Antibiotics Commonly Used to Treat Mastitis and Respiratory Burst of Bovine Polymorphonuclear Leukocytes

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ABSTRACT

The in vitro effects of six doses (2 × 10⁻³ to 2 × 10⁻⁸ M) of antimicrobial drugs that are frequently used in udder infusions on the capacity of bovine blood polymorphonuclear neutrophilic leukocytes to generate reactive oxygen species were studied by the measurement of luminol-dependent chemiluminescence after stimulation with phorbol 12-myristate 13-acetate. All drugs, except cloxacillin, significantly decreased chemiluminescence at the highest dose. Doxycycline induced the most severe inhibition, followed by neomycin and dihydrostreptomycin. The effect of ampicillin was due to the scavenging of reactive oxygen species and interactions with luminol. The inhibition observed with oleandomycin, neomycin, lincomycin, and dihydrostreptomycin was not due to direct effects on the production of oxidative metabolites but rather to interference with other components involved in the production of light, such as interference with the interaction between luminol and the myeloperoxidase-H₂O₂-halide system. The deleterious effects of doxycycline can be explained by several factors: decreased production of superoxide, yellow color, the scavenging of reactive oxygen species, and Ca²⁺ chelating effect. In conclusion, the results of this study show that antibiotics may affect neutrophil function at concentrations that are reached in the mammary gland after local and repeated administration.

(Key words: antibiotics, respiratory burst, polymorphonuclear neutrophilic leukocytes)

Abbreviation key: CL = chemiluminescence, DPBS = Dulbecco’s phosphate-buffered saline, MPO = myeloperoxidase (EC 1.11.1.7), PMA = phorbol 12-myristate 13-acetate, PMN = polymorphonuclear neutrophilic leukocytes, ROS = reactive oxygen species.

INTRODUCTION

Because of the large selection of cows used to increase milk production during the last 50 yr, the morbidity and mortality of cows diagnosed with various infectious diseases (e.g., mastitis caused by Escherichia coli and Streptococcus uberis, metritis) and metabolic diseases (e.g., hypocalcemia, ketosis, fatty liver syndrome, abomasal dislocation) during the periparturient period have been increased enormously. According to Erskine et al. (7), 40% of all cases of mastitis caused by E. coli are observed during the 1st mo after parturition. The higher incidence of these diseases is related to an impaired function of polymorphonuclear neutrophilic leukocytes (PMN) after parturition (4). Indeed, a reduction of several functional activities of PMN during the periparturient period has been observed by many investigators (4, 16, 18). Moreover, these functions are generally much lower in milk PMN than in blood PMN (20, 21, 28), which is especially important in cases of mastitis caused by E. coli because PMN play a pivotal role in the pathogenesis of and the defense against this type of mastitis (3, 13, 19, 22).

Mastitis is mostly treated by intramammary administration of antibiotics. These antibiotics may affect body defenses directly as well as indirectly via changes in microorganisms (9, 30) (e.g., changes in the bacterial cell membrane and inhibition of protein synthesis). Efficient removal of infectious bacteria requires both effectiveness of the drug against the microorganism and proper functioning of the defense mechanisms of the body. However, high producing dairy cows have an impaired immune system shortly after parturition (3). During mastitis caused by E. coli, the function and the number of circulating mature neutrophils are decreased, and the number of immature neutrophils is increased (4, 13). Therefore, drugs administered to treat infectious diseases after parturition must not impede the competence of the immune cells. The purpose of this study was to investigate the effects of some antibiotics that are commonly included in commercial udder infusions on the respiratory burst of PMN isolated from blood.
MATERIALS AND METHODS

Cows

Seven high producing Holstein cows in mid lactation were selected from the dairy herd at the University of Ghent (Merelbeke, Belgium). The cows were of different ages and were completely healthy.

Antimicrobial Drugs

Seven commonly used antimicrobials were monitored. All drugs were dissolved in Dulbecco's phosphate-buffered saline (0.9%) solution (DPBS; Gibco BRL, Life Technologies Inc., Gaithersburg, MD). The drugs were ampicillin, cloxacillin, dihydrostreptomycin, doxycycline, lincomycin, neomycin, and oleandomycin. All drugs (Sigma Chemical Co., St. Louis, MO) were tested at six different final concentrations from $2 \times 10^{-8}$ to $2 \times 10^{-3}$ M.

