

Effect of Sodium Diclofenac on Serum and Tissue Concentration of Amoxicillin and on Staphylococcal Infection

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The effect of sodium diclofenac on serum and tissue amoxicillin concentration as well as their effect against staphylococcal infection was observed. Four polyurethane sponges were placed in the back of thirty rats. After 14 d, two granulomatous tissues received 0.5 ml of 10^8 cfu/ml (*Staphylococcus aureus*). Two days later, the rats were divided into five groups: group 1 received amoxicillin 50 mg/kg/p.o., group 2 received amoxicillin 25 mg/kg/p.o., group 3 received sodium diclofenac 2.5 mg/kg/i.m. and amoxicillin 50 mg/kg/p.o., group 4 received sodium diclofenac 2.5 mg/kg/i.m., and group 5 (control group) received NaCl 1 ml/p.o. After six hours of drug administration, blood serum (10 μ l) and noninfected granulomatous tissues were placed on Mueller–Hinton agar inoculated with 10^8 cfu/ml (*S. aureus*). Infected tissues were dispersed in a sonic system and were spread (10 μ l) on salt mannitol agar. Microorganisms were counted and the inhibition zones were measured after 18 h of incubation at 37 °C. Amoxicillin tissue concentration was 6.27 μ g/g for group 1, 2.18 μ g/g for group 2, and 0.72 μ g/g for group 3. The serum concentrations were 11.56 μ g/ml for group 1, 5.36 μ g/ml for group 2, and 1.34 μ g/ml for group 3. No differences were observed among group 1, 2, and 3 regarding staphylococci counts (Kruskall–Wallis test $p > 0.05$). Group 4 reduced ($p < 0.05$) staphylococci counts comparing to group 5. It was concluded that sodium diclofenac reduced serum and tissue amoxicillin concentration and, even in large doses, amoxicillin was not effective in eradicating the staphylococcal infection after 6 h of administration.

Key words amoxicillin; sodium diclofenac; *Staphylococcus aureus*; staphylococcal infection

Amoxicillin is a widely prescribed aminopenicillin, primarily for oral use.¹ Ninety percent of the administered dose is absorbed without molecular modifications.² It provides human serum concentration ranging from 7.6 μ g/ml to 10.8 μ g/ml when 500 mg/p.o. is used^{3,4}; 15.1 μ g/ml when 15.4 mg/kg/p.o. is used,¹ and 14.5 μ g/ml when 40 mg/kg/p.o. is used.⁵ The serum concentrations in rats were 28 μ g/ml and 13 μ g/ml when amoxicillin doses of 40 mg/kg/p.o. and 7.0 mg/kg/p.o. were administered, respectively.^{6,7}

Amoxicillin has plasma protein binding ranging from 17 to 20%.⁸ Food interferes with neither absorption nor plasma concentration.⁵ Minimal inhibitory concentration (MIC) against susceptible *S. aureus* ranges from 0.06 to 1 μ g/ml.^{8,9}

Several methods can be used to measure amoxicillin concentration in body fluids and tissue. They basically depend on the degree of precision, the place where antimicrobial agent is present (capsules, tissue, blood), and the type of equipment. The microbiological method is effective in measuring amoxicillin concentrations in body fluids and tissue.^{10,11} Granulomatous tissue was proposed as a method to study concentration of antimicrobial agents in tissue.⁷

Diclofenac is an effective nonsteroidal anti-inflammatory drug (NSAID), analgesic, and antifebrile agent.¹² After oral administration, the absorption is rapid and complete, binding extensively to plasma albumin. It is metabolized to glucuronidated and sulphate metabolites,¹³ and excreted in urine. Little amount of diclofenac is eliminated unchangedly.¹⁴ In addition, it shows antimicrobial properties (MIC ranging from 50 to 100 μ g/ml) against gram-positive and gram-negative microorganisms.¹⁵

Drug interactions were verified when diclofenac was associated with aspirin, lithium, digoxin, methotrexate, cyclo-

sporin, cholestyramine, and colestipol.¹⁴

Drug interactions between amoxicillin and sodium diclofenac were not found in literature. However, both are commonly used together. The purpose of this study was to verify serum and tissue concentrations of amoxicillin and the effect of sodium diclofenac on these concentrations. Antimicrobial activity of amoxicillin and sodium diclofenac against a staphylococcal infection model in rats was also evaluated.

MATERIALS AND METHODS

Pharmacological Agents Amoxicillin trihydrate (Sigma Chemical Co., St Louis, MO, U.S.A.) was used in aqueous suspensions of 50 mg/ml and 25 mg/ml. Sodium diclofenac (Voltaren[®]-Novartis Co., Rio de Janeiro, Brazil) was used as a 2.5 mg/ml injectable solution. Physiological saline solution (0.9% NaCl) was used in all solution and in the control.

