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Cross-interactions between Norepinephrine, Methicillin-Resistant *Staphylococcus aureus* and Human Osteoblast Cells in Culture Conditions

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ABSTRACT

Objective: The role of norepinephrine (NE) on growth, adhesion and invasion of methicillin-resistant *Staphylococcus aureus* (MRSA) ATTC 43300 was examined in human osteoblast (HOB) cells. The effects of NE and/or MRSA on the viability and cell death pathways of HOB cells were also investigated. Furthermore, the alterations of bacterial response to oxidative stress (H_2O_2) were analyzed in the presence/absence of NE.

Materials and Methods: Bacterial growth was detected spectrophotometrically. The colony counting method was examined for adhesion-invasion. The alteration of HOB cell viability was determined by methyl thiazolyl diphenyl-tetrazolium bromide (MTT) assay. The death pathways of HOB cells were examined microscopically using acridine orange-ethidium bromide dual staining and dichlorofluorescein-diacetate (DCF-DA) dye. The bacterial response to H_2O_2 was investigated by agar dilution.

Results: The growth of bacterium was not affected in the presence of NE. Bacterial adhesion was decreased by NE ($p<0.0001$) while high-level NE induced invasion ($p=0.013$). HOB cell viability was reduced by MRSA and/or NE ($p<0.001$). MRSA and co-existence of MRSA and NE caused necrosis more than apoptosis in HOB cells ($p<0.05$). NE did not alter the bacterial response to oxidative stress.

Conclusion: Norepinephrine has different effects on the biological properties of both MRSA and HOB cells.

Keywords: Norepinephrine, MRSA, growth, adhesion-invasion, oxidative stress response, HOB cell viability, apoptosis-necrosis

INTRODUCTION

During an infection process, a microorganism enters the host tissues whereby host factors including hormones are accepted as the microbe's environment. Microbial endocrinology provides a new avenue of approach to infection biology via understanding bi-directional interactions between host and microorganisms. It appears that host hormones can be recognized by microorganisms and regulate their behaviors such as growth, virulence, antimicrobial susceptibilities and gene expressions (1–3). The effects of stress hormones on microbial behaviors during the infectious process have been investigated in many studies (1, 4-12).

Staphylococcus aureus is responsible for a number of conditions ranging from minor skin infections to serious ones including septicemia, endocarditis, pneumonia, or bone joint infections including osteomyelitis (13). In the present study, human osteoblast cell line (HOB) was used as

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an infection model. In this model, we investigated the roles of norepinephrine (NE) in the regulation of growth, adhesion and invasion properties of a methicillin resistant *Staphylococcus aureus* (MRSA) strain, viability and cell death pathways of HOB cells. Moreover, the response of MRSA to oxidative stress (in the existence of hydrogen peroxide; H_2O_2) was examined in tryptic soy agar (TSA) medium in the presence of NE.

MATERIALS AND METHODS

Bacterium, Medium and Hormone

MRSA ATCC 43300 strain was used in our experiments. The overnight culture of the bacterium was prepared in tryptic soy broth and incubated at 37°C for 24 hours. The bacterial suspension was prepared to arrange the initial bacterial count to 10^7 CFU/mL.

Physiological levels of norepinephrine (NE); (Sigma) in a healthy individual were taken into consideration to decide the two concentrations (low concentration: 0.0017 µg/mL and high concentration: 0.04 µg/mL).

Human Osteoblast Cell Culture

Due to the fact that *Staphylococcus aureus* may cause bone destruction and loss (14), we used HOB cell line (Sigma-Aldrich) in our experiments. High-glucose Dulbecco's modified eagle medium (DMEM; Sigma) was used for the cell culture. We added 10% fetal bovine serum (FBS; Biowest), 1% 2mM L-glutamine (Biological Industries) and 1% penicillin/streptomycin (Biological Industries) into DMEM.

For detecting bacterial growth, HOB cell viability and HOB cell death pathway, HOB cells were seeded at 1×10^4 density per well; for bacterial adhesion and invasion assays, the seeding density was 5×10^4 per well.

All plates were incubated to obtain confluent monolayers with cell densities 4×10^4 in 96-well plate and 2.4×10^5 in 24-well plate.

