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ORIGINAL ARTICLE

# Ibuprofen worsens *Streptococcus pyogenes* soft tissue infections in mice

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**KEYWORDS**
Animal model;
Nonsteroidal anti-inflammatory drugs;
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*Background:* Group A streptococcus (GAS) is a common cause of soft tissue infection. Nonsteroidal anti-inflammatory drugs have been reported to worsen GAS soft tissue infections.
*Methods:* A mouse model of GAS soft tissue infection was developed. The extent of cutaneous lesions, tissue damage, release of inflammatory cytokines, and survival rates were compared between mice with and without ibuprofen administration after GAS soft tissue infection.
*Results:* All twelve mice without ibuprofen administration survived for at least 10 days. In contrast, mortality rate of 14 mice with ibuprofen therapy was 72.5%. Ibuprofen-treated mice exhibited more evident macrophage infiltration and tissue damage in the GAS-infected soft tissues. In GAS-infected mice, tissue levels of interleukin 6 and tumor necrosis factor alpha were significantly higher in ibuprofen-treated mice than those in the control group.
*Conclusions:* The results supported the concept that ibuprofen use in GAS soft tissue infections might induce the development of severe necrotizing infections and increase mortality rate.


## Introduction

Group A streptococcus (GAS), *Streptococcus pyogenes*, is one of the most common pathogens of soft tissue infections. About 5—10 % of GAS soft tissue infections might evolve to necrotizing fasciitis (NF). Of note, the associated mortality rates of such invasive GAS infections ranged from 20% to 50%.[1,2] Underlying comorbidities were predisposing factors of NF caused by GAS infections.[3]

Use of nonsteroidal anti-inflammatory drugs (NSAIDs) is a common clinical practice because doctors would prescribe those drugs as analgesics or antipyretics to relieve sufferings in patients with soft tissue infections. NSAID use is common in clinical practice as an analgesic or antipyretic for symptomatic relief in patients with soft tissue

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infections. Recently, it has been reported that NSAIDs use may worsen the severity of GAS infections, even in previously healthy patients.[4–6] In some studies involving pediatric population, the use of NSAIDs in those with varicella-zoster virus infections has been associated with an increased risk of invasive GAS infections.[7,8] In addition, NSAIDs had been considered to be involved in the pathogenesis of streptococcal and staphylococcal toxic shock syndrome, which posed high mortality rates in the affected individuals.[9] However, previous observational studies were inconclusive regarding whether NSAIDs were a risk factor for NF.[2–4] Those studies were limited by small sample sizes, retrospective characteristics, and the lack of a control group. In particular, the existence of "indication bias," which suggests that NSAID may be given as a response to infectious diseases in patients with severe soft tissue infections, rather than being a cause of the severity of illness, appeared in many studies.[5–7]

The exact role of NSAIDs in disease progression of GAS infections is not clearly understood. Several authors have proposed that the use of NSAIDs could prevent GAS infections if GAS infections were diagnosed early, or on the contrary, would accelerate the infectious course as a result of their suppressive effects on host immune response.[10–12] In addition, a rabbit model examining the role of NSAIDs in GAS soft tissue infections did not find the evidence that NSAIDs would adversely affect the course.[13] In our study, we intended to establish a murine model of GAS soft tissue infection and to examine the effect of ibuprofen in mice with GAS soft tissue infections.

## Materials and methods

### Animals and bacterial strains

Six to 10 week-old female *BALB/cByJ* mice weighing 18—22 g were obtained from National Laboratory Animal Center. Animals were housed in a pathogen-free environment using 12 hours alternating periods of light and dark until the initiation of experiments. The study protocol was approved by the Institutional Animal Care and Use Committee of Chi Mei Medical Center, Taiwan.

Three clinical bacteremic isolates of *S pyogenes* (GAS-13, GAS-19, GAS-Jack) from patients with NF and septic arthritis were obtained from the microbiological laboratory in Chi-Mei Medical Center. The organisms were stored at −70°C in protected bacterial preservers (Technical Service Consultants, Heywood, UK). Each *S pyogenes* isolate was first cultured in Luria-Bertani broth (Difco Laboratories, Detroit, MI, USA) and then on nutrient agar (Difco) at 37°C overnight. A single colony was chosen and cultured overnight in freshly prepared Mueller-Hinton broth at 37°C. Four hundred microliters of each bacterial suspension was individually added to 20 mL Mueller-Hinton broth and incubated at 37°C for 4 hours. Bacteria were collected by centrifugation at 6,000 revolutions per minute for 10 minutes at 22°C. Pellets were resuspended and diluted to a final bacterial concentration of $2 \times 10^9$ colony forming units (CFU)/mL before injection into the mice. The concentration of the inoculum was confirmed by subsequent growth on agar plates.

