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The influence of ultrasound on the fluoroquinolones antibacterial activity

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ABSTRACT

In this work, the antibacterial effect of fluoroquinolones (FQs) upon *Escherichia coli* (*E.coli*) was measured with and without application of 40 kHz ultrasound (US) stimulation. The research results demonstrated that simultaneous application of 40 kHz US apparently enhanced the antibacterial effectiveness of FQs. That is, the synergistic effect was observed and the bacterial viability was reduced when FQs and US were combined. In addition, various influencing factors, such as FQs drug concentration, US irradiation time and solution temperature, on the inhibition of *E.coli* were also investigated. The antibacterial activity was enhanced apparently with increasing of FQs drug concentration, US irradiation time and solution temperature. Furthermore, we discussed preliminarily the mechanism of US enhanced antibacterial activity. Results show that US can activate FQs to produce reactive oxygen species (ROS) indeed, which are mainly determined as superoxide radical anion ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH$).

1. Introduction

With increasing antibiotic resistance among bacterial species, it becomes important to explore some novel approaches to overcoming the changing resistance. It was reported that the electric field dramatically enhanced the efficacy of antibiotics in killing biofilm bacteria [1,2]. Moreover, the number of viable *Pseudomonas aeruginosa* was decreased significantly after combined exposure to a magnetic field and gentamicin antibiotic [3]. Some researchers have recently started to pay attention to photodynamic therapy as a possible treatment for localized infections, because of the well-known increase in resistance among pathogenic microbes [4]. Some evidence suggests that multi-antibiotic resistant strains are as easily killed by photodynamic antimicrobial chemotherapy (PACT), and that bacteria will not readily develop resistance to PACT [5]. In a similar approach, low-frequency ultrasound (US) has been demonstrated to increase the efficacy of antibiotics against biofilm bacteria, known as the bioacoustic effect [6–8]. Focused US can penetrate into tissue more deeply than light and can be focused into a small region to activate sonosensitizers. Ma et al. hypothesized that sonodynamic antimicrobial chemotherapy (SACT) being different from traditional antiviral therapy, which utilizes US and sonosensitizers may be a promising novel antimicrobial strategy to against bacteria [9].

Fluoroquinolones (FQs) are potent, broad spectrum antimicrobial agents and have a very good safety and tolerability profile. Our previous study demonstrated that ciprofloxacin (CPFX) and levofloxacin (LVFX) showed obvious sonodynamic activity, that is, the addition of them to the liquid could accelerate reactive oxygen species (ROS) generation under US irradiation [10]. So, our work focused on combining CPFX and LVFX with a safe and accepted level of US to render a bacterium more susceptible to the antibiotic [11].

In this paper, the experiment was carried out in two steps. Firstly, the synergistic antibacterial effect of FQs and US were investigated. Meanwhile, the influencing factors, such as FQs drug concentration, US irradiation time and solution temperature, were also studied systemically. Secondly, the mechanism of the synergistic antibacterial effects was investigated by means of oxidation and extraction photometry [12]. Maybe, this report can offer a valuable reference to promote the synergistic antibacterial effects of FQs and US and simplify the detection of ROS.

2. Experimental

2.1. Materials

Escherichia coli (*E.coli*, JM 109) were provided by Bioscience Department, Liaoning University. Twenty-four hours before an experiment, an inoculum was transferred from a colony on the agar plate to 10 mL of nutrient agar (NA) and grown overnight at 37 °C. After 24 h, 0.01 mL of the culture was transferred to 9.99 mL of sterile NA and grown at 37 °C in 50 mL Erlenmeyer flasks on a rotary shaker. The number of cfu/mL suspension was measured by serial dilutions in physiological saline solution (PSS)

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and plating onto nutrient agar (NA) using the spread plate method. Plates were incubated for 24 h at 37 °C and counted.

Levofloxacin (LVFX, purity >98 %) and Ciprofloxacin (CPFX, purity >98%) were provided from Jinan Dachpharm Development Co., Ltd., China and were used without further purification (shown in Fig. 1). 10 mg drug was dissolved respectively in 25 ml distilled water to produce a 0.4 mg/ml solution; 1.0 ml of this solution was diluted to 10 μg/ml with distilled water and filter-sterilized. Dilutions of antibiotic used in the experiment were taken from this stock solution and diluted with sterile distilled water. Diphenylcarbazide (DPCI) as trapping agent and sodium azide (NaN_3), 2,6-Di-tert-butyl-4-methylphenol (BHT) and Vitamin C (VC) as quenchers of reactive oxygen species (ROS) were obtained from Sinopharm Chemical Reagent Co., Ltd., China. All solutions were prepared using doubly distilled water.

2.2. Apparatus

US exposure was generated with a Controllable Serial-Ultrasonics apparatus (KQ5200DB, Kunshan Ultrasonic Instrument Co., Ltd. China) shown in Fig. 2 operating at 40 kHz with a power density of about 1 W/cm^2. The US bath was filled with approximately 6.5 L distilled water and maintained at 37 °C by recirculation.

