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Ultra-low power laser stimulation impairs the adhesion of *Staphylococcus aureus* to primary human cells, and interferes with the expression of staphylococcal pathogenic factors

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SUMMARY

Lasers are commonly used in several fields of medicine as a complementary therapy for internal medicine, surgery and also diagnostics. The efficacy of ultra-low level laser therapy (ULLLT) at power levels around 0.15 mW/cm^2 has been demonstrated both in *in vitro* experiments and in the clinical environment. This work used an ULLLT laser source to analyze its efficacy on *Staphylococcus aureus* adhesion to cells and on its ability to produce pathogenic factors. Laser stimulation succeeded in impairing the binding of *S. aureus* to primary human cells in culture and in inhibiting the expression of coagulase, one of the main staphylococcal pathogenic factors. The importance of the extracellular matrix (ECM) and the modification of the ECM redox potential in these activities were also evidenced.

KEY WORDS: Laser therapy, Extracellular matrix, *S.aureus*, Pathogenic factors.

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INTRODUCTION

Complementary therapies are becoming more and more popular in medicine, and LASERs (Light Amplification by Stimulated Emission of Radiation) are now widely used for several medical, surgical and diagnostic purposes (Baratto *et al.*, 2000; Baratto *et al.*, 2011; Chow *et al.*, 2009). Therapeutic efficacy mainly depends on light wavelength, irradiation duration and laser power (Bumah *et al.*, 2013; Giuliani *et al.*, 2003; Kim *et al.*, 2013; Monteforte *et al.*, 2003; Monteforte *et al.*, 2005). Another important parameter is the depth of laser light penetration into the biological tissues; this parameter depends on incident radiation intensity, wavelength and tissue optical properties (Giuliani *et al.*, 2004; Giuliani *et al.*, 2009; Lorenzini *et al.*, 2010; Chaiswing *et al.*, 2008).

Over the last ten years, an increasing number of both experimental and clinical studies have demonstrated that ultra-low level laser stimulation (ULLLT, wavelength 633nm to 670nm, but also between 400 and 500nm, or around 800nm) at extremely low power (around 0.15 mW/cm^2), is able to induce significant biological effects, which can also involve the extracellular matrix (ECM) (Baratto *et al.*, 2000; Dai *et al.*, 2012; Enwemeka, 2013; Giuliani *et al.*, 2004; Giuliani *et al.*, 2009; Topaloglu *et al.*, 2013).

The ECM is an active and biologically dynamic tissue, which acts as a structural and nutritional agent for tissues and body organs (Zamir & Geiger, 2001; Karu *et al.*, 2001). It is composed of several proteins, namely fibronectin, collagen, laminin, and also glucosaminoglycans. This substance is often employed as a substrate for cell culture since *in vitro* it reproduces the basal membrane (Karu *et al.*, 1996; Karu *et al.*, 2008; Bolognani *et al.*, 1994). ECM is also

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important in infections because it is the substance that bacterial pathogens meet when they start an infection of the subepithelial tissues (Chaiswing *et al*., 2008; Haucka & Ohlsen, 2006; Chavakis *et al*., 2005).
Staphylococcus aureus is one of the most common infectious agents in humans. Several studies have confirmed the importance of the interactions between the EMC and *S. aureus* in causing human tissue infections (Haucka & Ohlsen, 2006; Chavakis *et al*., 2005; Agerer *et al*., 2003). To start an infectious process the pathogenic bacteria must adhere to host tissues by means of specific adhesins, which interact with some components of the ECM (Massey *et al*., 2001; Schwarz-Linek *et al*., 2004).
Human beings are a natural reservoir of *S. aureus*, which is the most common agent of skin and wound infections. Moreover, a series of exoenzymes, such as the staphylococcal coagulase, have a determinant role in the pathogenesis of inflammatory processes induced by *S. aureus* (Haucka & Ohlsen, 2006).
The aim of the present work was to study the effect of ULLLT treatments on the interactions between the human pathogen *S. aureus* and primary human cell cultures in vitro. We found that ULLLT can induce significant modifications in ECM redox potential and in *S. aureus's* ability to bind to cell monolayers and to produce important pathogenic factors.

