An. R. Acad. Nac. Farm., 2009, 75 (2): 217-231

ARTÍCULO

(Cu)II *in vivo* interaction with cefotaxime

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Recibido el 9 de diciembre de 2008.

ABSTRACT

The presence of Cu(II) in penicillin and cephalosporin solutions, has been proved, to promote *in vitro* the antibiotic degradation to the corresponding acid derivates. HPLC studies provided an additional evidence for the reaction mechanism. The mechanisms of Cu(II) catalysis involve a ternary complex. This work was undertaken to study the consequences of this degradation *in vivo* upper the pharmacokinetic, pharmacodynamic and activity of the antibiotic cefotaxime. It is one of the most used «third-generation» cephalosporin in the world, this is because of that the interaction cefotaxime-metal deserved our attention. Our results remarked a lower concentration of free cefotaxime in blood, liver, spleen, kidney, lung and heart in organs from animals suffering Cu-intoxication. The differences more significant between intoxicated and control rats were observed in liver, lung and kidney. In addition cefotaxime linked to copper lose most of the microbicidal activity against bacterial strains of *Bacillus subtilis* CECT 356, *Escherichia coli* CECT 434, *Escherichia coli* CECT 616, *Staphilococus aureus spp aureus* CECT 435, *Staphilococus aureus spp aureus* CECT 239, in plate tests. It means that cefotaxime would become ineffective as antibiotic in metal poisoned patients.

Key words: Copper(II); Cefotaxime; Pharmacodynamic; Pharmacokinetic; HPLC.

RESUMEN

Interacción *in vivo* del Cu(II) con cefotaxima

Este trabajo estudia la reacción del cobre II con una cefalosporina, la cefotoxima, *in vivo*; los mecanismos catalíticos que implican la presencia de complejos ternarios, su farmacocinética y su farmacodinamia que afectan a la actividad del antibiótico. Las diferencias más significativas entre las ratas intoxicadas y control fueron observadas en riñón, pulmón e hígado. También hemos estudiado la actividad microbicida en *Bacillus subtilis* CECT 356, *Escherichia coli* CECT 434, *Escherichia coli* CECT 616, *Staphilococus aureus spp aureus* CECT 435, *Staphilococus aureus spp aureus* CECT 239.

Palabras clave: Cobre(II); Cefotaxima; Farmacodinamia; Farmacocinética; HPLC.

1. INTRODUCTION

The transition metal interactions to penicillins (1-5) and cephalosporins (6) were studied previously by spectrophotometric and potentiometric methods. In the case of cefotaxime, as impurity, also for HPLC (7).

In those studies it was observed that the effect of Cu(II) on the penicillins and cephalosporins was to promote their degradation to coordination complexes of Cu(II) and the corresponding penicilloic and cephalosporanic acids, (see Figure 1). Other authors have studied the formation of the complex *in vitro* between Cu(II) and penicillins and cephalosporins, by means of the reaction mechanisms, evaluating the stability constants for the interaction between Cu(II) and these antibiotics (2, 6).

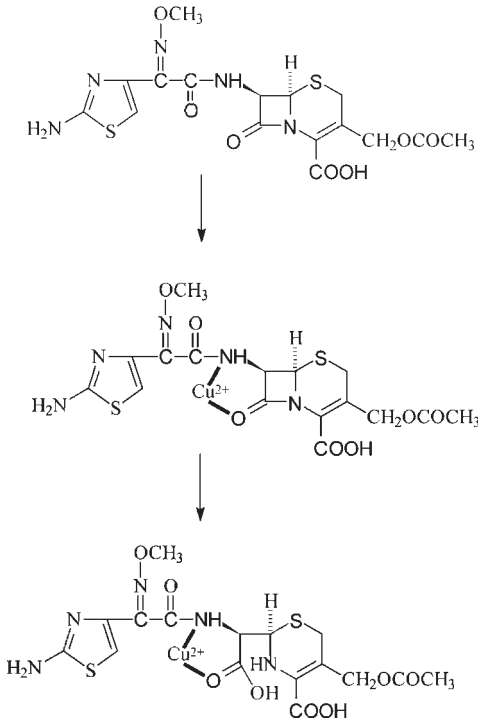

Figure 1. Mechanism of the hydrolytic reaction of Cefotaxime with addition of Copper.

