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IN VITRO STUDY ON THE IMMUNE RESPONSES OF PHAGOCYTIC CELLS INOCULATED WITH *STAPHYLOCOCCUS AUREUS* IN ASSOCIATION WITH ENROFLOXACIN TREATMENT

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ABSTRACT

In this study, we examined the in vitro interaction of *Staphylococcus aureus* (*S.aureus*) with culture of peripheral blood monocyte macrophage cells (PBMC) and polymorphnuclear leukocytes (PMNs). Also we studied the possible effects of enrofloxacin against *S.aureus* inoculation. In the experimental groups, culture supernatants of non-stimulated and *Staphylococcal*-stimulated PBMC were collected after 1hr, 2hr and overnight incubation to each of the 3 treatments. Measures evaluated for cultured PBMC at each interval were nitric oxide (NO), lysozyme, while cultured PMNs function was assessed after 30min of inocubation by cytochrome-c reduction assay. Moreover, samples of cultured PBMC were also taken for the determination of DNA fragmentation. The study showed that the interaction between *S. aureus* and cultured monocytes resulted in significant increase of nitric oxide and lysozyme but in stead this effect is declined following enrofloxacin treatment. Fragmentation of DNA of cultured *Staphylococcal*-stimulated monocytes was quantified colorimetrically by diphenylamine (DPA) assay exhibited significant DNA fragmentation after overnight incubation compared with low fragmented DNA pattern with enrofloxacin treatment. Data concerning the production of superoxide anion within PMNs by cytochrome c reduction assay only appear to induce significant elevation upon treatment with enrofloxacin. The results of the current experiment suggest that enrofloxacin appears to be effective in vitro killing against *S.aureus* isolate as can be indicated through modulation of bactericidal phagocytic mediator level and activity. The present data may, therefore, raise interesting questions about potential, expected consequences of a prolonged use of fluoroquinolones for therapeutic or prophylactic purposes in induced bacterial infections.

Key words *Bovine monocytes, polymorphnuclear cells, staphylococcal in vitro inoculation, enrofloxacin*

INTRODUCTION

Macrophages and neutrophils play several key roles in the innate immunity by the process of phagocytosis, subsequent formation of intracellular phagosomes and the destruction of the internalized pathogen. (Zwahlen and Roth, 1990; Sanchez *et al.*, 1993; Smits *et al.*, 1997 and Bochsler and Slauson, 2002). During engagement and internalization, phagocytes use several mechanisms to ensure effective killing of pathogens, including the use of antimicrobial peptides, broad spectrum proteases, bacteriolytic enzymes such as lysozyme, antimicrobial fatty acides, toxic oxygen- or -nitrogen containing molecules, the bacteriolytic complement components and further mechanisms with indirect impacts on bacterial multiplication (Kraus and Peschel, 2008). The generation of reactive oxygen species (ROS) by the NADPH oxidase complex is thought to play an important role in both direct and indirect killing of several species of bacteria and fungi (Bochsler and Slauson, 2002; Cross and Segal, 2004 and Fang, 2011). *S. aureus*, which often causes chronic or relapsing diseases is reported to persist as an opportunistic intracellular organism both in vitro and in vivo (Hebert *et al.*, 2000; Jett and Glimore, 2002; Bishayi and Sengupta, 2003 and Brouillette *et al.*, 2004).

It is generally accepted that extracellular bacteria, like *S. aureus*, are efficiently phagocytosed and killed within PMN and PBMC and this phenomenon is frequently used as a standard assay to examine the phagocytic cell function (Perticarari *et al.*, 1991 and Hampton and Winterbourn, 1995). Nowadays, antimicrobial resistance has become a major public health problem in many countries due to the constant circulation of resistant bacterial strains in the environment and possible foodstuff contamination. Consequently, antimicrobial agents should be able to penetrate bacterial cells and, most importantly, should

maintain their activity inside the cell (Tulkens, 1991). Enrofloxacin is a fluoroquinolone exclusively developed for farm animals. It is potent against many bacteria and has good pharmacokinetic properties (Brown, 1996; Hannan *et al.,* 1997; Richez *et al.,* 1997; Watts *et al.,* 1997 and Ganiére *et al.,* 2001). However, data from in vitro experiments that have evaluated the antimicrobial efficacy of enrofloxacin are scarce, although much research was done to study the antimicrobial actions of fluoroquinolones in bacterial of several species (Craven and Anderson, 1980 a,b; Craven and Anderson, 1983; Leijh *et al.,* 1986; Hoeben *et al.,* 1997; Nielsen and Gyrdhansen, 1997). The purpose of this in vitro study was to assess the antimicrobial mechanisms of PBMC and PMNs in response to *S. aureus* and to observe how the treatment with enrofloxacin can influence the effect of *S.aureus* on phagocytic functions.

