EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 6
31-35
Year: 2017

ANALYSIS OF GENOTOXICITY OF CEFTRIAXONE IN HUMAN LYMPHOCYTE CULTURES

Azra Metović* and Jasmin Mušanović

Department of Biology and Human Genetics, Medical Faculty, University of Sarajevo, Bosnia and Herzegovina.

***Author for Correspondence: Dr. Azra Metović**

Department of Biology and Human Genetics, Medical Faculty, University of Sarajevo, Bosnia and Herzegovina.

Article Received on 24/03/2017 **Article Revised on 15/04/2017** **Article Accepted on 05/05/2017**

ABSTRACT

I**ntroduction**: Genotoxic effect of ceftriaxone, a antibiotic of the third generation cephalosporins, have been evaluated. **Metods:** In vitro analysis included evaluation of the genotoxic and cytotoxic potential of different test ceftriaxone in concentrations of 0.15, 0.25 and 0.50mg/mL. Genotoxic potential was evaluated by using the cytokinesis block-micronucleus cytome assay in cell of cultivated human peripheral blood lymphocyte cells. **Results:** Results of the analysis on the presence of micronuclei, nuclear buds and nucleoplasmic bridges in 1000 binuclear cells per sample showed that the relative frequency of these indicators increased with increasing concentrations of ceftriaxone in lymphocyte cultures, while there are significant differences in their frequencies relative to controls as determined for each ceftriaxone concentration. **Conclusions**: Based on these results it can be assumed that ceftriaxone has a genetic potential. The frequency of micronuclei, nucleoplasmic bridges and nuclear buds in cytokinesis-blocked cultures of human peripheral blood lymphocytes increases with increasing concentrations of Ceftriaxone, with a significant difference in their frequencies relative to controls as was determined for each of the concentrations. Ceftriaxone present genotoxic, cytostatic and cytotoxic activity in the applied in vitro cytogenetic tests. Ceftriaxone in lymphocyte culture affects the inhibition of cells proliferation, as confirmed by the decrease of NDI (nuclear division index - indicator of cytostatic effect) and NDCI (nuclear division cytotoxicity index - indicator of cytotoxic effects) compared to the controls.

KEYWORDS: human lymphocytes, genotoxicity, micronuclei.

INTRODUCTION

The discovery and use of antibiotics in treating infections is one of the greatest achievements of medicine in the twentieth century. This is a group of drugs that prolong the lives of people on our planet and made some fatal diseases treatable.

In modern medicine, antibiotics are in one of the most important group of drugs due to the high efficiency in the treatment of infections in primary health care and complicated infections in hospitals. Ceftriaxone belongs third generation of cephalosporin antibiotics. Like other drugs of this class, it has broad spectrum of activity against Gram-positive and Gram-negative bacteria. Generally, cephalosporins are a group of antibiotics that shows no genotoxic potential.[1,2,3]

Results of some studies, which included the Ames test, micronucleus test and a test for chromosomal aberrations in cultured human lymphocytes *in vitro*, do not show genotoxic potential and mutagenic effect of Ceftriaxone in individual therapeutic doses.[4,5]

In this regard, the topic of this study was cytogenetic analysis of selected concentrations of Ceftriaxone genotoxicity on human lymphocyte culture.

The study in line with the objectives set is performed using cytokinesis - cytokinesis block micronucleus assay (CBMcyt) *in vitro*, which is included in the standard battery of genotoxicity tests. Cytome is a comprehensive term for the measurement of DNA damage, cytostasis and cytotoxicity.

