 Int J Hum Genet, 11(1): 23-29 (2011)

The Assessment of Cytotoxicity and Genotoxicity of Tetracycline Antibiotic in Human Blood Lymphocytes Using CBMN and SCE Analysis, *in Vitro*

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KEYWORDS Genotoxicity. Tetracycline. Sister Chromatid Exchange. Cytokinesis Block Micronucleus. Mitotic Activity. Cytokinesis Block Proliferation Index

ABSTRACT Tetracycline antibiotic, a widely used antimicrobial drug was tested for nuclear DNA damage in cultured peripheral blood lymphocytes in terms of chromosome alterations. The extent of cytogenetic damage, expressed as chromosome breakage and chromosome loss, was evaluated employing the Cytokinesis Block Micronucleus method (CBMN), the mutagenic effect of these antimicrobial drugs was investigated using Sister Chromatid Exchange (SCE) assay in cultured peripheral blood lymphocytes. Cytotoxicity was measured by evaluating mitotic activity (MA) and cytostatic effect was designated by measuring Proliferation Index and Nuclear Division index (NDI) in cultures. In this study,five different concentrations were used for tetracycline antibiotic. All the concentrations led to decrease in mitotic activity ($p<0.05$). SCE frequency was significantly different from negative control, ($p<0.05$) except at the concentration of 0.1µg/ml. All the concentrations induces the increase in MN frequency, but there is no significant difference between negative control and all the tested concentrations of tetracycline for micronucleus frequency ($p>0.05$). Tetracycline antibiotic has a moderate genotoxic from the point of view MN induction. Our results indicate that tetracycline has a positive response and is potentially more toxic than being genotoxic in the present study.

1. INTRODUCTION

Tetracyclines (TCs) are the broad-spectrum antibiotics commonly used in the treatment of various bacterial infections and acne. If tetracycline is administered during fetal development or permanent tooth formation, permanent discoloration and inadequate calcification of deciduous and permanent teeth are commonly seen (Orientrich et al. 1961; Gordon et al. 1981).

TCs have been used extensively since the late 1940s as broad-spectrum inexpensive antibiotics that are effective against a wide variety of diseases in humans, animals, and plants. The TC constitutes a group of antibiotics which are obtained naturally by fermentation with some fungi or by semi-synthetic processes. They are effective against different microorganisms and are frequently used in an indiscriminate manner. However, resistance to tetracycline has increased dramatically since the first appearance of resistance in 1953 in *S. dysenteriae* (Chopra and Roberts 2001). The emergence of bacteria resistance has limited its use. Inspite of this, however, in 2004, according to the National Ambulatory Medical Care Survey of visit to office-based physicians in the United States, tetracycline among other counterpart drugs was prescribed either as a new medication or as a continued medication (National Ambulatory Care Survey Data base 2007). Moreover, the drug has gained wide acceptance in the third world countries because of its easy accessibility, availability and cost effectiveness. As such, the drug can be abused due to self-prescription, which is rife in this part of the world. Among a wide variety of pharmaceutical compounds, antibiotics are of special concern due to their extensive use in human and veterinary medicine. Because of the increasing amount in the environment of antibiotics, their biological impacts have been widely discussed (Lindsey et al. 2001; Kolpin et al. 2002; Giger et al. 2003; Gao and Pedersen 2005). The presence of traces of this kind of pharma-ceutical in the environment can induce the development of antibiotic-resistant pathogens, causing serious problems for human health (Hirsch et al. 1999; Bautitz and Nogueira 2007). There are many antibiotics showing similar effects as TC. It is shown that these antibiotics also have toxic effects on non-target organisms. It is determined that erythromycin, lincomycin, clarithromycimn antibiotics lead the toxic effects on non-target organisms such as aquatic organisms (Isidori et al. 2005).

Genotoxicity testing of pharmaceuticals prior to commercialization is obligated by regulatory agencies worldwide. For the most part, a three or

four-test battery including bacterial mutagenesis, *in vitro* mammalian mutagenesis, *in vitro* chromosome aberration analysis and *in vivo* chromosome stability assay are required (Synder and Green 2001). Therefore, it is important to determine the genotoxic potential of a drug that will be used in therapy, particularly in native human cells. In general, CAs, SCEs and micronuclei are considered to be essential markers of genotoxicity in in vitro studies.

