects on *S. aureus*. However, the results indicated that vancomycin and gentamicin were unable to reach measurable levels in the exudates. In accordance with this penetration problem, these antibiotics had no effect on the bacterial count in the exudate. To the best of our knowledge, there has been no study conducted with gentamicin or any other aminoglycoside antibiotics in the air pouch infection model. However, vancomycin has been studied in this model by several investigators. In one of these studies, vancomycin was administered for three days with a dose of 10 mg/kg/day [12]. This schedule of vancomycin administration reached a measurable level at the infection site, which was nevertheless below its MIC values as assessed by bioassay. Consequently, it has been found to be ineffective on bacterial count. However, as one would expect, relatively high doses of vancomycin (given i.m. in single or divided doses of 100 mg/kg daily) have been found to decrease exudates’ bacterial count [4].

In contrast to vancomycin and gentamicin, fluoroquinolone group antibiotics, ciprofloxacin and levofloxacin, which are known for their high penetration capacity to tissues and body cavities, decreased bacterial count in a dose-dependent manner (Figures 1 and 2). We also evaluated the pharmacokinetic profile of levofloxacin and found that levofloxacin was well absorbed when given i.p. (Figure 5). After a 5 day-treatment, the exudate levofloxacin levels were found to be higher than its MIC value.

It is well established that fluoroquinolone antibiotics eradicate microorganisms in a concentration-dependent manner, as the antibacterial efficacy of fluoroquinolones has been found to be correlated with AUC/MIC or $C_{\text{max}}$/MIC [11]. In a clinical study, Preston et al reported that clinical response and microbiological eradication of pathogenic microorganism by levofloxacin was correlated with its $C_{\text{max}}$/MIC ratio [15]. Alternatively, Forrest et al. and Ambrosse et al. have reported that the clinical and microbiological effects of ciprofloxacin [16]...
and levofloxacin [17] were correlated with AUC/MIC ratio. Together, the latter observations suggest that a single daily dose should be more effective than equivalent divided doses, as the former protocol provides relatively high C_{max} or AUC values. However, in the present study we found that a single daily administration of levofloxacin was less effective than equivalent divided doses (Figure 3). The fact that the divided-dose protocol provides a better pharmacokinetic profile at the infection site (Figure 4) is actually consistent with the latter observation. This observation is in accordance with the finding that the efficiency of fluoroquinolone-induced bacterial eradication is best correlated with the time that the drug concentration spends over the MIC value [18]. Considering that dose and administration protocols are critical to determining the antibacterial efficacy of fluoroquinolones, the present model may prove to be a useful method for assessing the contribution of such factors in different bacterial infections.

In conclusion, the air pouch infection model provides relevant means to investigate the penetration of antibiotics to the infection site and to determine the correlation between antibacterial effect in the infection site and the plasma concentrations of tested antibiotics. However, due to the limitation of the observation period of 5 days, the method seems to be limited when the objective is to investigate the development of resistance in vivo.

Acknowledgements
Z.S., H.E., S.M., Ö.E. and M.M. were all involved in conducting the study and writing the manuscript. The authors would like to thank Professor Ümit Yaşar and Mustafa T. Gökras, M.D. from Hacettepe University, School of Medicine, Department of Medical Pharmacology, for the levofloxacin measurement by HPLC. The authors would also like to thank Professor Ongun Onaran for his helpful criticism of the manuscript.

Funding: This study was supported by a grant from TÜBİTAK, The Scientific and Technological Research Council of Turkey [Project No: 107S230 (SBAG-3724)].

References