Bacterial Inoculation Into HOB Cell Line

DMEM medium was aspirated and replaced with an antimicrobial solution-free medium. Two concentrations of NE were added to each well (20µL for each well/96-well plate and 50µL for each well/24-well plate) for the experiments. Cell cultures without hormones were used as controls.

After hormone addition, the microplates were incubated at 37°C for one hour. Human osteoblast cell culture was inoculated with an overnight culture of MRSA and bacterium was grown at 37°C for different periods in HOB cells. The length of the period depended on the experiment where three, six and 24 hours were given for bacterial growth, 1 hour for bacterial adhesion, three hours for bacterial invasion assays, and four hours for HOB cell viability and HOB cell death pathway at 37°C in CO_2 (15-18).

Bacterial Growth Assay

The wells containing HOB cells with/without NE at two different concentrations were inoculated with MRSA strain (bacterial counts as 10^7 CFU/mL) and incubated for three, six and 24 hours. The effect of NE was determined by comparing the absorbance of growth (600nm) in infected HOB cell culture with/without hormone by the spectrophotometric method at 600 nm (19).

All experiments were repeated independently three times and conditions were analyzed thrice.

Bacterial Adhesion and Invasion Assays

Bacterial adhesion and invasion stages were followed as reported previously (15). After inoculation for one hour at 37°C, phosphate buffer saline (PBS) was used to wash the HOB cells to distract unbound bacteria. The cells were lysed with 500 µL Triton X-100 (0.025%) and the culture dishes were incubated for 5 minutes at 37°C under 5% CO_2 conditions. After homogenization, TSA was used for inoculation of cell lysates and incubated for 24 hours at 37°C. The adhered-bacteria numbers were detected by colony counting.

Invasive bacterial numbers were detected as mentioned above with minor variation. PBS was added into the wells for washing, after HOB cells were incubated with bacteria at 37°C for three hours. To destroy extracellular bacteria, a fresh medium containing 200 µg/mL gentamycin was added. The microplates were incubated at 37°C for one hour. For quantification of invasive bacteria, Triton X-100 was used for lysing of HOB cells. Homogenized cell lysates were inoculated as mentioned above.

The colony counting method was used to detect the adhered and invasive bacterial counts (as colony forming units (CFU)/mL) obtained from inoculated HOB cell lysates.

All conditions were repeated three times and each experiment was performed thrice.

HOB Cell Viability Assay

We used methyl thiazolyl diphenyl-tetrazolium bromide (MTT) to detect HOB cell viability. MTT stock solution (12 mM; Neofrox 3580 MTT) described by Mosmann was used (16). The contents were aspirated from the wells after 4 hours of incubation at 37°C, and 70µL of dimethyl sulfoxide (DMSO) was added to the wells for 10 minutes. The composed formazan crystals showing the metabolic activity of HOB cells were measured spectrophotometrically at 540 nm. Then, cell viability was calculated according to the equation: Viability % = (Treated cells absorbance / Non-treated cells absorbance) × 100.

The effects of MRSA and/or NE at different concentrations on HOB cell viability were detected by comparing control conditions' absorbance data.

Each experiment was repeated twice and all samples were evaluated in duplicates.

HOB Cell Death Assays

Acridine Orange/Ethidium Bromide (AO/EB) Dual Staining

After labeling all nuclei with acridine orange (AO) and ethidium bromide (EB), the frequency of cell death (apoptotic or necrotic

mechanism) was determined by fluorescent microscopy as described by Tapajós et al. (17). The dye was applied directly onto the cells without removing them by trypsinization from the wells. AO/EB staining solution (1µL) (dye mixture containing 100 µg/mL AO and 100 µg/mL EB) was added to the cells. H_2O_2 (0.2 mM) treated cells were used as the positive control for four hours.

After the addition of AO/EB dye (20 minutes), the cell morphology was examined under fluorescent microscope (Carl-Zeiss/Axio Observer 3, Zen 2.3 Blue Edition software).

Visual analysis were used to assess apoptosis or necrosis as well as cell survival according to criteria described previously, namely, alive cells have a homogeneous green nucleus and red-orange cytoplasm; apoptotic cells have chromatin condensation and an uneven green nucleus; and necrotic cells have no cell membrane (uniformly red-stained cell nuclei) (17).

At least 200 cells were counted for statistical analysis. The experiments were repeated independently three times.