Isolation of PMN from Blood

Blood (40 ml) was collected aseptically from the external jugular vein with evacuated tubes containing heparin as an anticoagulant (Laboratoire EGA, Nogent-le Roi, France). The PMN were isolated according to a method previously described (14, 15). Briefly, after the plasma layer was removed, the buffy coat and the top layer of the packed red blood cells were discarded. After washing with 0.9% NaCl, the remaining red blood cells were lysed by the addition of 80 ml of double-distilled water and gentle mixing for 1 min. After restoration of the isotonicity by the addition of 40 ml of 2.7% NaCl and washing, the final cell pellet was resuspended in 1 ml of DPBS. For the chemiluminescence (CL) assay, 1 mg/ml of gelatin was added to the DPBS. After isolation, the cells were enumerated using an electronic cell counter (Coulter Counter ZF; Coulter Electronics Ltd., Luton, England). The viability of PMN immediately after isolation was determined by trypan blue dye exclusion, and differential cell counts were performed on smears stained with eosin-Giemsa stain (Hemacolor®; E. Merck, Darmstadt, Germany). On average, 95% of the isolated cells were PMN, and viability was 97%. After counting and differentiating the cells, cell suspensions were adjusted to a concentration of $4 \times 10^5$ viable PMN/ml and stored on ice until use.

CL Assay

Cellular CL that was dependent of luminol was used to quantify the respiratory burst activity of isolated PMN as previously described by Hoeben et al. (14, 15). The CL assay was performed at 26°C with a liquid scintillation counter (Rackbeta Spectral 1219; LKB Wallac Oy, Turku, Finland) using the out-of-coincidence mode and the tritium channel. The total volume used in the vials was 2 ml. One milliliter of the cell suspension ($2 \times 10^5$ cells/ml, final concentration) was preincubated with DPBS (with 1 mg/ml of gelatin) and 20 µl of the antibiotics during 30 min at 26°C. After this incubation, 0.1 mM luminol and 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) were added, and CL was immediately registered in duplicate for 30 min. The area under the curve was calculated for the registered impulse rates (counts per minute) over the entire period, and a comparison was made for cells that were incubated with antibiotic versus those that were incubated with a sham treatment. This quotient was multiplied by 100, which formed the CL index.

Effects of Antibiotics on CL in a Cell-Free System

The CL in a cell-free system was measured in duplicate according to the method of Briheim and Dahlgren (1) as modified by Hoeben et al. (14, 15). Briefly, the supernatant (25 µl) of sonicated PMN, 0.1 mM luminol, and 0.1 mM H$_2$O$_2$ were incubated with six concentrations of the antibiotic or DPBS, and CL was measured during 30 min at 26°C with a microtiter plate luminometer (LB 96P; EG&G Berthold GmbH & Co., Bad Wildbad, Germany). Sonication of PMN was performed according to the method described by Hoeben et al. (14, 15).

Effect of Antibiotics on Myeloperoxidase Activity

The effect of the antibiotics on myeloperoxidase (EC 1.11.1.7) (MPO) activity was measured in duplicate according to the method previously described by Somersalo et al. (26) and modified by Hoeben et al. (14, 15) in terms of oxidation of o-dianisidine by neutrophil extract containing H$_2$O$_2$ in the presence or absence of the drugs. Sonicated cells (25 µl of supernatant after sonication) of one cow, DPBS, and antibiotics (20 µl) were incubated at 26°C for 5 min. After incubation, 0.1 mM H$_2$O$_2$ and 0.8 mM o-dianisidine were added, and the absorption was measured in a spectrophotometer (Multiskan Plus Type 314; Labsystems Oy, Helsinki, Finland) at 450 nm.
Effect of Antibiotics on CL from Hypochlorite and Luminol

The CL from added hypochlorite was measured in duplicate according to the method previously described by Hoeben et al. (14, 15).

Effect of Antibiotics on Generation of Superoxide Anions by Stimulated PMN

The effect of antibiotics on the production of superoxide radicals was measured by means of the cytochrome c reduction test as described earlier by Hoeben et al. (14, 15).

