Inhibition of neutrophil inflammatory mediator expression by azithromycin and amoxicillin

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

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Abstract

**Background:** Peri-implant healing is susceptible to complications because peri-implant tissues exhibit a more vigorous inflammatory response to wounding than periodontal tissues. While a single dose of amoxicillin (AMX) prior to implant surgery reduces the risk of early healing complications, a recent study suggested that patients taking preoperative azithromycin (AZM) exhibit faster resolution of postoperative inflammation.

**Objectives:** This study compared the effects of AZM and AMX on neutrophil expression of several inflammatory mediator genes involved in the early phase of peri-implant healing (IL-1β, TNF-α, IL-6, IL-8, G-CSF and GM-CSF).

**Methods:** We compared the effects of AZM and AMX on induction of IL-1β, TNF-α, IL-6, IL-8, G-CSF and GM-CSF mRNA. Neutrophils were isolated from healthy human donors and pre-incubated with AZM (4 or 8µg/ml) or AMX (2 or 4µg/ml). Cells were then incubated with LPS (1µg/ml), TNF-α (10ng/ml), or medium alone (negative control) for 1, 2 and 4-hrs. Total RNA was isolated and reverse transcribed into cDNA, which was analyzed to quantify changes in the expression of these six cytokines.

**Results:** LPS and TNF-α induced a similar pattern of IL-1β mRNA expression, with peak expression at 1 hr. Thus, for the other five inflammatory mediators, the 1-hr time point was selected for study. Induction in neutrophils activated by LPS was markedly reduced in a dose-dependent manner by AZM and AMX for several markers. Real-time PCR proved
that full therapeutic dose of AZM was able to statistically reduce the expression of inflammatory markers of neutrophils tested in this study. AMX was effective only in a few cases and under certain conditions. Therefore, AZM was more potent in its direct anti-inflammatory action.

**Conclusion:** AZM produces similar or more potent inhibition of neutrophil inflammatory mediatory mRNA expression in comparison to AMX. Given that a single dose of AZM produces higher and more sustained concentrations in periodontal tissues than a single dose of AMX, AZM has greater potential to inhibit inflammatory mediator expression at a wound site than AMX. This is in agreement with a previous report that pre-operative AZM enhances resolution of inflammation after implant placement surgery to a greater extent than AMX.
Acknowledgments

I would like to acknowledge the efforts and patience of my advisor Dr. John Walters without whom this work would not have been possible. I would also like to thank my committee members for their input in this study. I specially want to thank Dr. Dimitris Tatakos, my program director for his guidance. Last but not the least, I want to thank all the faculty, staff and my co-residents in the department of Periodontology for their constant encouragement and support.
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Fields of Study

Major Field: Dentistry
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Chapter 1: Introduction

While replacement of teeth with dental implants has become one of the most predictable and sought after procedures in dentistry,\(^1,2\) implant failures do occur.\(^3,4\) Failing implants typically require immediate removal. Implant removal jeopardizes the clinician’s effort to accomplish satisfactory function and esthetics. For the patient, loss of an implant is nearly always associated with additional procedures and costs.

Diabetics, smokers and patients with poor bone conditions have a higher risk of complications with implant integration.\(^5-7\) Implants can undergo early failure when there are complications in wound healing after placement, leading to formation of scar tissue or inhibition of bone apposition around the implant.\(^8\) Data from a recent study suggests that peri-implant healing could be more susceptible to complications than periodontal healing. During healing after implant placement surgery, the levels of some proinflammatory cytokines are significantly higher in the peri-implant crevice than in the crevices of adjacent natural teeth.\(^9\) Thus, it appears that peri-implant tissues, when compared to periodontal tissue, exhibit a more vigorous inflammatory response to injury or infection.\(^9-11\) Many studies have looked at approaches to minimizing or preventing early implant failure by controlling inadvertent tissue damage.
The typical rationale for use of a prophylactic antibiotic is to produce transient suppression of bacteria in blood and at wound sites in individuals who are susceptible to infection. Several studies have shown that a single dose of antibiotic prior to implant surgery can reduce the risk of early complications after implant placement.\textsuperscript{5, 12, 13} In an early study that followed 2973 implants for 36 months after placement, there was a significantly higher implant survival rate in patients who had taken pre-op antibiotics.\textsuperscript{12} Recent evidence-based reviews reinforce these findings. A review of randomized clinical trials concluded there was evidence that 2 g of Amoxicillin (AMX) given orally 1 hour prior to implant surgery significantly reduces failures of dental implants placed in ordinary conditions.\textsuperscript{5, 8} A more recent review of eight clinical trials indicated that a single pre-op dose of antibiotics has a beneficial effect on reducing implant failure.\textsuperscript{13} In light of this evidence, pre-op prophylaxis with AMX is a standard procedure in many clinics that place implants. Thus, there is evidence that prophylactic antibiotics can help control localized infections and facilitate a more predictable outcome. However, it is uncertain which class of antibiotics produces the most favorable protective effects.