Dichlorofluorescein-Diacetate (DCF-DA) Staining

By staining with DCF-DA, the degree of reactive oxygen species (ROS) was determined [DCF-DA (D6883) 50mg; Sigma-Aldrich] under a fluorescence microscope. The amount of green fluorescence is proportional to the amount of ROS. This approach for measuring ROS in living cells is frequently utilized (18). It is known that ROS can cause apoptosis in different kinds of cell types. Therefore, to determine whether generation of ROS has any role in cell death, the degree of ROS was measured.

HOB cells were treated as explained above for four hours. After the treatment, the cells were rinsed with PBS (pH 7.4). The positive control was H_2O_2 (0.2 mM) treated cells.

DCF-DA stock solution (20 mM) was prepared and then diluted in DMSO (0.1 mM culture medium). DCF-DA staining was performed as described previously by application of the dye directly onto the cells without removing them by trypsinization from the wells (18).

The cells were then stained with DCF-DA (0.1 mM) and covered with aluminum foil for 30 minutes at 37°C. Finally, the cells were rinsed in PBS and were examined using fluorescent microscope (Carl-Zeiss/Axio Observer 3, Zen 2.3 Blue Edition software).

A single 300 ms exposure was used to capture fluorescence photos, followed by differential interference contrast (DIC) images in the same field of view. The each cell's fluorescence intensities were measured in a 60 x 60-pixel box by Zen 2.3 Blue Edition software, and the average of at least 100 cells per well was taken (18).

Bacterial Response to Oxidative Stress

To detect the influence of NE on the sensitivity of MRSA strain to H_2O_2, an agar dilution method was used as previously described (20). The initial suspension of MRSA strain was adjusted to $2x10^8$ CFU/mL from the overnight culture.

TSA including 2, 1, 0.5, 0.2 and 0.1 mM H_2O_2 and/or two different concentrations of NE were prepared. The spot inoculation (3 µL) method was used and agar plates were incubated for 24 hours at 37°C. The results were evaluated considering the growth/no growth of MRSA.

Statistical Analyses

Differences between the results of experimental and control conditions were statistically analyzed. The growth alterations were assessed using one-way ANOVA (Dunnett's multiple comparisons test). Two-way ANOVA followed by Dunnett's multiple comparisons test was used for the analysis of adhesion and invasion results. Multiple comparisons were examined by Tukey's procedure for MTT and microscopic analyzed on GraphPad Prism 8.3.0. All results were presented as mean±standart deviation. The significant differences were considered as p values, less than 0.05.

RESULTS

The Alteration of MRSA Growth in the Presence of NE

Norepinephrine, at two concentrations, was shown to have no effect ($p>0.05$) on the growth of MRSA strain at three-, six- and 24-hour periods in cell culture conditions (Figure 1). Even though the alterations were not statistically significant, the growth of bacterium was decreased in three and six hours of incubation. However, if the incubation was prolonged to 24 hours, the growth was shown to be increased in the presence of both two NE concentrations.

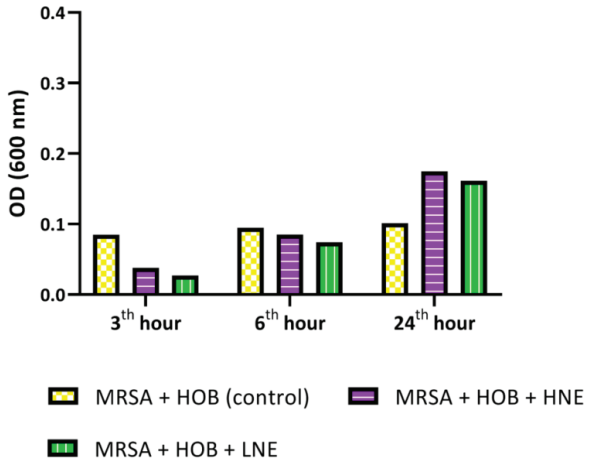

Figure 1. The alteration of MRSA growth in the presence of NE.

The growth of MRSA in HOB cell culture with/without NE was analyzed using one-way ANOVA (Dunnett's multiple comparisons test). HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL)

The Alteration of Adhesion-Invasion of MRSA in the Presence of NE

Both concentrations of NE statistically significantly ($p<0.0001$) decreased the adhesion of MRSA. However, the invasion of MRSA was increased (p: 0.013) only in the presence of high-level NE (Figure 2).

