



Evaluating the Safety of Sodium Hypochlorite Usage as an Irrigation Solution in Acute Osteomyelitis: an Experimental Study in Rat Tibia via a Renewed Osteomyelitis Model

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Abstract

Objective: The ideal irrigation solution for osteomyelitis surgery has not yet been clarified. The aim of this study is to evaluate the therapeutic effect and the optimum concentration of sodium hypochlorite (NaOCl) solution for the mechanical treatment of acute osteomyelitis.

Methods: Forty tibias of 20 male Wistar rats were used. The medullas were percutaneously inoculated with *Staphylococcus aureus* ATCC 25923 to induce acute osteomyelitis in tibias. One week later, rats were randomly assigned to 3 treatment groups and irrigated with 0.5%, 1% and 5% NaOCl. Irrigation with saline was used at positive control group and any treatment was not given to negative control group. All rats were sacrificed 24 hours after NaOCl irrigation. The degree of bacterial density, necrosis, inflammation and edema were evaluated histologically as; none (0), mild (1), moderate (2) and severe (3). Kruskal-Wallis and Dunn tests were used for statistical evaluations.

Results: Acute osteomyelitis developed in all tibias. NaOCl treatment no matter the concentration reduced the bacterial density compared to negative control group. The decrease in bacterial density and inflammation was significant at 0.5% NaOCl group compared to positive group (respectively; $p=0.019$, $p=0.045$), while the pairwise comparisons were statistically insignificant in terms of necrosis and edema. There was not any statistically difference between positive and 1%-5% NaOCl groups in terms of bacterial density.

Conclusion: In conclusion, irrigation with 0.5% NaOCl was more therapeutic than saline, 1% NaOCl and 5% NaOCl concentrations for acute tibia osteomyelitis at rats while 0.5% NaOCl group was same with saline group in terms of safety to tissue.

Keywords: Acute osteomyelitis model, experimental animal model, rat, sodium hypochlorite irrigation, treatment

INTRODUCTION

Acute osteomyelitis, a disease of bone due to the infection, rare but serious condition, is great concern in orthopedic surgeries. The most cause of the acute osteomyelitis is the hematogenous

spread of the causative bacteria into the bone, and less cause by surgery as open fracture surgery, or bone surgery including joint replacements/osteosynthesis (1). It develops rapidly over a period of 7 to 10 days, and in some specific situations (i.e. presence of heart failure, immunosuppressant medications) can



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be life treating (1,2). In the treatment, debridement and irrigation with several solutions during the surgery, and antibiotic usage are the first line options with the aim to control and remove the infection (2). The people infected might need to get their antibiotic medications for several weeks and in more serious osteomyelitis, surgery to remove the infected tissue and bone might be required.

The irrigation with solutions is commonly performed in orthopedic surgeries to keep patient from risk of infection. Isotonic saline, disinfectants or antibiotic solutions may be used as irrigation solution (3-6). In most circumstances saline is preferred for the irrigation of surgical site but it does not have any effect on microorganisms (4). The usage of antibiotic solutions is very limited because of its higher cost and antibiotic resistance but disinfectants remain as an option (4). The appropriate solution for irrigation has not been clarified thus far (2). Owing to rising antibiotic resistance in recent years, the importance of irrigation solutions has increased further (1). This, in turn, has revealed the importance of the antimicrobial effects of irrigation solutions (2).

To perform a most proper treatment for acute osteomyelitis, animal studies are done by using foreign body (7). Because the difficulty of performing infection at rats, the majority of osteomyelitis performing models use foreign objects as sclerosing material, heat or necrosis (7-9). The common source for acute osteomyelitis is known to be *Staphylococcus aureus* due to hematogenous transmission (1,2). Due to the usage of foreign objects as sclerosing material, heat or necrosis at osteomyelitis models, we think these performed techniques don't reflect exactly the acute osteomyelitis.

Sodium hypochlorite (NaOCl) is common used disinfectant in general purpose and health care services. It has lethal effect on viruses, fungi and wide spread of bacteria, and hence it is used for tap water disinfection (10). NaOCl is reported to be toxic to cell even at very lower concentration (0.01%) *in vitro* (11). Though the *in vitro* studies, in management of dental infections, 0.5% to 5% NaOCl solutions are used for irrigation with success (12-14). However, NaOCl solutions are not routinely used as an irrigation solution for osteomyelitis surgeries.

In the present study, we aimed to investigate the safety and the efficacy of different concentrations (0.5 to 5%) of NaOCl due to a renewed acute osteomyelitis model of rat tibia.

METHODS

The study was conducted upon the permission of local ethics committee for animal experiments (HADYEK, 15.12.2016,

2016/16). Twenty male Wistar albino rats weighing 200-250 g were used. All animals involved in experiments have received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (15). Rats were fed ad libitum; the temperature was 22+/-2°C with the 50% humidity. The rats were kept at 12/12h dark and light cycles. To prevent unnecessary animal sacrificing, both tibias of all animals were used.

Fluids Used for Irrigation and Creating the Groups

At the surgical processes under anesthesia, 2 rats were died. The remaining 18 rats were randomly divided into 5 groups. The 1st group was the negative control group (group 1) (n=3 rats, n=6 tibias) for which no treatment was applied. The 2nd group was the positive control group (group 2) (n=3 rats, n=6 tibias) which was treated with saline (0.9% NaCl isotonic) as it is routinely used in surgery. The other three groups were treatment groups and were named as; 0.5% NaOCl (group 3) (n=4 rats, n=8 tibias), 1% NaOCl (group 4) (n=4 rats, n=8 tibias) and 5% NaOCl (group 5) (n=4 rats, n=8 tibias). A brief summary of groups and applied treatments are given at Table 1.