This test is a good indicator of genotoxicity, it identifies the chromosome and genome mutations, is applicable on many cell types, and to determine aneurogenic and clastogenic effects of the test compounds commonly used.[6,7,8,9,10] In addition, this test allows the determination of the number of cells in apoptosis and necrosis,as well as the nuclear division index (NDI), which is an indicator of cell proliferation rate and indicates the duration of the cell cycle *in vitro*.[11]

Micronuclei are independent chromatin structures that are completely separate from the nucleolus. Genotoxic

agents can induce the formation of micronuclei and the similar structures in the exposed organism through different mechanisms[12,13,14] Their presence indicates the existence of aberrations in the previous cell division, and therefore the frequency of micronuclei can be used as a quantitative measure of structural and numerical chromosomal aberrations in cells, both *in vitro* and *in vivo* under the influence of different genotoxic agents[15]

DNA damage is accumulated in cells that have undergone one division (binuclear cells) and include[16]

a) Micronuclei as a biomarker of chromosome breakage and/or loss of whole chromosomes;
b) Nucleoplasmic bridges, biomarkers of DNA miss repair and/or fusion of the ends of telomeres;
c) Nuclear buds, biomarkers of enhanced DNA elimination and/or DNA repair.

Cytostatic effects are measured through mitotic status (proportion of mono-, bi- and multinucleated cells), and cytotoxicity by determining the status of viability (necrosis and/or cells apoptosis)[11]

This test has been successfully used for biomonitoring *in vivo* genotoxic exposure, *in vitro* genotoxicity testing in different research areas[17]

The aim of this study was to evaluate the genotoxic and cytotoxic potential of different test ceftriaxone in concentrations of 0.15, 0.25 and 0.50mg/mL.

MATERIAL AND METHODS

Ceftriaxone ($\underline{C}_{18}\underline{H}_{18}\underline{N}_{8}\underline{O}_{7}\underline{S}_{3}$) is a white crystalline powder easily soluble in water, moderately soluble in methanol and very little soluble in ethanol. Ph of 1% water solution is approximately 6.7.

The structural formula is.

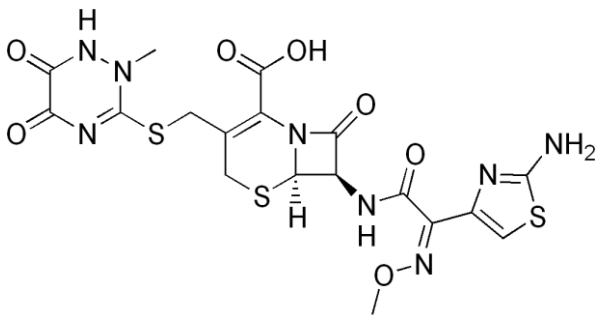

Figure 1: Structural formula of Ceftriaxone.

Ceftriaxone belongs to third generation of cephalosporin antibiotics. Like other drugs of this class, it has broad spectrum activity against Gram-positive and Gram-negative bacteria. Ceftriaxone is often used (in combination with macrolide and/or aminoglycoside antibiotics) for the treatment of mild to moderate pneumonia.[18]

For cytogenetic analysis of genotoxicity in this paper Ceftriaxone was used, as pure substance in powder, which was dissolved in the solvent for parenteral use, by aqua pro injection solution which provided concentrations of: 0.15mg /mL, 0.25mg/mL and 0.50mg/mL.

TREATMENT OF CELL CULTURES

Cytogenetic analysis of genotoxicity Ceftriaxone was performed on peripheral blood samples of 10 healthy volunteer donors (5 men and 5 women) aged 30-40 years at the time of sampling and all were nonsmokers.

For the purposes of this research were chosen healthy subjects whose occupation is not linked with exposure to chemical and physical agents that could affect the value of cytogenetic findings. The blood was collected by venipuncture in sterile vacutainers with sodium heparin (BD Vacutainer Systems, Plymouth, UK).

For each control (without exposure to drug) and experimental treatment with the selected test concentrations of Ceftriaxone: 0.15, 0.25 and 0.50mg/mL, was seeded whole blood under sterile conditions, and in the shortest period in the sterile conditions are formed cell cultures.

Cultivation of peripheral blood lymphocytes was performed using standard procedures[19] with certain modifications.
For culturing human peripheral blood lymphocytes was used complete culture medium PB-MAX ™ Kariotyping Medium (GIBCO-Invitrogen). To 5mL of this medium was added 300µl of whole blood of the donor and 0.5 ml of the following selected test Ceftriaxone concentrations: 0.15, 0.25 and 0.50mg/mL. Cultures were maintained in an incubator at 37^{o}C.