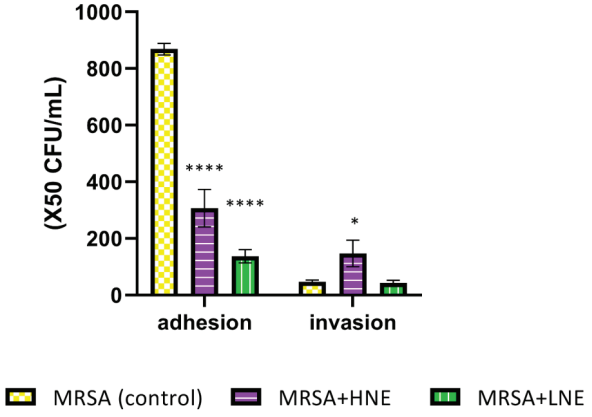

Figure 2. The alteration of MRSA adhesion and invasion in the presence of NE.

The adhesion and invasion of MRSA to HOB cell culture with/without NE were examined using two-way ANOVA (Dunnett's multiple comparisons test). HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL) (****, *p<0.0001 and p=0.013, respectively)

The Alteration of HOB Cell Viability in the Presence of NE and/or MRSA

The MTT experiment revealed that the HOB cell viability was reduced in the presence of both NE concentrations and/or MRSA (p<0.001; Figure 3).

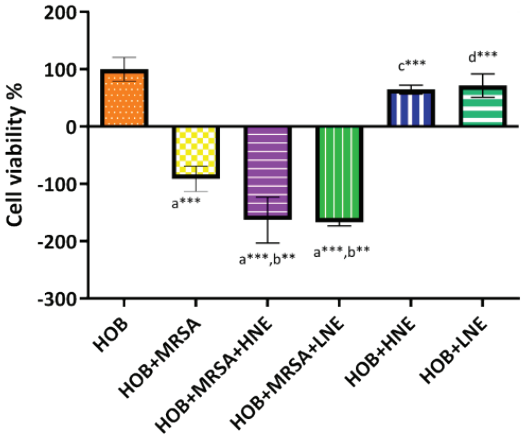

Figure 3. The alteration of cell viability in the presence of NE and/or MRSA.

The cell viability with/without NE and/or MRSA were examined using one-way ANOVA (Tukey multiple comparison tests) (**, ***p<0.0001). HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL) The characters showed comparions of experimental groups' statistically significant results as mentioned in Figure 3 are: **a:** HOB (non-treated negative control), **b:** HOB-HOB+MRSA inoculated group, **c:** HOB- HOB+MRSA+HNE inoculated group, **d:** HOB- HOB+MRSA+LNE inoculated group

Cell Death in the Presence of NE and/or MRSA

As shown in Figure 4, AO/EB staining demonstrated that MRSA and MRSA+HNE/LNE caused mainly necrosis in HOB cells (necrosis: 98.33±1.16% by MRSA; 98.67±1.15% by MRSA+HNE; 97.67±0.58% by MRSA+LNE) when compared to the negative control (0.55±0.33) (mean±standart deviation) (p<0.001). Besides, there was no significant difference in the percentage of apoptotic and necrotic cells between HOB+MRSA (apoptotic: 1.66±1.15; necrotic: 98.33±1.16), HOB+MRSA+HNE (apoptotic: 1.33±0.88; necrotic: 98.67±1.15) and HOB+MRSA+LNE (apoptotic: 2.33±0.57; necrotic: 97.67±0.58).

However, the presence of NE at two concentrations did not cause cell death (neither apoptosis nor necrosis) significantly (p>0.05). There was also no significant difference in the percentage of apoptotic and necrotic cells between HOB+HNE (apoptotic: 5±1; necrotic: 0.58±0.33) and HOB+LNE (apoptotic: 2.67±1.5; necrotic: 0.58±0.33). Microscopic images stained by AO/EB are shown in Figure 5.

As illustrated in Figure 6, according to DCF-DA experiment results, it was found that MRSA and/or NE did not cause ROS production in HOB cells significantly (p< 0.05).

Figure 7 shows representative microscope images from the DCF-DA assay sample.

Bacterial Response to Oxidative Stress in the Presence of NE

MRSA strain was resistant to all H_2O_2 concentrations tested. The presence of both two NE concentrations did not alter the susceptibilities.