Thus, the aim of present study was to evaluate the genotoxic and cytotoxic effects of tetracycline, an antibiotic, on peripheral blood lymphocytes using the CBMN, SCE test and measuring MA and PRI or/and NDI respectively *in vitro.*

2. MATERIALS AND METHODS

2.1. The Collection of Blood Samples

The study was carried out using human peripheral lymphocytes from fresh blood samples. Blood samples were obtained from five young non-smoking healthy donors. All donors were healthy and non-smokers. They were not exposed to X-ray or to treatment with any drugs. Approximately, 10 ml of blood was collected, by venipuncture, into syringes containing sodium heparin as anticoagulant. Lymphocyte cultures were used for the CBMN test and SCE assay.

2.2. The Preparation of Antibiotic Solutions

Tetracycline hydrochloride (minimum purity 95 %, from SIGMA, 64-75-5) plasma concentration was considered for determination of doses of tested antibiotic. It is reported that the free blood concentrations of this drug is 1 µg/ml after usual therapeutic dose (Sakellari et al. 2000). In this study, dose range is 0,1-2 µg/ml.

2.3. The Preparation of Lymphocytes Cultures for SCE and CBMN Test

1. Sister Chromatid Exchange (SCE)
2. Cytokinesis Block Micronucleus Method (CBMN).

1. Sister Chromatid Exchange (SCE) Analysis: The study was carried out by using blood samples from five healthy non-smoking female donors, aged 22, 24, 24, 26 and 24 years. In the five donors, results of clinical routine laboratory analyses were in normal range, and the absence of exposure to known genotoxicants was considered. Briefly, lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood to 4.5 ml of RPMI 1640 chromosome medium supplemented with 15% heat-inactivated fetal calf serum, antibiotics (penicillin and streptomycin) and L-glutamine (all obtained from Gibco, Eragny, France). Lymphocytes were stimulated by 1% phytohaemagglutinin (PHA, Gibco). Test chemical (TC) and 5-Bromo-2-deoxyuridine (9 mg/mL, BrdU, Sigma) was added to cultures at 24 h after phytohaemagglutinin stimulation the at 37°C for SCE analysis. Lymphocytes were cultured in the dark for 72 h, and metaphases were blocked during the last 1.5 h with Colcemid at final concentration of 0.2 mg /mL. The cells were harvested by replacing the culture medium with KCl (0.075 M) in which cells were incubated for 20 min. at 37°C. The cells were fixed in Carnoy's fixative (methanol: acetic acid, 3:1 v:v) five times, and slides were kept at room temperature overnight. Air-dried slides were stained according to Fluoresence-Plus Giemsa method by Perry and Wolf (1974) with slight modification. Two slides were prepared for each concentration. The number of SCEs was counted in 50 second-metaphase cells from each of the cultures on coded slides. Thus, 100 cells were scored in blind per dose for SCEs. Mitomycin C (2 mg /mL) was used as positive control.

2. Cytokinesis Block Micronucleus Method (CBMN): The CBMN assay was carried out using the Standard technique proposed by Fenech (1993), with slight modifications. Cytochalasin-B (Cyt-B, Sigma), at a final concentration of 3µg/ml was added at 44 h after the cultures were established, to arrest cytokinesis of dividing cells. Binucleated lymphocytes were harvested 72 h after culture setting. The test chemical (tetracycline) was added at 24 h after phytohaemmaglutinin stimulation. The cells were collected by centrifugation and washed with hypotonic solution (0.075 M KCl) at room temperature using vortex for two minutes. The induction of MN was evaluated by scoring a total of 1000 BN cell with well-preserved cytoplasm at 1000 x magnification. Only BN cells were included in the microscopic analysis.

