Cyclophosphamide and Doxorubicin but Not Methotrexate Responsible For Rapid Development of the Resistance to Ciprofloxacin in Uropathogenic *Escherichia Coli* in *Vitro*

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ABSTRACT:

BACKGROUND:

The effect of anticancer chemotherapies on mammalian cells had been studied previously but few studies are performed to study the reaction of the bacterial cell toward mutagenic chemotherapies. We sought to study the effect of some anticancer chemotherapies on the rapid development of resistance to ciprofloxacin in ciprofloxacin susceptible uropathogenic *E.coli* (UPEC) in vitro.

METHODS:

Two methods of mutation induction are performed, modified Ames test and modified tube method. Cyclophosphamide, doxorubicin and methotrexate (MTX) at plasma concentration tested as mutagenic drugs towards ciprofloxacin resistance in UPEC. Disc diffusion test for antibiotic susceptablity is also used. Chi-square test test and pooled t test are used for statistical analysis.

RESULTS:

- Antibiotic susceptibility test; Multidrug resistance (70%) was found in the UPEC isolates and is often associated with the isolates of leukemic patients ($P<0.05$). About 90% of these isolates showed resistance to antibiotics which had been received previously by the leukemic patients during the administration of anticancer chemotherapies ($P<0.01$).
- Mutation induction: In modified Ames test, a growth zone of 25mm and 24mm around the cyclophosphamide and doxorubicin containing discs respectively are developed. But no bacterial growth is noticed around both MTX and control discs. In modified tube method, ciprofloxacin susceptible UPEC developed resistance to it gradually during 72 hours. UPEC in doxorubicin, MTX containing broth and control broth still susceptible.

CONCLUSION:

We conclude that the mutagenic effect of cyclophosphamide and doxorubicin may be responsible for the relapsing during antibiotic treatment of urinary tract infections in patients treated with these drugs and perhaps other mutagenic anticancer chemotherapies.

KEYWORDS: Cyclophosphamide-Doxorubicin-Methotrexate-Ciprofloxxacin resistance- Uropathogenic *Escherichia coli*- Mutation-Leukemia.

INTRODUCTION:

Cyclophosphamide is an alkylating agent used as anticancer drug. Alkylating agents are found to be covalently bound to a wide variety of biological molecules including, nucleic acids[16] . It produce interstand and intrastrand DNA-DNA crosslinks. This drug used for the treatment of some types of leukemias.[9] Doxorubicin is an anthracyclines, active as anticancer drug, it is employed in the treatment of carcinoma of lungs, breast cancer, acute lymphocytic leukemias and many other cancers. Methotrexate (MTX) is a synthetic drug (diaminopyrimidine derivatives) act as a structural analogue of folic acid and which bind to and inhibits the dihydrofolate reductase (DHFR). Choriocarcinoma, acute lymphocytic leukemia and Burkitt's lymphoma are highly sensitive to MTX[5].

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The reduction of the number of neutrophils and other leukocytes due to the toxic effect of these drugs, lead also to reduce granulocyte-macrophage colony stimulating factor (GM-CSF) leading to opportunistic infections in different body sites with Gram negative and Gram positive bacteria[13, 15 and 17].. Bacterial infections are indication of the antibacterial drugs administration. Many types of antibiotics are used including fluoroquinolones (FQs). They inhibit many types of bacteria, these drugs are highly active against Enterobacteriaceae (Yu et. Al., 1999), and are generally effective in urinary tract infections (UTIs)[10 and 12]. In the leukemic patients with UTIs, who are treated with anticancer chemotherapies and antibiotics, the bacteria exposed to both of the drugs at plasma concentration just like the mammalian cells or even more when the drugs are excreted via urinary tract, the reaction of mammalian cells toward chemotherapies is well studied to many of drugs

specially cyclophosphamide, doxorubicin and MTX[5]. But few studies are performed to study the reaction of the bacteria cell toward chemotherapies[14]. In this study, we sought to study the effect of chemotherapies on the rapid development of resistance to ciprofloxacin in uropathogenic *E.coli* (UPEC) in vitro.