MATERIALS AND METHODS

Bacteria and ECM
The *S. aureus* ATCC 25293 collection strain was used for the microbiological experiments. It was isolated in mannitol salt agar and grown in BHI medium for cell counting. The Cultrex gel (C-ECM, BD Bioscience, USA) was used as an analog of ECM; stock solutions of C-ECM were kept at -80°C until use. 200 µl of C-ECM were injected onto a 48 well plate, which was then incubated at 37°C for 30 min for complete polymerization.

Ultra-low level laser source
The laser device employed in the experiments was a Biolite, a medical device offering a square wave modulated coherent red light from a very-low power diode laser (Baratto *et al*., 2011). Biolite LP020 is an internationally patented medical device designed and produced by RGMD (Genoa, Italy). Biolite emission characteristics are as follows:
- Spot Area Ø 4 mm =12.6 mm^2 =0.13 cm^2
- Peak Power (P_p) 3 mW
- Mean Power 1 (P_{M1}) =0.03 mW ($Pulsed_1$, at 100 Hz - Duty Cycle 1%)
- Mean Power 2 (P_{M2}) =0.015 mW ($Pulsed_1$, as above AND $pulsed_2$ at 1 Hz - Duty Cycle 50%)
- Peak Power Density (D_{Pp}) =23 mW/cm^2 =2.3 W/m^2
- Mean Power 1 Density (D_{Pm1}) =0.23 mW/cm^2 =0.023 W/m^2
- Mean Power 2 Density (D_{Pm2}) =0.13 mW/cm^2 =0.013 W/m^2
- Energy per point (20 sec flashed emission) (E_p) =0.3 mJ
- Energy Density per point (D_{Ep})= 2.3 mJ/cm^2 =0.23 J/m^2
- Total Energy per Therapy Session (TETS) =E_p X N_p
- (Number of treated points: under 10) <3 mJ.

The Ton/(Ton+Toff) ratio, namely the duty cycle (DC), allows fast calculation of the average emitted power definition (P_A) from the device peak power (P_P): P_A=Pp x D_C. The emission frequency (F) is 1/(Ton+Toff) ratio. Thus the Ton and Toff combinations selected for this test provide frequencies consistently around 100 Hz, but at different power levels. In fact, in this study the working parameters were adjusted, as indicated in Table 1. A typical Biolite application envisages energy doses of about 0.002 J/cm^2, which are far below those normally used for physiotherapy purposes (Baratto *et al*.,

TABLE 1 - *Biolite working parameters used in this study*

Code	*Ton*	*Toff*	*F (Hz)*	*DC (%)*	*Pot (mW)*	*Dose 1*	*Dose 2*	*Dose 3*
A	0,1	9,9	100	1	0,03	20 sec	40 sec	80 sec
B	1	9,9	92	9	0,28			

2000; Baratto *et al*., 2011; Chow *et al*., 2009; Giuliani *et al*., 2003; Monteforte *et al*., 2003; Monteforte *et al*., 2005).

Detection of pH and redox potential of C-ECM
Biolite activity on the C-ECM pH and redox potential (RP) was determined to define the working power to be used in the experiments. The C-ECM was diluted 1:25 and was poured onto a 24 multiwell plastic plate with 1 ml of PBS per well. The RP was measured in a HI 8417 apparatus (Hanna Inst, USA). All the tests were performed at least twice and three different Biolite doses could be assayed in the various experiments (Table 1).

S. aureus growth and adhesion to human primary cell cultures
Human umbilical vein cells (HUVEC, Life Technologies, Italy) were cultured in Medium 200 supplemented with LSGS (Life Technologies). Twenty-four multiwell plates were used. A 1:25 dilution of C-ECM was injected into some of the wells. About 1,000 cells were cultured in each well for 24 h at 37°C in a CO2 incubator. Then the cells were washed, and antibiotic-free medium was added.
The cells underwent a laser treatment either before or after the addition of the *S. aureus* suspension. About 1,000 CFU of *S. aureus* were added to the wells, and incubated for 2 h at 37°C. After this time, the non-adherent bacteria were removed with three washes in PBS, and the cell monolayer was lysed with a solution of 0.25% triton X100 in distilled H_2O. With this system, both surface adherent and internalized bacteria were collected and counted on BHI agar plates.