Absorption of Antibiotics at 405 to 630 nm

Because luminol emits light at 425 nm, absorption at wavelengths ranging from 405 to 630 nm in the presence or absence of antibiotics was monitored using a spectrophotometer (Multiskan Plus Type 314). The effect of the colored antibiotic doxycycline on CL was studied as follows: a vial with a control was inserted into a larger vial containing the antibiotic at the highest concentration. The CL reaction of this sample was compared with that of another control sample without antibiotic.

Statistical Analyses

Statistical analysis of the results from the CL assay was performed for each antibiotic using a two-way analysis of variance (24); the concentration of the antibiotics was a fixed factor, cows (n = 7) were randomized factors, and their interaction was the error term. Comparisons of means were performed via least significant differences (24). A logarithmic transformation of the absolute values of the CL data of the calculated area under the curve was used. A Bartlett's test of equal variances was used to study the equality of variances of the different groups. The results of this test allowed us to use the analysis of variance.

Statistical analyses of the results from the cell-free assay, the MPO assay, the assay with luminol and sodium hypochlorite, and the cytochrome c reduction test were performed for each antibiotic using a one-way analysis of variance (n = 2). For statistical analysis, a logarithmic transformation of the absolute values of the results from the different assays was used. Comparison of means was performed via least significant differences. A Bartlett's test of equal variances was used to study the equality of variances. The Statistix® program package (27) was used. Significant differences were determined at P < 0.05, P < 0.01, and P < 0.001.

RESULTS

Effects of Antibiotics on Cellular CL

Except for cloxacillin, all antibiotics reduced (P < 0.001) cellular CL at a concentration of 2 × 10⁻⁵ M (Figure 1; Table 1). Dihydrostreptomycin, doxycycline, and neomycin already showed an inhibitory effect (P < 0.001) at concentrations of 2 × 10⁻⁴ and even at 2 × 10⁻⁵ M. No other antibiotics had a significant effect on cellular CL at these concentrations. Doxycycline was the only drug that significantly reduced CL at even lower concentrations (2 × 10⁻⁶

<table>
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<th>Antibiotic</th>
<th>2 × 10⁻⁸ M</th>
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<th>2 × 10⁻⁶ M</th>
<th>2 × 10⁻⁵ M</th>
<th>2 × 10⁻⁴ M</th>
<th>2 × 10⁻³ M</th>
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<td>94.8 ± 4.0</td>
<td>44.0***</td>
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<td>99.7 ± 3.6</td>
<td>94.3 ± 0.6</td>
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<td>94.2 ± 2.5</td>
<td>98.8 ± 3.9</td>
<td>83.1* ± 3.7</td>
<td>69.7*** ± 6.5</td>
<td>20.7*** ± 3.1</td>
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<td>74.3 ± 4.2</td>
<td>54.2*** ± 8.7</td>
<td>31.4*** ± 6.8</td>
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<td>100.2 ± 6.0</td>
<td>80.3 ± 3.9</td>
<td>27.5*** ± 3.9</td>
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Data are means (±SEM) of seven cows.

1Area under the curve (AUC) of the antibiotic - incubated cells/AUC of cells incubated with sham treatments × 100.

**P < 0.05.

***P < 0.001.
Figure 1. Influence of different doses of antibiotics on chemiluminescence (CL) of polymorphonuclear neutrophilic leukocytes as induced by phorbol 12-myristate 13-acetate. Values are the means of data for seven cows, and error bars represent the standard errors of the means. The CL index = [area under the curve (AUC) of the antibiotic ± incubated cells] / AUC of cells incubated with sham treatments × 100. Legend: ampicillin (◊), cloxacillin (⁄), dihydrostreptomycin (o), doxycycline (ÿ), lincomycin (π), neomycin (…), and oleandomycin (∫).

Figure 2. Influence of different doses of antibiotics on chemiluminescence (CL) in a cell-free system. The CL index = [area under the curve (AUC) of the antibiotic ± incubated cells] / AUC of cells incubated with sham treatments × 100. Legend: ampicillin (◊), cloxacillin (⁄), dihydrostreptomycin (o), doxycycline (ÿ), lincomycin (π), neomycin (…), and oleandomycin (∫).