PATIENTS, MATERIALS AND METHODS:

Patients: Sixty UPEC isolates were obtained from two groups of patients at Al-Kadhymia Teaching Hospital-Baghdad. 30 isolates were obtained from leukemic patients suffering from UTls, their ages ranging from 25-61years. All of them receive triple combination chemotherapies with two or more, antibactieral drugs. The chemotherapies were vincristine, cyclophosphamide, doxorubicin, 6-mercaptopurine, MTX, 6-thioguanidine. They received these chemotherapies for three weeks to three years, 25 of them receive antibactial drugs which were co-trimazole, gentamicin, ampicillin, ciprofloxacin and cloxacillin. The receiving duration of such drugs ranging from 14 to 22 days, many of them (19) suffering from relapsing during UTIs treatment. The second group of isolates (30 isolates) were obtained from patients had UTIs, their ages ranging from 20-65 years, they didn't had predisposing factors of infection .

MATERIALS:

Anticancer chemotherapies; cyclophosphamide and MTX (MEHECO-China), and doxorubicin (Serum Institute of India) were used. Ciprofloxacin (MBO-Syria), Brain heart infusion BHI (Oxoid-UK) and Nutrient agar (Oxoid-UK) are used as well as 12 antibiotic susceptibility discs which are ampicillin, cloxacillin, cephalexin, cefotaime , co-trimazole, amikacin, gentamicin , tetracycline, norfloxacin and chloramphenicol (Al-Razi-Iraq), ciprofloxacin and nitrofurantoin (SDI-Iraq).

METHODS:

A- A modification of Ames test[4]. For mutagenesis, was used by substitute the histidine and *Salmonella* in the original test with an antibiotic (ciprofloxacin) and *E.coli* 278 (ciprofloxacin susceptible isolate). Nutrient agar contains ciprofloxacin at minimum inhibitory concentration (MIC) was incubated with 10^8 cell/ml of cultured BHI broth with UPEC 278 isolate[18]. By spreading the cultured BHI broth on the surface of the agar, three discs each one contain one of the three chemotherapies at plasma concentration (MTX= 6 μM , doxorubicin= 10 μM and cyclophosphamide = 450nM) were distributed on the surface of the agar with control discs contain normal saline, then the agars were incubated at 37^0C.

B- Amodification of a tube method of Garrod *et al* (1973) is another method for mutation induction was used. Four test tubes each one contain one of the three chemotherapies at plasma concentration in BHI broth, and each contain ciprofloxacin at concentration lower than MIC (2 μg/ml), the fourth contain only ciprofloxacin at 2 μg/ml in BHI to be used as control. All of these tubes were inoculated with *E.coli* 278 culture at final concentration of 10^8 cell/ml in BHI broth. After overnight incubation 500 $\mu\ell$ of broth from each tube were obtained and diluted with sterile normal saline, compared with 0.5 MacFarland standard tube to reach 10^8 cell/ml, then the diluted broth was submitted to ciprofloxacin susceptibility test. The rest broth in four test tubes were incubated again overnight and tested for ciprofloxacin susceptibility, twice , after 24 and 48 hours.

C- Disc diffusion test was used for antibiotics susceptibility[18 and 19].

D- Statistical analysis: The data were analyzed by chi-square test which was applied to find an association between the antibiotics receiving by the leukemic patients, who were suffering from relapsing during UTIs treatment and the resistance to these antibiotics in their UPEC isolates. Pooled t test was used for analysis of antibiotic susceptibility differences between the leukemic and non-leukemic UPEC isolates[8] .

RESULTS:

1- Antibiotic susceptibility test: Resistance to eight and more antibiotics was found in 42 (70%) of UPEC isolates. This feature, multidrug resistance, is often associated with the isolates of leukemic patients ($P<0.05$). About 90%of these isolates showed resistance to antibiotics which had been received previously by the leukemic patients during the administration of anticancer chemotherapies ($P<0.01$).