ULLLT treatment of staphylococcal coagulase
A 18 h BHI culture of *S. aureus* was used to study the effect of Biolite treatment on the staphylococcal coagulase. 200 µl of diluted C-ECM were poured onto a 48 multiwell plate; this was kept at 37°C for 30 min to allow polymerization. 250 µl of a bacterial suspension were added to each well, as well as 250 µl of sterile rabbit plasma (Microbiol, Italy, cod. M 1032). The plate was incubated in a CO_2 incubator at 37°C and coagulase activity was checked every 40 min up to 24h, so as to detect the complete plasma coagulation.

RESULTS

Determination of pH and redox potential of C-ECM after treatment with ULLLT emissions
When the C-ECM was treated with Biolite, the pH solution remained practically unchanged between 7.2 and 7.4 (data not shown).
However, the redox potential showed very significant modifications depending on the energy dose; in actual fact, the potential was equal to -4.8 mV in the untreated control, whereas in the samples treated with Biolite 20 s (DOSE

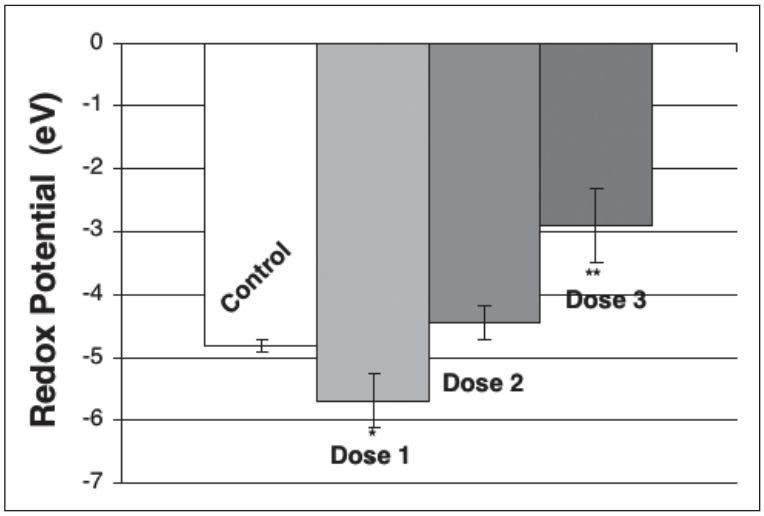

FIGURE 1 - *The effect of Biolite treatment on C-ECM redox potential. The C-ECM was treated in vitro with different energy doses of low power 100 Hz pulsed laser (3 mW Peak Power, Duty Cycle 1%). The ratio between "DOSE 2" and the control was not significant, whereas the ratio between "DOSE 1"* and control was significant (p= 0.03) and the ratio between "DOSE 3"** control was highly significant (p<0.01).*

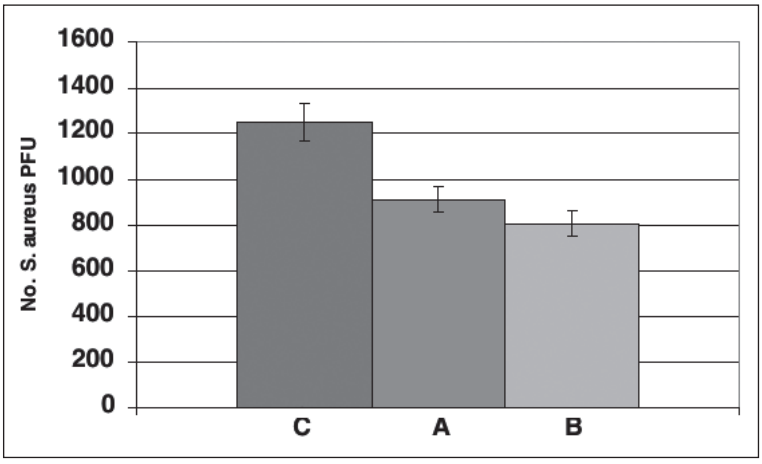

FIGURE 2 - *Effect of Biolite treatment on S. aureus adhesion to primary HUVEC cells\ in vitro. C: control. A: Biolite treatment (DOSE 3) was performed only on cells immediately before S. aureus injection; B: Biolite treatment (DOSE 3) was performed after S. aureus injection on cell monolayers. PFU: Colony forming units. Statistics: A versus C: p=0.0048; B versus C: p=0.0016.*