2.4. Cell Proliferation Kinetic (CPK) and Mitotic Activity

The mitotic activity (MA) was calculated as proportion of metaphases among the total cell

population by counting a total of 1000 cells. The cell proliferation kinetics was defined as the proportion of the relative frequency of first division metaphases (M1, identifiable by uniform staining of both the sister chromatids), second-division metaphases (M2, identifiable by differential staining of the sister chromatids), and third and subsequent division metaphases (M3, identifiable by non-uniform pattern of staining). Replication index or proliferation index (RI) was calculated according to Ivett and Tice (1982). RI is the average number of replications completed by metaphase cells and is calculated as follows:

PRI = 1x(first division metaphases) + 2x(% second division metaphases) + 3x(% subsequent division metaphases)/500

In the MN study, toxicity was evaluated by classifying 500 cells according to the number of nuclei. The well-known cytotoxicity index was used: an index for measuring the cell proliferation kinetics, called the Nuclear Division Index (NDI) which was calculated following the expression: where MI–MIV represent the numbers of cells with one to four nuclei, respectively, and MIII and MIV are equally considered to be in their third cell cycle. As previously demonstrated, the NDI is an accurate and biologically relevant index in detecting cellular toxicity or cell-cycle delay (Fenech 2007).

NDI = MI +2 MII +3(MIII, MIV)/500

2.5 Statistical Analysis

Data were compared by one-way analysis of variance. Statistical analysis was performed using the SPSS for Windows 9.05 package program. Multiple comparisons were performed by Student Newman-Keuls (SNK) test. Statistical decisions were based on a significance level of 0.05.

3. RESULTS

The results of the frequency of SCEs, MA, PRI values and MN, NDI, for each dose and control groups are depicted in table 1 and table 2.

Table 1 shows the results obtained for the SCE frequencies and for the PRI and MA after treatments lasting for 72 h in blood peripheral blood lymphocyte cultures set up from five healthy donors (1-5) and Table 2 represents the results obtained for the MN frequencies and for the NDI after treatments lasting for 72 h in blood peripheral blood lymphocyte cultures set up from five healthy donors (1-5). Table 3 shows the mean ±SE values for all the parameters.

These results reveal that tetracycline slightly induces a dose-dependent increase in the frequency of binucleated cells with MN (BNMN) in five donors (Fig. 1). But, this increase statistically is not significant between concentration groups and control or /and within concentration groups. There is no significant difference between negative control and all tested concentrations of tetracycline for micronucleus frequency ($p>0.05$).

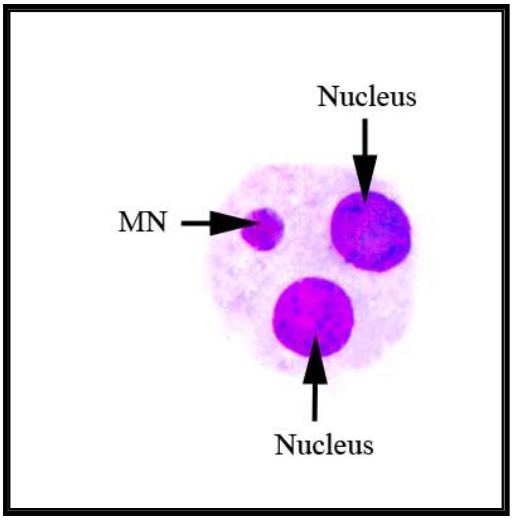

Fig. 1. Giemsa-stained binucleated lymphocytes in human peripheral blood treated with Tetracycline. Arrows indicate micronucleus (MN) and main nucleus

These results show that tetracycline has genotoxic or mutagenic activity and induces a dose-dependent increase in the frequency of SCE in five donors, compared negative control at all the concentrations. SCE frequency was significantly different from negative control, ($p<0.01$, $p<0.001$) except at the concentration of 0.1µg/ml ($p=0.230$). There is no significant difference between concentration of 0.5 µg/ml and 1 µg/ml of tetracycline antibiotic ($p=0.125$). The percentage increase in SCE frequency are shown in Table 3.The percentage increases in SCE frequency were expressed versus negative control.