### Murine model of GAS soft tissue infections and ibuprofen therapy

To correlate the effects of drug exposure between mouse and human, the dose in mice is equal to human doses multiplied by human *Km*/mice,[15,16] and therefore the ibuprofen dose of 30 mg/kg/d in human, the usual dosage for pain and fever control,[14] is about 50 mg/kg/d in mice. GAS strains (GAS-13, GAS-19, or GAS-Jack) were intramuscularly injected into right thigh of 26 mice with the inoculum of $10^8$ CFU. To determine the effect of ibuprofen in the course of GAS soft tissue infection, 14 of 26 mice were treated with ibuprofen, and the other 12 with saline (control group). After GAS infection, ibuprofen (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline was orally fed through an oral tube at a dose of 50 mg/kg/d divided into three doses for 7 days.

### Measurement of infected areas

Wound surface areas were measured daily from Day 1 to Day 10. Images obtained by a Lumix DMC-FX9 digital camera (Panasonic, Tokyo, Japan) were analyzed by the Image-Proplus 5.0 software (Media Cyberbetics, Bethesda, MD, USA). Wound areas tracing the wound margins with a fine resolution computer mouse were calculated. Measurements were performed in duplicate, and mean values of consecutive tracings were computed.

### Histopathology of the infected lesions

At 7 days after injection of *S pyogenes*, mice were euthanized with isoflurane, and infected tissues were obtained from each animal via skin biopsies that included dermis and underlying soft tissues. Each tissue sample was fixed in 10% buffered formalin before embedding in paraffin. Formalin-fixed tissues were sectioned and stained with hematoxylin and eosin stain (Sigma-Aldrich) by standard methodology. An Olympus model BX51 microscope equipped with a Q-FIRE camera (Olympus, Tokyo, Japan) was used for image capture.

### Serum and tissue levels of interleukin 6 and tumor necrosis factor alpha

The concentrations of tumor necrosis factor alpha (TNF-$\alpha$) and interleukin 6 (IL-6) in the serum and infected tissue were determined. Two cytokines were measured on 1, 3, 5, and 7 days after intramuscular inoculation of GAS using commercial enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. These assays were performed in duplicate.

### Statistical analyses

Continuous variables were described as medians (interquartile ranges) and compared by a Mann-Whitney U test because of the small sample sizes. Survival curves were determined by Kaplan-Meier survival analysis, and the

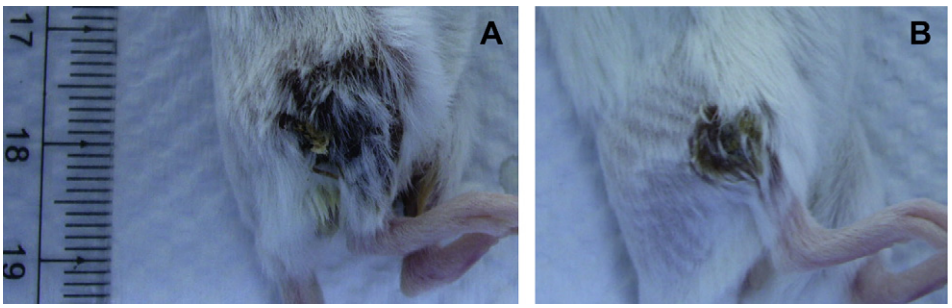


**Figure 1.** Wound size of ibuprofen-treated and control mice at Day 5. (A) The ibuprofen-treated wound extended to the other side of the thigh with a dark-red discharge, with gangrene and necrotic discharge evident at the wound margin. (B) The control mice show pink color and scar formation.

log-rank test was used to compare the differences. SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA) was used for the analysis. All statistical assessments were two sided, and a $p$ value less than 0.05 was considered to be statistically significant.

## Results

### Adverse effect of ibuprofen in GAS soft tissue infections

At Day 2 of GAS infection, the extent of lesions and production of exudates were greater in mice treated with ibuprofen than the control group (Figs. 1 and 2). Of note, ibuprofen was discontinued on Day 7 in the experimental group, and thereafter the wounds of the surviving mice begun to heal. Thirteen days later, the wounds completely healed with residual scars. In the control group, the time of wound healing ranged from 3 days to 7 days (median 5 days), which was significantly shorter than those in the mice treated with ibuprofen (median 8 days) ($p < 0.001$).