MATERIALS and METHODS

Bacterial strain and growth condition

S. aureus isolation was done from mastitis-susceptible cows with no history of medical treatment for mastitis. In brief, each milk sample of quarters of mastitis-susceptible cows was cultured on mannitol salt agar (specific media), blood agar (for detection of haemolysis) and nutrient agar (Oxoid media) and incubated at 37°C for 48h. Bacterial colonies presumptively identified as *staphylococci* by colony characteristics, and by catalase, oxidase, urease, phosphatase test (Quinn *et al.,* 2002), coagulase test (Koneman *et al.,* 1988), sugar fermentation, nitrate reduction test, (Cruickshank *et al.,* 1975) and Ornithine decarboxylase test (kloos *et al.,* 1991). Those isolates were analyzed further using the API staph system (Biomerieux, France).

Preparation of bacterial suspension for in vitro inoculation

S. aureus isolate was grown for 18 h on sugar broth, washed twice with a large volume of saline, and opsonized for 30 min at 37°C in the presence of 5% heat-inactivated bovine serum. After additional washing, the density of bacteria was measured spectrophotometrically at 540 nm, and the number of cells was calculated by using a previously determined standard curve based on traditional counting plate (CFU counts). Finally, the concentration of bacteria was adjusted to 10^9/ml in phosphate-buffered saline (PBS) by (Verbrugh *et al.,* 1979).

Enrofloxacin:

Enrofloxacin was obtained as pure substance from (Bayer AG, Leverkusen, Federal Republic of Germany). For enrofloxacin treatment in the present work, a fresh stock solution of 10 mg/ml was prepared as 50 ug/ml of in RBMI-1640 according to Gorla *et al.* (1999) for use in bioassays.

Isolation and culturing of Peripheral Bovine Mononuclear Cells (PBMC)

Blood-derived Mononuclear was isolated and cultured from buffalo as described briefly by Toshinobu *et al.* (2003) with some modification. PBMC were isolated from heparinized blood using Ficoll-Hypaque and centrifuged at 400 *xg* for 30 minutes. The interface layer (lymphocytes and monocytes) was collected and washed 3 times by HBSS balance salt solution (HBSS) and centrifuged at 600xg for 10 minutes per time. Monocytes were permitted to differentiate by culturing the suspended cells in growth RPMI-1640 with 10% fetal bovine serum medium in 96 well tissue culture plate as 1.5 x 10^6 cells/ml for 3 hours under the standard culture condition (at 37 °C, 5% CO_2 and 90% humidity). The non adherent cells as well as the culture medium were removed and a new growth RPMI-1640 was added and the plate re-cultured for obtaining macrophage sheet under the standard culture condition for 5 days.

Treatments of PBMC.

PBMC were suspended in RPMI 1640 supplemented with 10% fetal calf serum (Sigma-Aldrich Fine Chemicals, St. Louis, Mo.). Bacterial culture were incubated in tissue culture medium prior treatment for 16 to 18 hrs. After that, a group of monocytes incubated with opsonized suspension of *S .aureus* was co-cultured with enrofloxacin suspension while another infected group was cultured without enrofloxacin suspension. Finally, control group of monocyte culture was neither exposed to *S.aureus* nor enrofloxacin. Culture supernatant of monocytes from each group was collected after 1hr, 2hrs and overnight incubations for the assessment of the subsequent immune parameters.

Determination of nitric oxide

The concentration of NO in PBMC supernatants was quantified colorimetrically using the Griess reaction. Briefly, 100 *u*L of PBMC culture supernatant from each group was combined with an equal volume of Griess reagent (1 % sulfanilamide/ o.l % naphthylethylene-diamine dihydrochloride in 2.5% H3P04) and incubated for 10 minutes at room temperature as described previously (Moshage and Jansen, 1998). The nitrite concentrations were determined at 570 nm using an automated ELISA plate reader and the results are expressed in ng/ml.

