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RESEARCH ARTICLE

Effects of ceftiofur sodium liposomes on free radical formation in mice

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ABSTRACT

To examine the effects of ceftiofur sodium liposomes on the free radical formation in liver of mice, 24 mice were assigned randomly into three groups, i.e., 1) ceftiofur sodium; 2) ceftiofur sodium liposomes and 3) physiological saline. Treatments were applied via intraperitoneal injections for 7 days. At the end of the treatment period, animals were euthanized and liver collected for analysis of superoxide dismutase (SOD) activity and malondialdehyde (MDA) contents and the ability of liver tissue to suppress hydroxyl radical formation. Ceftiofur sodium liposomes-treated mice had higher activity of SOD than ceftiofur sodium- and saline-treated mice; however, MDA content and the ability of liver tissue to suppress hydroxyl radical formation did not reach statistical significance among groups. It was concluded that ceftiofur sodium liposomes can improve the SOD activity compared to ceftiofur alone in mice.

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INTRODUCTION

Liposome is a microscopic spherical particle formed by a lipid bilayer enclosing an aqueous compartment. Liposomes were first produced in England in 1961. Liposome can be used as a vehicle for administration of nutrients and pharmaceutical drugs. As of 2008, 11 drugs with liposomal delivery systems have been approved and six additional liposomal drugs were in clinical trials (Zhang *et al.*, 2008). Due to their physical and chemical structural properties, liposomes can entrap hydrophilic compounds in their inner aqueous compartment and lipophilic compounds in the phospholipid bilayers (Torchilin, 2005). Because of desirable biocompatibility, biodegradability, and low toxicity, liposomes have been widely used as carriers to enhance efficacy and reduce toxicity of pharmaceuticals (Lian and Ho, 2001). Therefore, liposomes can be used to improve delivery systems of pharmaceutical agents.

Ceftiofur sodium is a third generation broad spectrum cephalosporin which is widely used clinically to treat respiratory diseases in livestock. Ceftiofur is a highly-effective antibiotic and acts by inhibiting synthesis of bacterial cell wall leading to bacterial death (Prescott *et al.*, 1993). Free radicals that include oxygen are commonly referred to as reactive oxygen species (ROS) and include superoxide anion, hydrogen peroxide, and hydroxyl radicals (Acworth and Bailey, 1997). In our earlier study (Liu *et al.*, 2010), we observed that Ceftiofur sodium encapsulated in liposomes provided therapeutically-effective plasma concentrations of longer duration compared to non-encapsulated ceftiofur. Therefore, interval between doses could be extended if using liposome-encapsulated ceftiofur. However, the effects of ceftiofur encapsulated in liposomes on free radical formation are unknown. In the current study, we examined the effects of ceftiofur sodium delivered in liposome on superoxide dismutase (SOD) activity, malondialdehyde (MDA) content and the ability of suppression on the hydroxyl radical in liver of mice. We hypothesize that liposomes have superior resistance to and greater capability of scavenging free radicals compared to non-encapsulated ceftiofur sodium.

MATERIALS AND METHODS

Reagents and chemicals: Ceftiofur sodium was purchased from Wuhan Fuxin Chemical Co., Ltd. (Hubei, China), ceftiofur sodium liposomes (containing 10% ceftiofur sodium) and blank liposomes (containing no ceftiofur sodium) were prepared by the Laboratory of Clinical Veterinary Medicine of Huazhong Agricultural University (Wuhan, Hubei, China) as reported previously (Liu *et al.*, 2010). Ceftiofur sodium was encapsulated in

liposome using reverse phase evaporation. Ceftiofur sodium liposomes appear as a milky, light yellow suspension. The shape of the liposomes is uniformly spherical or nearly spherical, and the mean particle size is approximately 100 nm (Liu *et al.*, 2010). Coomassie brilliant blue protein assay kit (20090821), SOD activity assay kit (20090915), MDA activity assay kit (20090901) and hydroxyl radical activity assay kit (20090910) were all purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). All other chemicals were analytical reagent grade purchased from Sinopharm Chemical Reagent Co., Ltd.