For patients who are allergic to penicillin, azithromycin (AZM; 500 mg) is recommended as a prophylactic regimen for dental procedures.\textsuperscript{14} AZM, along with other macrolides, produces potentially useful immunomodulatory effects. Recently, AZM has been known to produce anti-inflammatory effects in gingiva.\textsuperscript{15} In contrast to AMX, AZM concentrations in gingival crevicular fluid (GCF) are typically 40 times higher than in serum and are also sustained for up to 2 weeks.\textsuperscript{16} A randomized comparison of the effects
of AZM and AMX found that a single dose of AZM before implant placement produced more profound inhibition of inflammatory mediator production and yielded more sustained antimicrobial activity in periimplant crevicular fluid (PICF) than AMX during the postoperative course. Azithromycin was available at the surgical site for a longer period of time than amoxicillin, and patients taking azithromycin exhibited lower levels of specific proinflammatory cytokines and chemokines in GCF and PICF. Thus, preoperative azithromycin enhances resolution of post-operative inflammation to a greater extent than amoxicillin.

Studies have shown that AZM inhibits inflammatory mediator expression by several different types of cells. This includes inhibition of IL-8 production by LPS-stimulated oral epithelial cells, bronchial epithelial cells and human alveolar macrophages. Also, clarithromycin inhibits IL-8 production by LPS-stimulated peripheral blood monocytes. AZM is known to inhibit IL-6 and IL-12 production and enhance IL-10 production by activated mouse macrophages. There is substantial evidence that the anti-inflammatory effects are mediated through suppression of NF-κB activation. In contrast, AMX does not appear to possess direct anti-inflammatory properties.

Taking these studies into consideration, we hypothesized that therapeutic levels of AZM produce more profound inhibition of inflammatory mediator production by host cells in peri-implant tissue than AMX, primarily by inhibiting NF-κB activity. During postsurgical wound healing, neutrophils and epithelial cells play a major role in pro-
inflammatory cytokine production during the early stages of healing, while fibroblasts and mononuclear phagocytes play a major role during the intermediate stages.

This study focuses on human neutrophils, which are the primary effector cells in the acute inflammatory response. They are rapidly recruited in large numbers from the bloodstream by the processes of adhesion to the vascular endothelium, trans-endothelial migration and/or chemotaxis to a local inflammatory site.\textsuperscript{24} Once localized, neutrophils phagocytose particles such as bacteria and immune complexes bearing immunoglobulin-G (IgG), via Fc receptors (FcRs), or complement components.\textsuperscript{24} During the inflammatory process, IL-1\textbeta and TNF-\alpha produced by neutrophils serve numerous functions, including stimulation of phase reactant synthesis, augmentation of T- and B-cell activation and induction of other regulatory cytokines such as IL-6, IL-8 and GM-CSF. In addition, IL-1\textbeta and TNF-\alpha production by neutrophils has important autocrine and paracrine effects, including stimulation of further IL-1\textbeta production and local induction of vascular endothelial cell adhesion molecules, thereby promoting recruitment of additional neutrophils as well as monocytes and lymphocytes. Along with GM-CSF, G-CSF and IL-6, IL-1\textbeta produced by neutrophils plays a vital role in increasing the pool of phagocytes by inducing the growth and differentiation of hematopoietic progenitors. IL-1\textbeta, TNF-\alpha, GM-CSF, G-CSF and IL-6, also stimulate mature neutrophil functions, including phagocytosis and superoxide generation.\textsuperscript{24} In addition, IL-8 produced by neutrophils and a variety of other cells is a potent chemotactic agent for neutrophils and serves as an angiogenic factor.\textsuperscript{24} For these reasons, we decided to look the influence of AMX and AZM on inflammatory mediator
production by neutrophils. The objective of this study is to determine whether neutrophil
cytokine and chemokine production could potentially be modulated by AZM or AMX.
This approach will highlight similarities and differences in sensitivity to modulation of
biological mediator expression by cells that play an essential role in early healing after
implant placement surgery.
Neutrophil isolation and antibiotic incubation

