Ciprofloxacin but not amoxicillin significantly elevated serum peroxynitrite level in patients with enteric (typhoid fever): In vitro study

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J Fac Med Baghdad 2006; Vol. 48, No.3
Received March 2005
Accepted Sep. 2005

Summary:

Peroxynitrite is one intermediate of reactive nitrogen species with bactericidal and cylotoxic effects. Fluoroquinolones. drugs used for salmonella infections, are interacted v\ith nitrogen species and their baeterieida effect is influenced by these species. This study aims to assess serum peroxynitrite level in patients with enteric (typhoid) fever and. to investigate the effect of ciprofloxacin or amoxicillin on serum peroxynitrite level as well as in aqueous buffer solution in vitro. Thirty patients with enteric fever diagnosed clinically and serologically and twenty healthy individuals served as controls were admitted in this study. None of our sample was received anli-salmonellosis agents. Our results show that serum peroxynitrite level tended to be significantly less in patients with typhoid fever in comparison with controls. In in vitro experimental model, ciprofloxacin but not amoxicillin at 6.25 ug elevate significantly serum peroxynitrite level. In aqueous solution, the ability of ciprofloxacin to produce peroxynitrite is higher than that of amoxicillin. We conclude that Ciprofloxacin . as bactericidal agent against salmonellosis, may act via producing or elevating peroxynitrite level.

Introduction:

Salmonella species are gram negative motile, facultative intracellular bacilli, and their invasion of, and multiplication within mononuclear phagocytic cells in the liver, spleen, lymphnodes and peyer's patches are the hallmark events of typhoid fever[1].

Peroxynitrite (ONOO-) is a strong oxidant and nitrating agent produces cytotoxic action against various microbes includind salmonella [2,3] Possibly, it acts through disintegration and chemical modification of various biomolecules such as lipids. proteins and DNA[4]. The evidence for bactericidal effect of peroxynitrite against salmonella is the formation of nitrotyrosine [5] This effect is influenced by salmonella strains. Peroxynitrite rarely colocalized within wild-type salmonella. It is localized in the vicinity of the *S. typhimuriii SPh* mutant strain-infected macrophages [6]. Therefore, the intracellular salmonella is protected from reactive nitrogen intermediates. Moreover, the LT2 strain was much more susceptible to the bactericidal effect of peroxynitrite than the Gifu strain suggesting that peroxynitrite resistance may contribute to salmonella pathogenicity [7]

Recently, it has been found that **ciprofloxacin,** an antimicrobial agent for enteric (typhoid) fever, is interacted with reactive nitrogen intermediates [8] Therefore, it is worth trial to assess the serum level of peroxynitrite of patients with enteric fever, and to investigate the effect of ciprofloxacin and amoxicillin on the peroxynitrite status in serum as well as in. *in vitro,* aqueous buffer solution.

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Subjects and methods:

This study is conducted in Department of Pharmacology - College of Medicine in cooperation with Basic Sciences Department-Biochemistry. College of dentistry - Al-Mustansiriya University during the summer 2004.

Subjects

The subjects of this study are:

1. Patients group: a total number of thirty patients (1 8 males and 12 females with enteric fever were allocated from one public clinic at Al-Thawra city in Baghdad. Our patients were diagnosed clinically and serologically (Widal test) as enteric (typhoid) fever. All patients enrolled in this study.had serum anti-0 > 1:160 for *S.typhi* and/or *S.paratyphi A* or *S. paratyphi B.*
2. Control group: a total number of twenty apparent healthy subjects (13 males and 7 females) were allocated from the same public clinic. All of them showed negative serological Widal test for salmonellosis.

Methods:

1. Assessment of serum peroxynitrite (ONOO)

Five milliliter of venous blood was obtained from each subject. The sera were separated by centrifugation (3000 rpm for 2 minutes) and kept in freezer at -20°C for later assay of peroxynitrite (ONOO).

Peroxynitrite level was determined in biological samples according to the method described by Beckman *el a!* 1992 [9] , cited from the reference

VanUffelen et al1998 [10]

The procedure of peroxynitrite assay is based on peroxynitrite -mediated nitration of phenol resulting in nitrophenol formation. In brief. 10 uL of serum was placed in a glass test tube and 5 mM phenol in 50 mM sodium phosphate buffer was added to a final volume of 2 mL and mixed well. The mixture was incubated for two hours at 37°C. then 1 5 uL of 0.1 NaOH was added and mixed. The absorbance *of* the sample at 412 ntn was then immediately recorded by SpeCol spectrophotometer (PGI1 Radio Fernesehen Elektro. DDR). The yield of nitrophenol was calculated from e = 4400 M^{-1} cm^{-1}.