M). None of the tested antibiotics induced a significant stimulatory effect. Taken together, we concluded that doxycycline induced the most pronounced effect on the CL as induced by PMA at all concentrations, followed by neomycin and dihydrostreptomycin. Cloxacillin had no significant effect; however, lincomycin showed ample influence.

Effects of Antibiotics on CL in a Cell-Free Assay

All antibiotics impaired CL in the cell-free assay (Figure 2). These negative effects were generally more pronounced than were the effects observed in the cellular CL assay. Oleandomycin, however, showed an inhibitory effect (P < 0.001) (64%) only at a concentration of 2 × 10^{-3} M. Cloxacillin and dihydrostreptomycin inhibited cell-free CL at concentrations of 2 × 10^{-4} and 2 × 10^{-3} M (62% (P < 0.01) and 91% (P < 0.001), respectively, for cloxacillin and 69% (P < 0.001) and 99% (P < 0.001), respectively, for dihydrostreptomycin). In the cellular assay, dihydrostreptomycin showed significant effects at a concentration of 2 × 10^{-5} M. Ampicillin and lincomycin caused significant inhibitory effects on CL in the cell-free assay at concentrations of 2 × 10^{-5}, 2 × 10^{-4}, and 2 × 10^{-3} M [35% (P < 0.01), 81% (P < 0.001), and 90% (P < 0.001) respectively, for ampicillin, and 18% (P < 0.01), 64% (P < 0.001), and 86% (P < 0.001), respectively, for lincomycin]. In the cellular assay, lincomycin reduced CL only at the highest concentration. Doxycycline and neomycin inhibited CL in a cell-free assay at concentrations of 2 × 10^{-6} M (P < 0.05) and at 2 × 10^{-5}, 2 × 10^{-4}, and 2 × 10^{-3} M (P < 0.001) [39, 95, 98, and 99%, respectively, for doxycycline and 23, 42, 77, and 85%, respectively, for neomycin]. The most pronounced effects were again observed with doxycycline, followed by neomycin, ampicillin, and dihydrostreptomycin.

Effects of Antibiotics on MPO Activity

As shown in Figure 3, only two antibiotics showed a significant inhibitory effect on MPO activity. Dihydrostreptomycin decreased the activity by 18% (P < 0.05) at a concentration of 2 × 10^{-3} M. Doxycycline reduced the reaction by 19% (P < 0.01) at a concentration of 2 × 10^{-4} M and by 40% (P < 0.001) at a concentration of 2 × 10^{-3} M. The inhibitions at the highest concentration were lower than those observed in the former two CL assays. The largest depressions were again noted with doxycycline, followed by di-
hydrostreptomycin. The other drugs had much lower effects that were not significant: a decrease of 12% by ampicillin, of 8% by doxycycline, of 6% by neomycin, and of 5% by lincomycin.

**Effect of Antibiotics on CL of Hypochlorite and Luminol**

Only effects at concentrations of $2 \times 10^{-6}$ M and higher were shown (Figure 4) because lower concentrations exerted no significant effects on CL. All antibiotics reduced the CL response to the reaction of luminol and hypochlorite. Ampicillin, dihydrostreptomycin, and oleandomycin reduced the response at doses of $2 \times 10^{-4}$ and $2 \times 10^{-3}$ M [75 and 99%, respectively, for ampicillin ($P < 0.001$); 53 and 100%, respectively, for dihydrostreptomycin ($P < 0.001$); and 16 and 54%, respectively, for oleandomycin ($P < 0.001$)]. The other antibiotics showed significant effects at concentrations of $2 \times 10^{-5}$ M. Cloxacillin decreased the response at concentrations of $2 \times 10^{-5}$, $2 \times 10^{-4}$, and $2 \times 10^{-3}$ M [13% ($P < 0.05$) and 33 and 77% ($P < 0.001$), respectively]. Doxycycline, lincomycin, and neomycin also decreased the reaction at those same concentrations [12% ($P < 0.05$) and 78 and 100% ($P < 0.001$), respectively, for doxycycline; 54, 75, and 98% ($P < 0.001$), respectively, for lincomycin; and 43, 96, and 100% ($P < 0.001$), respectively, for neomycin].

