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Preparation and Investigation of In Vitro Effect of Liposomal Ceftazidime on the Resistant Pseudomonas Aeruginosa

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Abstract

The resistance of bacteria to β-Lactam antibiotics produces many problems in the treatment of bacterial infections. Although Ceftazidime is an extended antibiotic spectrum, it becomes inactive against β-Lactamase produced of Pseudomonas aeruginosa. This study clears how Liposome protects Ceftazidime hydrolysis by β-Lactamase.

For preparing Large Unilamellar Vesicles, Phosphatidylcholine and Cholesterol are solved in Ether and then Ceftazidime is added to this mixture, and after affecting this mixture by Sonicator, the uniform suspension is produced and then evaporates under vacuum condition in Rotary Evaporator until the thin layer is remained around the basin. It is used from dialysis method for isolating this drug which is entered in Liposome of outside drug and these Large Unilamellar Vesicles are seen by the lighting microscope. This bacterium is standard strain of Pseudomonas aeruginosa (ATCC 27853).

Ceftazidime Minimum Inhibitory Concentration (MIC) for standard Pseudomonas aeruginosa is 2μg/ml that shows high resistance of bacteria to Ceftazidime, but liposomal Ceftazidime MIC decreases to 0/5μg/ml. All the tests were done for 3 times and every time the result approved the right effect of the before result. Furthermore the deduction of growing bacteria in existence of liposomal Ceftazidime was observed in its natural curve.
This study shows the positive effects of these entered β-Lactam antibiotics inside Liposomes in vitro condition. Attending resistance of these bacteria that produce β-Lactamase, this method can be used for better treatment in the future.

Keywords: ceftazidime, cholesterol, liposome, MBC, MIC, phosphatidylcholine

1. Introduction

Pseudomonas aeruginosa is a member of Pseudomonas great family which is Gram-negative bacillus, mobile and oxidase-positive. It is an aerobic compulsive organism growing easily across many of culture media in a temperature of 37 to 42°C [1, 2, 3]. B-Lactamase enzyme generated by Pseudomonas aeruginosa hydrolyzes the circle of Ceftazidime β-Lactam. Ceftazidime is a 3rd generation of Cephalosporins and a β-Lactam antibiotic, which has an antibacterial effect in treating infections, resulted from Pseudomonas aeruginosa [4, 5, 6]. To protect Ceftazidime antibiotic against enzyme, we sought a medicine delivery system to prevent Ceftazidime hydrolysis. Liposome is a small global vesicle, composed of a two-layered membrane containing phospholipids and Cholesterol. The most common natural phospholipid involved in preparing Liposomes is Phosphatidylcholine, an amphipathic molecule known as Lecithin [7]. Also Cholesterol thickens and stabilizes the lipid membrane. It is proved that Cholesterol has an outstanding effect in organizing lipid hydrocarbon chain [8, 9]. Liposomes accommodate water-solute medicines in their aquatic phase, and lipid-solute medicines inside their lipid membrane. This enables them to carry both hydrophilic and hydrophobic molecules. To carry the molecules to the point of action, two-layered lipid interacts with other two-layered lipids including cell membrane and thereby the Liposome contents are forwarded into the cell [10]. Due to their unique features, Liposomes are used to carry drugs, DNA, toxicity decline and maximize the stability of the encapsulated drug. They also increase the influence and therapeutic index [10, 11]. Liposomes have numerous clinical applications, in bacterial, parasitic and fungal infections, arthritics, diabetes, storage-related diseases, myocardium infarct, toxicosis, etc. [12].