2. **Effect of Ciprolloxaein or Amoxicillin on serum peroxvnitrite level in *vitro* model**

Serum level of peroxynitrite was determined after incubation of serum sample with final concentration of 6.25 ug of ciprofloxacin or amoxicillin at room temperature for 10 minutes prior to proceed for peroxvnitrite assay.

3. **Determination peroxvnitrite (ONOO-) level produced by ciprolloxaein or Amoxicillin**

The propcrity of producing peroxynitrite by ciprofloxacin or amoxieillin in aqueous buffer solution was assessed in this study. The same procedure, as mentioned above, was followed but instead of serum, an equal volume of ciprofloxacin or amoxicillin in a final concentration ranged from 5-50 ug.

Chemicals and drugs

All the chemicals used in this study were of analar grade. Ciprolloxaein FIC1 (pure substance) was generously obtained from Dofar pharmaceuticals, Iraq and amoxicillin generously obtained from Arab Company of antibiotics Industries (ACAI). Both drugs were dissolved in distilled water and prepared freshly at the time of assay.

Statistical analysis

The data are presented as means ± SD of number of observations. Data analysis is achieved by using Student's "t" test taking $p < 0.05$ as the lowest limit of significance.

Results

Table 1 shows the characteristics of the study. There is insignificant difference between patients and control groups in respect to the age and gender factors. Family history of enteric (typhoid fever) was been found in 40% of our patients' sample (twelve out of thirty). Twelve out of thirty patients (40%) had previous history of enteric (typhoid fever) (table 1).

Serological tests (Widal) findings show that the most common infected pathogen is *S. typhi* followed by *S. paratyphi* (tables 2 & 3). Mixed infections were observed in fourteen patients (46.7%) (table 3).

Assessment of serum peroxvnitrite(0\QO)

Table 1. The characteristics of the study.

	Patients group	Control group
Number	30	20
Gender		
Male (No.)	18	13
Female (No.)	12	7
Age (year)	_	
Minimum		14
Maximum	56	5 3
Range	43	39
Mean :L SD	32.9	34.7 t 11.9
Median	27.5	31.0
Family history of enteric (typhoid) fever:		
Positive	12	-
Negative	18	20
Previous history of enteric (typhoid) fever:		
Positive	12	-
Negative	18	20

Table 2. Distribution of patients in respect to the serological (\Vidal 1 test.

Infected pathogen	Number of patients'_	
	Antibody titre 1:160	Antibody titre 1:X20
S. typhi	18	
S. paral pi A	9	-
S. pur•aiy.pi B	16 _	-

Table 3. Distribution of patients in respect to the infected pathogens.

Infected pathogen	Frequency
1. Single infected pathogen:	
S. typhi	12
S. pca•atypi A	4
S. paratyphi B	0
2. Mixed infected pathogens: S& typhi + S paratyphi B	5
S. paratyphi A + S. paratyphi B	5
S. typhi + S paratyphi A	2
S. typhi + S. paratyphi A + Sparatyphi B	2
Total	"0

Figure 1 shows that serum level of peroxynitite related to patients with enteic (typhoid) lexer is significantly lower than that of control group. It is 2.09 ± 0.427 umol while that of controls is 2.34 ± 0.305 umol ($t = 2.411, p < 0.02$). Effect of ciprofloxacin or amoxacillin on serum peroxynitrite in *in vitro* model

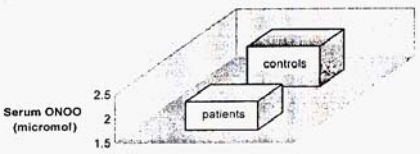


Fig. 1. Shows the significant ($p < 0.02$) low serum peroxynitrite level in patients with enteric (typhoid) fever.

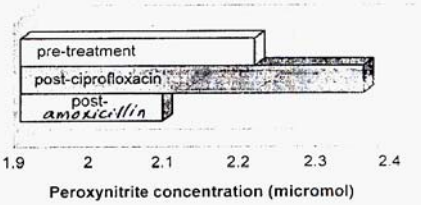
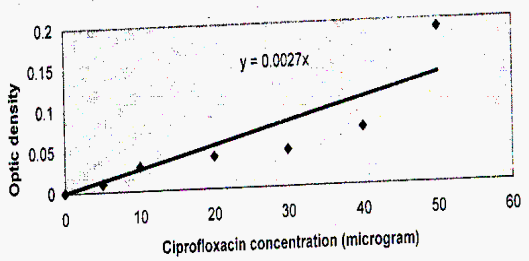
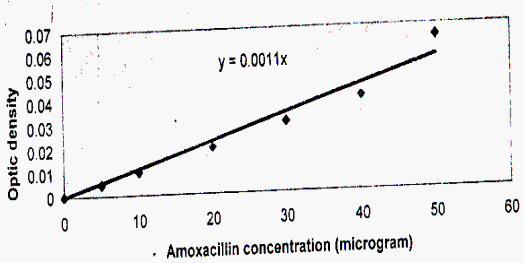


Fig.2. The effect of anti-salmonellosis agents (notably ciprofloxacin and amoxacillin) on the serum peroxynitrite level in vitro.