Animals Thirty adult male Wistar rats (*Rattus norvegicus-albinus*), 60 d of age and weighing 175 ± 25 g, were obtained from CEMIB-UNICAMP (Centro de Bioterismo-ICLAS Monitoring/Reference Center, Campinas, Brazil) where they were maintained under aseptic conditions. The Pharmacology Graduation Committee approved this study scientifically and ethically.

Bacterial Strain A penicillin-susceptible *S. aureus* strain (ATCC 25923) was used for the *in vitro* test to determine the MIC₁₀₀ and MBC₁₀₀ in Mueller–Hinton broth (Merck, Darnstadt, Germany) and Salt Mannitol agar (Merck, Darnstadt, Germany), respectively. *S. aureus* was used for the regression line assay, for the microbiological assay of amoxicillin concentration, and for the *in vivo* test as an infectious agent.

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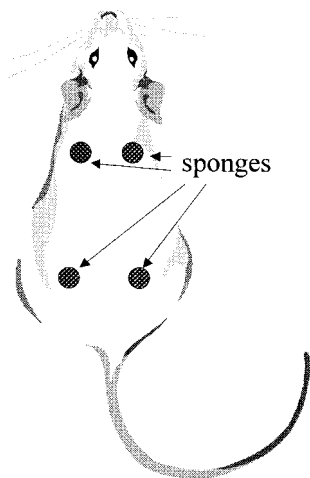


Fig. 1. Schematic Representation of Sponge Positioning

Induction of Granulomatous Tissue and Infection Procedures Granulomatous tissue was induced by implanting four sterilized polyurethane sponge discs (density=35 kg/m³) subcutaneously in the back of each rat. These sponge discs (Proespuma Com. & Ind. Ltd., Sao Paulo, Brazil) were 12 mm in diameter and 5 mm thick, weighing 11.94 ± 0.79 mg. Figure 1 shows the position of sponges.

After 14 d of sponge implantation, a careful antisepsis was carried out in the back of all animals and *S. aureus* ATCC 25923 was injected (0.5 ml of suspension of 10⁸ cfu/ml) into the two granulomatous tissue samples at the tail position.

Drug Administration Two days after infection, the rats were divided into five groups of six. Amoxicillin 50 mg/kg/p.o. was used for group 1, amoxicillin 25 mg/kg/p.o. for group 2, amoxicillin 50 mg/kg/p.o. and sodium diclofenac 2.5 mg/kg/i.m. for group 3, sodium diclofenac 2.5 mg/kg/i.m. for group 4, and 1.0 ml/p.o. of physiological saline (0.9% NaCl) for group 5 (control). All drugs were administered in single doses.

Surgical Procedures and Samples After six hours of drug administration and a rapid anesthetic induction with ethyl ether, all rats were killed by cutting their carotid plexus. After centrifugation, 10 µl of blood serum was placed on two dry discs of paper filter (6.25 mm). Microbiological assay (Mueller–Hinton agar plates inoculated with 10⁸ cfu/ml of *S. aureus* ATCC 25923) was used to obtain serum concentration.

Each infected granulomatous tissue sample was removed and separately placed in tubes with 10 ml of 0.9% NaCl solution. These tubes were weighed before and after the tissue insertion. Their content was dispersed using an ultrasonic system (Vibra Cell 400W, Sonics & Materials Inc., Danbury, CT, U.S.A.) and diluted 10 times and 100 times in saline solution. Ten microliters of the resulting suspension was spread on salt mannitol agar and incubated at 37 °C. Eighteen hours after incubation, the colonies were counted using a manual colony counter and submitted to the Kruskal–Wallis test (software Bioestat 1.0[®] for Windows[®]).

The noninfected granulomatous tissue samples of the control were weighed and analyzed by a histological routine technique (HE). In addition, wet-weight results were submitted to the Kruskal–Wallis test (software Bioestat 1.0[®] for

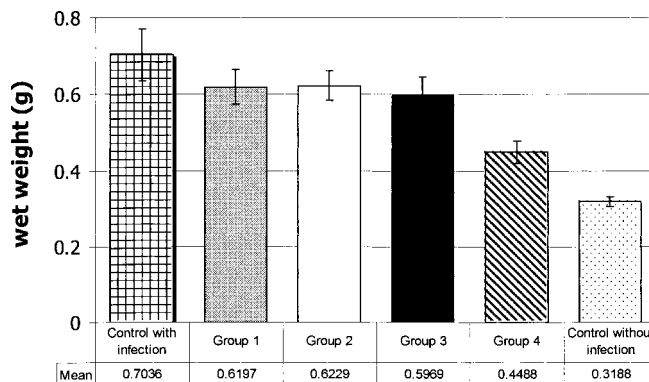


Fig. 2. Wet Weights (Mean±S.E.M.) of Granulomatous Tissue Samples Considering Each Group

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Two noninfected granulomatous tissue samples of the other groups were removed and placed on Mueller–Hinton agar inoculated with 10⁸ cfu/ml of *S. aureus* ATCC 25923. The inhibition zones were measured 18 h after incubation at 37 °C.