Animals: Twenty-four (12 each of female and male) KunMing mice (supplied by the Hubei research center of Experimental Animals, China) at 8 wk of age and weighing 20 g on average were used in these experiments. Males and females were kept in separate cages, and bedding was changed regularly. Mice had free access to feed (mouse chow, Hubei research center of Experimental Animals, China) and water during the experiment. The mice were acclimated to housing and environmental conditions (temperature, humidity, light, and ventilation) for at least 1 week prior to beginning of treatments. All procedures involving animals were approved by the Animal Care and Use Committee of Hubei Province, PR China.

Experimental design: Twenty-four mice were randomly assigned to one of three groups: 1) Control: mice were injected with 0.9% physiological saline (0.5 mL) daily for 7 days (n=8); 2) ceftiofur sodium: mice were administered non-encapsulated ceftiofur sodium (2.2 $mg \cdot kg^{-1}$) for 7 days; and 3) Encapsulated ceftiofur sodium: mice were administered liposome-encapsulated ceftiofur sodium (2.2 $mg \cdot kg^{-1}$) for 7 days. All treatments were applied via intraperitoneal (i.p.) injections. Each group had four female and four male mice. Mice were deprived from food for 8h after the last treatment injection and then euthanized by cervical dislocation. After the mice stop breathing and no audible heart beat, the mice were dissected and liver samples were collected for analysis.

Biochemical studies: Liver was collected, rinsed with cold 0.9% physiological saline, dried with absorbent paper, then weighed and put into a small beaker. Nine times the weight of liver tissue of 0.9% physiological saline (W:V=1:9) were added into beaker. Livers were minced, grinded and centrifuged at 3000 g for 10 min. The extracted supernatant, representing a 10% tissue suspension was stored at 4°C until processing.

The protein content in the liver samples was determined using the improved and optimized Coomassie Brilliant Blue G-250 method (Pink *et al.*, 2010). One percent tissue suspension was prepared by diluting the previously described 10% tissue suspension to 1:9 in 0.9% physiological saline and used to detect the protein content following Coomassie brilliant blue protein assay kit following manufacturer instructions.

The Xanthine oxidase method (Xu and Wen, 2011) was used to detect the SOD activity in 1% tissue suspension. Xanthine and xanthine oxidase generate superoxide anion radicals, which oxidate hydroxylamine to form nitrite. This method produces a purple reaction, and the absorbance is determined by ultraviolet and visible spectrophotometry (ZW1103080605, Shanghai Spectrum Instruments Co. LTD). The results were expressed as U/mg protein.

MDA contents were measured in 10% tissue suspension using Thiobarbituric acid (TBA) method (Raharjo *et al.*, 1993). This method is the most common method for measuring oxidative changes in biological samples and is based on a spectrophotometric quantitation of a red-violet complex reaction formed with MDA. Results were expressed as nmol/mg protein.

Hydroxyl radical suppression in the liver was determined by the Fenton's reagent method (Barbusinsky, 2009). Ten % tissue suspension was diluted to 0.5% in 0.9% physiological saline before assay. One unit of hydroxyl radical suppression ability was defined as a decrease of 1 mmol/L H_2O_2 per min for 1 mg tissue protein at 37°C and the results were expressed as U /mg protein.

Statistical analysis: Data were expressed as mean ± standard deviation (SD). Analysis of the data was performed by multivariate analysis of variance (MANOVA) and subsequent analysis was performed using the Tukey's test.

RESULTS

The results of SOD activity in liver tissue of mice are shown in Table 1. There was no effect of sex on mean SOD activity; therefore, data from males and females were combined for further analysis. Mean activity of SOD in the liver was lower (P=0.05) in Ceftiofur sodium treated mice than in liposome-encapsulated Ceftiofur sodium treated mice and controls (Table 1).