This communication stresses in how the formation of the complex between Cu(II) and cefotaxime affects on the concentration, distribution and stability of this cephalosporin. The importance of the formation of this complex stems from the lowest concentration of free cefotaxime in blood and organs only when takes place intoxication. This free-antibiotic concentration could be lower than the administration of the antibiotic required killing the micro-organism that causes the infection.

We compared the concentration, distribution and stability of the free cefotaxime in rats without toxic with poisoned rats, after 30, 60, 90, 120, 150, 180 and 210 min. after administrating this antibiotic. This comparison was achieved by measuring the concentration of free cefotaxime in blood, liver, spleen, kidney, lung and heart homogenates of poisoned rats and rats without the toxic. The

methodology applied for this purpose was HPLC since this technique facilitates the separation of the complex Cu(II)cefotaxime, the corresponding Cu(II)-cephalosporanic acid chelate, free cefotaxime and their compounds resulting from the degradation of cefotaxime.

This paper is also focused on how much microbial activity contains the compounds produced from the complexation metal-cefotaxime. In pursuit of that aim, we studied the biological activity of the complexes against *Bacillus subtilis* CECT 356, *Escherichia coli* CECT 434, *Escherichia coli* CECT 616, *Staphilococus aureus spp aureus* CECT 435 and *Staphilococus aureus spp aureus* CECT 239 compared to data from experiments with free cefotaxime and Cu(II) at physiological concentrations found in poisoned living beens.

This work should be a clue to reveal whether the complex antibiotic-metal loses the microbiological activity as would be hypothesised by former works done in *in vitro* conditions.

2. MATERIALS AND METHODS

2.1. Biology materials

Seventy five Wistar rats males of 250 to 300g weight were used throughout this work and supplied by the warehouse of the Complutense University of Madrid.

Animals were maintained in one of the rats room of the warehouse. The temperature was 20 ºC, the relative humidity was 55% and the intensity of the light inside the room was 400 LUX using cycles of 12 hours of light/darkness.

Animals were distributed by groups in order to assay different treatments. One group of 35 rats with copper (group A), which was poisoned with a nasogastric sound administering to their everyday 4 mL of a solution of 4 mg/mL of a copper (II). monohydrate. acetate during 8 days. The groups left were arranged one of them with 35 rats (group B), and the other one with 5 rats (group C), both of them without metal.

Groups A and B were put under treatment with an intramuscular administration of an only dose of 200 mg of cefotaxime.

Group C was used as a control to know the different substances which belonged to the animals in order to not to be confusing with the free cefotaxime throughout the study.

Five rats of the groups A and B, were sacrificed after 30, 60, 90, 120, 150, 180 and 210 minutes after the antibiotic administration. The blood, liver, spleen, kidney, lung and heart of those rats were extracted and after that, they were measuring their free cefotaxime concentration by HPLC.

Residual microbicide activity was tested by antibiogram experiments in agar plates using bacteria strains from the CECT (Spanish Type Culture Collection, Burjasot, Spain). Those strains were *Escherichia coli* CECT 434 = ATCC 25922, described as standard for sensibility, *Escherichia coli* CECT 616 = ATCC 8739, described for assays with liquid with metal, *Staphilococus aureus spp aureus* CECT 435, *Staphilococus aureus spp aureus* CECT 239 = ATCC 6538 and Bacillus subtilis CECT 356 = ATCC 6633 as «a priori» negative control since the species was not listed among the affectable bacteria by cefotaxime.

The strains were maintained in TSA media at refrigeration teemperature and precultured in tubes with 10 mL TSB medium at 37 ºC for 24 hours before the experiments.

2.2. Chemical and reagents

Cefotaxime sodium was supplied by the Teaching Hospital of Madrid. Copper (II) acetate was obtained from Merck. Trichloracetic acid was supplied by Scharlau, this substance was used in concentrations of 10% and 20%.

The mobile phase reagents used were HPLC grade methanol from Scharlau. Water used was from a Milli-Rho-Milli-Q system (Millipore, Bedford, MA, USA). Phosphate buffer (0.1 M) was prepared with phosphate monopotasic anhydride and o phosphoric acid supplied by Merck.

The agar media used were TSA, a general bacterial growth media, and blood agar, both cooked as recommended the Merck's recipes.