Preparation of Bacterial Suspensions

Standard Methicillin-sensitive *Staphylococcus aureus* (MSSA) ATCC 25923 isolate, stored in freezer at -80°C was brought to room temperature and then incubated overnight in 5% sheep's blood agar passaging at 37°C. The next day after purity check of bacteria, bacterial suspensions with McFarland 10 turbidity (approximately 3x10⁹ cfu/mL) were prepared in saline.

Induction of Osteomyelitis and Irrigation

All rats had intraperitoneal ketamine (50 mg/kg) (Ketalar; Eczacıbaşı, İstanbul, Turkey) and xylazine (10 mg/kg) (Rompun; Bayer, Leverkusen, Germany) for anesthesia. Knees were wiped with povidone iodine. Supplemental analgesia was provided by intraperitoneal injection of buprenorphine (0.3 mg/kg). The

| Group number | Group name | Number of tibias (n) | Treatment administered |
|--------------|------------------|----------------------|-------------------------------------|
| 1 | Negative control | 6 | No treatment |
| 2 | Positive control | 6 | Irrigation with 20 mL of saline |
| 3 | 0.5% NaOCl | 8 | Irrigation with 20 mL of 0.5% NaOCl |
| 4 | 1% NaOCl | 6 | Irrigation with 20 mL of 1% NaOCl |
| 5 | 5% NaOCl | 8 | Irrigation with 20 mL of 5% NaOCl |

NaOCl: Sodium hypochlorite

tuberositas tibia (TT) was palpated. Percutaneous drilling with a 22-gauge needle tip (without skin incision) from the TT to tibial medulla was performed (16). After drilling, a 26-gauge needle tip was used to inoculate the medulla percutaneously with 10 μ L (nearly 3×10^7 cfu) of the bacterial suspension of *Staphylococcus aureus*. During follow-up, 7.5 mg/kg paracetamol (Parol, Atabay, İstanbul, Turkey) was added to drinking water.

After 7 day of inoculation with bacterial suspension to the rat tibias, experimentations in all groups were performed under ketamine (50 mg/kg)/xylazine (10 mg/kg) anesthesia with buprenorphine (0.3 mg/kg) analgesia. After the knee joints were wiped with povidone iodine, 1 cm longitudinal incision was done to the TT. Using the previous entry point, the tibia medulla was manually drilled with 22-gauge needle tip. At the point where the needle entered the bone, soft tissues were excised to allow fluid flow out of the medulla. Then the tibias in the groups were irrigated with the relevant solutions for 30 seconds. After the irrigation, 4/0 no absorbable polypropylene sutures (Propilen; Doğan Tibbi Malzeme San A.Ş, Trabzon, Turkey) were used to close the skin. At follow-up, rats received 7.5 mg/kg paracetamol ad libitum in drinking water. After irrigation, 1 rat in the 1% NaOCl group died (3 rats died in sum).

Radiological Magnetic Resonance Imaging (MRI) Assessment of Osteomyelitis Induction

On the 7th day of the study, rats were anesthetized intraperitoneally with ketamine (50 mg/kg) and xylazine (10 mg/kg).

To determine whether osteomyelitis developed in rats with tibias inoculated with bacteria or not, contrast (0.1 mmol gadobutrol/kg) T1 MRI (Siemens Magnetom Aera 1.5T, Erlangen, Germany) was used (17). Contrast material was administered intraperitoneally. Thin sagittal sections of the tibia were taken (Figure 1). As tibias without contrast would not be included in the study, a radiologist, and an orthopedist assessed results during imaging.

Histopathology

On the 8th day of the study (24 hours after irrigation), the rats were sacrificed with high doses of ketamine (100 mg/kg) and xylazine (100 mg/kg) anesthesia. Both tibias of rats (total 34) were dissected, including the soft tissues surrounding the tibias, and placed into 10% formalin, and submerged in paraffin. Sagittal sections in 4 μ m thickness were taken with a microtome (Thermo Scientific Microm HM 340E). Paired consecutive sections from each tibia were stained with HE and two were stained with Giemsa. Two different pathologist under a light microscope (Olympus CX41) performed histopathological

assessments in blind. Bone and soft tissues were assessed for necrosis, inflammation, edema and bacterial density. For all assessments, scoring was none (0), mild (1), moderate (2) and severe (3) according to density and spread of the parameter examined (18).

In Vitro Investigation of Antibacterial Activity of NaOCl Against Standard Pathogenic Bacterias

An *in vitro* study was conducted to investigate the effectiveness of the NaOCl available. The aim of this investigation was to evaluate the antibacterial activity of NaOCl against four different standard bacteria with minimal inhibitor concentrations by alamar blue microdilution broth method. The standard obtained from the American type culture collection (ATCC); MSSA ATCC 25923, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853 isolates were used. Serial dilution of NaOCl was carried out in sterile microplates of 96 wells, ranging from 1/2 to 1/1024 in Mueller Hinton Broth (MHB, Merck) medium. Suspensions of standard bacteria (turbidity number 107-108 cfu/mL) adjusted to McFarland 0.5 turbidity were added 100 mL to wells and incubated at 37°C. The last two wells were used as a negative (only medium and NaOCl) and positive (only medium and bacteria) control. After 20 hours, 20 μ L of alamar blue (Sigma-Aldrich, USA) was added to the microplates and incubated for another 4 hours. The change of the alamar blue in the wells to pink color according to the color change was interpreted as the presence of reproduction or the absence of color change was interpreted as stopping the reproduction. In addition, samples were taken from the wells were cultivated and reproduction was