In this study is applied the generally accepted protocol for cytokinesis-block micronucleus cytome assay.[6,11] It includes a 72-hour cultivation of peripheral blood lymphocytes *in vitro*, wherein to inhibit cytokinesis during 45 hour to the culture was added 0.5mL of cytochalasin B. In this study, the solution of cytochalasin B was first dissolved in dimethylsulfoxide (DMSO) (Pancreac Quimica, Barcelona, Spain) to a concentration of 0.5mg/mL, and then diluted in sterile H_2O.

The technique is based on the use of cytochalasin B, actin polymerization inhibitor, which blocks cytokinesis, but not the division of the nucleolus. In this manner was possible the analysis of exclusively (binuclear) cells that have completed their first "*in-vitro*" division after the initiation of the culture.[6]

After the expiration of cultivation, the content was shaken and centrifuged for 10 minutes at 800rpm. After removal of the supernatant, the cells were treated with hypotonic for 5 minutes.

Afterwards, the fixation was performed, which was repeated three times. Milky white residue obtained after three times and resuspended in up to 0.5 fresh fixation agent and placed on the microscope glass slides. The slides were air-dried, and then stained with 5% Giemza solution (Carlo Erba-Antibiotics Spa, Rodeno, Italy) in phosphate Gurr buffer (Gibco-Invitrogen, Carlsbad, CA) for 7 minutes.

MICROSCOPIC ANALYSIS

Slides were analyzed under magnification of 900 times. Cytokinesis-block micronucleus assay Cito included determining the frequency and distribution: micronuclei in binuclear cells, binuclear cells with micronuclei, nuclear buds, nucleoplasmic bridges, apoptotic and necrotic cells and nuclear division index, by the analysis of 1000 cells for each control and sample treated with Ceftriaxone.

Identification and analysis of the above markers was conducted in accordance with the prescribed HUMN criteria.[11] The same samples were used in the analysis and 1000 cells and were determined the frequencies of mono-, di-, three- and tetra nuclear cells and calculated the nuclear division index (NDI) of peripheral blood lymphocytes, method proposed by (20) and the index of nuclear proliferation and cytotoxicity (NDCI).[21]

STATISTICAL ANALYSIS

Statistical significance of the difference between control and test cultures treated with Ceftriaxone concentrations, was tested by one-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparisons, using MedCalc version 12.5.0.0.significant level was set at $p<0.05$.

To analyze the correlation between the results of the conducted test was applied the Pearson coefficient of correlation (product of moment correlation coefficient).

RESULTS

Results of the analysis genotoxic effects of test concentrations of ceftriaxone in cytokinesis blocked lymphocyte cultures expressed by micronuclei frequency, nucleoplasmic bridges and nuclear buds as well as nuclear division index and nuclear division cytotoxicity index in human lymphocyte culture are presented in table 1 as the means ± standard deviation (SD).

Table 1: CBMN-cyt assay in human lymphocytes treated with ceftriaxone.

Treatment	MN	NB	NPB	NDI	NDCI
Control	2,1±0.738	0,3±0.675	0	2.178±0.052	2.178±0.052
0.15 mg/mL	3,1±0.994	2,6±1.265	0	2.097±0.056	2.096±0.057
0.25mg/mL	5,2±1.549	3,6±1.506	0,9±0.876	1.982±0.057	1.977±0.028
0.50 mg/mL	12,6±2.319	7,3±1.567	1,6±0.699	1.760±0.063	1.755±0.062

Values presented as the mean ±SD
Significantly different compared to controls $p< 0.05$.
MN – micronuclei; NB – nuclear buds; NPB – nucleoplasmic bridges;
NDI – nu clear division index; NDCI – nuclear division cytotoxicity index.

ANOVA accompanied by Student-Newman-Keuls test showed a significant increase in the frequency of micronuclei between cultures treated with ceftriaxone concentrations 0.25mg/mL and 0.5mg/mL compared to control . Although the frequency of micronuclei in cultures treated with Ceftriaxone 0.15mg/mL was increased compared to control, this increase was not statistically significant.