2.3. Measurements of bactericidal activity

Firstly, six 50 mL Erlenmeyer flasks, which contained 20.0 mL sterile broth, were marked with a–f, respectively. Six 0.2 mL *E.coil* storage solutions (10^6 cfu/mL) were taken exactly and put into the Erlenmeyer flasks respectively. Then, two 0.5 mL sterile distilled water were added to Erlenmeyer flasks a and b, respectively. After that, two 0.5 mL LVFX and CPFX storage solutions (0.4 mg/mL) were added to Erlenmeyer flasks c, d and e, f, respectively. Afterwards, b, d and f were placed in an ultrasonic apparatus (1 W/cm^2 at 20 °C). Others were only placed in an incubator in a separate room. After 45 min, six 100 μL sample solutions from Erlenmeyer flasks were plated onto nutrient agar and incubated the agar plates for 24 h. The bacteriostasis of drugs were measured by the numbers of bacteria on the agar plates.

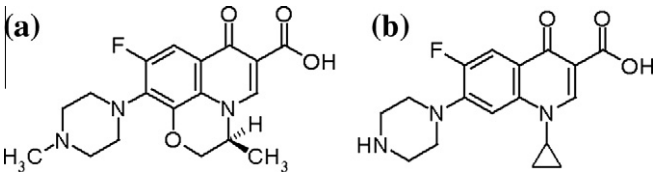

Fig. 1. Molecular structures of LVFX and CPFX.

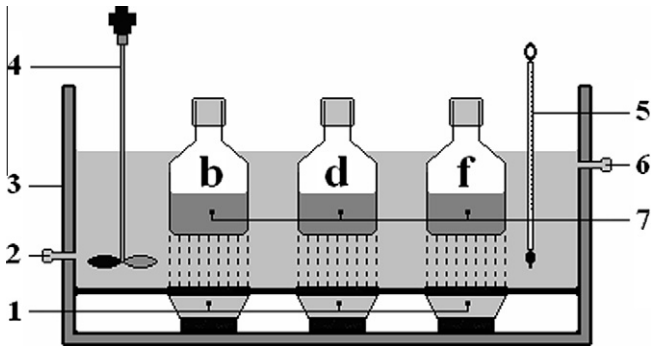

Fig. 2. The apparatus of ultrasonic irradiation (1: ultrasonic transducer; 2: intake; 3: flume; 4: stirrer; 5: thermometer 6: outlet; 7: reactor; b: only US; d: US + LVFX; f: US + CPFX).

In order to investigate the synergistic effect of US and FQs systematically, the effects of drug concentration, US irradiation time and solution temperature were also examined.

2.4. Identification of the reactive oxygen species (ROS)

Firstly, two aliquots of 2.0 mL DPCI and 2.0 mL LVFX were taken exactly and put into volumetric flask (a) and (b), respectively. Then, the solutions were diluted to 10.0 mL with Tris–HCl-NaCl solutions, making the final concentration of DPCI and LVFX were 5.0×10^{-3} M and 1.0×10^{-4} M, respectively. Afterwards, one of them was transferred into 50 mL conical flask and placed in an US irradiation apparatus, the other was kept in the dark. After 1.0 h, the solutions were extracted repeatedly with Benzene-CCl_4 (1:1) mixed solution extractant. The extraction liquids were diluted to 10.0 mL with the extractant and detected by UV–vis spectrophotometer. CPFX repeated the above operation. In order to investigate systematically the changes of ROS level under US irradiation, the effects of FQs drug concentration and US irradiation time were also studied. Furthermore, the quenching effects of NaN_3, BHT and VC [13–15] were investigated. All of the quenchers' concentrations were 0.05 M.

3. Results and discussion

3.1. The effect of FQs drug concentration

Fig. 3 shows the experiment results performed at different FQs drug concentrations. The inhibitory ratios (IR) of both LVFX and CPFX were obviously higher control group's, and all increased along with the increase of FQs drug concentration under US irradiation for 30 min. The IR can be defined as:

$$IR = 1 - N_t/N_0 \times 100\% \quad (1)$$

where N_0 and N_t were the colony number of the control group and drugs group, respectively. Moreover, the IR reaches 98 % for LVFX to 10 μg/mL concentration, while it is 83 % for CPFX. It suggests that the synergistic effect of LVFX and US had better effect than CPFX under US irradiation for 30 min.

3.2. The effect of US irradiation time

Fig. 4 shows experiment results performed with FQs at 8 μg/mL. These results also showed enhanced killing of more than antibiotic alone with the increase of US irradiation time. Moreover, whether or not US, the IR of LVFX was higher than the CPFX's, also suggesting that the synergistic effect of LVFX and US had better effect than that of CPFX and US. It may be because of the ROS produced by LVFX was higher than that by CPFX. But, the data also show that, without drugs exposure, US alone had no significant impact on the cell population.

3.3. The effect of solution temperature

To verify the effect of solution temperature, the IRs of LVFX and CPFX under US irradiation were measured at 20, 30, and 40 °C, respectively. Results (in Fig. 5) showed that the IR of LVFX is higher slightly than CPFX's at the same situation. Especially, the IRs of LVFX and CPFX are all close to 100 % when temperature becomes above 30 °C after US irradiation for 30 min. As the same time, a good bactericidal effect can be gotten even at 30 °C below. It may be because of the mechanism of synergistic effect of drug and US is mainly the non-thermal effects rather than thermal effects. The non-thermal effect is traditionally referred to that cavitation