With respect to the cytotoxic effects of the test compound on lymphocyte cultures, as measured by MA, NDI and PRI, a dose-dependent reduction in cell proliferation has also been observed in five donors, reaching statistical significance at all the

Table 1: The frequency of sister chromatid exchanges (SCE), the values of mitotic activity (MA) and proliferation index (PRI) in human blood lymphocytes treated with Tetracycline antibiotic for 72 h

Donor	*Treatment*	*SCE/Met*	*MA*	*M1*	*M2*	*M3*	*PRI*
1	NC	4.79	6.25	25	31	44	2.19
	0.1	4.90	6.00	65	26	9	1.44
	0.5	5.01	5.65	66	25	9	1.43
	1	5.22	5.22	67	24	9	1.42
	1.5	5.68	5.00	67	30	3	1.36
	2	5.92	4.65	69	25	6	1.37
	MMC (2µg/ml)	11.5	3.89	53	38	9	1.56
2	NC	4.81	6.26	25	33	42	2.17
	0.1	4.92	5.97	64	28	8	1.44
	0.5	5.15	5.70	65	28	7	1.42
	1	5.28	5.28	67	26	7	1.40
	1.5	5.79	5.01	68	27	5	1.37
	2	5.98	4.70	70	25	5	1.35
	MMC (2µg/ml)	12.1	3.78	55	40	5	1.50
3	NC	4.78	6.25	29	31	40	2.11
	0.1	4.91	6.01	66	25	9	1.43
	0.5	5.09	5.64	65	28	7	1.42
	1	5.29	5.32	65	26	9	1.44
	1.5	5.61	5.00	67	26	7	1.40
	2	5.90	4.62	69	28	3	1.34
	MMC (2µg/ml)	12.5	3.90	56	40	4	1.48
4	NC	4.82	6.20	27	33	40	2.13
	0.1	4.90	5.99	65	28	7	1.42
	0.5	5.20	5.61	63	30	7	1.44
	1	5.22	5.21	68	27	5	1.37
	1.5	5.74	5.00	66	30	4	1.38
	2	5.98	4.55	68	28	4	1.36
	MMC (2µg/ml)	12.3	3.78	54	37	9	1.55
5	NC	4.77	6.28	28	30	42	2.14
	0.1	4.96	6.02	64	28	8	1.44
	0.5	5.18	5.62	65	28	7	1.42
	1	5.42	5.28	65	26	9	1.44
	1.5	5.77	5.01	65	26	9	1.44
	2	6.00	4.59	68	28	4	1.36
	MMC (2µg/ml)	12.4	3.88	54	40	6	1.52

SCE: sister chromatid exchange; *Met:* metaphase*; MA:* mitotic activity*; M1*: first mitosis*; M2:* second mitosis; *M3:* third mitosis*; PRI:* Proliferation index; *NC:* negative control; *MMC:* mitomycin C

Table 2: Induction of micronuclei (MN) and cytokinesis block proliferation index values in human lymphocyte cultures treated with Tetracycline for 72 h

Donor	*Treatment*	*MN/1000 MNBN*	*One nucleated cell*	*Two nucleated cell*	*Three nucleated cell*	*NDI*
1	NC	1	100	175	225	2.25
	0.1	1	325	140	35	1.42
	0.5	2	320	165	15	1.39
	1	2	330	165	5	1.35
	1.5	2	340	150	10	1.34
	2	3	375	110	15	1.28
	MMC (2µg/ml)	62	275	175	50	1.55
2	NC	1	105	170	225	2.24
	0.1	2	325	140	35	1.42
	0.5	2	340	130	30	1.35
	1	2	340	135	25	1.37
	1.5	1	330	145	25	1.39
	2	2	320	170	10	1.38
	MMC (2µg/ml)	65	265	185	50	1.57
3	NC	1	95	150	255	2.32
	0.1	1	345	130	25	1.36
	0.5	1	330	135	35	1.31
	1	2	315	150	35	1.44
	1.5	2	330	160	10	1.36
	2	2	315	175	10	1.39
	MMC (2µg/ml)	57	270	185	45	1.55
4	NC	1	100	110	290	2.38
	0.1	1	310	145	45	1.47
	0.5	1	340	135	25	1.37
	1	2	340	130	30	1.35
	1.5	2	340	140	20	1.36
	2	2	330	150	20	1.38
	MMC (2µg/ml)	58	275	185	40	1.53
5	NC	1	100	105	295	2.39
	0.1	1	325	150	25	1.40
	0.5	1	320	175	5	1.37
	1	1	340	135	25	1.37
	1.5	2	305	195	0	1.39
	2	2	340	160	0	1.32
	MMC (2µg/ml)	58	260	195	45	1.57