2.3. Apparatus and instrument

Kontron high-pressure liquid chromatograph equipped with a Kontron 420 pump. An automatic injector with 6 valves Kontron Auto Sampler 460. A variable-wavelength UV detector of Kontron Uvikon 735 LC. Phenomenex C18 column (25 x 0.46 cm), fulled with particles of 5 mm, besides this column of separation, a precolumn and a protective column fulled with the same material that the first column were used. A Kontron Station Data with D450 software was used to control the detector and injector, on the other hand it allowed the integration of the chromatogram peaks and it cuantificated the results.

Paper Whatman discs, discs AA of 7 mm diameter were used to absorb the antibiotic solutions in the agar-plate experiments.

2.4. Analytical procedure

The extraction of cefotaxime in blood was carried out by means of protein precipitants in 10% trichloroacetic acid (8) 1.5 mL of this reagent was added to 0.5 mL of blood. This mixture was centrifuged at 3000 rpm for 5 min. The supernatant was collected through a Pasteur pipet and filtered through MFS disks of nylon of 0.4 mm of diameter. The clean supernatant was assayed by HPLC.

The extraction of cefotaxime in organs such as liver, spleen, kidney, lung and heart, was carried out by means of trichloroacetic acid 20%. 2 mL of this reagent were added to 0.5 g of dry weight of each organ. Each mixture was done homogeneous and was centrifuged at 3000 rpm for 10 min. The supernatant was collected through a Pasteur pipet and filtered through MFS disks of nylon of 0.4 mm of diameter. It was later assayed by HPLC.

Isocratic HPLC conditions used along this wak were: the mobile phase consisted of a 20:80% solution (v/v) of methanol in 0.01 M PO4H2K. The pH of the final solution was adjusted to 3.2 with phosphoric acid. The flow rate was 1 mL/min. Chromatograph was loaded with 40 µL of each sample. Chromatograms were run at room temperature. Samples were detected at 254 nm.

The susceptibility tests were as follows: One loop of each of the precultured strains was spread on to the whole surface of agar-plates. Immediately after the inoculation paper discs soaked with 20 ìL of the assays solutions were placed on to the agar media. The plates were kept in the fridge for 30 minutes to allow the liquid diffusion through out the agar media while the bacterial growth was arrested. Finally the plates were cultured for one day at 37 ºC and the microbicide effect of the solutions were measured by the size of the diameter of the growth inhibition zone (GIZ) surrounding each disc.

The assayed liquids were: free metal, free cefotaxime and the complex metal-antibiotic.

Standard solutions of free cefotaxime was prepared based on the Minimal Inhibitory Concentration (MIC) reported in literature (9), being the solutions 5 times over the MIC of each species: *for S. aureus spp aureus* and *E. coli*, the MICs were 3.3 µM and 0.12 µM respectively. In the assays with *B. subtilis*, since it was assumed to be unsensitive to the antibiotic, the mother solution was as *in S. aureus spp aureus*.

The highest concentrations of free metal assayed dissolved in water wee 5 times the maximum amount of Cu(II) found by us in poisoned rats (10). The source of Cu(II) was obtained from the acetate monohydrate salt at 44 µM.

To measure the activity of the complex antibiotic-metal was necessary one previous procedure to isolate the antibiotic bounded to copper. Free antibiotic and the metal salt were dissolved together at saturation, 0.4 M for both components, at room temperature to promote the complexation reaction. The complex was separated from the residual contamination by chromatophy in a HPLC column exactly as the conditions as we identified the free cefotaxime in rats. The chromatografied buffer containing the complex was collected in 1.5 ml eppendorfs.

The resulting complex was dissolved in a methanol buffer. To prevent the methanol toxicity, the alcohol was removed by dissecation in a vacuum trap. The final power debris was weighted, resuspended in water at saturation, homogenized and pooled. The dry amount of the complex antibiotic-Cu was 90 mg. We assumed all the dry weight to be the complex since the phosphate coeluted from the isolation buffer was negligible (5 µg).

The aqueous complex was prepared extemporarely at room temperate yielding 0.66 M cefotaxime-Cu final concentration. The assays were done with this concentration and four other more diluted in water.

3. RESULTS AND DISCUSSION

3.1. Concentration of cefotaxime

The concentration homogenates of free cefotaxime was measured in blood, liver, kidney, spleen, lung and heart of the killed rats from groups A and B at the 30, 60, 90, 120, 150, 180 and 210 min. after the administration of cefotaxime. As there were 5 rats by each time, the average concentration of free cefotaxime was calculated by each group of 5 rats that were killed at each time.