During the study period of 10 days, mice with GAS infection receiving ibuprofen treatment had a higher mortality rate than those without ibuprofen treatment (Fig. 3). Of 14 mice treated with ibuprofen, 10 (72.5%) died within 10 days, and in contrast, all 12 mice in the control group survived ($p < 0.001$). In addition, ibuprofen-treated mice were less active and had less appetite than those without ibuprofen treatment.

### Ibuprofen predisposed GAS NF

Inflammatory changes were significant in the muscle tissues of ibuprofen-treated mice. Microscopic examinations revealed that macrophage infiltration and swelling of subcutaneous and muscle tissues were much more severe in ibuprofen-treated mice (Fig. 4). In addition, there were complete dissolution of the dermis and fascia and polymorphonuclear infiltration and numerous bacteria within

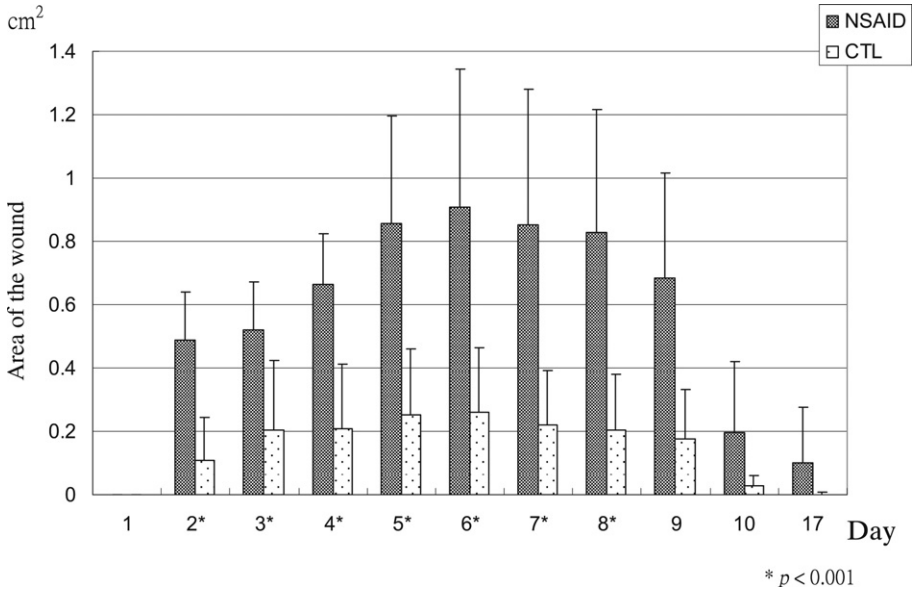


**Figure 2.** Wound areas in ibuprofen-treated and control mice within 17 days after infection. The maximum wound area in ibuprofen-treated and control mice (control) was 1.76 $cm^2$ at Day 6 and 0.6 $cm^2$ at Day 5, respectively. NSAID = nonsteroidal anti-inflammatory drug.

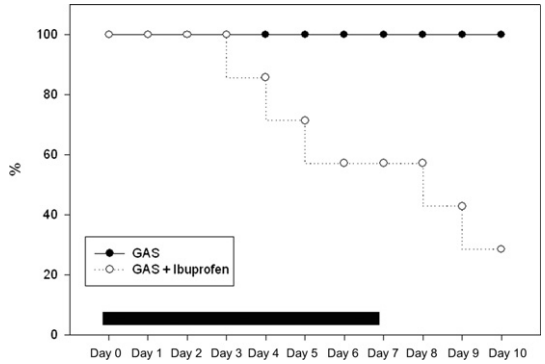


**Figure 3.** Kaplan-Meier survival analysis of GAS infections in control and ibuprofen-treated mice. At Day 10, survival rate of the control mice was 100%, whereas that of the ibuprofen-treated group was 27.5% ($p < 0.001$). As indicated by the bar, ibuprofen use was discontinued at Day 7. GAS = group A streptococcus.

the destroyed fascia, compatible with NF (Fig. 4A). In the control group, there were minimal tissue damage and few bacteria in the subcutaneous tissue (Fig. 4B). At Day 5, the resected tissue was submitted for culture; and in the ibuprofen-treated mice, GAS grew with a bacterial load of $2.47 \times 10^8$ CFU/g, which was 100-fold higher than that in the control group.