Citrated whole blood was collected from healthy adult human donors, who provided written informed consent under a protocol approved by the Ohio State University Biomedical Sciences Institutional Review Board (IRB# 2014H0245). PMNs were isolated through a procedure that utilized Ficoll/Hypaque density gradient centrifugation and dextran sedimentation.\textsuperscript{25} Residual erythrocytes were eliminated by hypotonic lysis. The remaining cells were washed three times with phosphate buffered saline. Cells isolated in this manner are generally \textgreater 99\% PMNs (based on cytospin preparations stained with Wright-Giemsa) and \textgreater 99\% viable (based on trypan blue exclusion).\textsuperscript{25} Prior to assays, PMNs were suspended at a density of 5 x 10\textsuperscript{7}/ml in RPMI medium 1640 (Gibco Life Technologies, Grand Island, NY) containing 0.5\% human serum albumin and incubated for 30 min at 37\(^\circ\) C. At the time of assay, cells were incubated for 20 minutes at 37\(^\circ\) C in the absence of antibiotics (control) or in the presence of AZM or AMX at concentrations similar to those found in blood and gingival crevicular fluid (GCF). Both half and full therapeutic dosages of antibiotics were used for comparison in this study. This consisted of 4 and 8 μg/ml concentrations for AZM as well as 2 and 4μg/ml concentration for AMX.\textsuperscript{16} Cells were stimulated with LPS from \textit{Porphyromonas gingivalis} strain 381 (1 μg/ml, purified as described by Preshaw et al)\textsuperscript{26} or TNF-α (10ng/ml, Peprotech, Inc, Rocky
Hill, NJ), or with medium alone (negative control) for 1, 2 and 4 hours. During the incubations, cells were maintained in suspension at 37º C by gentle rotation. At the conclusion of treatment, cell suspensions were pelleted at 500 x g and processed for RNA extractions.

**RNA extraction and cDNA conversion**

Total RNA was isolated from each supernatant using RNeasy mini kit (Qiagen; Germantown, MD) according to the manufacturer’s protocol. The RNA (1 µg/per sample) was reverse transcribed into first-strand cDNA using the QuantiTect Rev Transcription Kit (Qiagen). This protocol included elimination of genomic DNA as well. As an additional control measure, in the last step of reverse transcription, negative controls were set up which received all reaction components except the reverse transcriptase enzyme. This was done to ensure the amplification noted with reverse-transcription PCR (RT-PCR) was solely from the RNA-cDNA conversion and did not contain genomic DNA amplification products.

**RT-PCR Primer design**

The total RNA was isolated and reverse transcribed in cDNA, which was analyzed to quantify changes in the expression of several inflammatory mediators. We selected six target inflammatory mediator molecules that are characteristic in neutrophil expression. These six cytokines were –IL-1β (Interleukin-1 beta), TNF-α (Tumor Necrosis Factor alpha), IL-6 (Interleukin-6), IL-8 (Interleukin-8, CXCL8), G-CSF (Granulocyte Colony-
Stimulating Factor, CSF3), and GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor, CSF2). Human β-actin was as the internal control. The PCR amplification of the cDNA was then performed using a forward primer and a reverse primer for each of the six mediators mentioned above. The primers used for RT-PCR are described in Table 1. The PCR conditions were: initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 45 s. The amplified product was visualized on 1% agarose gel (Sigma; St. Louis, MO) stained with ethidium bromide and observed under ultraviolet light.