Free antibiotic was present in all of samples showing a peak after 30 min of the administration the highest concentration of cefotaxime was found in blood (four order of magnitude more cefotaxime than the solid organs).

After the first 30 min, the antibiotic concentration dropped from the maximum to the minimal values reached after 3 hours and half of the beginning of the experiment.

The declining plots depicted a «two-phase» kinetic, inespectively of the group of rats or the organ source of the samples, in good agreement with the conclusion of the decrease of free antibiotic found in adult humans with normal renal function (11, 12).

In the first 30-90 min cefotaxime disappeared from the sample slowly, afterwards the declining late accelerated.

We can establish conclusive results describing that the amount of antibiotic is much higher in control rats than in copper-toxified rats.

The differences more significant have been seen in liver, kidney and lung. In spleen and heart, those differences were less prominent, although they are very appreciable. In blood, concentrations of free cefotaxime were greater in rats without copper, but the differences between concentrations are not as rough as in the others samples

that have been analysed. So, the presence of copper is an influential factor in the concentration of free cefotaxime.

This observation has been supported with the help of a statistical analysis, here the means of concentrations of free cefotaxime, in blood, liver, spleen, lung, heart and kidney of rats without copper and of the rats with it, at 30, 60, 90, 120, 150, 180 and 210 min. after administration of this cephalosporin, have been compared. This comparison has been done by means of a T distribution with $p < 0.05$.

Therefore, in all those times is dismissed the nule hypothesis and the averages differences of cefotaxime concentrations were statistically significant. As deduced from these differences between the free cefotaxime concentration in rats with and without copper did not happen randomly, so those differences in the concentration could be due to the formation of antibiotic/metal complex, remaining a lesser concentration of free cefotaxime.

3.2. Distribution of cefotaxime

Tables 1 and 2 show the greatest concentration of cefotaxime in blood. In the assayed organs the concentration of this cephalosporin is decreasing in the sense: Kidney > liver > lung > spleen > heart. Therefore cefotaxime is mainly stored in kidney. This fact happened in both groups, so we induced, the metal is not an influential factor in the distribution of cefotaxime.

Table 1. **Average concentrations (mg/mL) of free cefotaxime in rats without copper**

	TIME (MIN)						
SAMPLES	**30**	**60**	**90**	**120**	**150**	**180**	**210**
BLOOD	0.80	0.46	0.25	0.14	0.08	0.06	0.02
LIVER	$6.03.10^{-5}$	$2.68.10^{-5}$	$2.16.10^{-5}$	$1.71.10^{-5}$	$1.42.10^{-5}$	$1.37.10^{-5}$	$1.24.10^{-5}$
SPLEEN	$2.70.10^{-5}$	$1.58.10^{-5}$	$1.45.10^{-5}$	$0.53.10^{-5}$	$0.41.10^{-5}$	$0.39.10^{-5}$	$0.38.10^{-5}$
KIDNEY	$24.2.10^{-5}$	$18.1.10^{-5}$	$10.6.10^{-5}$	$5.90.10^{-5}$	$5.01.10^{-5}$	$4.81.10^{-5}$	$3.17.10^{-5}$
LUNG	$7.19.10^{-5}$	$5.26.10^{-5}$	$3.52.10^{-5}$	$3.02.10^{-5}$	$1.33.10^{-5}$	$1.22.10^{-5}$	$0.46.10^{-5}$
HEART	$2.56.10^{-5}$	$1.87.10^{-5}$	$1.23.10^{-5}$	$1.14.10^{-5}$	$1.08.10^{-5}$	$0.86.10^{-5}$	$0.85.10^{-5}$

Table 2. **Average concentrations (mg/mL) of free cefotaxime in rats with copper**