### Ibuprofen potentiates the release of proinflammatory cytokines

The kinetics of tissue IL-6 levels was demonstrated in Fig. 5A. The level of IL-6 in infected tissue is significantly higher in the ibuprofen-treated group ($p = 0.0001$) (Fig. 5B). Tissue IL-6 peaked at Day 3 and declined gradually. Similarly, in the ibuprofen-treated mice, serum IL-6 level was higher ($p < 0.0001$). Peak serum level of IL-6 was noted at Day 3 and undetectable at Day 5. In the control group, serum IL-6 level is undetectable at Day 3.

The levels of TNF-$\alpha$ in the infected tissue of ibuprofen-treated mice were higher than those of mice without ibuprofen treatment ($p = 0.001$) (Fig. 5C). Tissue TNF-$\alpha$ levels in two groups peaked at Day 1 and decreased gradually. At Day 3, tissue TNF-$\alpha$ level in the ibuprofen-treated mice is 2.5-fold higher than that in the control group. However, serum TNF-$\alpha$ level was undetectable in two groups at the first day of GAS infection.

## Discussion

The murine model established in the present study showed that NSAIDs would worsen the GAS soft tissue infection, which was compatible with previous clinical observations. Several mechanisms may explain this phenomenon. One hypothesis is that NSAIDs have adverse influences by suppressing primitive functions of chemotaxis, oxidative burst, phagocytosis, and bacterial killing in granulocytes.[12] Data also demonstrate that NSAIDs may inhibit neutrophil aggregation or degranulation *in vitro* and *in vivo*, and so interfere with the function of lymphocytes and inhibit monocyte superoxide production.[17,18] Moreover, some studies provide the evidence that NSAIDs can augment production of certain cytokines, such as TNF-$\alpha$, IL-1, and IL-6, on infection,[12] which were similar to our findings in the mice. Our findings were of clinical importance and we suggest that frequent use of NSAIDs in patients with soft tissue infection should be cautious, at least in those with GAS infection. In addition, our study also provided the model for further research to understand the effects of NSAIDs on bacterial soft tissue infections.

The present study demonstrated that NSAIDs increased the mortality significantly in the GAS-infected mice. These findings can also be explained by the suppression of immune function of granulocytes and upsurge of proinflammatory cytokines mediated by NSAIDs. Previous studies indicate that an increase of proinflammatory cytokines predispose patients to severe sepsis.[19] Moreover, some clinical evidences show that use of NSAIDs in patients with GAS infection would possibly increase the risk of septic shock and multiorgan failure,[12,20] which leads to high mortality subsequently. In the present study, levels of TNF-$\alpha$ and IL-6 in the serum and infected tissue of mice treated with ibuprofen peaked after the third day of GAS infection, which were compatible with the finding that an increase of mortality and wound necrosis had been notified after that time.

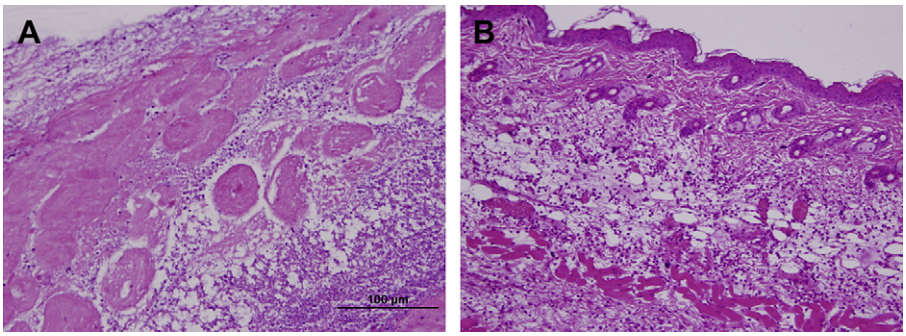


**Figure 4.** Pathological findings of infected soft tissues in ibuprofen-treated and control mice on Day 7. (A) Marked neutrophilic and histiocytic infiltrates and tissue necrosis in muscle tissue of ibuprofen-treated mice [hematoxylin and eosin (HE) stain; 100×]. (B) Intact fascia and muscle tissue structures with minimal neutrophil infiltration and few bacteria in subcutaneous tissue were noted in infected mice without ibuprofen therapy (HE stain; 100×).