Content of MDA in liver are shown in Table 1. There was no significant statistical difference in MDA content in liver neither among treatment groups nor between female and male mice.

The ability to suppress hydroxyl radical formation in liver samples was lower in liposome-encapsulated ceftiofur sodium-treated mice and ceftiofur sodium than in control-treated mice (Table 1). The ability to suppress hydroxyl radical formation did not differ between liposome-encapsulated and non-encapsulated ceftiofur sodium. Gender had no significant effect on suppression hydroxyl radical formation.

Table1: The effects of ceftiofur sodium liposomes on free radical formation in liver tissue of mice

Groups	SOD (U/mgprot)			MDA (nmol/mg protein)			Hydroxyl radicals (U/mgprot)		
	Female	Male	Total	Female	Male	Total	Female	Male	Total
Control	99.8±3.80	90.3±7.39	95.05±4.25^{A}	0.79±0.051	0.85±0.051	0.82±0.035	38.44±6.20	38.05±9.09	38.24±6.12^{A}
Ceftiofur sodium	74.79±4.53	76.05±5.44	75.42±3.29^{B}	0.90±0.10	0.91±0.081	0.90±0.062	16.94±3.57	18.49±4.71	17.71±3.13^{B}
Ceftiofur sodium liposomes	88.41±3.19	82.39±3.90	85.40±2.59^{A}	0.78±0.098	0.90±0.081	0.84±0.077	18.71±1.88	18.21±2.95	18.46±1.63^{B}

Values (mean±SD) bearing different letters in a column differ significantly (P<0.05). There were total 8 mice, 4 each of female and male.

DISCUSSION

Reactive oxygen species (ROS), such as superoxide anion, hydrogenperoxide (H_2O_2) and hydroxyl radical, are potentially toxic to cells and believed to contribute to the pathogenesis of a variety of diseases (Mohammad and Melissa, 1998). Reactive oxygen radicals are generated *in vivo* by multiple mechanisms, including the respiratory redox chain in mitochondria, the respiratory burst of phagocytes and the activities of various oxidases.

Superoxide dismutase (SOD) is metalloenzyme that catalyzes the dismutation of superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). SOD is an important enzymatic antioxidant enzyme in antioxidant systems and an intracellular compound that protects against oxidative processes initiated by the superoxide anion (Fang, 2002). The SOD converts superoxide radicals (O_{2-}) into H_2O_2 plus O_2, thus participating with other antioxidant enzymes in the enzymatic defense against oxygen toxicity. SOD protects cells from damage and plays an important role in the balance of production and scavenging of free radicals (Fang, 2002). Consequently, they provide an important defense mechanism against superoxide radical toxicity. SOD activities are higher in the liver than in other tissues. Liver SOD activity can be used as an indirect representation of the body's ability to scavenge oxygen free radicals (Yuan *et al.*, 2005). The results of our studies indicate ceftiofur sodium liposomes have the higher SOD activity than ceftiofur sodium. During preparation of liposomes, vitamin C and vitamin E are added to ceftiofur sodium liposomes (Liu *et al.*, 2010), and there was no vitamin C and vitamin E in the ceftiofur sodium. Vitamin C, or ascorbic acid, is a common enzymatic cofactor in at least eight enzymatic reactions in mammals used in the synthesis of collagen, and ascorbate is a powerful reducing agent capable of rapidly scavenging a number of reactive oxygen species (Hardie *et al.*, 1991), so ascorbate acts as an antioxidant by protecting the body against oxidative stress (Padayatty *et al.*, 2003). Vitamin E is a fat-soluble compound that includes both tocopherols and tocotrienols, and is used as antioxidant for inhibiting production of reactive oxygen species formed during lipid oxidation (Herrera and Barbas, 2001).