Determination of lysozyme activity

Lysozyme concentration was measured according to Schltz, (1987) Lysoplate was prepared as 1% agarose in 0.067 M PBS at pH 6.3. When the temperature of the dissolved agarose lowered to 60 °C, the bacterial uniform suspension (*Micrococcus lysocleikticus*) was added and mixed well to form homologous mixture. The mixture was poured in Petri dish at thickness 4 mm. Exactly, 25 µl of monocytes culture supernatant/ group was inoculated in a representative well and the

plate was incubated at room temperature for 18 hours. The cleared zone ring diameter that has been developed was measured in mm. The concentration of the standard was plotted on the logarithmic axis against the corresponding cleared zones diameter on the linear axis of bemilogarthemic graph. The diameter of the sample was plotted against the standard for obtaining the lysozyme concentration in µg/ml.

Quantification of DNA fragmentation

A diphenylamine colorimetric assay (Liles *et al.*, 1995; Squier and Cohen, 1997) was used to quantify DNA fragmentation. Briefly, 0.5 ml of 4 X 10^6 target cells ml were centrifuged at 300 xg for 10 min to separate supernatant from cell pellets. The cell pellets were lysed with 0.5 ml of a hypotonic solution containing 10 mM Tris±HCl, pH 7.4, 1 mM EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 10 min at 13 000 x g to separate high molecular weight, intact chromatin (pellet, designated as B). From cleaved, low molecular weight DNA (top solution, designated as T). The supernatant (S), low molecular weight (T) and high molecular (B) fractions were precipitated overnight with 0.5 ml of 25% trichloroacetic acid (TCA) (Sigma, St Louis, MO), sedimented at 13 000 x g for 10 min, and hydrolyzed in 80 ml of 5% TCA at 90°C for 15 min. All fractions were incubated with 160 ml of diphenylamine (Fisher Scientific, Pittsburgh, PA, USA) reagent at 378C for 4 hrs, in which 150 mg of diphenylamine was dissolved in 10 ml of glacial acid, then 150 ml of concentrated sulfuric acid and 50 ml of acetaldehyde solution were added and mixed well. The amount of DNA in each sample was estimated from its absorbance at 570 nm in a plate spectrophotometer. The results were calculated by following equation:

% DNAfragmentation . $[(S + T)]/(S +T +B)] \times 100$

Where S is the amount of DNA in the supernatant, T the amount of low molecular weight, cleaved DNA in the top solution and B the amount of high molecular weight, intact chromatin DNA.

Isolation and treatment of PMNs

Blood samples were collected from healthy buffalo via jugular vein puncture, and neutrophils were isolated as described by (Fajt *et al.*, 2000). Cell viability exceeded 95% based on trypan blue staining. Samples were suspended with HBSS to a concentration of 2.5×10^6 cells/ml. Treatment of neutrophil culture medium was similar to the previously mentioned groups (incubation for 30min) and cytochrome cell reduction assay was performed according to the method described by (Roth and Kaeberle, 1981a.).

Statistical analysis:

Data were subjected to statistical analysis according to Snedecor and Cochran (1982) by one way ANOVA employing a completely randomized design.

RESULTS

Nitric oxide production in stimulated PBMC.

Nitric oxide generation in the supernatants of *Staph aureus* infected PBMC without enrofloxacin treatment was increased significantly ($p<0.05$) which noted between 2hr and overnight incubation in comparison to their respective control group. On the other hands, values recorded remarkable declines under enrofloxacin treatment (table.1).

Table 1: Concentrations of nitric oxide (ng/ml) in monocyte culture supernatants in response to *staphylococcal* inoculation and enrofloxacin treatment.

Experimental groups	1hr post inculation	2hr post inculation	Overnight post inculation
Negative control	8.25±0.18	8.10±0.17	8±0.1
Staphylococcal stimulated monocyte enrofloxacin-free medium	12.74±0.30*	238.10*±3.22	236.9*±36.65
Staphylococcal stimulated monocyte and treated with enrofloxacin	8.88±0.42	11.68±3.8	26.38±1.87

Values are mean ± S.D. Mean values with astrik in a row are statistically significant from each other at P<0.05.