1, CODE A, Tab. 1), the mean value decreased to -5.7 mV; with Biolite 40 s (DOSE 2, CODE A) the values slightly increased to -4.4 mV, and finally with Biolite 80 s (DOSE 3,CODE A) a further increase in the redox potential could be observed up to a mean value of -2.9 mV.
Figure 1 shows the media of three experiments; the media differences are significant for DOSE 1 of treatment (p=0.038) and for DOSE 3 compared to the control (p=0.013).
The difference between the sample treated with DOSE 2 and the control was not significant.

Effect of ULLLT on S. aureus adhesion to primary cells in culture

Biolite treatment (DOSE 3, CODE A, Table 1) has been shown to significantly interfere with the capacity of the cultured Huvec cells to bind and keep living staphylococci adherent. This result concerns all the bacteria attached to the cells, and no difference was considered among adherent or internalized bacteria. As shown in Figure 2, in all cases, either when the cells were irradiated before or after *S. aureus* injection, the treatment with ULLLT resulted in a

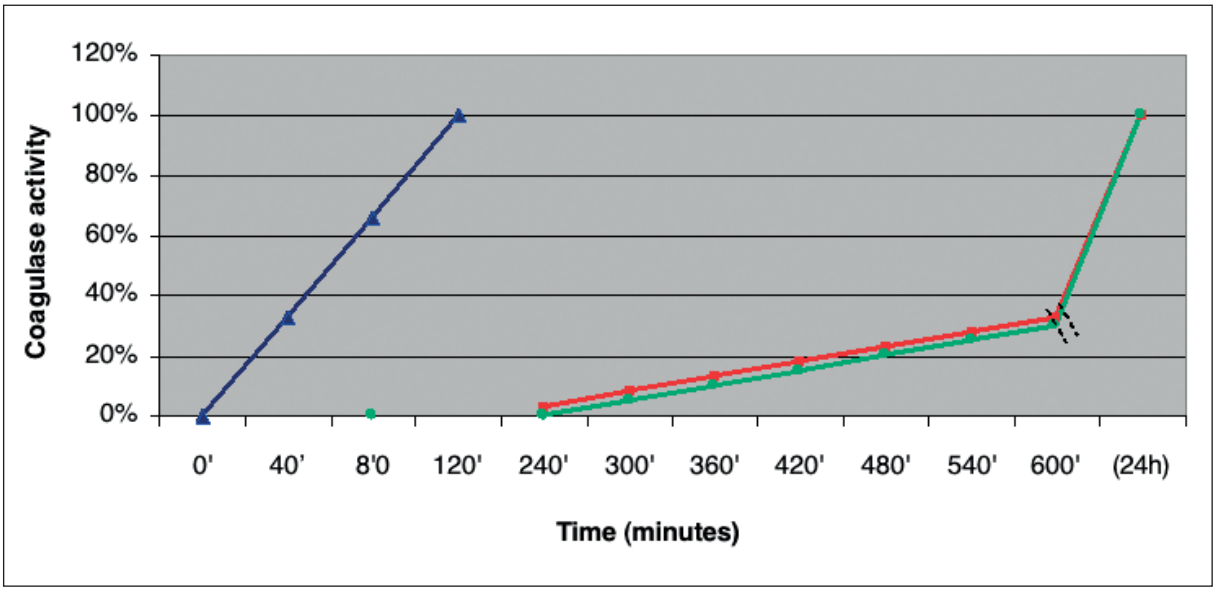

FIGURE 3 - *Effect of Biolite treatment on staphylococcal coagulase. Blue line: untreated control; green line: Biolite treatment, DOSE 3 (CODE A); red line: Biolite treatment, DOSE 3 (CODE B). Each sample was performed in triplicate. Statistics: red line versus blue line: p<0.01; green line versus blue line: p<0.01.*

significant reduction of the number of bacteria adherent to the cultured cells. Biolite treatment before bacterial inoculation induced a decrease in bacterial adhesion by about 30% (p=0.0048), whilst with treatment after bacterial inoculation, adherent bacteria were even lower, decreasing by as much as 35% (p=0.0016).