2. Method

To prepare Liposome containing Ceftazidime, 9ml of distilled water was added to 1ml of Ceftazidime stock solution with concentration of 1280µg/ml to obtain the concentration of 128µg/ml. Also to prepare Liposomes 30µmol of Phosphatidylcholine and 30µmol of Cholesterol were weighed with ratio of 1 to 1 and 3ml of Ether was added under a hood. Then 2ml of Ceftazidime with concentration of 128µg/ml was added to mixture. Nitrogen gas was added to prevent oxidation too. Then the two-phase system was exposed to sonic waves for 8 to 10 minutes in a bath-like Sonicator, under 0-5°C. Then the mixture was poured and evaporated in the Rotary Evaporator in reversed phase evaporation method and with rotation of 180 to 200rpm in the vacuum condition. As a result of this evaporation, at first the sample foams and then jellifies. After a while, this jell is transformed and gets a more liquid form, called Liposome suspension in water [13, 14]. Then this Liposome suspension was observed and photographed under electronic microscope with magnification of 1000. After that dialysis bag used to separate the encapsulated Ceftazidime from the Liposomes, the Liposome samples were dialyzed thrice and each time for 15 minutes against 100 PBS (Phosphate-Buffered Saline). Then the sample was centrifuged for 20 minutes with ultra-centrifuge device and with rotation of 12000rpm. In doing so, the Cholesterol was not solved but was deposited. To understand the photonic absorption of the Ceftazidime released in the solution, Blank (Lecithin, and 20% Triton x-100 in PBS) and standard solutions were prepared for spectrophotometry. The blank solution was considered as 0, and then considering the Ceftazidime calibration curve, photonic absorption of Ceftazidime was gained consequently, according to Ceftazidime calibration curve formula, and considering the dilution of the primitive Liposome sample, amount and concentration of the encapsulated Ceftazidime in Liposomes were gained. This was replicated thrice and considering the average of densities, the encapsulation efficiency percentage was calculated. Then considering the macro-broth dilution method [15], MIC was calculated. Minimum Bactericidal Concentration (MBC) is the least concentration of antibiotic in which 99.99% of a number of live cells in the primitive inoculum volume unit (1×10^8 CFU/ml) are killed. Considering the fact that Ceftazidime is a bactericide antibiotic, MBC criterion is regarded for MIC too. Finally, kinetics determination of bacterial mortality in the minimum fatal concentration of antibiotic was done and after determination of MBC, we prepared a suspension of free and liposomal drugs in MBC concentration and then put it inside greenhouse. In 0, 2, 4, 6, 8, 10, and 24-hour intervals after incubation of bacteria, we sampled that and after preparing the dilution, we cultured in Muller Hinton Agar culture media and put them in greenhouse for 24 hours. Then we counted the number of bacteria with different intervals. After 24 hours, we counted the number of colonies in the first countable plate and put them in the following formula, so that the amount of bacteria in 1ml of culture medium is gained. (Number of bacteria= counted colonies× reversed dilution× 20).

With this method it is possible to examine the effect of free and Liposome-encapsulated drugs on a given bacterium.

3. Results

To determine the concentration of Liposome-encapsulated Ceftazidime, the photonic absorption of Ceftazidime was measured thrice and the concentrations were measured thrice according to Ceftazidime calibration curve. Then they were averaged. Subsequently, according to the gained average, encapsulation efficiency percentage got about 50%. Thus of 128µg/ml concentration used in formation of Liposomes, about 64µg/ml was encapsulated. After that with the Liposome-encapsulated concentration, MIC of Ceftazidime was calculated against standard Pseudomonas aeruginosa in free and liposomal modes. Ceftazidime MIC against these bacteria in a free form was 2µg/ml and in the liposomal form was declined to 0.5µg/ml. Also, Ceftazidime MBC in a free form against standard Pseudomonas aeruginosa was 2µg/ml, and in the liposomal form was declined to 0.5µg/ml. Then the natural curve of bacterial mortality in MBC of Ceftazidime antibiotic was drawn. The result was that growth in culture medium lacking antibiotic ascended within 24 hours and growth in the culture medium containing free antibiotic reached to 0 within 24 hours, and finally growth in culture medium containing Liposome-encapsulated antibiotic reached to 0 within 10 hours.
Then the natural curve of bacterial mortality in the Minimum Bactericidal Concentration (MBC) of Ceftazidime antibiotic was drawn. The result was that growth in culture media lacking antibiotic ascended within 24 hours and growth in the culture media containing free antibiotic reached to 0 within 24 hours, and finally growth in culture medium containing Liposome-encapsulated antibiotic reached to 0 within 10 hours. Ceftazidime Minimum Inhibitory Concentration (MIC) for standard Pseudomonas aeruginosa is 2µg/ml that shows high resistance of bacteria to Ceftazidime but Liposomal Ceftazidime MIC decreases to 0.5µg/ml. All the tests were done for 3 times and every time its result showed the right effect of the before result. Furthermore the deduction of growing bacteria in existence of Liposomal Ceftazidime was observed in its natural curve.