Intracellular lysozyme content in stimulated PBMC.
Co-incubation of opsonized *S.aureus* suspension and PBMC supernatants resulted in significant elevated lysozyme content after 2hr and overnight incubation respectively whereas those associated with enrofloxacin treatment had low lysozyme concentrations. (Table 2)

Table 2: Concentrations of lysozyme (ug/ml) in monocyte culture supernatants in response to *staphylococcal* inoculation and enrofloxacin treatment

Experimental groups	1hr post inoculation	2hr post inculation	Overnight post inculation
Negative Control	75.01±7.35	70.5±7.04	72.3±6.3
Staphylococcal stimulated monocyte Enrofloxacin – free medium	111.78±12.75	200.04±25.5*	119.14±7.34
Staphylococcal stimulated monocyte treated with enrofloxacin	97.07±7.36	148.56±7.35	111.22±21.88

Values are mean ± S.D Mean values with a strike in a row are statistically significant from each other at P<0.05.

Determination of Monocyte Apoptosis by DNA fragmentation
Quantitative DNA fragmentation in all groups of monocytes culture supernatant was evaluated by diphenylamine (DPA) assay spectrophotometrically. Monocyte cultures incubated with *S.aureus* suspension caused significant DNA fragmentation only after overnight incubation in comparison to treatment with enrofloxacin.

Table 3: Quantitative estimation of DNA fragmentation (%) using DPA assay in the control and experimental groups

Experimental groups	1hr post inoculation	2hr post inoculation	Overnight post inoculation
Negative control	0.593±0.93	0.563±0.033	0.607±0.008
Staphylococcal stimulated MO enrofloxacin free medium	0.687±0.110	0.594±0.018	0.847±0.011*
Staphylococcal stimulated MO treated with enrofloxacin	0.561±0.038	0.634±0.041	0.491±0.030

Values are mean ± S.D
Mean values with astrik in a row are statistically significant from each other at P<0.05.

Cytochrom –C reduction assay:
Superoxide anion (O^{-}_{2}) activity was significantly elevated (*at* $P < 0.05$) in PMC supernatants co-cultured with *S.aureus* suspension combined with enrofloxacin treatment as compared to other two groups. (Table 4).

Table 4: Superoxide anion released from staphylococcal–stimulated PMNs after 30 minutes inculation and measured by cytochrom c reduction assay:

Experimental groups	Superoxide anion
Negative control	0.098±0.004
Staphylococcal stimulated PMNs enrofloxacin free medium	0.173±0.004
Staphylococcal stimulated PMNs treated with enrofloxacin	0.182±0.003*

Values are mean ± S.D
Mean values with astrik in a row are statistically significant from each other at P<0.05

DISCUSSION

S. aureus has long been considered as an extracellular pathogen, resulting However, *S. aureus* may in prolonged and recurrent infections occasionally become intracellular, at least within monocytes, macrophages, and polymorphonuclear neutrophils when host defense mechanisms are activated (Paillard *et al.,* 2002). Our present report showed that PBMC is susceptible to *S. aureus* inoculation through significant generation of nitric oxide and lysozyme and their interaction was minimized after treatment with enrofloxacin. It is well known that fluoroquinolones are accumulated by phagocytes (Carlier *et al.,* 1987b; Pascual *et al.,* 1989; Carlier *et al.,* 1990), and are reported to be active against several sensitive intracellular bacteria (Emson *et al.,* 1986; Carlier *et al.,* 1989; Scorneaux *et al.,* 1989; Sárközy, 2001). These findings are agreed with Nielsen *et al.* (1997) who attributed. The mechanisms of intracellular killing to intracellular accumulation, where the antibiotic can kill the bacteria directly or make them more susceptible to the bactericidal effect of the granulocyte.

The data presented in table (3) show that PBMC become apoptotic after *S.aureus* inoculation which has been documented by the significantly increased DNA fragmentation and is brought back near to control after enrofloxacin treatment. Baran *et al.,* 1996 suggested that after phagocytosis of bacteria, macrophages have reduced viability which correlated with changes in their morphology, indicating apoptosis. Indeed, the significant release of a free radical NO in this work can inflict damage on all classes of cellular macromolecules and eventually leading to cell death. Free radicals may also lower the threshold of antiapoptotic defense within monocytes, which explains why monocytes that ingested bacteria preferentially show DNA strand breaks (Baran *et al.,* 1996; Subhankari *et al.,* 2011). Thus, enrofloxacin treatment may indirectly protect monocytes *staphylococcal*-infection induced cellular changes leading to amelioration of fragmented DNA which may support the explaination (Hoeben *et al.,* 1997).