**Real time PCR**

Real-time PCR was carried out to estimate and quantify the relative levels of the inflammatory mediator expression between the control and test groups. For quantitative comparison, the same amount of cDNA (1 µg/µl) was used as the template. The primers used for real-time PCR were customized for the six cytokines mentioned above (Qiagen). Customized qPCR primer assays (Qiagen) were standardized and used for the Real time PCR reactions. The real-time PCR reactions were performed using SYBR Green Master Mix (Qiagen) and the iCycler Real-Time PCR Detection System (Bio-Rad). β-actin was again used as the control. The PCR conditions were: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 30 s. The PCR product accumulation was monitored by the increase in fluorescence intensity caused by the binding of SYBR Green to double-stranded DNA. Triplicate analyses were performed and compared statistically.
Chapter 3: Results

This study consisted of ten conditions per experiment, including 5 negative controls that were not exposed to either LPS or TNF-α but received antibiotic treatment with either AZM or AMX; one positive control that had no antibiotic treatment but was stimulated with LPS or TNF-α, and four tests which were exposed to either a full or half therapeutic dose of AMX or AZM and stimulated with LPS or TNF-α. All ten conditions were repeated to test the expression of six inflammatory mediators. Peak expression of IL-1β mRNA occurred at the 1-hour mark (Fig.1). For this reason, experiments with the other five mediators were limited to the 1-hour peak expression interval. Interestingly, induction in neutrophils activated by LPS and TNF-α was markedly reduced in a dose-dependent manner by AZM, and to a lesser extent by AMX.

**RT-PCR expression**

The RT-PCR analyses showed that both LPS and TNF-α induced a similar pattern of IL-1β mRNA expression, with peak expression at 1 hr (Fig.1). Induction by LPS and TNF-α was markedly reduced in a dose-dependent manner at all time points in the presence of AZM (Fig.1). At the 1 hr timepoint, IL-1β mRNA expression in the presence of 8µg/ml AZM was comparable to the negative control (Fig. 1A). In neutrophils treated with LPS, AMX also produced dose-dependent inhibition of IL-1β expression, but to a lesser extent.
than AZM (Fig. 1A). This effect was most evident at 4 hrs (Fig. 1E). In neutrophils treated with TNF-α, AMX produced comparatively little inhibition of IL-1β expression (Figs. 1B, D and F).

LPS-stimulated expression of TNF-α at 1 hour was not inhibited by AMX and appeared similar to the positive control (Fig. 2A). In contrast, AZM inhibited the expression of TNF-α in a dose dependent manner to a level similar to the negative controls (Fig. 2A). With regard to induction by TNF-α, AZM inhibited gene expression in a dose-dependent fashion, but AMX was less inhibitory (Fig. 2B).

In both LPS and TNF-α activated neutrophils, IL-6 expression appeared to be inhibited only at 8 µg/ml AZM (Fig. 2C and D). No other treatment seemed to alter the expression of IL-6 as compared to the positive control. In a similar fashion, induction of IL-8 expression by LPS was reduced only in the presence of 8µg/ml AZM (Fig 3A). IL-8 induction by TNF-α was reduced by both AMX and AZM, with 8 µg/ml AZM producing the most inhibition (Fig 3B).

Finally, regardless of whether neutrophils were activated by LPS or TNF-α, 8 µg/ml AZM was the only treatment that appeared to inhibit expression of GM-CSF (Fig. 3C and D) and G-CSF (Fig. 3E and F). Under other treatment conditions, expression levels appeared similar to the positive control. Overall, AZM consistently inhibited the expression of a
broad spectrum of inflammatory mediators. AMX also appeared to inhibit the expression of some mediators, but to a lesser extent.

**Relative expression level**

Results from RT-PCR were extended with real-time PCR. AZM (8 µg/ml) produced statistically significant treatment effects on induction of IL-1β gene expression by both TNF-α and LPS (Fig. 4), whereas AMX (4 µg/ml) significantly altered induction of IL-1β gene expression by LPS (Fig.4). Both concentrations of AZM inhibited induction of TNF-α expression by LPS and TNF-α (Fig. 5), and AZM produced very similar results with respect to IL-6 expression (Fig.6). Induction of IL-8 (Fig. 7) and GM-CSF (Fig. 8) expression was significantly altered only in the presence of 8 µg/ml. TNF-α induction of G-CSF gene expression was significantly reduced under all treatment conditions with AMX or AZM (Fig. 9). However, only AZM produced statistically significant inhibition of LPS-induced G-CSF expression (Fig. 9).