DISCUSSION

Many studies have shown the effects of mammalian hormones on the regulation of physiological properties of both mammalian cells and microorganisms. Microbial endocrinology concept has provided a basic understanding of the inter-kingdom interactions. It is well known that catecholamines can regulate the immune system. Norepinephrine is known as a neurotransmitter that can affect the inflammatory process directly or indirectly (21). Furthermore, the level of norepinephrine secretion can be stimulated under stress conditions (22). As previously reported, up-taking of iron in limited conditions, hormone-mediated induction of auto-inducers, acting as quorum sensing compounds have been reported to be the possible action mechanisms of NE (1-3). At present, it is commonly known that during the evolutionary process, microorganisms improved their abilities to modulate the growth, metabolic pathways, virulence and antimicrobial susceptibilities in a host (1-3).

Considering this view, this study determined the role of norepinephrine in the modulation of some biological properties (growth, adhesion, invasion and response to oxidative stress) of methicillin-resistant *Staphylococcus aureus* strain inoculated into human osteoblast cell culture imitating the host environment as much as possible. Furthermore, we investigated the influences of MRSA infections and/or NE on HOB cell viability and cell death pathway.

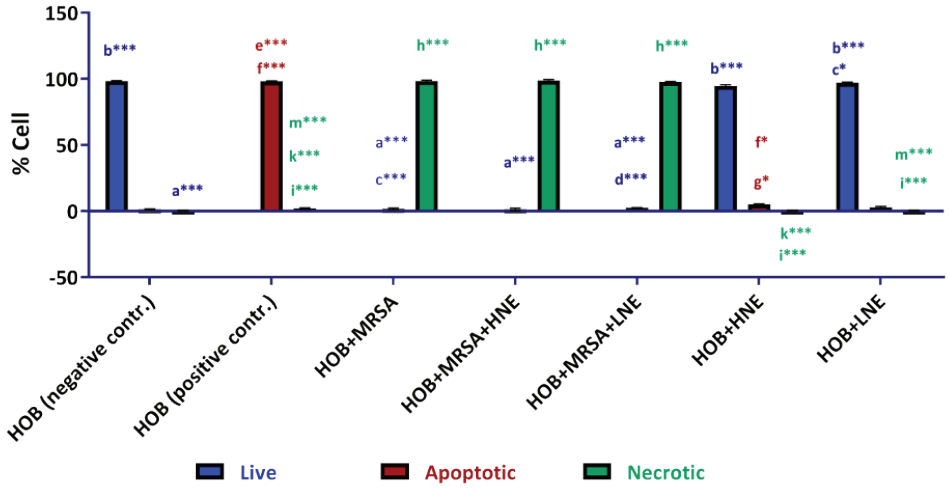

Figure 4. The alteration of cell death frequency in the presence of NE and/or MRSA.

MRSA and/or NE induce necrosis. The percentage change of the proportion of necrotic cells and the untreated negative control was compared, and one-way ANOVA (Tukey multiple comparison test) was used.
(*, **, *** $p<0.05$, indicates a significant difference compared with the negative control, n=3).
H_2O_2 (0.2 mM)- treated HOB cells (positive control), non-treated HOB cells (negative control)
HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL)
The characters showed comparions of experimental groups' statistically significant results as mentioned in figure 4 are:
a: HOB live cell numbers (non-treated negative control), **b:** HOB live cell numbers (H_2O_2-treated positive control), **c:** the live cell numbers of HOB+HNE, **d:** the live cell numbers of HOB+LNE, **e:** apoptotic cell numbers of H_2O_2-treated positive control, **f:** apoptotic cell numbers of non-treated negative control, **g:** apoptotic cell numbers of HOB+MRSA+HNE, **h:** necrotic cell numbers of non-treated negative control, **i:** necrotic cell numbers of HOB+MRSA, **k:** necrotic cell numbers of HOB+MRSA+HNE, **m:** necrotic cell numbers of HOB+MRSA+LNE.

According to some previous studies, norepinephrine decreased the growth of various microorganisms such as *Prevotella intermedia, Porphyromonas gingivalis,* some *Actinomyces* strains, *Escherichia coli (E. coli)* in microbiological culture media (4, 5). However, others showed that the growth of *Staphylococcus epidermidis, Helicobacter pylori, Streptococcus (S) pneumoniae, Vibrio harveyi* and *Campylobacter jejuni (C. jejuni)* was stimulated by NE (1, 6-8). In some studies, there was no alteration of microbial growths in the presence of NE (2, 23, 24).