MN: Micronucleus; *MNBN:* micronucleated binucleated cell; *NDI:* cytokinesis Block proliferation index; *MMC:* mitomycin C; *NC:* negative control

concentrations tested. All the concentrations of tetracycline antibiotic inhibited the mitotic division and therefore it caused the decrease of MA. Compared with negative control group there is significant difference ($p<0.05$). It is found that significant difference between all tested concentrations and negative control for NDI values ($p<0.001$). There is no significant difference among concentrations of tetracycline antibiotic. PRI values were affected from tetracycline antibiotic compared with negative control values. It is found that there is statistically a significant difference between negative control and all the concentrations ($p<0.001$). There is no significant difference between concentration of 0.5 µg/ml and 1 µg/ml of tetracycline antibiotic and 0.1 and 0.5 µg/ml µg/ml or 1 µg/ml and 1.5 µg/ml concentration of tetracycline antibiotic (respectively,

Table 3: The frequency of SCE, MN and the values of MA, PRI and NDI in human blood lymphocytes treated with Tetracycline antibiotic for 72 h

Treatments	*MN/1000 MNBN Mean±SE*	*NDI Mean±SE*	*MA(%) Mean±SE*	*SCE/Met Mean±SE*	*PRI Mean±SE*	*% increase of SCE*
NC	1,00±0.00	2.31±0.03	6.24±0.01	4.79±0.01	2.14±0.01	-
Tetracycline µg/ml0.1	1.20±0.20	1.41±0.01***	5.99±0.00**	4.91±0.01	1.43±0.00***	2,5
0.5	1.40±0.24	1.35±0.01***	5.64±0.01***	5.12±0.03**	1.42±0.05***	6,88
1	1.80±0.20	1.37±0.01***	5.26±0.02***	5.28±0.03***	1.41±0.01***	10,02
1.5	1.80±0.20	1.36±0.01***	5.00±0.00***	5.71±0.04***	1.39±0.02***	19,20
2	2.20±0.21	1.35±0.02***	4.62±0.03***	5.95±0.02***	1.35±0.01***	24,21
PC-MMC (2µg/ml)	60.0 ±1.51***	1.55±0.01***	3.84±0.03***	12.16±0.17***	1.52±0.04***	153,86

**** p<0.001, **p<0.01, MN:* Micronucleus; *MNBN:* micronucleated binucleated cell; *NDI:* cytokinesis Block proliferation index; *MA:* mitotic activity; *SCE:* sister chromatid exchange; *Met*: metaphase; *PRI*: Proliferation index;

p=0.451, p=0.614, p=0.137).Therefore, the results of our study indicate that tetracycline can exert cytotoxic or cytostatic effects in human peripheral blood lymphocyte cells.

4. DISCUSSION

In the present day world, man is exposed to a variety of chemicals environmentally, occupationally, therapeutically or due to change in the lifestyle. The studies carried out in bacteria, mammalian systems and cell cultures showed that some chemicals interacted with the genetic material and caused DNA damage (Mirian 2004).

According to the data presented in this study, tetracycline antibiotic lead to increase in MN frequency and tetracycline antibiotic is a mutagenic agent on the basis SCE assay. The abovementioned cytogenetic effects were accompanied by a significant decrease of the three indices, PRI or NDI and MA, which is indicative of cytostatic and cytotoxic activity respectively. This data can be explained by the fact that tetracycline is a bifunctional alkylating agent that probably needs much higher concentrations to induce chromosomal aberrations, compared with the levels that induce an increase in SCE (Lawley and Brookes 1963). Bifunctional alkylation of DNA by synthetic cross-linking agents has been shown to be profoundly more cytotoxic than alkylation by a corresponding monofunctional agent (Brendel and Ruhland 1984; Pratt et al. 1994). As a result, DNA interstrand cross-linking is regarded as a highly lethal type of DNA damage, and it has been hypothesized that cross-links at the replication fork may pose a more severe impediment to replication than a comparable monofunctional drug-DNA adduct, which is localized to only one strand. An alternative (or additional) hypothetical rationale for the high cytotoxicity of cross-link-type damage is that the cellular repair mechanism of the cross-linked DNA is less efficient than that of monofunctional lesions (Tomasz and Palom 1997).