A significant is also the difference in the frequency of micronuclei between each of the selected cultures treated with Ceftriaxone concentrations (0.15, 0.25 and 0.50mg/mL).

ANOVA, followed Student-Newman-Keuls test, also showed a significant increase in the frequency of nuclear buds in all tested concentrations of the drug as compared to control.

There was also a significant difference in the frequency of nuclear buds in a concentration of 0.50 mg/mL compared to the concentration of 0.15mg/mL and 0.25 mg/mL.

Although is observed increased frequency of nuclear buds in a concentration of 0.25 mg/mL versus the concentration of 0.15mg/mL, that difference is not statistically significant.

The analysis of variance (ANOVA) and a Student-Newman-Keuls test at a significance level of $p <0.05$, confirmed the significance of differences in frequencies of nucleoplasmic bridges between cultures treated with a concentration of Ceftriaxone of 0.25 mg/mL and 0.50mg/mL.

By the analysis of simple linear regression is demonstrated a statistically significant association between the nuclear division index (NDI) and the applied concentration of Ceftriaxone in lymphocyte cultures ($p=0.006$, $t =-12.51$). Low p value indicates that there is a linear relationship between NDI and the concentration of Ceftriaxone and negative t value that the increase in the concentration of the drug causes a decrease of the nuclear division index (NDI) (figure 1)

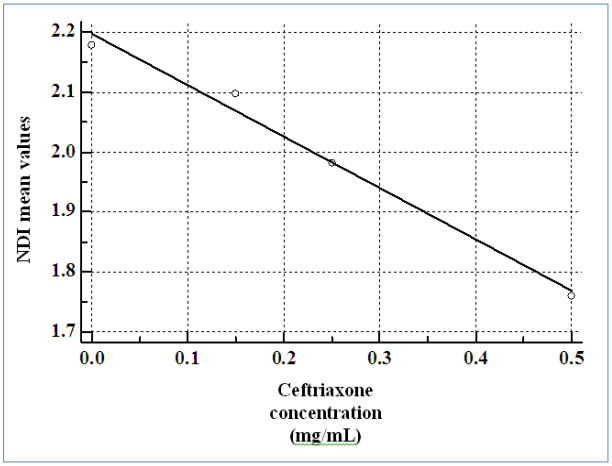

Figure 1: Average NDI values.

Results of the analysis of simple linear regression between the index of the nuclear division cytotoxicity (NDCI) and applied concentration of Ceftriaxone in lymphocyte cultures (p=0.006, t=-12.48), also indicate a strong linear correlation between the values NDCI and test concentrations of the drug, and direction of the regression line shows that this relationship is inversely proportional (figure 2).

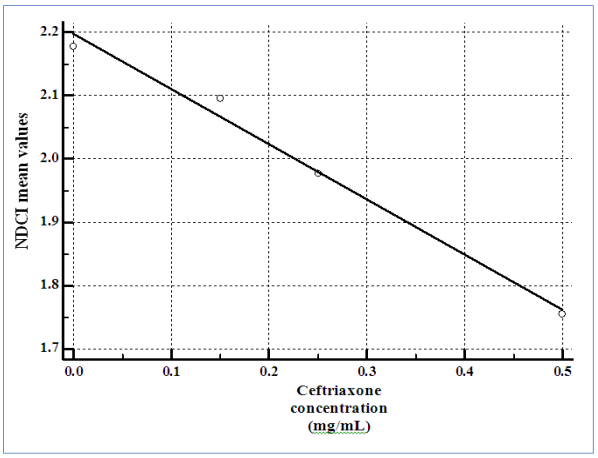

Figure 2: Average NDCI values.

Results of correlation between micronuclei (MN), nuclear buds (NB), nucleoplasmic bridges (NPB), NDI and NDCI obtained by using Pearson's correlation test are shown in tabele 2 (above the diagonal are shown p values and r values below the diagonal)

Table 2: Results of correlation between the frequency of MN, NB, NPB, NDI and NDCI.