Regression Line Amoxicillin suspensions of 0.03, 0.05, 0.1, 0.3, 0.5, 0.7, 1.0, 3.0, 5.0, 7.0, 10.0, 13.0 and 15.0 µg in 10 µl of blank serum were placed on paper filter discs (6.25 mm). Three discs of each concentration were placed on Mueller Hinton agar inoculated with 10⁸ cfu of *S. aureus* ATCC 25923. The resulting inhibition zones were measured (mm) after 18 h of incubation at 37 °C. These zones and the amoxicillin concentrations were used to obtain the regression line (Excel 97[®] for Windows[®]–Microsoft Corporation). Serum and tissue amoxicillin concentrations were submitted to the Kruskal–Wallis test (software Bioestat 1.0[®] for Windows[®]).

RESULTS

MIC₁₀₀ and MBC₁₀₀ of amoxicillin against *S. aureus* ATCC 25923 were 0.1 µg/ml and 1.9 µg/ml, respectively.

The wet weight values (mean±S.E.M.) of the granulomatous tissue samples are shown in Fig. 2. No statistically significant differences (*p*>0.05) were observed among groups 1, 2, 3 and the infected control concerning the wet weight values. However, statistically significant differences were observed when these groups were compared to group 4 and the noninfected control (*p*<0.05). Statistically significant differences (*p*<0.05) were observed between group 4 and the noninfected control.

In all samples, the histological analysis showed a delimited fibrous capsule involving the sponges. Fibroblasts, mesenchymal cells and capillaries were verified in large scale. Infectious exudate was not observed in noninfected control.

The detection limits of the regression line ranged from 12 mm (0.03 µg) to 32.8 mm (15.0 µg). Amoxicillin concentrations were calculated by using the following formula (*R*²=0.988), inhibition zone=3.2103×Ln(µg of antimicrobial agent)+24.169, which resulted from the regression line.

Figure 3 shows amoxicillin serum and tissue concentrations for all groups. Amoxicillin serum and tissue concentrations observed in group 3 were at least 8.5 times lower than those in group 1 (*p*<0.05) and at least 3 times lower when

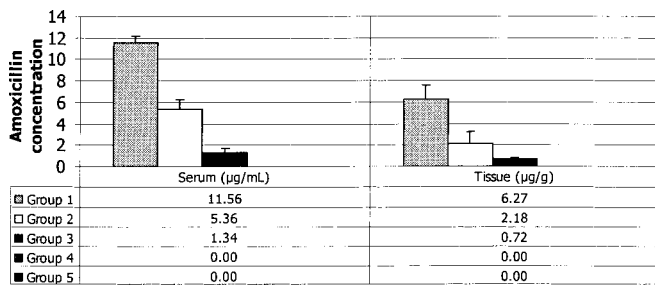


Fig. 3. Amoxicillin Serum and Tissue Concentrations of all Groups

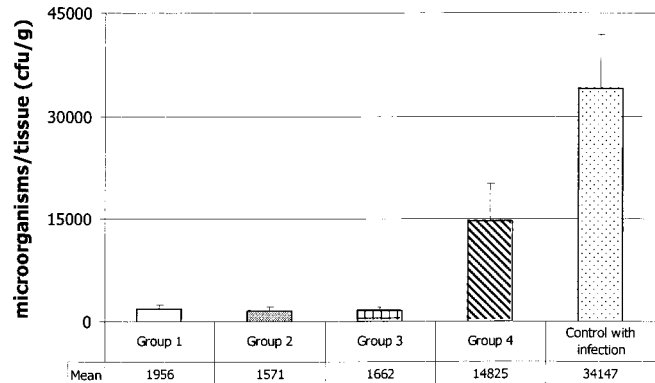


Fig. 4. Microorganism Counts (cfu/g) Concerning Infected Granulomatous Tissue Samples for Each Group

compared to group 2 ($p < 0.05$). Amoxicillin serum and tissue concentrations in group 1 were at least 2 times higher than those in group 2 ($p < 0.05$).

Figure 4 shows the microorganism counts (cfu/g) concerning the infected granulomatous tissue samples for each group. No statistically significant differences ($p > 0.05$) were observed among groups 1, 2, and 3. However, these groups showed statistically significant differences when compared to group 4 and the infected control ($p < 0.05$). Statistically significant differences ($p < 0.05$) were observed between group 4 and the infected control.