4. Discussion

The general mechanism of bacterial resistance against β-Lactam antibiotics is production of enzymes called wide-spectrum β-Lactamses able to hydrolyze the β-Lactam circle of these drugs. This mechanism is the dominant mechanism in Gram-negative bacteria [4, 16]. To confront with this bacterial resistance, a drug delivery system called Liposome was used with the ability to encapsulate an expansive scope of drugs, either solved in water or solved in lipid [10]. As it was mentioned in the previous sections, in reversed phase evaporation method, at first the reversed aquatic phase in Ether solution of Liposome-forming lipids with the influence of sonic waves gets the form of water emulsion in organic phase. Lecithin and Cholesterol as emulsifiers surround the drops of Ceftazidime solution in

water, and then Liposomes are formed through Ether phase evaporation. It is necessary to state that Lecithin phospholipid is used in preparation of Liposomes in this study. The emulsion part of the sample gets stable more rapidly compared to the samples that only contain Lecithin [8].
During these studies, the efficiency of encapsulating the Liposomes containing Ceftazidime was reported to be 50% and it was concluded that Large Unilamellar Vesicles with average encapsulation efficiency and appropriate size can be used as drug carriers. It can also be concluded that reversed phase evaporation method is an appropriate method to prepare Large Unilamellar Liposomes as drug carriers [17]. Finally the result of the effect of Ceftazidime bactericidal against Pseudomonas aeruginosa with a decrease in MIC and MBC of Ceftazidime antibiotic from 2µg/ml in free form to 0.5µg/ml in liposomal form implies that MIC and MBC of Ceftazidime antibiotic in liposomal form has decreased by two degrees. Liposomes stable for targeting, through selection of appropriate phospholipid and appropriate amount of Cholesterol. With such drug carriers, it is likely to decrease the resistance of Pseudomonas aeruginosa against third generation Cephalosporins including Ceftazidime [18].

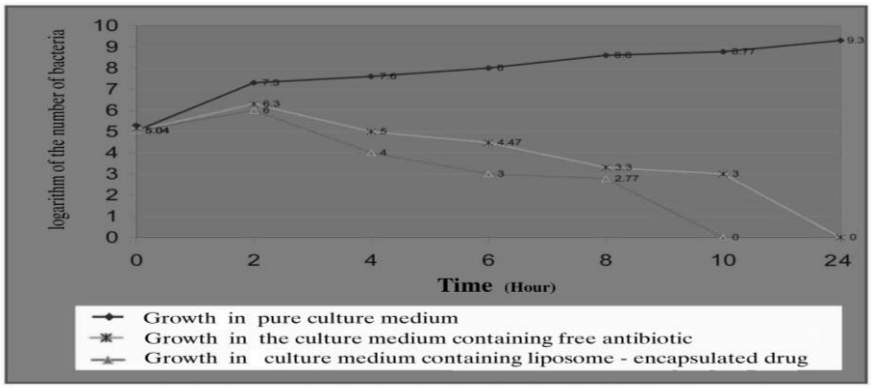

Fig1. The curve of comparison of bacterial growth prevention by different forms of drug at different times

5. Conclusion

The present research indicated the positive impacts of the entrance of β-Lactam antibiotics into Liposomes in vitro condition. This method may be used for better treatment in the future with respect to the resistance of such bacteria which produce β-Lactamase.

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