Thus, the results from real-time PCR demonstrated that AZM (8 µg/ml) consistently produced statistically significant inhibition of inflammatory mediator gene expression by neutrophils. AMX was inhibitory only in a few cases (IL-1β, TNF-α and G-CSF). Therefore, AZM was more potent in its direct anti-inflammatory action.
Chapter 4: Discussion and Conclusion

Although the primary purpose of a prophylactic antibiotic is to transiently suppress bacteria in blood and at wound sites in individuals who are susceptible to infection, the findings of this study suggest that there are other potential benefits. The study demonstrates that therapeutic concentrations of AZM strongly inhibit induction of cytokine and chemokine gene expression by neutrophils. AZM effects were observed in all groups and under both LPS and TNF-α stimulation. AMX also inhibited expression of some of these genes, but to a lesser extent. Previous studies have suggested that AMX does not possess any direct anti-inflammatory properties.\textsuperscript{23}

The present study’s main finding is consistent with a previous report that preoperative AZM enhances resolution of post-surgical inflammation to a greater extent than AMX.\textsuperscript{17} AZM is available at the surgical site for a longer period of time than AMX.\textsuperscript{15} It is interesting to note that AZM appeared to alter several potentially important aspects of acute inflammation, which is vital to early healing after implant surgery. Although recruitment of neutrophil cells plays an essential role in healing, reduction of excessive infiltration is associated with accelerated wound closure in mice and could potentially contribute to a lower incidence of complications in healing.\textsuperscript{27}
Upon wounding, a sequence of events is set in motion culminating in tissue repair. Three overlapping phases can be distinguished: an inflammatory phase, characterized by neutrophil infiltration; a proliferative phase, dominated by collagen deposition and angiogenesis; and a maturation phase, involving resolution of inflammation. As part of the inflammatory phase, a massive infiltration of neutrophils, which is greatest at day 1 post-injury, can be observed. Starting within minutes after injury, neutrophils are thought to protect the host from infection by combating an invading microorganism and clearing cellular debris during this process. However, activated neutrophils secrete a battery of pro-inflammatory cytokines, bioactive substances, such as proteases and reactive oxygen intermediates, which contribute to serious tissue damage. This is most clearly demonstrated in non-healing wounds, in which resolution of inflammation is often delayed. In the absence of infection or an underlying medical condition in a wounded individual, neutrophils are considered neutral to healing. Neutrophil-secreted IL-1β and TNF-α play important roles in wound healing and trigger the release of other cytokines that act as mediators of inflammation in response to any wound. Both play a central role in the recruitment of neutrophils and mononuclear phagocytes to a wound site. In combination with IL-6, GM-CSF and G-CSF (also produced by neutrophils), IL-1β stimulates production of new neutrophils and mononuclear phagocytes. IL-8 plays a major role in attracting neutrophils to wound sites. Most of these agents also prime or directly stimulate the release of cytotoxic superoxide and granule-associated enzymes from neutrophils. Although recruitment and activation of neutrophils helps in healing, reduction of excessive neutrophil infiltration is associated with accelerated wound closure. Reduction of
cytokine and chemokine gene expression by AZM could potentially accelerate wound healing. In comparison to AZM, AMX consistently produced less inhibition of gene expression by neutrophils. Given that AZM has a half-life of up to 68 hr (versus 1-2 hr for AMX), a single prophylactic dose of AZM has greater potential to inhibit inflammatory mediator expression at a wound site than a single dose of AMX.

Clinical relevance
Based on this study we discovered that AZM concentrations similar to those found in gingival crevicular fluid inhibit induction of neutrophil inflammatory mediator expression more profoundly than AMX. This is consistent with previous observations suggesting that preoperative AZM enhances resolution of post-surgical inflammation to a greater extent than AMX. Thus, preoperative azithromycin may enhance resolution of postoperative inflammation to a greater extent than amoxicillin.