	MN	NB	NPB	NDI	NDCI
MN		<0.0001	<0.0001	<0.0001	<0.0001
NB	0.8911		<0.0001	<0.0001	<0.0001
NPB	0.6846	0.693		0.0006	0.0005
NDI	-0.660	-0.611	-0.517		<0.0001
NDCI	-0.668	-0.604	-0.526	0.929	

Higher values of correlations between micronuclei in binuclear cells and nuclear buds (r=0.89, p<0.0001) indicate their stronger interconnection with respect to the correlation between the frequency of the total number of micronuclei in binuclear cells and nucleoplasmic bridges (r=0.68, p<0.0001)

Statistically significant, moderate, negative correlation exist between the total number of micronuclei in binuclear cells and nuclear division index with values of r=0.660, p<0.0001. Negative and moderate correlation was detected between NDCI and the total number of micronuclei in binuclear cells, with values r=-0668, p<0.0001.

DISCUSSION

Ceftriaxone expressed its effect by cytological (according to Environmental Mutagen Informatic Center-EMIC index).[22] Cytological effects are manifest by appearance of micronuclei, which are certain indicator of the loss of genetic material by changing the morphology of the nucleolus, and the emergence of multiple nucleoli.

According to the results it is observed that with increasing concentrations of Ceftriaxone in cytokinesis-blocked cultures of peripheral blood lymphocytes, also is increased the frequency of micronuclei per 1000 binuclear cells.
In particular are emphasized (statistically significant difference in frequency of micronuclei and structural aberrations compared to controls) genotoxic effects of Ceftriaxone in cultures of peripheral blood lymphocytes at a concentration 0.50mg/mL, which also showed the strongest antiproliferative or cytostatic and cytotoxic activity.

Determined is also increased frequency of apoptosis and necrosis in cytokinesis-blocked lymphocyte cultures treated with Ceftriaxone in concentrations of 0.50 mg/mL, indicating the need for more detailed analysis of its potential apoptosis - inducing effect, since the induction of apoptosis is the mechanism of action of some drugs.[23]

In contrast to our results, which indicate a genotoxic potential of the tested antibiotic, other antibiotics such as tetracycline[24] and amoxicillin[25] do not show any genotoxic effects in lymphocyte cultures.

CONCLUSIONS

Ceftriaxone express genotoxic, but also the cytostatic and cytotoxic activity on the applied cytogenetic *in vitro* tests. Frequency and expressiveness of observed effects are directly related with applied concentrations of Ceftriaxone.

Not all types of damage can be presented as micro nucleuses because of which it is wrong to assume that the cytokinesis-block micronucleus assay could replace detailed analysis by the standard analysis of chromosome aberrations.

Applicable significance of this research arises from the fact of vague, and until now, insufficiently explored genotoxic potential of Ceftriaxone. For this reason, our findings provide exact data on the possible harmful effects of this drug on the basis of which it will be possible to derive a general conclusion about its eventual share in the genetic load of human populations.

Based on all the above, this research should contribute to its clearer positioning in a broad spectrum of substances with the antibiotic potential and the possible need to rationalize its application.

DECLARATION OF INTEREST

The authors report no declarations of interest. The authors confirm no conflicts of interest and are alone responsible for the content and writing of the paper.