	TIME (MIN)						
SAMPLES	**30**	**60**	**90**	**120**	**150**	**180**	**210**
BLOOD	0.65	0.48	0.25	0.12	0.06	0.05	0.03
LIVER	$4.47.10^{-5}$	$2.46.10^{-5}$	$1.24.10^{-5}$	$0.88.10^{-5}$	$0.84.10^{-5}$	$0.61.10^{-5}$	$0.25.10^{-5}$
SPLEEN	$2.59.10^{-5}$	$1.49.10^{-5}$	$0.95.10^{-5}$	$0.50.10^{-5}$	$0.35.10^{-5}$	$0.27.10^{-5}$	$0.23.10^{-5}$
KIDNEY	$20.6.10^{-5}$	$9.03.10^{-5}$	$5.16.10^{-5}$	$4.01.10^{-5}$	$3.28.10^{-5}$	$2.80.10^{-5}$	$2.64.10^{-5}$
LUNG	$3.93.10^{-5}$	$3.46.10^{-5}$	$2.57.10^{-5}$	$2.16.10^{-5}$	$1.24.10^{-5}$	$1.01.10^{-5}$	$0.26.10^{-5}$
HEART	$1.61.10^{-5}$	$1.39.10^{-5}$	$1.21.10^{-5}$	$0.98.10^{-5}$	$0.87.10^{-5}$	$0.66.10^{-5}$	$0.29.10^{-5}$

3.3. Stability of cefotaxime

The decrease rate constants of the concentration of free cefotaxime in treated and control rats are of first-order, according to the best values of correlation coefficient. The absolute values of the logarithmic of the observed constants K are shown in Table 4, where the highest value of log K is in heart for the 2 groups of rats, therefore the cefotaxime is more stable in heart than in the others studied organs.

In rats without copper the stability of cefotaxime in organs is decreasing in the sense: heart and liver > kidney and spleen > lung. In rats with copper the stability is: heart > kidney > liver, spleen and lung.

In blood it was observed the lowest stability of cefotaxime in the 2 groups of rats.

Therefore the highest and lowest stability were unaffected by the presence of copper.

3.4. Activity of cefotaxime

It is not only important the presence of copper in the availability of cefotaxime, but also the presence of the metal results to be crucial in the effectiveness of the cephalosporin dosage administrated to the

animals because of the decrease of the free cefotaxime concentration due to the metal.

Table 3. **Differences more significant in concentrations of free cefotaxime between rats with copper and without it**

SAMPLES	TIME (MIN)	P	VALUE OF T
BLOOD	150	0.04	2.34
	30	0.02	2.7
	90	0.02	2.9
	120	$1.47.10^{-4}$	6.73
LIVER	150	0.02	2.8
	180	$1.57.10^{-3}$	4.68
	210	$6.36.10^{-8}$	18.9
	90	$7.27.10^{-3}$	3.57
	150	0.02	2.9
SPLEEN	180	$1.14.10^{-4}$	6.98
	210	$2.50.10^{-4}$	6.23
	60	$4.34.10^{-4}$	5.74
	90	$1.12.10^{-7}$	15.57
KIDNEY	120	5.10^{-4}	5.61
	150	$5.46.10^{-3}$	3.77
	180	$4.88.10^{-4}$	5.64
	30	$4.93.10^{-5}$	7.86
	60	$1.39.10^{-4}$	6.8
LUNG	90	$1.35.10^{-3}$	4.8
	120	$5.46.10^{-3}$	3.77
	180	0.04	2.45
	210	$1.28.10^{-3}$	4.84
	30	$6.39.10^{-4}$	5.4
HEART	120	$6.16.10^{-3}$	3.68
	180	$8.72.10^{-3}$	3.44
	210	$1.19.10^{-6}$	12.9

Table 4. **The absolute values of the logarithmic of the observed constant K**

	RATS WITHOUT COPPER	RATS WITH COPPER
BLOOD	1.73	1.79
LIVER	2.16	1.86
SPLEEN	1.93	1.86
KIDNEY	1.93	1.93
LUNG	1.86	1.86
HEART	2.16	2.16

Copper would provoke a fall down of free cefotaxime to values even below of those corresponding to the MIC (minimum inhibitory concentration) to kill the microorganism that would cause the infection, so that the activity of cefotaxime will decrease or even the cephalosporin will not have activity in many cases. So, in poisoned patients with copper, it is very important to bear in mind this fact when they have to follow a course of treatment with Cefotaxime.

In order to determinate and compare the biological activity of the complex, it was necessary to know the impact in the cell viability of the free cefotaxime as well as the metal by it-self in the same experimental conditions.

Dissolved copper in media culture has not a deleterious effect at all on growing cells (data not shown) even a mM scale in the discs, concluding that if the complex had any biological activity, it should not be due to rests of metal in the complex.