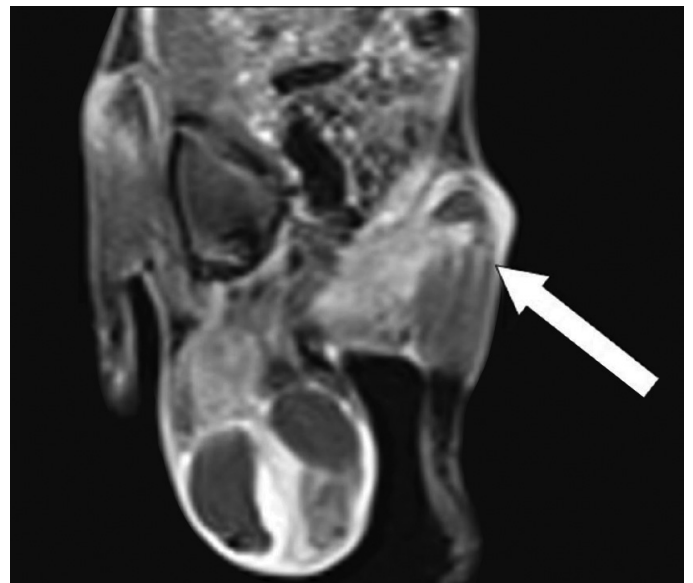


Figure 1. Contrast T1 MRI showing significant contrast enhancement MRI: Magnetic Resonance Imaging

checked. The concentration of stock NaOCl used was 5%. The concentrations were reduced by half and half.

Statistical Analysis

Statistical analyses were performed using R 3.5.1 Statistical Software (r-project), a free software environment for statistical computing and graphics. ICC estimates and their 95% confidence intervals were calculated using SPSS statistical package version 17.0 (SPSS Inc, Chicago, IL) based on a mean-rating (k=2), consistency and 2-way random-effects model. ICC values less than 0.5, between 0.5 and 0.75, between 0.75 and 0.9, and greater than 0.90 were indicative of poor, moderate, good, and excellent reliability, respectively (19).

The variables in the study were measured at an ordinal level thus the baseline characteristics of the groups were presented as median and interquartile range. The Kruskal-Wallis test was used as an omnibus test to compare medians of groups (20). Dunn multiple comparison test (21) was used after significant Kruskal-Wallis test (20). DunnTest function in FAS package of the R program was used for multiple comparisons. P values adjusted with the False Discovery Rate method were given.

RESULTS

Comparing the two observers to one another, the intra class correlation for edema, inflammation and necrosis were 0.90 (95% confidence interval (CI): 0.77-0.912), 0.914 (95% CI: 0.754-0.981) and 0.843 (95% CI: 0.617-0.940), respectively.

It was of interest to test whether there was a significant difference among negative control, positive control, 0.5% NaOCl, 1% NaOCl, and 5% NaOCl groups in terms of necrosis, inflammation in soft tissues and bones, edema in soft tissues, and bacterial density in bones. Descriptive statistics of variables for each group and the associated p values of the Kruskal-Wallis tests are shown in Table 2. Significant differences were found between the groups in terms of inflammation in soft tissues, necrosis, inflammation and bacterial density in bones with p values of 0.0241, 0.0239,

0.00022 and <0.001, respectively (Table 2). The adjusted p values of post hoc tests are shown in Table 3.

Percutaneous *Staphylococcus Aureus* Inoculation to Rat Tibias Caused Acute Osteomyelitis

With the percutaneous bacteria inoculation method, we identified the success rate for acute tibial osteomyelitis to be 100% in rat tibias. Investigated with contrast T1 MRI, 34 tibias of 17 rats were identified to have acute osteomyelitis. Contrast material involvement was observed in all of the 34 tibia medullas and in soft tissues surrounding tibias (Figure 1). After sacrificing; necrosis, acute inflammation and edema in surrounding soft tissues, bone marrows and soft tissues in all tibias with HE staining, and bacterial colonization with Giemsa staining were observed in histopathological investigations.

Irrigation with NaOCl Reduced Bacterial Density

While the density of bacteria was assessed histopathologically with Giemsa staining, a variety of dimensions and densities of bacterial colonization were observed and classified (Figure 2). Significant differences were found between groups in terms of bacterial density in bones. No significant differences were found between negative and positive control groups in pairwise comparisons (p=0.388) (Table 3). The 0.5% NaOCl, 1% NaOCl and 5% NaOCl groups had significantly lower amount of bacterial density in bones than that of the negative control group (negative control vs. 0.5% NaOCl, p<0.001; negative control vs 1% NaOCl, p=0.039; negative control vs 5% NaOCl, p<0.001). The 0.5% NaOCl group had a significantly lower amount of bacterial density in bones than that of the positive control group (Table 3) (positive control vs 0.5% NaOCl, p=0.019). The 1% NaOCl and 5% NaOCl groups had no significant difference compared to the positive control group (Table 3) (positive control vs 1% NaOCl, p=0.192; positive control vs 5% NaOCl, p=0.186).

These findings show that NaOCl usage reduced the density of bacteria. With 0.5% NaOCl usage, bacterial density was significantly reduced compared to the positive control group.