**Effect of Antibiotics on the Production of Superoxide Radicals**

Only doxycycline significantly affected the production of superoxide radicals as measured by the cytochrome c reduction test. Production was decreased 14% ($P < 0.05$) at a concentration of $2 \times 10^{-5}$ M, and production was decreased 83% at concentrations of $2 \times 10^{-4}$ and $2 \times 10^{-3}$ M ($P < 0.001$).

**Absorption by Antibiotics at Wavelengths of 405 to 630 nm**

Strong increases in absorption were only observed with doxycycline at the highest concentration. Doxycycline has a yellow color, which made it easy to observe. The highest absorptions were measured at a wavelength of 405 nm (0.685 vs. 0.015). As wavelengths increased (up to 630 nm), the absorption by this drug decreased (0.035 vs. 0.015).

**DISCUSSION**

The respiratory burst activity of bovine PMN isolated from blood has been measured by means of CL as induced by PMA and enhanced by luminol. Phorbol 12-myristate 13-acetate is a soluble stimulant of respiratory burst, which easily penetrates the cell membrane. This feature is valuable for the study of
effects of drugs on respiratory burst, because it eliminates probable interferences of processes related to phagocytosis. Phorbol 12-myristate 13-acetate acts directly on intracellular protein kinase C (EC 2.7.1.37) and mimics the function of diacylglycerol. Because of this direct activation, protein kinase C can be considered a receptor for PMA, which renders PMA as a very potent activator of NADPH oxidase, the key enzyme of respiratory burst. Upon activation of the protein kinase C by PMA, different components of NADPH oxidase become phosphorylated, and the burst enzyme becomes activated.

Because CL is a very sensitive method, additional experiments were performed to study subcellular interactions. Chemiluminescence in a cell-free system was studied to see whether the effects observed in the cellular assay were due to effects on the cell membrane (NADPH oxidase). Because the MPO-H2O2-halide system is necessary to yield luminol CL, the MPO assay and the CL with sodium hypochlorite were studied, and, because the first step of the burst is the formation of superoxide radicals, the cytochrome c reduction test was performed.

Blood PMN were used because all PMN in the mammary gland originate from the blood. As mentioned previously, the function of milk PMN is much lower than that of blood PMN. Because previous studies (14, 15) have shown that there is almost no difference in the effects of drugs on blood PMN and milk PMN, because blood PMN produce less variable results, and because the viability and the integrity of PMN isolated from blood are much higher than those of PMN isolated from milk, we decided to use PMN isolated from blood.

According to Briheim et al. (2), the single peak of CL that was enhanced by luminol and that we perceived after stimulation with PMA was mainly due to intracellular events (2). Intracellularly generated CL depends very much on the diffusion of luminol into the cells (2). Not only the diffusion of luminol, but also the penetrability of the antibiotics into the cells, is important. Because most antibiotics penetrate PMN very poorly (29, 30), it is obvious that the effects were more pronounced in the cell-free assay than in the cellular assay. Aminoglycosides, such as neomycin and dihydrostreptomycin, penetrate very slowly into PMN. These drugs are not free in the cytosol but are localized within vacuoles (29). The β-lactam antibiotics ampicillin and cloxacillin also penetrate very poorly into PMN. The ratio of intracellular to extracellular concentration is less than 0.1 (30). Clindamycin accumulates very well in PMN, but lincomycin does not accumulate at all (29) and is not taken up by an active transport system. However, after penetration, both clindamycin and lincomycin show a bimodal distribution in the cells, namely, in the cytosol and in the granules. Because doxycycline has a relatively good degree of lipid solubility, it penetrates the cell membranes more easily than does oxytetracycline (10, 11). Because macrolides such as erythromycin and oleandomycin penetrate PMN very well with intracellular to extracellular ratios of 10 and higher (29, 30), the effect in the cell-free assay was similar to that in the cellular assay. Because the effects of the other drugs were more pronounced in the cell-free assay than in the cellular assay, effects on the membrane-bound NADPH oxidase seemed to be very doubtful.