An important factor to consider in the decision to use a prophylactic antibiotic is the possible side effects the agent may cause. Although healthy adults treated with azithromycin (AZM) do not appear to have a significant risk of cardiovascular death, studies have suggested that a multiday course of AZM can promote cardiac arrhythmias and increase the risk of cardiovascular death in individuals with a high baseline risk of cardiovascular disease.\textsuperscript{30-32} It is appropriate to be cautious about prescribing AZM to patients with this type of risk. However, AZM (500 mg) is recommended as a one time prophylactic regimen for dental procedures in patients who are allergic to amoxicillin.\textsuperscript{14}
For patients who are not affected by cardiovascular disease or allergy to AZM, the risks associated with prescribing a single 500-mg dose of AZM are low. The most common side effects are diarrhea, nausea, vomiting, and abdominal pain.\(^3\) With AMX, allergic reactions (typically skin rashes) are the side effects of greatest concern, but AMX can also produce gastrointestinal upsets.\(^3\) Patients should always be advised of potential adverse medication effects, and these risks should be balanced with the expected benefits. Fortunately, single-dose prophylactic antibiotics have less potential than multi-dose regimens to induce adverse side effects.

**Study limitations**

Although anti-inflammatory effects appear to contribute to the effectiveness of macrolides in treating chronic inflammatory airway diseases, the mechanisms by which macrolides inhibit production of inflammatory mediators are not fully understood.\(^28\) Macrolides inhibit the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are known to regulate the expression of IL-8, IL-6, TNF-α, and IL-1β and other pro-inflammatory cytokines.\(^21,29\) Macrolides may also inhibit mitogen-activated protein kinase (MAPK) and extracellular-regulated kinase (ERK), resulting in a decrease in IL-8 production.\(^20\) It is conceivable that azithromycin works through similar mechanisms to inhibit the expression of cytokine and chemokine genes by neutrophils, but this hypothesis has not yet been tested. A logical extension of these experiments would be to examine the effects of AZM and AMX on inhibition of NF-κB activation in neutrophils. The finding
that AMX inhibits neutrophil gene expression was unexpected, and the mechanism by which this occurs is unclear

**Conclusion**

Results from this study show that AZM concentrations similar to those found in gingival crevicular fluid inhibit induction of neutrophil generated inflammatory marker expression more profoundly than AMX. This is consistent with previous observations suggesting that preoperative AZM enhances resolution of post-surgical inflammation to a greater extent than AMX. These reports, coupled with the previous study that evaluated the effect of AZM on clinical and biochemical parameters of healing around newly placed dental implants suggest that AZM has a favorable influence on healing, but further studies are indicated to fully characterize the extent to which AZM alters the complex processes associated with the inflammatory response.
<table>
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**Table 1. RT-PCR Primers**
Figure 1. Effect of AMX and AZM on induction of neutrophil IL-1β gene expression

**Figure 1:** Effect of AMX and AZM on induction of neutrophil IL-1β gene expression by LPS (A, C and E) or TNF-α (B, D and F) at 1, 2 and 4 hours respectively. Purified neutrophil suspensions were pre-incubated with the indicated concentrations of AMX or AZM, then activated with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Neutrophil mRNA was extracted and converted to cDNA, which was electrophoresed on a 1% agarose gel and stained with ethidium bromide. Lane 1 – Positive control; lane 2 - 2 µg/ml AMX; lane 3 – 4 µg/ml AMX; lane 4 – 4 µg/ml AZM; lane 5 – 8 µg/ml AZM.
Figure 2. Effect of AMX and AZM on induction of neutrophil TNF-α and IL-6 gene expression

Figure 2: Effect of AMX and AZM on induction of neutrophil TNF-α (A and B); and IL-6 (C and D) gene expression by LPS or TNF-α respectively. Neutrophils were pre-incubated with the indicated concentrations of AMX or AZM, and then activated with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Neutrophil mRNA was extracted and converted to cDNA, which was separated on a 1% agarose gel and stained with ethidium bromide. Lane 1 – Positive control; lane 2 - 2 µg/ml AMX; lane 3 – 4 µg/ml AMX; lane 4 – 4 µg/ml AZM; lane 5 – 8 µg/ml AZM.
Figure 3. Effect of AMX and AZM on induction of neutrophil IL-8, GM-CSF and G-CSF gene expression