Table 2. Descriptive statistics and Kruskal-Wallis test results

| | | Negative control | Positive control | 0.5% NaOCl | 1% NaOCl | 5% NaOCl | Test statistic (p) |
|--------------|-------------------|------------------|------------------|------------|----------|----------|--------------------|
| Bone marrows | Bacterial density | 3 (0) | 2 (0.75) | 1 (0.25) | 2 (0.75) | 1 (0) | 25.7 (<0.001*) |
| | Necrosis | 2 (0) | 1 (0.75) | 1 (0.25) | 1.5 (1) | 2 (0) | 12.95 (0.02*) |
| | Inflammation | 3 (0) | 2.5 (1) | 2 (0) | 2 (0) | 2 (0) | 18.66 (0.002*) |
| Soft tissues | Necrosis | 1 (0.75) | 1 (0.75) | 0 (0.25) | 0.5 (1) | 1 (1.25) | 4.49 (0.48) |
| | Edema | 2 (0) | 2 (0) | 2 (1) | 2 (0) | 2 (0.25) | 7.06 (0.21) |
| | Inflammation | 3 (0.75) | 3 (0) | 2 (0.5) | 2 (0.75) | 2 (0) | 12.9 (0.02*) |

Data are expressed as median with interquartile range in parentheses, NaOCl: Sodium hypochlorite, *p<0.05

Irrigation with NaOCl Reduced Inflammation

Inflammation was assessed with HE staining. During assessment of inflammation, neutrophil density in bone marrows was observed to decrease (Figure 3). No significant difference was

found in bone marrows between negative and positive control groups in terms of inflammation ($p=0.383$) (Table 3). The 0.5% NaOCl, 1% NaOCl and 5% NaOCl groups had significantly lower amounts of inflammation in bone marrows than that of the

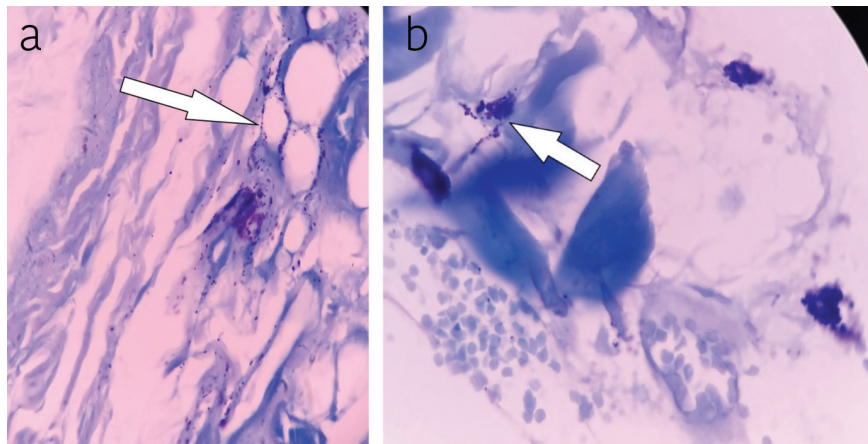


Figure 2. Bacterial density (Giemsa staining, 40x100 magnification). Arrows indicate the bacteria; a) 0.5% NaOCl group, score 1, b) positive control group, score 3

NaOCl: Sodium hypochlorite

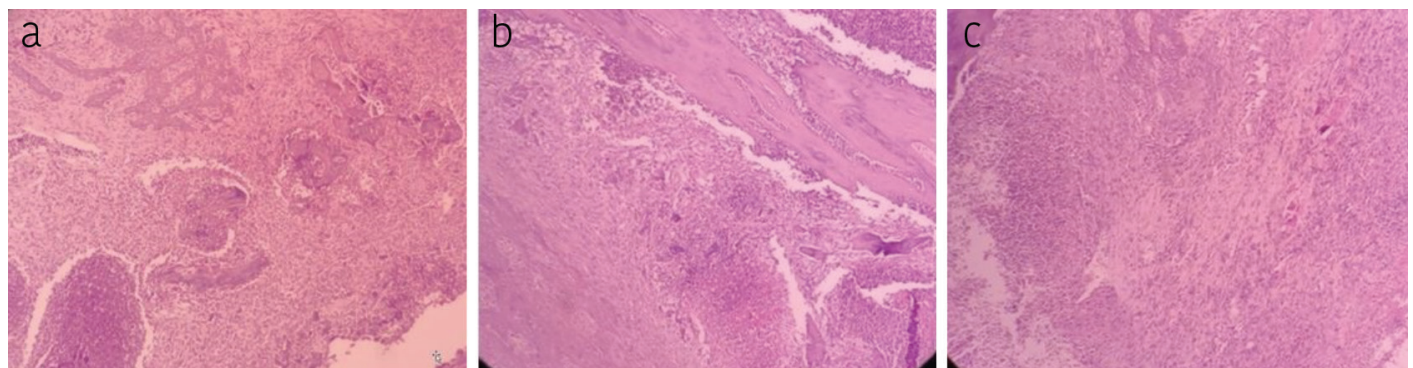


Figure 3. Inflammation and necrosis of soft tissues and bones (H & E 10x100); a) 0.5% NaOCl (irrigation with 0.5% NaOCl), necrosis score 1, inflammation score 3, b) positive control (irrigation with saline, necrosis score 1, inflammation score 2, c) negative control (no irrigation) necrosis score 2, inflammation score 3

NaOCl: Sodium hypochlorite, H & E: Haematoxylin and eosin

| Table 3. Multiple comparisons for significant tests (adjusted p values) | | | | |
|--|-----------------------------------|---------------------------------|-------------------------------------|-------------------------------------|
| Comparison | Bacterial density in bones | Necrosis in bone marrows | Inflammation in bone marrows | Inflammation in soft tissues |
| Negative control - positive control | 0.388 | 0.108 | 0.383 | 0.575 |
| Negative control - 0.5% NaOCl | <0.001 | 0.086 | 0.011 | 0.182 |
| Negative control - 1% NaOCl | 0.039 | 0.334 | 0.045 | 0.511 |
| Negative control - 5% NaOCl | <0.001 | 0.891 | 0.036 | 0.189 |
| Positive control - 0.5% NaOCl | 0.019 | 0.964 | 0.094 | 0.045 |
| Positive control - 1% NaOCl | 0.192 | 0.555 | 0.285 | 0.153 |
| Positive control - 5% NaOCl | 0.186 | 0.081 | 0.325 | 0.031 |
| 0.5% NaOCl - 1% NaOCl | 0.326 | 0.528 | 0.727 | 0.650 |
| 0.5% NaOCl - 5% NaOCl | 0.703 | 0.076 | 0.549 | 0.914 |
| 1% NaOCl - 5% NaOCl | 0.209 | 0.252 | 0.872 | 0.674 |

NaOCl: Sodium hypochlorite

negative control group (negative control vs 0.5% NaOCl, $p=0.011$; negative control vs 1% NaOCl, $p=0.045$; negative control vs 5% NaOCl, $p=0.036$) (Table 3). No significant difference was found in terms of inflammation in bone marrows between the positive control group and 0.5% NaOCl, 1% NaOCl and 5% NaOCl groups (positive control vs 0.5% NaOCl, $p=0.094$; positive control vs 1% NaOCl, $p=0.285$; positive control vs 5% NaOCl, $p=0.325$) (Table 3).