REFERENCE

1. National Committee for Clinical Laboratory Standards, Supplemental Tables. NCCLS document, 2000; M100-S 10(M7): (ISBN 1-56238-309-9).
2. Kondo K, Takase S, Nishimoto Y, Miyajima H, Shiratori O, Miyake YJ. Genotoxicity studies of cefmatilen hydrochloride hydrate (S-1090). Toxicol Sci, 2001; *26*(1): 243-54.
3. Agarwal SK, Bhatnagar U, Rajesh N. Acute and genotoxic profile of a dimeric impurity of cefotaxime. International Journal of Toxicology, 2004; *23*(1): 41-45.
4. Berkowitz RL, Coustan DR, Mochizitki TK. *Handbook for Prescribing Medications During Pregnancy*. Little, Brown & Co. Boston, MA, 1981.
5. Brogden RN, Ward A. Ceftriaxone: a reappraisal of its antibacterial activity and pharmacokinetic properties, and an update on its therapeutic use with particular reference to once-daily administration. Drugs, 1988; *35*: 604-645.
6. Fenech M, Morley AA. Measurement of micronuclei in lymphocytes. Mutat. Res, *147:* 29–36.
7. Norppa H, Luomahara S, Heikanen H, Roth S, Sorsa M, Renzi L, Lindholm C. Micronucleus assay in lymphocytes as a tool to biomonitor human exposure to aneuploidogens and clastogens. Environ Health Persp, 1993; *101*(3): 139-43.
8. Kirsch - Volders M, Elhajouji A, Cundari E, Vanhumelen P. The *in vitro* micronucleus test- a multiendpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction. Mutation Research, 1997; *392*: 19-30.
9. Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M. Ishidate M, Lorge E, Norrpa H, Surrales J, Von der Hude W, Wakata A. Report from the In Vitro Micronucleus Assay Working Group. Environ Mol Mutagen, 2000; *35*: 167-72.
10. Fenech M, Crott JW. Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. Mutation Research, 2002; *504*: 131-6.
11. Fenech M. Cytokinesis-block micronucleus cytome assay. Nature Protocols, 2007; *2*: 1084-104.
12. Lindholm C, Norppa H, Hayashi M ,Sorsa M. Induction of micronuclei and anaphase aberrations by cytochalasin B in human lymphocyte cultures. Mutat. Res, 1991; *260*: 369–375.
13. Fučić A, Mijić A. Micronucleus method in vitro and in vivo. Arh hig rada toksikol, 1999; 50(*3)*: 299–306.
14. Falck G.C.M., Catalán J., Norppa H. Nature of anaphase laggards and micronuclei in female cytokinesis-blocked lymphocytes. Mutagenesis, 2002; *17*: 111–117.
15. Norppa H, Falck GC. What do human micronuclei contain? Mutagenesis, 2003; 18*(*3): 221-33.
16. Savage JRK. Micronuclei: Pitfalls and Problems. Atlas of Genetics and Cytogenetics in Oncology and Haemotology. 2000 URL:http: // AtlasGeneticOncology.org/Deep/MicronucleiID20016.html
17. Thomas P, Fenech M. Cytokinesis- block micronucleus cytome assay in lymphocytes. Metod in Molecular Biology, 2011; *682*: 217-234.
18. Gladwin M. *Clinical Microbiology Made Ridiculously Simple 4th ed.* Miami, FL: MedMaster, Inc, 2007; 67.
19. Moorehead PS, Nowell PC, Mellman WJ, Battips DMA, Hungerford DA. Chromosomal preparations of leucocytes cultured from human peripheral blood. Experimental. Cell Research, 1960; 20: 613-616.
20. Eastmond D A. Tucker J D. Identification of aneuplodyinducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. Environ Mol Mutagen, 1989; *13*: 34–20.
21. Fenech M, Chang W, Kirch-Volders M, Holland N, Bonassi S, Zeiger E. HUMN project: detailed description of de scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutation Research, 2003; *534*(1-2): 65-75.
22. Wassom JS. *The storage and retrieval of chemical mutagenesis information*. In: Alečević M.,ed *.Progres in Environmental Mutagenesis*. Elsevier/ North Holand, Amsterdam, New York, Oxford, 1980.
23. Graziano MJ, Spoon TA, Cockrell EA, Rowse PE, Gonzales AJ. Induction of apoptosis in rat peripheral blood lymphocytes by the anticancer drug CI- 994 (acetyldinaline). Journal of Biomedicine and Biotechnology, 2001; 1*(2)*: 56-61.
24. Celik A, Eke D. The assessment of cytotoxicity and genotoxicity of tetracycline antibiotic in human blood lymphocytes using CBMN and SCE analysis, in vitro. Int J Hum Genet, 2011; 11(1): 23-29.
25. Istifli ES, Topaktaş M. Cytogenetic genotoxicity of amoxicillin. Environ Mol Mutagen, 2010; 51(3): 222-8.