2- Mutation induction: In the modified Ames test (Figure 1) growth zones of 25mm and 24 mm around the cyclophosphamide and doxorubicin containing discs respectively are developed. These zones of growth were appear after 24 hours incubation at 37^0C, while a few colonies distributed irregularly on all other agar plates with MTX and control discs.

Figure 1: The development of E.coli growth zone around the disc contain cyclophosphamide at plasma concentration in media contain ciprofloxacin at minimum inhibitory concentration.

Modified tube method of Garrod *et al* (1973) was performed also to induce mutation by thechemotherapies. In this method ciprofloxacin susceptible *E.coli* 278 in ciprofloxacin-cyclophosphamide containing broth was submitted to ciprofloxacin susceptibility test. In the first 24 hours of incubation, the inhibitory zone around ciprofloxacin disc was 20mm. It was 10mm after 48 hours. After 72 hours of incubation the zone disappears. Neither doxorubicin nor MTX containing culture broth show any changes in the inhibitory zone during these three days of incubation. *E.coli* 278 still susceptible in control .

DISCUSSION:

We induce mutation using antibiotic resistance as a simple feature that can be easily detected and reflect the effect of anticancer chemotherapies. This mutation induction may be given an idea about possible other mutations toward other features may develop during the years of the treatment with mutagenic anticancer chemotherapies. *E.coli* is used as a model for such induction because it is the most common causative agent of UTIs and was the most common one in UTIs in luekemic patients in our study.

Furthermore, we use ciprofloxacin to induce resistance to it because it is the antibiotic that is used for treatment of the most clinical cases of UTIs recently in adults in both leukemic and non leukemic patients. On the base of the obtained results of modified Ames test. The concentration of 4 μ g/ml , which is the MIC of ciprofloxacin.[6], play the major role in 24 hours mutation induction. Baquero and Negr (1997) explain such result in that the antibiotic resistance mutant is expected to have a maximum at one particular antibiotic concentration close to MIC for the bacteria, beyond this concentration, antibiotic concentrations may be able to reduce or suppress in an equivalent way the growth of both susceptible and variant populations and therefore the selection of a particular antibiotic resistance variant may happened only in a narrow range of drug concentrations and that define as selective window and that mean that when the antibiotic concentration rises , the number of selectable mutants decreases and the mutations by this method at MIC concentration will develop. Bacteria growing in vivo are frequency under stress, because they are starve, under antibiotic challenge and under stress of defense mechanisms. So the frequencies of mutation are probably much higher in the coarse of infective process than those that have been determined by in vitro analysis.[4] Chemotherapies, as mutagenic drugs, will be able to be more mutagenic in starving laboratory conditions, in modified tube method, without any addition of nutrient or correction to the PH of the Medium and may be resemble to that in vivo.

In modified tube method, the isolate in cyclophosphamide containing tube became resistant gradually. In first 24 hours *E.coli* isolate became a moderately susceptible to ciprofloxacin, with inhibitory zone of 20mm.

After another 24 hours of incubation with cyclophosphamide and ciprofloxacin, the bacteria became resistant but the inhibitory zone was 10mm in diameter. The zone was disappear in the next 24 hours of incubation. This can be explained according to Martinez and Baquero (2000) who are found that, the changes in at least seven positions in *gyr* A gene that result in a quinolone resistance

Phenotype or changes in only three positions in the *par*C (gene, that encode for topoisomerase IV) also can result in such phenotype. The mutation in one or another of these changes may produce low level resistance (moderately susceptible). Whereas, high level resistance frequently required mutation in more than one gene (cooperative mutations).

Successive mutations in *gyr* A, *par*C and in regulatory sequences of efflux pumps as well, may responsible for a complete resistance to ciprofloxacin in the third day of incubation.

Most of anticancer chemotherapies had been reported as mutagenic and carcinogenic agents in Ames test and in vivo studies of carcinogensis, table 1 show these facts.