In assessment of inflammation, neutrophil density in soft tissues was observed to decrease. No significant difference was found in soft tissues between negative and positive control groups in terms of inflammation ($p=0.575$) (Table 3). Also no significant difference was found in soft tissues in terms of inflammation between negative and 0.5% NaOCl, 1% NaOCl, and 5% NaOCl groups (negative control vs 0.5% NaOCl, $p=0.182$; negative control vs 1% NaOCl, $p=0.511$; negative control vs 5% NaOCl, $p=0.189$). The 0.5% NaOCl and 5% NaOCl groups had significantly lower amounts of inflammation in soft tissues compared to the positive control group (positive control vs 0.5% NaOCl, $p=0.045$; positive control vs 5% NaOCl, $p=0.031$) (Table 3).

With these findings, it is possible to suggest that irrigation of osteomyelitis with NaOCl reduces inflammation in bone marrows and soft tissues in osteomyelitis disease.

Soft Tissue Edema and Necrosis Rates were Comparable in Each Group

Edema in soft tissues was assessed with the increase in intercellular space and neutrophil infiltration. No difference was observed between the groups. When groups were assessed in terms of soft tissue edema, no statistical difference was observed ($p=0.21$) (Table 2).

In all groups, necrosis was observed in bone marrows and lamellar bones along with soft tissues (Figure 3). Although, a significant difference was found between the groups with respect to necrosis in bone marrows (Table 2) ($p=0.02$), pairwise comparisons could not detect any pairwise significant difference at the 0.05 significance level ($p>0.05$) (Table 3). Thus, it can be concluded that the groups did not differ in terms of necrosis in bone marrows ($p>0.05$) (Table 3). There was no difference observed between the groups in terms of necrosis in soft tissues also (Table 2) ($p=0.48$). These findings show irrigation of osteomyelitis with NaOCl did not increase necrosis in bone marrows and soft tissues.

In Vitro Results of Antibacterial Activity of NaOCl Against Standard Pathogenic Bacterias

Briefly the *in vitro* results were given at Figure 4. The 1/64 (0.078% NaOCl) dilution of NaOCl was effective against all bacteria other

than *Escherichia coli*. The dilution of NaOCl at 1/128 (0.039% NaOCl) was effective against *Escherichia coli*.

DISCUSSION

Previously, many experimental osteomyelitis models have been described and any foreign material is used to perform osteomyelitis models in rats (7,22,23). Acute osteomyelitis in humans is generally observed with the hematogenous route without presence of foreign objects. With the aim of simulating the completely acute osteomyelitis scene, we did not use foreign objects in the acute osteomyelitis induction model unlike the osteomyelitis models which were performed by using foreign materials as bone wax, sclerosing agents, K wires etc. (7,22,23). The standard MSSA ATCC 25923 isolate was used as it is the most common pathogen associated with implants and is successful in inducing infections (24,25). The dose used for bacterial inoculation was above the minimal required dose (10^7 CFU) recommended by Worlock et al. (26). During preliminary studies, before beginning the experiment, the drilling and bacterial inoculation procedure were applied with an incision above the TT. However, in this preliminary study, the inoculated bacterial suspension drained out of the bone medulla through the hole which we drilled for needle entry, before we could close the hole by bone wax. We predicted the reason for this drainage was the pressure of intramedullar hemorrhage formed in the tibias. Therefore, we chose the percutaneous method as a research technique, and drilled the tibias percutaneously similar to the method Rissing et al. (16). used sclerosing agents at rats but we did not use sclerosing material as Gaudin et al. (27) method that performed at rabbits, with the concerns of not using foreign material to mimic the acute osteomyelitis model more exactly. Additionally, as there was no skin incision in our percutaneous



Figure 4. The *in vitro* results of NaOCl against 4 different bacterial pathogens. The used stock NaOCl concentration was 5%. The concentrations of in the *in vitro* investigation were reduced half and half

NaOCl: Sodium hypochlorite

technique, there was no need to use foreign materials like bone wax to close the hole formed in the bone. T1 contrast MRI taken 1 week after bacterial inoculation revealed contrast involvement in all tibias and soft tissues surrounding the tibias (Figure 1) (17). Histopathologically, osteomyelitis was observed in all tibia sections (Figure 2). Previously described methods have reported lower osteomyelitis development rates as 90% (7,24). The probable reasons for developing acute osteomyelitis at all tibias in the present study may include 1) success of the bacterial strain (MSSA ATCC 25923) inoculated into the medulla for inducing infections (24), 2) applying bacterial inoculation above the required minimum dose (10^7 CFU) for osteomyelitis development (26), and 3) keeping all of the bacterial suspension within the tibia medulla thanks to percutaneous inoculation.