Over the last decades, SCE techniques in peripheral blood lymphocytes have been extensively applied as biomarkers of early cytogenetic damage caused by several environmental hazards or therapeutic agents (Çelik and Akbaş 2005; Aydemir et al. 2005). DNA strand-breaks caused by several genotoxic agents elicit repair processes, whose effects may become apparent as morphologically altered chromosomes or SCE. The morphological consequence of DNA damage depends on the cell-cycle phase during induction of the lesion and its persistence. When damage is induced prior to DNA synthesis (S) it may undergo inter-chromosomal or intra-chromosomal repair processes that, upon DNA replication, produce chromosome-type aberrations such as dicentrics, rings or acentric fragments. On the other hand, if the damage is induced during S phase, or persist until that stage, it may initiate chromatid-type aberrations, such as, chromatid exchanges/interchanges or SCE. Therefore, several genotoxic agents may induce only CAs or SCE, whereas other agents may, under appropriate conditions, induce both types of damage, with SCE being more sensitive at lower concentrations (Dillehay et al. 1987).

The simplicity of scoring and the wide applicability of the in vitro micronucleus test in different cell types make it an attractive tool to assess cytogenetic abnormality. The micronucleus assay is a useful endpoint and has a wide application in the assessment of chromosomal damage in different organisms, plant, animal and human in *vitro* and *in vivo* caused by different

mutagens (Major et al. 1998; Çelik et al. 2003; Ünyayar et al. 2006; Çelik 2006; Çavaş and Könen 2007).

In various researches, the contradictory results were obtained related with tetracycline antibiotic. In a study performed by Hagiwara et al (2006), it is indicated that tetracycline failed to induce chromosome aberrations in Syrian Hamster embryo cells regardless of exogenous metabolic activation. Again in the same study, it decreased the mitotic index dose-dependent. In the present study also all the concentrations of tetracycline decreased the mitotic index of human blood peripheral lymphocytes *in vitro.* In this study, used TC concentrations compared free blood concentration of TC, 1ig/ml, these results show that this antibiotic is cytotoxic under in vitro conditions. Although cell type is different used in two studies it is remarkable formation of a dose-dependent effect for mitotic index. In another study, it is determined that tetracycline failed to induce morphological transformation and UDS in Syrian hamster embriyo cells (Suzuki 1987). However, as indicated by Tsutsui et al. (1976) clastogenic and mutagenic activities of tetracycline was observed in a mouse mammary carcinoma cell line FM3A.

The antibiotics such as methicillin, bacitracin, rifamycin and tetracycline have been shown to undergo oxidation, and particularly, in the presence of transition metal ions, these drugs lead to the formation of highly reactive oxygen species (Khan and Musarrat 2002; Quinlan and Gutteridge 1991). In the study performed by Khan and Musarrat (2002), it is determined that the tetracycline-Cu(II) induced DNA damage occurs due to (1)significant binding of tetracycline and Cu(II) with DNA, (2) methyl group transfer from tetracycline to nitrogenous bases on DNA. Tetracycline does carry one methyl group at C-6 on the ring adjacent to the aromatic ring and two methyl group on nitrogen atom at C-4 of the last ring system. Probably, the methyl groups at activated sites are released and captured by N-7 and N-3 of the guanine and adenine bases. N-7 position of a purine ring is affected from methylation. Therefore, the modification occurs in nitrogenous bases at this site and modified bases may undergo tautomerization. The alkylation of bases is known to disturb the normal base pairing and changes the structure of the phosphate backbone. The damage in the secondary structure of DNA leads to the accumulation of defects and to delay in repair system. Since SCE and MN have been shown to be a sensitive indicator of DNA damage, these results indicate that tetracycline antibiotic can induce genetic damage in human peripheral blood lymphocyte cells under in vitro conditions.

5. CONCLUSION

On the whole, when we consider our results together with other published data, this study indicated that the antimicrobial drug, tetracycline, is able to induce both cytotoxic and moderate genotoxic effects in cultured human blood lymphocytes. Hence, it is advisable to be careful of the potential hazards of this antibacterial drug as they may become capable of attacking the genetic material.

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