Figure 3: Effect of AMX and AZM on induction of neutrophil IL-8 (A and B); GM-CSF (C and D) and G-CSF (E and F) gene expression by LPS or TNF-α respectively. Neutrophils were pre-incubated with AMX or AZM, then activated with TNFα (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Neutrophil mRNA was extracted and converted to cDNA, which was separated on an agarose gel and stained with ethidium bromide. Lane 1 – Positive control; lane 2- 2 µg/ml AMX; lane 3 – 4 µg/ml AMX; lane 4 – 4 µg/ml AZM; lane 5 – 8 µg/ml AZM.
Figure 4. Effect of AMX and AZM on induction of neutrophil IL-1β gene expression

Figure 4: Effect of AMX and AZM on induction of neutrophil IL-1β gene expression by TNF-α or LPS. Neutrophils were pre-incubated with the indicated concentrations of AMX or AZM, then activated with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Relative fold changes in expression levels were determined by real-time PCR utilizing SYBR Green master mix with β-actin as the housekeeping gene. The positive control was set at 1 and levels of other conditions were derived in relation to it. Data are presented as mean and SEM of three experiments. Legend: * denotes significant differences (P<0.05) from control as assessed by repeated measures ANOVA and Holm-Sidak post-hoc test, while + denotes a significant difference from control indicated by repeated measures ANOVA on ranks and Dunn’s post-hoc test.
Figure 5. Effect of AMX and AZM on induction of neutrophil TNF-α gene expression

Figure 5: Effect of AMX and AZM on induction of neutrophil TNF-α gene expression by TNF-α or LPS. Neutrophils were pre-incubated with the indicated concentrations of AMX or AZM, then activated with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Relative fold changes in expression levels were determined in triplicate by real-time PCR, utilizing β-actin as the housekeeping gene. Legend: * denotes significant differences (P<0.05) from control, as assessed by repeated measures ANOVA and Holm-Sidak post-hoc test.
Figure 6: Effect of AMX and AZM on induction of neutrophil IL-6 gene expression by TNF-α or LPS. Neutrophils were pre-incubated with the indicated concentrations of AMX or AZM, then activated with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Relative fold changes in expression levels were determined in triplicate by real-time PCR, utilizing β-actin as the housekeeping gene. Legend: * denotes significant differences (P<0.05) from control, as assessed by repeated measures ANOVA and Holm-Sidak post-hoc test.
Figure 7: Effect of AMX and AZM on induction of neutrophil IL-8 (CXCL8) gene expression by TNF-α or LPS. Neutrophils were pre-incubated with the indicated concentrations of AMX or AZM, then activated with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Relative fold changes in expression levels were determined in triplicate by real-time PCR, utilizing β-actin as the housekeeping gene. Legend: + denotes significant differences (P<0.05) from control indicated by repeated measures ANOVA on ranks and Dunn’s post-hoc test.
Figure 8: Effect of AMX and AZM on induction of neutrophil GM-CSF (CSF2) gene expression by TNF-α or LPS. Neutrophils were pre-incubated with the indicated concentrations of AMX or AZM, then activated with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Relative fold changes in expression levels were determined in triplicate by real-time PCR, utilizing β-actin as the housekeeping gene. Legend: * denotes a significant difference (P<0.05) from control as assessed by repeated measures ANOVA and Holm-Sidak post-hoc test, while + denotes a significant difference from control indicated by repeated measures ANOVA on ranks and Dunn’s post-hoc test.
Figure 9: Effect of AMX and AZM on induction of neutrophil G-CSF gene expression by TNF-α or LPS. Neutrophils were pre-incubated with the indicated concentrations of AMX or AZM, then activated with TNF-α (10 ng/ml) or LPS (1 µg/ml) for 1 hr. Relative fold changes in expression levels were determined in triplicate by real-time PCR, utilizing β-actin as the housekeeping gene. Legend: * denotes significant differences (P<0.05) from control as assessed by repeated measures ANOVA and Holm-Sidak post-hoc test, while + denotes significant differences from control indicated by repeated measures ANOVA on ranks and Dunn’s post-hoc test.