As commonly known, NaOCl has cytotoxic effects. Therefore, whether the possible necrosis we could observe at results was due to the cytotoxic effect of NaOCl or the drill technique we applied was one of our major concerns. It is well known that high torque drilling or using motors perform necrosis because of heat effect (9). So, attempts were made to standardize the irrigation procedure because of the potential necrosis performing of the drilling method. Before the irrigation procedure, all tibias were kindly and slowly pitted with an 18-gauge needle tip and irrigation solutions were administered intramedullary by using a 22-gauge needle tip. The present study did not identify a significant difference in terms of necrosis of soft tissues ($p=0.48$) (Table 2) and bone marrows ($p>0.05$) (Table 3) between the groups (Figure 3). The lack of difference for bone marrows and soft tissues in the groups in terms of necrosis abides by the principle of "not harming the tissue" required for the ideal irrigation solution (2). Besides, at our preliminary study which we compared the saline and NaOCl irrigation in terms of necrosis and safety after drilling the tibia, there was not any difference between saline and 0.5% NaOCl groups (unpublished data). So we think, unlike the osteomyelitis methods those use high torque motors to perform necrosis (9), current low torque drilling method made by manually did not cause additional necrosis.

The importance of acute osteomyelitis due to orthopaedic implants is increasing worldwide. The treatment of acute and chronic osteomyelitis is different from each other. The irrigation of the infected area accelerates the improvement (28). Besides if there is an implant at the body, the most important criteria was the time after the implantation surgery for the differential diagnosis of acute/chronic infection. If the time after the implantation was more than one year, the case was accepted as chronic infection. But currently the most important criteria

are the time to start the complaints up to 3 weeks (29). To apply surgery and irrigation at the treatment of implant related acute infections, the irrigation solution gains more important (28).

Different solutions are commonly used in clinical practice, in order to irrigate the orthopedic infections during the surgery (3-6). Saline and castile soap solution were compared for irrigation of open fractures, the saline was found to be more effective (30). Azzam et al. (31) found 44% success with the application of antibiotic serum irrigation for patients with infected prosthesis. George et al. (5) recommended the irrigation with chlorhexidine in order to prevent infection in total joint prosthesis. Dakin's solution, contains 0.5% NaOCl, is used in diabetic foot and osteomyelitis treatments (6,32,33). Before the widespread use of antibiotics, Dakin's solution is used for the continuous irrigation-suction treatment method for chronic osteomyelitis (6,34). We investigated the NaOCl, because the NaOCl has the advantage of effectiveness on all resistance bacteria, viruses, prions and fungi compared to other disinfectant (10). In the *in vitro* part of the current study NaOCl is germicidal even at lower concentrations (Figure 4). As NaOCl with concentrations from 0.5% to 5% are used for treatment purposes in humans (12-14,32,33), we researched this concentrations interval for irrigation of acute tibial osteomyelitis in the current study.

There are numerous *in vitro* studies with different irrigation solutions in the presence of infection (35-37). Van Meurs et al. (36) recommended povidone iodine as the ideal irrigation solution in an *in vitro* study. Usage of chlorhexidine with orthopedic implants was shown to reduce biofilm formation *in vitro* (37). Ernest et al. (35) reported that NaOCl reduced biofilm and bacterial concentration via *in vitro* studies. The suggested concentrations of NaOCl at *in vivo* (6,32-33) and *in vitro* (11,35,38,39) studies are different each other. The suggested concentrations of NaOCl is lower (0.01 NaOCl) at *in vitro* (11) studies than *in vivo* and human (0.5-5% NaOCl) (6,12-14,32,33) studies. This is most probably because of the lack of defense system of living organism, as white blood cell, macrophages, immune system, hormones etc, at *in vitro* conditions.

Irrigation alone is not sufficient for osteomyelitis treatment (40). However, it is an important stage in surgery. The criteria (2) for the ideal irrigation fluid are as follows: 1) It should reduce the number of pathogens mechanically by irrigating the surgical site 2) it should reduce the number of pathogens by antimicrobial effect 3) it should not cause side effects such as damaging the tissues. In our study we found: 1) Soft tissue (Table 2) and bone marrow (Table 3) necrosis were insignificant between the groups, abiding by the lack of harm condition for the ideal irrigation

fluid, 2) the reduction in bacterial density with the use of NaOCl abides by the condition of reducing the density of bacteria mechanically and through antimicrobial effect, and 3) the reduction of inflammation in soft tissues and bones with the use of NaOCl may be interpreted as NaOCl to be a marker that regresses the disease. This study revealed the histological results of NaOCl irrigation in acute osteomyelitis of the long bones.

In terms of treatment efficacy, the most important aspect of this study was the decrease in bacterial density. When compared with the negative control group, all three NaOCl groups were observed to have significantly reduced bacterial density ($p < 0.001$, $p = 0.039$ and $p < 0.001$, respectively) (Table 3). When NaOCl groups were compared with the positive control group in terms of bacterial density, only the 0.5% NaOCl group had a significant reduction in bacterial density ($p = 0.019$). But when the 1% NaOCl and 5% NaOCl groups were compared with the positive control group in terms of bacterial density, no statistical difference was observed ($p = 0.192$, $p = 0.349$ and $p = 0.186$, respectively) (Table 3). Higher concentrations of NaOCl can be predicted to be more bactericidal. But this situation is in accordance with the literature; it is known that lower concentration of alcohol is more bactericidal than that of higher concentrations (41,42). A recently study made by our study group revealed that H_2S , a toxic hormone at higher concentrations, can be therapeutic at physiological low doses (43). Besides, Wong and Cheung (44) applied 0.5% and 3% concentrations of NaOCl for irrigation and searched for bacterial density in an *ex vitro* study made with extracted human teeth. The 0.5% NaOCl and 3% NaOCl groups were found to be statistically insignificant in reducing bacterial density. In our study, statistically compared with both the negative and positive control groups, the only treatment group that significantly reduced bacterial density was the 0.5% NaOCl group (Table 3). This feature of 0.5% NaOCl abides by the principle (2) of antimicrobial effect desired in the ideal irrigation solution.