Malonaldehyde (MDA) is the organic compound with the formula $CH_2(CHO)_2$. MDA, the major small aldehyde produced in lipid oxidation (Ishii *et al*., 2006), is a reactive aldehyde, and is one of the many reactive electrophile species that cause toxic stress in cells (Farmer and Davoine, 2007). This aldehyde is used as a biomarker to measure the level of oxidative stress in an organism (Del *et al*., 2005). In the current study, similar trends with SOD activities were detected on MDA, but the slightly lower of MDA contents of ceftiofur sodium liposomes was not statistically significant. Lipid peroxidation in liver microsomes can form MDA, but MDA is unstable and can be metabolized quickly. Therefore, although lipid peroxidation *in vivo* may occur, MDA content is usually low. Although lower MDA contents were observed in ceftiofur sodium liposomes compared with ceftiofur sodium groups, these changes displayed some interindividual variability and the low values of MDA contents, which prevented them from reaching statistical significance.

The hydroxyl radical is the neutral form of the hydroxide ion (OH^-), which can initiate a damaging chain reaction of lipid peroxidation in the unsaturated lipids within cell membranes. Hydroxyl radicals are highly reactive and have a half-life of approximately 10^{-9} seconds. It can easily oxidize into a variety of organic and inorganic compounds. This oxidative reactivity makes free hydroxyl radicals toxic to organisms (Reiter *et al.*, 1997), causing oxidative damage with potential changes in lipid and amino acids structures, and damage to nucleic acids (e.g., DNA mutations; Gerald and Denver, 1999). Hydroxyl radicals are difficult to detect because they are highly reactive and have short half-life in the reaction. Based on the results in this study, the ability to suppress hydroxyl radical formation does not differ between encapsulated and non-encapsulated ceftiofur sodium.

Because SOD activity was greater in saline-treated mice than in ceftiofur sodium-treated mice, it appears that 7 days of ceftiofur treatment seems to cause some degree of liver damage and impair SOD function. The ability to suppress hydroxyl radical formation was greater in physiological saline-treated mice than in liposomes and ceftiofur sodium, it may have the similar reason with SOD activity. The liver is considered the body's detoxification organ, and long-term use of ceftiofur and liposomes may cause liver damage. As the average encapsulation efficiency of liposomes was 57.16%, there was free ceftiofur sodium outside the liposomes (Liu *et al.*, 2010), so the liposomes can only partly reduce the toxicity but not avoid the damage completely.

One free-radical species produced by the body, the superoxide anion (O_2^-) (Thom, 1992), is possibly the main factor to cause oxygen toxicity. The other oxygen free radicals are derived from the superoxide anion. SOD is the specificity enzyme to scavenge the superoxide anion *in vivo*, and vitamin E has a strong inhibition on the superoxide anion radical. Thus, it is reported that vitamin E can improve the SOD activity (Liang *et al.*, 2007). For MDA and hydroxyl radical, they are mainly caused by the lipid peroxidation, because the small amount of vitamin C and vitamin E in the liposomes, the liposomes did not show their advantage on the scavenging of MDA and hydroxyl radical. However, liposome-encapsulated ceftiofur presents an advantage on minimizing superoxide anion radical formation. In addition, consistently to previous report (Chen *et al.*, 2009), gender does not seem to interact with the ability of liposome-encapsulated ceftiofur to suppress radical formation.

Conclusion: The SOD activity of liposomes in this study was higher than ceftiofur sodium, it is indicated that ceftiofur sodium liposomes minimizes superoxide anion radical formation in liver cells. This effect may be related to the antioxidant actions of vitamins C and E present in the liposome preparation. Thus, liposome-encapsulated ceftiofur seems to provide added resistance to scavenging superoxide anion radical compared to ceftiofur alone in mice. Application of liposome delivery systems of antimicrobial agents such as ceftiofur may improve efficacy and minimize toxicity of ceftiofur treatment in animals.

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