Table 1: The effect of some antineoplastic agents in three systems of carcinogensis (Chabner, 1982)

Agent	Ames test	Sister chromatid Exchanges	Animal Studies
Cyclophosphamide	+	+	+
Doxorubicin	+	+	+
6-Mecaptopurine	+	-	-
Vincristine	-	±	-
Methotrexate	-	+	-
Nitomycin	+	+	+

+ = positive result - = no positive result reported

cyclophosphamide has the effect of the inhibition of the DNA synthesis by producing interstand and intrastrand crosslink[9] . Figure 2 reveals cyclophosphamide structure.

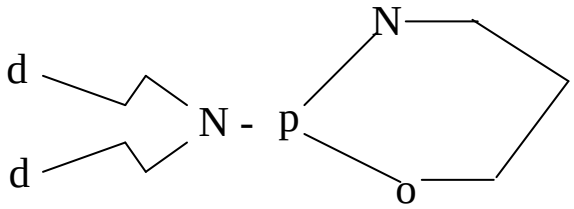

Figure 2 : Cyclophosphamide Structure (Fox and Fox, 1984)

Doxorubicin in general composed of Tetracyclinc chromophores and possess a single sugar (Figure 3). The Chromophore appears to insert itself between base pairs perpendicular to the long axis of the double helix of DNA. The major interaction coming between the B and C rings of the drug and the base above and below them, the A and D rings protrude out of either side of the helix.[3] .

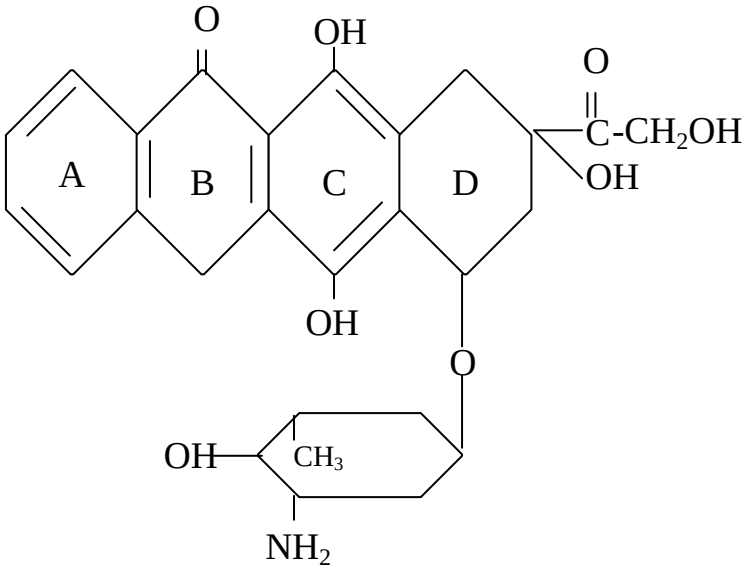

Figure 3: Doxorubicin structure (Fox and Dox, 1984).

As shown in table 1, and also in our study MTX didn't show mutagenic effect. MTX is a structural analogue of folic acid which bind to and inhibit the DHFR. In our study, cyclophosphamide shows the strongest effect among the three chemotherapies as mutagenic drug. Doxorubicin reveals mutagenic effect just with modified Ames test.

But MTX didn't show such effect as reported previously. Most of the UPEC isolates of the leukemic patients who received antibiotics and anticancer chemotherapics were resistance statistically to the antibiotics which were received. From these facts we conclude that the mutagenic effect of these drugs and other mutagenic anticancer drugs may be responsible for the relapsing during treatment of UTIs in patients treated with mutagenic anticancer chemotherapies. The mutations toward antibiotic resistance may be induced under stress condition at inflammatory sites in the presence of chemotherapies.

These mutations increase in he presence of both stress factors and mutagenic drugs as compare with the presence of stress factors only.

Therefore, UPEC isolates of leukemic patients show multidrug resistance more than those of UTIs only, and were resistant to the most of the 12 antibiotics. We recommend that, the mutations towards other features must be studied well, whether it get benefit to the host or to the bacterial cells. The position of gene mutations must be determined in such cases. On the other hand, and to overcome the treatment problems, antibiotic susceptibility test must be done and the antibiotics must be given with high does to the mutagenic drug treated patients.

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