The results from ampicillin suggested a scavenging effect on reactive oxygen species (ROS) such as H2O2 and ·OCl but probably no effect on MPO. An effect on the production of superoxide radicals could not be observed. Gunther et al. (12) also found that ampicillin scavenged ·OCl, H2O2, and OH·. Those researchers (12) also observed no effect on the production of superoxide radicals, no scavenging effects on these radicals, and no interference with the activity of the MPO. According to Lagercrantz (17), the β-lactam ring of ampicillin can be cleaved by the H2O2 that results in the formation of a quasistable nitronoe species. Our results are in agreement with those of Gunther et al. (12) and those of Briheim and Dahlgren (1), who suggested that interference with the peroxidase was unlikely but that ampicillin and also penicillin G may scavenge not only H2O2, ·OCl, and OH· but also singlet oxygen. The reduced CL was not due to decreases in the viability of the PMN (1) or to an increased absorption of light emitted by luminol, as was shown in our absorption assays and was demonstrated by Briheim and Dahlgren (1). Because every time luminol was present in the incubation mixture the reaction was reduced, interference with this compound may also be involved. This suggestion was also made by Briheim and Dahlgren (1). The lack of access of ampicillin to all cellular compartments in which ROS were generated has been mentioned previously by Gunther et al. (12). The lack of effects at low concentrations and inhibitory effects at high and even supratherapeutic concentrations are in agreement with results that have been reported previously (6, 8, 25, 31).

The results for cloxacillin and lincomycin suggested that these drugs have no effect on the activity of MPO as did all tested antibiotics except doxycycline. Because of the very poor penetration in the PMN, effects were more pronounced in the cell-free assay, indicat-
ing that these drugs probably have no effect on the activity of the membrane-bound NADPH oxidase nor on the production of superoxide radicals but probably do have an effect on the interaction of luminol and the MPO-H2O2-halide system. These effects were also suggested for the penicillins, ampicillin and penicillin G (1), and for most other tested drugs.tolokisova and Lokaj (25) observed no effect of lincomycin on CL of human PMN at therapeutic and higher doses as induced by phagocytosis.

Although dihydrostreptomycin, oleandomycin, and neomycin penetrate very poorly into PMN, large effects were observed in the cellular assay. These drugs had comparable effects in the cellular and the cell-free assays. Because CL enhanced by luminol provides an idea of the production of H2O2 by the cells, this production could be decreased without a decreased production of superoxide. A decreased catalase (EC 1.11.1.6) activity could play a role as could increased superoxide dismutase (EC 1.15.1.1) activity. However, these suggestions cannot explain the effects observed in the cell-free assay and in the assay of luminol and hypochlorite. Because large effects in these assays were noticed, interference with the interaction between luminol and the MPO-H2O2-halide system seems more likely. These drugs may reduce the penetrability of luminol into PMN by binding the luminol outside the cell.

We demonstrated that the inhibitory effects of doxycycline and oxytetracycline (14, 15) on CL were partially due to absorption of the blue light emitted by luminol at 425 nm. Siegel and Remington (23) showed the same result with oxytetracycline and doxycycline, which are yellow substances. The enormous effects of doxycycline were due to the decreased production of superoxide radicals, to its yellow color, to the scavenging of ROS, and to its Ca2+ chelating effect. The most pronounced inhibitory effects were observed with tetracyclines, which have the best lipid solubility and the best chelating activity. Therefore, tetracyclines induce more pronounced effects on CL than does doxycycline, and doxycycline induces more pronounced effects than does oxytetracycline (10, 11). Indeed, because doxycycline penetrates much better into PMN than does oxytetracycline, this drug can bind intracellular Ca2+ and induces more pronounced effects than does oxytetracycline (14, 15). This action inhibits the CL because PMA stimulates the cells by decreasing the ratio of bound intracellular Ca2+ to free intracellular Ca2+. The extracellular concentration of Ca2+ plays no role in the generation of CL after stimulation with PMA (5). The intracellular concentration, however, is very important in the generation of CL, which was also observed by Glette et al. (10).

From all drugs tested, doxycycline was the only drug that did not affect CL in the tested concentration range. The inhibitory effects of the remaining drugs were only significant at concentrations that are normally not obtainable in vivo. However, after local and repeated administration of these drugs in the mammary gland, these high concentrations may be achieved, especially near the rosette of Fürstenberg. The vast majority of PMN is thought to enter the mammary gland via this venous ring. As a consequence, the antibiotics that are administered intramammary may affect the function of these PMN, resulting in a decreased killing of the invaded bacteria.

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