In the present study, NE did not affect the growth of MRSA independently from the incubation period and the concentration of the hormone in HOB cell culture.

It has also been reported that host hormones can influence some infectious stages such as adhesion and invasion. It seems that in the presence of NE, the adhesion of EHEC O157:H7, adherent-invasive *E. coli, C. jejuni* and *Enterococcus (E) faecalis* strains to various host tissues (HeLa cells, Caco-2/TC-7 cells and Caco-2 cells) have been induced (8-11) but consistent with our results, the adhesion of *S. pneumoniae* to A549 cells is reported to be reduced (12). Most of the studies showed that NE acts as an inducer for the adhesion stage of different kinds of microorganisms as mentioned above, but in the frame of our results, it is possible to suggest that NE plays an inhibitory role in MRSA adhesion to HOB cells.

There are limited number of studies investigating the roles of NE on the invasion to host cells showing that NE acted commonly as an inducer for invasion of adherent-invasive *E. coli and C. jejuni* (8, 9). Our results show that HNE concentration stimulated MRSA invasion of HOB cells correlated with previous studies.

Some studies have reported that cell viability decreases with the increase of NE concentration and high hormone concentrations may cause an increase in bone destruction by inhibiting osteoblast differentiation (25, 26). Suzuki et. reported that NE has a stimulator role for MC3T3-E1 osteoblast-like cells via DNA synthesis, dependent on the increasing of NE dose (27). In our study, the administration of NE inhibited HOB cell viability is consistent with Xue-Min et al. and Grassel's findings (25, 26).

Similary, MRSA significantly decreased the cell viability, consistent with the results of a previous study (28).

To our knowledge, there are no studies to determine the influences of NE on MRSA-infected HOB cells. Furthermore, Beata et al. indicated that NE has effects on the interaction between adherent-invasive *E. coli* and Caco-2 cells which strengthens bacterial virulence (9).

In the present study, when NE was added to MRSA-infected HOB cells, the viability was also reduced significantly. In sum-