[A]



[B]

Fig.3. Standard drug concentration-generating peroxynitrite response for ciprofloxacin [A] and Amoxacillin [B].

Figure 2 shows that ciprofloxacin (6.25ug) but not amoxicillin (6.25ug) is significantly ($p < 0.05$) elevated the serum peroxynitrite level when it incubated at 6.25ug with serum. The serum peroxynitrite levels are 2.355 ± 0.37 and 2.21 18± 0.3818 umol for ciprofloxacin and amoxicillin respectively as compared with pre-incubated peroxynitrite level of 2.09 ± 0.427 umol.

Determination of peroxvnitrite (ONOO)level produced by ciprofloxacin or amoxicillin

Figure 3 shows the standard drug-conccntralion-generating peroxynitrite response curve for cuprofloxacin (A) and amoxicillin (B). From these curves, the calculated yield of peroxynitrite produced by an equal concentration of 6.25 ug for ciprofloxacin and amoxicillin are 1.659 and 1.186 umol respectively.

Discussion

The results of this study show that untreated patients with enteric (typhoid) fever have significant low level of the bactericidal nitrogen species; peroxynitrite. Also this study demonstrates, for the first time, that ciprofloxacin which is anti-salmonellosis agent significantly produces and elevates peroxynitrite level. Such effect may lead us to suggest that fluoroquinolones act as bactericidal agents by promoting the production of peroxynitrite. Coban and Durupinar 2003 [8] demonstrated that the effects of fluoroquinolones (ofloxacin, ciprofloxacin. and pefloxacin) are decreased via activation of soxRS (a regulon associated with resistance to fluoroquinolones) by nitric oxide in *S.enteric serovar Typhimurium.* On the other hand Ciccone *et al* 2003 found that adding a nitric oxide moiety to the fluoroquinolone ciprofloxacin displayed a marked activity at low nanomolar concentration against mycobacterium tuberculosis H37Rv strain [11] . Moreover, inhibitors of nitric oxide synthase exacerbate infection *in vitro* and *in vivo* against salmonella species [12]. Also, salmonella species protect itself from the effect of nitric oxide by the presence of inducible flavohaemoflobin[13].

Therefore, the bacterial haemoglobin may represent a cellular protective mechanism against nitrosative stress exerted by reactive nitrogen species. Add to this inducible nitric oxide synthase is required to control the proliferation of *S. typhimurium* in infected organs [14] and within infected macrophages [15] . Nitric oxide *per se* is not a potent cytotoxic molecule and most bactericidal effect of it appears to be via a reaction with superoxide anion to yield peroxynitrite [3].

Studies, *in vitro,* confirmed that peroxynitrite is cytotoxic to parasite whereas nitric oxide is cytostatic. Add to this, some peroxynitrite is decomposed to the hydroxyl free radical. Therefore peroxynitrite and derived radicals are likely to be important macrophage-derived cytotoxin[15] Also Fristsche *el al* 2001 *i\n\nd* that peroxynitrite , which is formed after chemical reaction of nitric oxide with superoxide anion. appears to be the principal effected molecule for macrophage-mediated cytotoxicity toward intracellular parasite [16]. Several studies showed that both nitric oxide and superoxide anion contribute critically to host defense against *serovar*

typhimurium [17. 18]

Our findings are in consistent with that findings reported by Wong *et al* 2000 [19]. Those researchers showed that, *in vitro* model, ciprofloxacin caused an increase in the levels of nitrite (an end product of nitric oxide synthase) when it incubated with *S. (Hireus* - infected macrophages.

The efficacy of amoxicillin in producing peroxynitrite is less than that observed with ciprofloxacin. This observation may be partly explained the reason why amoxicillin is less effective than ciprofloxacin in management of salmonellosis [2() 21].

We conclude that serum peroxynitrite level is significantly reduced in salmonellosis, and ciprofloxacin as bactericidal agent against salmonellosis. may act via producing or elevating peroxynitrite. Peroxynitrite itself is a bactericidal as well as cytotoxic agent.

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