Osteomyelitis does not only affect bone tissues but also the surrounding soft tissues (45). Hence, when bones recover from the disease, soft tissues start to recover as well. We detected inflammation to be reduced in bone marrows ($p = 0.002$) and soft tissues ($p = 0.02$) in the first 24 hours after irrigation of osteomyelitis with NaOCl (Figure 3) (Table 2). This situation may be interpreted as NaOCl irrigation of bone with acute osteomyelitis may have therapeutic effects on bones and surrounding soft tissues.

Based on the results of the first 24 hours, there was no statistical difference identified between the groups in terms of soft tissue edema ($p = 0.21$) (Figure 3) (Table 2). The edema formed by NaOCl

in soft tissues disappears within 24-48 hours in clinic (12). This finding may be assessed as irrigation with NaOCl does not cause additional edema in soft tissues.

CONCLUSION

In conclusion, 1) experimental acute osteomyelitis developed in all tibias by using the percutaneous bacterial inoculation 2) the findings indicate that 0.5% NaOCl is beneficial for irrigation of acute osteomyelitis and there is a need for further studies on the topic.

Ethics

Ethics Committee Approval: The study was conducted upon the permission of local ethics committee for animal experiments (HADYEK, 15.12.2016, 2016/16) Republic of Turkey Ordu University Animal Experiments Local Ethics Committee.

Informed Consent: Animal experiments.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: K.A., C.Z.E., Design: K.A., C.Z.E., Data Collection or Processing: A.D., İ.Ç., F.Y.A., Ş.D., Analysis or Interpretation: C.Z.E., E.A., Literature Search: A.D., F.Y.A., Writing: K.A., S.T., C.Z.E.

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REFERENCES

1. Kapukaya A, Subaşı M, Kaya H, Kesemenli C, Sarı İ, Kandıya E. Akut osteomyelit tedavisinde granuloocyte macrophage-colony stimulating factor (GM-CSF)'ün etkisi. *Acta Ortop Traumatol Turc* 1998;32:325-8.
2. Tiemann AH, Hofmann GO. Wound irrigation within the surgical treatment of osteomyelitis. *GMS Interdiscip Plast Reconstr Surg DGPW* 2012;1.
3. Campbell ST, Goodnough LH, Bennett CG, Giori NJ. Antiseptics commonly used in total joint arthroplasty interact and may form toxic products. *J Arthroplasty* 2018;33:844-6.
4. Conroy BP, Anglen JO, Simpson WA, Christensen G, Phaup G, Yeager R, et al. Comparison of castile soap, benzalkonium chloride, and bacitracin as irrigation solutions for complex contaminated orthopaedic wounds. *J Orthop Trauma* 1999;13:332-7.
5. George J, Klika AK, Higuera CA. Use of chlorhexidine preparations in total joint arthroplasty. *J Bone Jt Infect* 2017;2:15-22.
6. Taylor AR, Maudsley RH. Instillation-suction technique in chronic osteomyelitis. *J Bone Joint Surg Br* 1970;52:88-92.
7. Patel M, Rojavin Y, Jamali AA, Wasielewski SJ, Salgado CJ. Animal models for the study of osteomyelitis. *Semin Plast Surg* 2009;23:148-54.