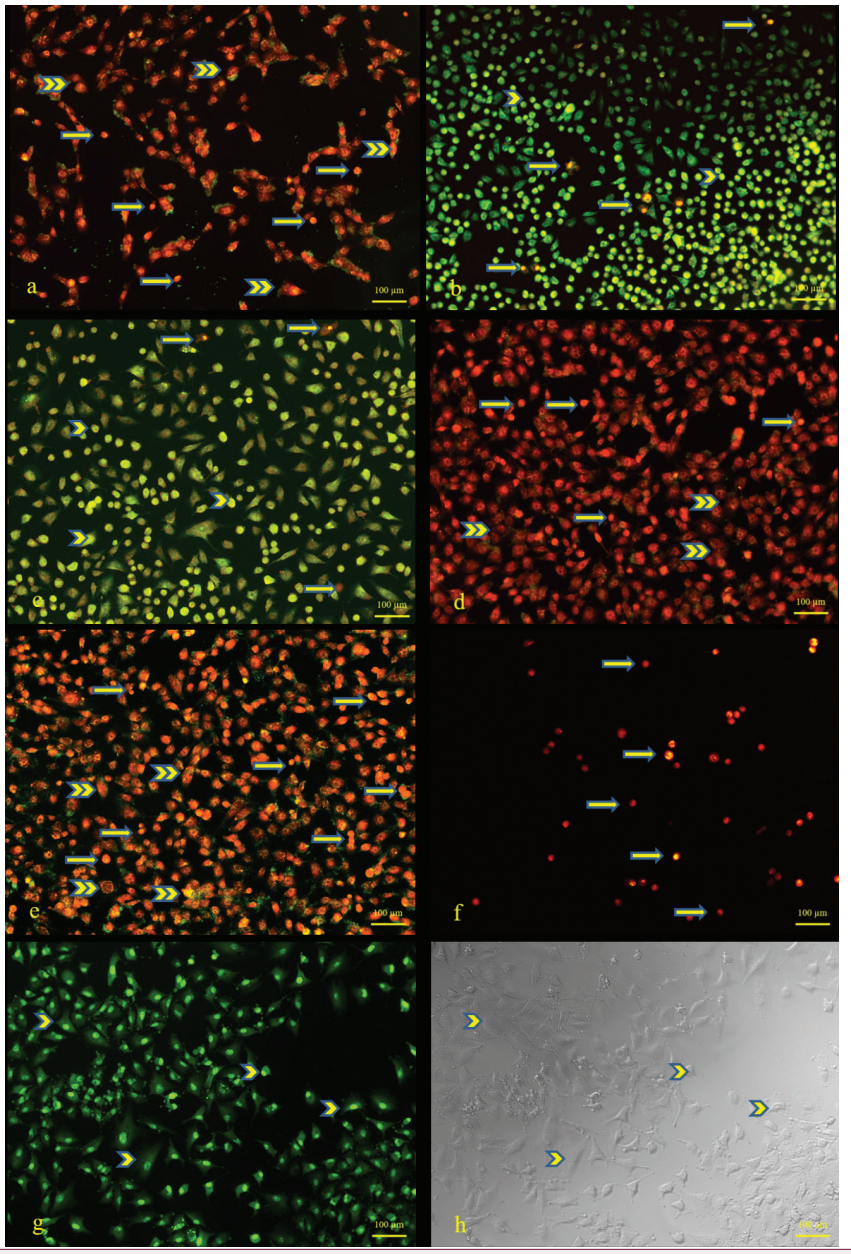

Figure 5. AO/EB dual staining of HOB cells under the microscope.

a) HOB+MRSA b) HOB+HNE **c)** HOB+LNE **d)** HOB+MRSA+HNE **e)** HOB+MRSA+LNE **f)** H_2O_2 (0.2 mM)-treated HOB cells (positive control); **g)** non-treated HOB cells (negative control) **h)** image **g**'s brightfield (Magnifications: 10x).
HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL); Apoptotic cells are shown by arrows, necrotic cells by arrowheads, and living cells by double arrowheads.

mary, our results showed that NE and/or MRSA decreased the viability of HOB cells. It is well known that NE acts as an anti-inflammatory for the host. Thus, it is possible to suggest that both NE and the presence of MRSA infection may be responsible for reducing HOB cell viability.

It was reported that NE modulates cell proliferation and apoptosis pathway on chondrocytes and reduces the rate of apoptosis (29). Ning et al. found that *Staphylococcus aureus* caused apoptosis in HOB cells during *in vitro* conditions (30). However, Choi et al. showed that MRSA had distinct effects on bone cells (*in vitro* or *in vivo*). Detecting significant necrosis rates in rats, they suggested that bone necrosis was dose dependent (31). According to our AO/EB dual staining results, cell death increased significantly in the presence of MRSA, MRSA+HNE and MRSA+LNE and this cell death occurred more with the necrosis

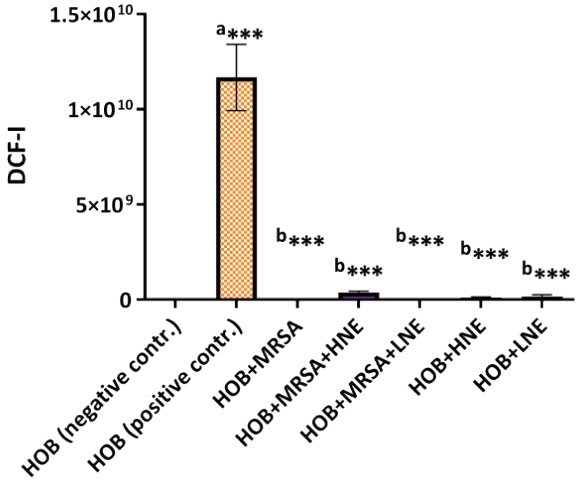

Figure 6. Cellular ROS production of HOB cells was examined by DCF-DA showing ROS detection.

DCF-I: ROS intensity averages for at least 100 cells from each well of each study group.
Used one-way ANOVA (Tukey's multiple comparison test) to analyze statistical significance [***p<0.0001 indicates a significant difference compared with control groups (H_2O_2 (0.2 mM)- treated HOB cells as positive control, non-treated HOB cells as negative control)].
HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL)

rather than apoptosis pathway (necrosis: 98.33±1.16% by MRSA; 98.67±1.15% by MRSA+HNE; 97.67±0.58% by MRSA+LNE). However, the induced pathway leading to necrosis and details of this mechanism is another subject worth investigating.