It is evident from the result of the present trial that there is a significant increase in superoxide anion in the inoculated PMNs supernatants and treated with enrofloxacin compared to enrofloxacin-free medium. The increased enzymatic activity by enrofloxacin could act directly on the granulocytes enhancing their oxygen-dependent or oxygen-independent killing mechanisms and a direct action of enrofloxacin on PMNs to generate superoxide anion might explain its higher intracellular activity (Neilson *et al.,* 1997). Furthermore, There are reports that optimal intracellular efficacy of various quinolones is obtained when PMNs have an intact oxidant-generating system (Van Rensburg *et al.,* 1990), but this does not exclude the possibility that oxidants act first on bacteria to increase their susceptibility to the bactericidal action of the drugs. Previous reports on the quinolones and their influence on the respiratory burst have been contradictory. Some investigators found that the respiratory burst was enhanced following incubation of the granulocytes with quinolones, whereas others found no effect or even an inhibitory effect (Forsgren and Bergkvist, 1985; Boogaerts *et al.,* 1986; Aoki *et al.,* 1994; Kubo *et al.,* 1994; Capecchi *et al.,* 1995). In conclusion, in the study described here, for enrofloxacin in general, no adverse effects on phagocyte function were described. Another impact of enrofloxacin on the immune responses is the possible shortening of infection, resulting in a reduction in a protective specific response and immunologic memory, which enables the host to resist subsequent challenge. With the gradual increase in our knowledge of the ambivalent role of phagocytes and other immune effectors (defense versus destruction), an approach to stimulate the phagocyte to decrease the harmful while retaining

the beneficial potential of these cells is urgently required. However, some innate immune responses have been generated from in vitro studies and need to be verified in vivo.

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دراسة معملية على الاستجابات المناعية للخلايا البلعمية المحقونة بميكروب العنقود الذهبى والمعالجة بالانروفلوكساسين

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أجريت هذه الدراسة معمليا لفحص التفاعل بين سائل الطائف النقى لخلايا الدم البلعمية وميكروب العنقود الذهبى والتاثير المحتمل للانروفلوكساسين ضد الميكروب. جمعت عينات سائل الطائف النقى لخلايا الدم البلعمية من المجموعا ت الضابطة والمعاملة بعد ساعة وساعتان وطوال الليل من التحضين. اجري قياس لاكسيد النيتريك والليزوزيم على سائل الطائف النقى لخلايا الدم وحيدة النواة بينما تم قياس ايون فوق الاكسيد على سائل الطائف النقى لخلايا الدم البلعمية متعددة الانوية. كما تم قياس تجزئة الحمض النووى على سائل الطائف النقى لخلايا الدم البلعمية وحيدة النواة . وقد أسفرت الدراسة عن وجود زيادة معنوية لكل من اكسيد النيتريك والليزوزيم فى المجموعة المحقونة فقط بالميكروب بينما ادى العلاج بالانروفلوكساسين الى تاثير اقل. اظهر اختبار قياس تجزئة الحمض النووى لخلايا الدم البلعمية وحيدة النواة تقليل لهذه التجزئة بعد التحضين مع الانروفلوكساسين طوال الليل عند مقارنته بالمجموعة المحقونة بالميكروب فقط. كما يبدو ان ايون فوق الاكسيد يزداد فقط مع التحضين بالانروفلوكساسين. وتقترح نتائج التجربة الحالية بان للانروفلوكساسين تاثير قاتل معمليا ضد ميكروب العنقود الذهبى حيث يتضح من خلال تعديل الانشطة المضادة للبكتريا للخلايا البلعمية. ولذلك ربما تعلى تلك البيانات الاهتمام بالاسئلة حول تاثير والتوقعات المتاعقبة للاستخدام الممتد للكينولونز لاغراض علاجية او وقائية لمسببات العدوى البكتيرية.