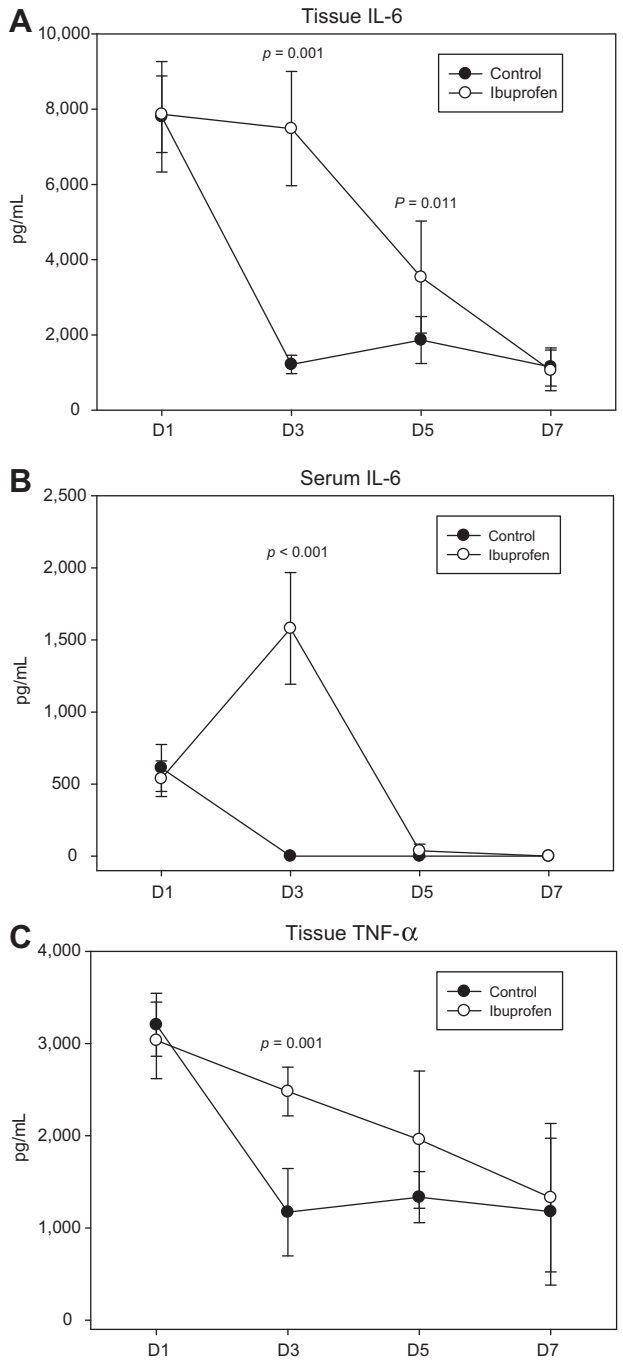


**Figure 5.** Dynamics of serum and tissue levels of IL-6 and TNF-α in mice with GAS infection with and without ibuprofen therapy: (A) Tissue IL-6; (B) Serum IL-6; (C) Tissue TNF-α. GAS = group A streptococcus; IL-6 = interleukin 6; TNF-α = tumor necrosis factor alpha.

A mouse model of GAS NF has been established.[18] However, animal studies focus on the role of NSAIDs in modulating the infection process is rare. GAS NF in a rabbit model suggest that NSAIDs do not adversely affect the outcome.[17] Nonetheless, the rabbit model has some limitations. The rabbits do not have muscular fascia. In addition, GAS is not naturally pathogenic to rabbits as in humans. The picture similar to NF in humans has only been seen in rabbits injected with the combination of GAS as well as staphylococcal alpha toxin.[13] Additionally, the inconsistencies between previous studies and our results may be explained by the different NSAID regimen. In our study, ibuprofen (3 mg/kg body weight) was administered three times per day orally consecutively for 7 days. In contrast, two doses of NSAIDs (diclofenac 4 mg/kg body weight) intramuscularly before and after GAS infection was administrated in the rabbit model.[21] Therefore, the current model has a potential to become a template to study the nature of NSAIDs in the infection process of GAS NF. Moreover, it merits further study to reveal the negative effects of NSAIDs in soft tissue infections caused by pathogens other than GAS, such Gram-negative organisms or *Staphylococcus aureus*.

Although the effect of NSAIDs in human GAS infections remains unclear, the present data suggest that the use of NSAIDs predisposes affected patients to NF and subsequently increases mortality. Therefore, clinical use of NSAIDs as analgesics or antipyretics in patients with skin and soft tissue infection should be used with caution.

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