Effect of ULLLT treatment on S. aureus coagulase activity in vitro

The activity of Biolite treatment on staphylococcal coagulase was evaluated by means of an *in vitro* test with the *S. aureus* collection strain. Two different treatment methods with laser sources were used; in the first experiment, the Biolite power was set at Ton 0.1 and Toff 9.9 (DOSE 3, CODE A), in the second test, treatment intensity was Ton 1.0 and Toff 9.9 (DOSE 3, CODE B). In both cases, Biolite treatment induced a consistent modification of the staphylococcal coagulase activity, as compared to the control. While coagulase clot formation was complete in the control within 40-120 min, in the laser-treated samples, the clot formation started slowly after about 4 h, and was only complete around 24 h after the start of the experiment. As shown in Figure 3, the difference between the control and the samples treated with ULLLT was statistically significant ($p<0.05$). However, no difference was found between CODE A or CODE B treatment, suggesting a likelihood of frequency-related effectiveness, rather than a power-energy related effectiveness.

DISCUSSION AND CONCLUSIONS

ULLLT, as used in this study, is able to induce important modifications in some pathological properties of *S. aureus* in culture *in vitro*. *S. aureus* partly loses the ability to adhere to and colonize human cultured endothelial cells. This effect is obtained if cell laser treatment occurs either before or after bacteria inoculation. Since we found that laser treatment, at the wavelengths used in this study (630-670nm), does not alter staphylococcal viability, unlike blue or near infrared laser light (Bumah *et al.,* 2013; Topaloglu *et al.*, 2013), it is probable that Biolite laser treatment, as found in our experiments, mainly exerts its activity on cell structures necessary for bacterial adhesion and colonization. Bacterial adhesion is the first step in the development of an infectious disease. After adhesion, the microorganisms can replicate and colonize the tissues, inducing tissue injury directly or by producing pathogenic factors. In actual fact, previous experiments have demonstrated that laser treatment can interfere with staphylococcal coagulase activity, which has a decreased enzymatic activity, and requires as much as 24 h to produce a complete plasma clot formation. Since the staphylococcal coagulase is an important *S. aureus* pathogenic factor, we can conclude that ULLLT is able to interfere with the pathogenicity of this bacterium.

It is known that LLLT can have significant effects on cells and tissues. Laser treatment acts on cell mitochondria and influences the cell oxidative state, inducing a reduction of the mitochondrial potential and involving calcium metabolism, the production of nitric oxide and reactive oxygen species, which exert important biological effects on cells and tissues (Baratto *et al.*, 2011; Chow *et al.*, 2009; Karu *et al.*, 2008). This work has also shown that several doses of ULLLT cause a significant increase in C-ECM redox potential from -4.8 to -2.9 mV, whereas a single dose induces a small but significant decrease in redox potential. This effect was unexpected, but not surprising, since Calabrese & Baldwin (2002) had already described some typical biphasic responses (the phenomenon of hormesis) after laser treatment. This study showed that ULLLT is also able to induce a biphasic response, depending on the different doses of laser employed. Moreover, redox potential is known to influence the activity of some proteins and enzymes (Baratto *et al.*, 2011; Chaiswing *et al.*, 2008; Haucka & Ohlsen, 2006). Considering that the cultured cells interact with the C-ECM for monolayer formation and binding to solid surfaces, the modification of the C-ECM redox potential in a system of cell-bacteria interaction could therefore be at least partly responsible for the altered adhesion of *S. aureus* to the cell surface and reduced coagulase activity. It is also important to note that the best results were obtained with ULLLT CODE A DOSE 3: gen-

erally a duty cycle of Ton 0.1 and Toff 9.0 was sufficient to obtain some significant results, and increasing the power of laser treatment did not induce better results.

In conclusion, the reported data allow us to consider ULLLT a complementary therapy for superficial staphylococcal infections, where inflammation, edema and pain are prevalent symptoms, in addition to the classical therapy, particularly when conventional therapies have limited efficacy.

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