DISCUSSION

MIC₁₀₀ and MBC₁₀₀ of amoxicillin against *S. aureus* ATCC 25923 strain confirmed its susceptibility as observed in previous studies.^{6,7,9,16}

In the present study, the surface of the granulomatous tissue allowed a staphylococcal infection development since the bacterial surface adherence is one of the most important phenomena for infection establishment.¹⁷ Inoculated tissues showed purulent exudate, confirmed by viable bacterial counting concerning groups 4 and control.

The microbiological method was accurate enough to measure amoxicillin concentrations in this study, as observed in previous studies.^{6,7} This method is as precise as HPLC assay^{10,11}; it has been widely used to determine amoxicillin concentration.^{18,19}

The tissue wet weight values revealed differences among groups. The reduction in the tissue wet weight verified in group 4 might be due to the high sodium diclofenac anti-inflammatory activity,¹² and inhibition of both leukocyte migration²⁰ and superoxide production.²¹ No reduction in wet

weight was observed for groups 1, 2 and 3. It is suggested that amoxicillin is likely to affect the anti-inflammatory action of sodium diclofenac in group 3. In spite of the high bactericidal activity observed in the amoxicillin groups, the host defense system was not able to remove all the dead cells increasing the tissue wet weight. The wet weight mean values of noninfected granulomatous tissue were similar to those observed in a previous study.⁷

Two days after infection course (control), the colony forming units displayed low mean values (3.4×10^4 /g), in spite of the large inoculum initially used (5×10^7 cfu/ml). Usually large inocula are required to overcome host defenses and establish a reproducible infection in rats, which have a highly efficient defense system.²² Therefore, the low mean values verified in the control might be due to the rat's defense system.

Amoxicillin intestinal absorption has a significant passive diffusion component.²³ Besides, the absorption to some extent of penicillins has been shown to involve the intestinal di, tri or oligopeptide transporter system, which is present mainly in kidney and intestine.²⁴ It has been suggested that the intestinal and renal peptide transporter systems are two homologous peptide transporters, PEPT1 and PEPT2, which were found mainly in the small intestine and in the kidney, respectively. Amoxicillin showed higher affinity for PEPT2 which suggests that tubular reabsorption could be important.²⁵

Intestinal absorption of diclofenac occurs mainly by its excretion in the bile.²⁶ Peptide transporters were not usually associated with diclofenac absorption. However, other drugs, such as amiloride²⁷ and enalapril,²⁸ having a high affinity for peptide transporters, caused a significant decrease in amoxicillin concentration.

A significant increase in the elimination half-life of ceftriaxone combined with diclofenac was previously observed.²⁹ Diclofenac increased ceftriaxone biliary excretion significantly.

The absorption mechanism of amoxicillin is saturable, which has implications for high oral amoxicillin doses.³⁰ In the present study, plasma and tissue concentrations showed linearity, despite the administration of large amoxicillin doses (50, 25 mg/kg). The amoxicillin concentrations were affected only when amoxicillin was associated with sodium diclofenac.

Although sodium diclofenac has high plasma protein binding (about 99%), amoxicillin has poor binding (from 17 to 20%),⁸ suggesting that the protein binding is not responsible for the low amoxicillin concentrations observed in the present study.

Sodium diclofenac is rapid and extensively biotransformed in the liver.¹³ Its enzymatic induction in the liver could be responsible for the low serum and tissue amoxicillin concentrations. However, amoxicillin is metabolized into penicillinoic acid to a limited extent, which is then excreted in urine. About 60% of an oral dose of amoxicillin is excreted unchangedly in urine after 6 h by glomerular filtration and tubular secretion.³¹

Amoxicillin decreases the renal clearance of methotrexate, probably due to a competition at the common tubular secretion system.³² However, the renal excretion of sodium diclofenac is negligible.⁸ Thus, an increase in the amoxicillin

renal clearance caused by diclofenac could be improbable.

In spite of a great reduction in serum and tissue amoxicillin concentrations caused by sodium diclofenac, the amoxicillin had the same efficacy in all groups. Amoxicillin concentrations higher than the MIC (*S. aureus*), even six hours after drug administration, might be responsible for this efficacy.

Bactericidal properties of sodium diclofenac against gram-positive and negative microorganisms were previously demonstrated *in vitro*. The MIC of sodium diclofenac (50 to 100 µg/ml) against a large number of microorganisms might be easily obtained in serum.¹⁵⁾ These facts might explain the significant reduction in *S. aureus* counts caused by sodium diclofenac.

Even in large doses, used in the present study, amoxicillin did not eradicate the infectious agent. A slow process of bacterial multiplication in the infectious site might affect the efficacy of penicillin.³³⁾

It was concluded that sodium diclofenac significantly reduced serum and tissue amoxicillin concentrations.

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