8. McPherson JC 3rd, Runner RR, Shapiro B, Walsh DS, Stephens-DeValle J, Buxton TB. An acute osteomyelitis model in traumatized rat tibiae involving sand as a foreign body, thermal injury, and bimicrobial contamination. *Comp Med* 2008;58:369-74.
9. Korkusuz F, Uchida A, Shinto Y, Araki N, Inoue K, Ono K. Experimental implant-related osteomyelitis treated by antibiotic-calcium hydroxyapatite ceramic composites. *J Bone Joint Surg Br* 1993;75:111-4.
10. Center for Disease Control and Prevention (2016). Disinfection By-Products. Available from: <https://www.cdc.gov/safewater/chlorination-byproducts.html> Accessed 20 February 2019.
11. Heling I, Rotstein I, Dinur T, Szwec-Levine Y, Steinberg D. Bactericidal and cytotoxic effects of sodium hypochlorite and sodium dichloroisocyanurate solutions in vitro. *J Endod* 2001;27:278-80.
12. Pashley EL, Birdsong NL, Bowman K, Pashley DH. Cytotoxic effects of NaOCl on vital tissue. *J Endod* 1985;11:525-8.
13. Zan R, Kutlu G, Hubbezoglu I, Sumer Z, Tunc T, Mutlu Z. Bactericidal effects of various irrigation solutions against *Staphylococcus Aureus* in human root canal. *J Istanbul Univ Fac Dent* 2015;49:19-26.
14. Mathew ST. Risks and management of sodium hypochlorite in endodontics. *Journal of Oral Hygiene Health* 2015:1-5.
15. www.nap.edu/catalog/5140.html Accessed 20 February 2019
16. Rissing JP, Buxton TB, Weinstein RS, Shockley RK. Model of experimental chronic osteomyelitis in rats. *Infect Immun* 1985;47:581-6.
17. Lee YJ, Sadigh S, Mankad K, Kapse N, Rajeswaran G. The imaging of osteomyelitis. *Quant Imaging Med Surg* 2016;6:184-98.
18. Tiemann A, Hofmann GO, Krukemeyer MG, Krenn V, Langwald S. Histopathological Osteomyelitis Evaluation Score (HOES)-an innovative approach to histopathological diagnostics and scoring of osteomyelitis. *GMS Interdiscip Plast Reconstr Surg DGPW* 2014:3.
19. Koo TK, Li MY. A guideline of selecting and reporting intraclass correlation coefficients for reliability research. *J Chiropr Med* 2016;15:155-63.
20. Kruskal WH, Wallis WA. Use of ranks in one-criterion variance analysis. *J Am Stat Assoc* 1952;47:583-621.
21. Dunn OJ. Multiple comparisons using rank sums. *Technometrics* 1964;6:241-52.
22. Reizner W, Hunter JG, O'Malley NT, Southgate RD, Schwarz EM, Kates SL. A systematic review of animal models for *Staphylococcus aureus* osteomyelitis. *Eur Cell Mater* 2014;27:196-212.
23. Lucke M, Schmidmaier G, Sadoni S, Wildemann B, Schiller R, Stemberger A, et al. A new model of implant-related osteomyelitis in rats. *Journal of Biomedical Materials Research* 2003;67:593-602.
24. Harrasser N, Gorkotte J, Obermeier A, Feihl S, Straub M, Slotta-Huspenina J, et al. A new model of implant-related osteomyelitis in the metaphysis of rat tibiae. *BMC Musculoskeletal Disorders* 2016;17:152.
25. Haenle M, Zietz C, Lindner T, Arndt K, Vetter A, Mittelmeier W, et al. A model of implant-associated infection in the tibial metaphysis of rats. *ScientificWorldJournal* 2013;2013:481975.
26. Worlock P, Slack R, Harvey L, Mawhinney R. An experimental model of post-traumatic osteomyelitis in rabbits. *Br J Exp Pathol* 1988;69:235-44.
27. Gaudin A, Amador Del Valle G, Hamel A, Le Mabeque V, Miegerville AF, Potel G, et al. A new experimental model of acute osteomyelitis due to methicillin-resistant *Staphylococcus aureus* in rabbit. *Lett Appl Microbiol* 2011;52:253-7.
28. Kuiper JW, Vos SJ, Saouti R, Vergroesen DA, Graat HC, Debets-Ossenkopp YJ, et al. Prosthetic joint-associated infections treated with DAIR (debridement, antibiotics, irrigation, and retention) Analysis of risk factors and local antibiotic carriers in 91 patients. *Acta Orthop* 2013;84:380-6.
29. Li C, Renz N, Trampuz A. Management of periprosthetic joint infection. *Hip Pelvis* 2018;30:138-46.
30. FLOW Investigators, Bhandari M, Jeray KJ, Petrisor BA, Devereaux PJ, Heels-Ansdell D. A trial of wound irrigation in the initial management of open fracture wounds. *N Eng J Med* 2015;373:2629-41.
31. Azzam KA, Seeley M, Ghanem E, Austin MS, Purtill JJ, Parvizi J. Irrigation and debridement in the management of prosthetic joint infection: traditional indications revisited. *J Arthroplasty* 2010;25:1022-7.
32. Duarte B, Cabete J, Formiga A, Neves J. Dakin's solution: is there a place for it in the 21st century? *Int Wound J* 2017;14:918-20.
33. Cornwell P, Arnold-Long M, Barss SB, Varnado MF. The use of Dakin's solution in chronic wounds: a clinical perspective case series. *J Wound Ostomy Continence Nurs* 2010;37:94-104.
34. Michelinakis E. Treatment of chronic osteomyelitis with the continuous irrigation-suction method. *Acta Orthop Scand* 1972;43:25-31.
35. Ernest EP, Machi AS, Karolcik BA, LaSala PR, Dietz MJ. Topical adjuvants incompletely remove adherent *Staphylococcus aureus* from implant materials. *J Orthop Res* 2018;36:1599-604.
36. Van Meurs SJ, Gawlitta D, Heemstra KA, Poolman RW, Vogely HC, Kruyt MC. Selection of an optimal antiseptic solution for intraoperative irrigation: an in vitro study. *J Bone Joint Surg Am* 2014;96:285-91.
37. Schwechter EM, Folk D, Varshney AK, Fries BC, Kim SJ, Hirsh DM. Optimal irrigation and debridement of infected joint implants: an in vitro methicillin-resistant *Staphylococcus aureus* biofilm model. *J Arthroplasty* 2011;26:109-13.
38. Hidalgo E, Bartolome R, Dominguez C. Cytotoxicity mechanisms of sodium hypochlorite in cultured human dermal fibroblasts and its bactericidal effectiveness. *Chem Biol Interact* 2002;139:265-82.
39. Sawada K, Caballe-Serrano J, Bosshardt DD, Schaller B, Miron RJ, Buser D, et al. Antiseptic solutions modulate the paracrine-like activity of bone chips: differential impact of chlorhexidine and sodium hypochlorite. *J Clin Periodontol* 2015;42:883-91.
40. Azzam K, Parvizi J, Jungkind D, Hanssen A, Fehring T, Springer B, et al. Microbiological, clinical, and surgical features of fungal prosthetic joint infections: a multi-institutional experience. *J Bone Joint Surg Am* 2009;91(Suppl 6):142-9.
41. Center for Disease Control and Prevention (2008) Chemical Disinfectants. Available from: <https://www.cdc.gov/infectioncontrol/guidelines/disinfection/disinfection-methods/chemical.html> Accessed 20 February 2019.
42. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* 1999;12:147-79.
43. Aytekin K, Erhan SŞ, Erişgin Z, Esenyel CZ, Takır S. Intra-articular injection of hydrogen sulfide decreased the progression of gonarthrosis. *Can J Physiol Pharmacol* 2018;97:47-54.
44. Wong DT, Cheung GS. Extension of bactericidal effect of sodium hypochlorite into dentinal tubules. *J Endod* 2014;40:825-9.
45. Öztuna V. Osteomyelit Patofizyolojisi ve Tedavi Prensipleri. *TOTBİD Dergisi* 2005;4:63-71.