According to our ROS detection results, there was no significant increase in ROS production in the presence of NE and/or MRSA which confirms that cell death does not occur via the apoptosis pathway. In fact, it was previously reported that MRSA strains did not induce oxidative stress in osteoblast-like cells (32). However, some studies show that infection of osteoblast cells with MRSA induces apoptosis pathway that can disrupt host cells' defense barrier, actin cytoskeleton reorganization and cell proliferation (33, 34).

When an infection occurs, microorganisms encounter several environmental stress factors; one of them is H_2O_2. Oxidative molecules, such as H_2O_2, released by immune system cells destroy microorganisms (35, 36). Some microorganisms resist oxidative stress and some are susceptible. In our study, MRSA was resistant to H_2O_2 and NE did not alter its resistance.

In our study, the most important finding was the significant inhibition of HOB cell viability and induction of cell death via necrosis, by NE and/or MRSA in cell culture.

In conclusion, this study provides the knowledge about the possible influences of NE on the biological properties of bacteria and host cells, hence on the infectious process.

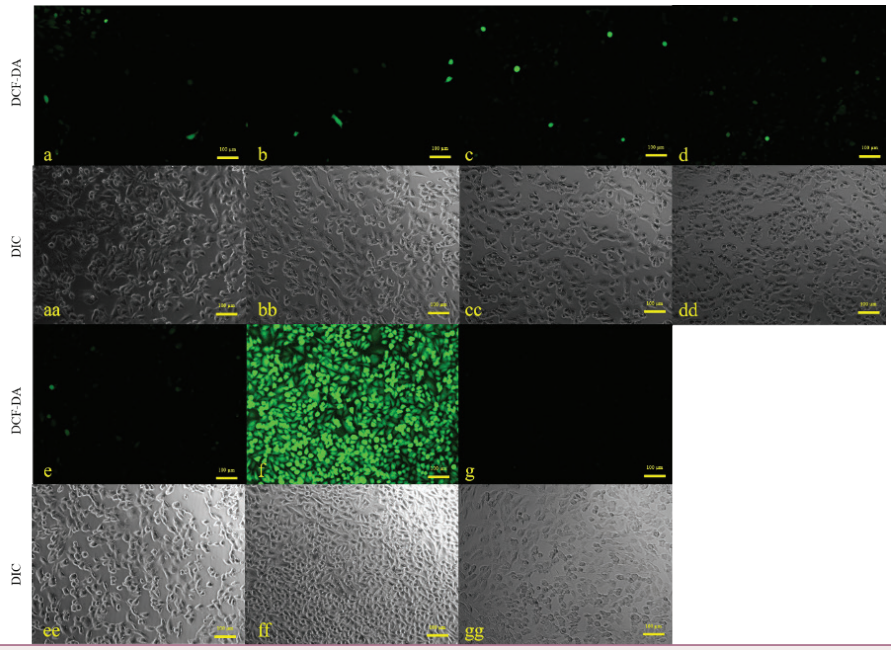

Figure 7. Representative microscope images from DCF-DA assay with fluorescence filter.

DIC brightfield's are included to show the same field with the presence of all the cells in the well.
a) HOB+MRSA **b)** HOB+HNE **c)** HOB+LNE **d)** HOB+MRSA+HNE **e)** HOB+MRSA+LNE **f)** H_2O_2 (0.2 mM)- treated HOB cells (positive control)
g) non-treated HOB cells (negative control)
HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL)
All images of brightfield are given as DIC (aa, bb, …gg). The amount of green fluorescence is proportional to the quantity of ROS (Magnifications: 10x).

Ethics Committee Approval: This study was performed in cell culture and using a standard bacterial strain. For this reason, ethics committee approval was not obtained.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study - F.K.Y., D.G., G.İ.G.; Data Acquisition - F.K.Y., D.G., G.İ.G., A.U.Ö., N.E.; Data Analysis/Interpretation - F.K.Y., D.G., G.İ.G.; Drafting Manuscript - F.K.Y., D.G., G.İ.G., M.A.; Critical Revision of Manuscript - F.K.Y., D.G., G.İ.G., M.A.; Final Approval and Accountability - F.K.Y., D.G., G.İ.G., M.A.

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