The detailed results of the assays with cefotaxime are shown in Table 5 and 6.

Shocking the *B. subtilis* CECT 356 strain (Table 5), which was thought to be a not susceptible bacteria (1, 9), arose as the most sensitive strain to cefotaxime, much more that even the well known sensitive strains of *E. coli* used by us. However *B. subtilis* was not sorted out in the past among the cefotaxime targeted species, perhaps due to be not a clinical bacteria, we strongly ask to favour their use as standard in sensitivity tests in the future.

Table 5. **Activity of cefotaxime against *B. subtilis* CECT 356 and *E. coli* CECT 434 and CECT 516**

	Concentration of cefotaxime in Moles	Diameter of the halo in mm
	$16.5.10^{-6}$	47
	$3.3.10^{-6}$	32
***B. subtilis* CECT 356**	$1.65.10^{-6}$	33
	$0.82.10^{-6}$	31
	$0.33.10^{-6}$	25
	$0.655.10^{-6}$	21
	$1.125.10^{-6}$	16
***E. coli* CECT 434**	$0.062.10^{-6}$	16
	$0.031.10^{-6}$	15
	$0.012.10^{-6}$	12
	$0.655.10^{-6}$	22
***E. coli* CECT 516**	$0.062.10^{-6}$	18
	$0.031.10^{-6}$	16
	$0.012.10^{-6}$	14

Table 6. **Activity of cefotaxime against *S. aureus spp aureus* CECT 239 and CECT 435**

	Diameter of the halo in mm			
	TSA Media		Agar-blood Media	
Concentration of cefotaxime	***S. aureus* spp *aureus* CECT 239**	***S. aureus* spp *aureus* CECT 435**	***S. aureus* spp *aureus* CECT 239**	***S. aureus* spp *aureus* CECT 435**
$16.5.10^{-6}$	33	37	27	30
$3.3.10^{-6}$	27	29	21	24
$1.65.10^{-6}$	27	27	18	23
$0.82.10^{-6}$	24	25	15	20
$0.33.10^{-6}$	19	23	12	17

As expected, cefotaxime was active against *E. coli* studied (Table 5), but some differences would be observed: strikingly *E. coli* CECT

434, described as standard of sensitivity likely was less affected than *E. coli* CECT 516, used in resistance to metals.

In the *S. aureus spp aureus* cultures, two different media were used: TSA and blood agar. The later provided an environment similar in somewhat manner to the mammalian tissues, because is a protein-rich medium containing cellular rests. cefotaxime was effective against those strains of *S. aureus spp aureus*, but the sensitivity was increased in the TSA cultures. It could be justified because an antibiotic sequestration by protein binding occurred in the blood agar medium. cefotaxime is reported to bind proteins, so the amount of free antibiotic would be 27-38% reduced in the presence of proteins (11). It means that the amount of functional antibiotic was lesser in the blood agar. Our findings agreed the reported data, because the GIZ in blood agar had mean values 25% smaller than in TSA cultures.

As observed in *E. coli*, the two strains assayed did not show the same results regarding the level of sensitivity to cefotaxime. However the experimental results were not enough to build statistical analysis, it seemed, comparing the data of the *E. coli* and *S. aureus spp aureus* strains, that the intensity of the antibiotic aggression could have a strain dependent component in each of the sensitive species.

As above mentioned, the importance of this work is to know whether the complex, antibiotic-metal has biological activity against the strains of microorganisms studied.

The cefotaxime-copper complex (Table 5) had some activity against *B. subtilis* CECT 356 and *E. coli* CECT 516 only at the highest concentration used, being harmless at lower concentrations.

The results would induce misleading conclusions. Apparently, there waas a moderate microbicide activity against the model species, but a matter of scale should be addressed. cefotaxime was fully active at μM concentrations or lower, whereas the complex derivate required a concentration six orders of magnitude higher to affect slightly the cell viability. The maximum concentration *in vivo* of cefotaxime reported in adult patients with a normal renal function is 55 μM (1,11), so the microbicide effect of the complex lacked of a practical relevance in physiological terms.

Finally, we wish to warm about the clinical outcome of our finding: antibiotic treatment based on cefotaxime in copper poisoned patients

with a bacterial sickness would be completely counterproductive, because the infecting microorganisms could progress without opposition.

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