The frequency of sister chromatid exchange and micronuclei in evaluation of cytogenetic activity of Kombucha on human peripheral blood lymphocytes

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SUMMARY

Kombucha is a refreshing beverage obtained by the fermentation of sweetened black tea with a „tea fungus" (symbiotic culture of acetic acid bacteria and yeasts). It is consumed due to its potential beneficial effects on human health. The aim of this study was to investigate activity of Kombucha on human peripheral blood lymphocytes in vitro. We analyzed Kombucha made from different substrates: Camellia sinensis and Satureja montana, and effects of substrates alone. The frequencies of sister chromatid exchange (SCE) and micronuclei (MN) were scored as genetic endpoints and mitomycin C was used as model mutagen. Kombucha from Camellia sinensis and Camellia sinensis substrate increased frequency of MN and SCE on mitomycin C-treated and -untreated peripheral blood lymphocytes. However, Kombucha from Satureja montana reduced incidence of MN on mitomycin C-treated and -untreated peripheral blood lymphocytes, while SCE frequency was higher than control value. In our pilot study we showed for the first time that Kombucha from different substrates induced different effects on mitomycin C-treated and -untreated peripheral blood lymphocytes.

Key Words: *Lymphocytes; Cells, Cultured; Beverages; Tea; Yeasts; Sister Chromatid Exchange; Micronuclei, Chromosome-Defective; Mitomycin*

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Abbreviations:
SCE – Sister chromatid exchanges; MN – Micronuclei; PBL – Peripheral blood lymphocytes; MMC – Mitomycin C; Cs – Camellia sinensis and Sm – Satureja Montana; KSm – Kombucha from Sm; KCs – Kombucha from Cs

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INTRODUCTION

Kombucha is traditionally prepared by fermenting of sweetened (sucrose) black tea (*Camelia sinensis* L.). This medium is usually inoculated with cellulose pellicle formed during the previous cultivation, what is popularly known as a ″tea fungus″ (1). This so-called ″tea fungus″ is actually a symbiosis of acetic acid bacteria (*Acetobacter xylinum, Acetobacter aceti*, *Gluconobacter oxydans*) (2) and yeasts (*Saccharomyces sp.*, *Zygosaccharomyces sp.*, *Torulopsis sp.*, *Pichia sp.*, *Brettanomyces sp.*) (1,3). The final product is a sour, slightly carbonated, acidic beverage which has been consumed worldwide as a healthy, refreshing drink for a very long time especially in China, Russia and Germany (4).

Research conducted in Russia at the beginning of the 20th century indicates that Kombucha can improve resistance against cancer, prevent cardiovascular diseases, promote digestive functions, stimulate the immune system, reduce inflammatory problems, and can have many other benefits (5). The beneficial effects of Kombucha are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, amino acids, antibiotics and a variety of micronutrients produced during fermentation (6). Although there are numerous claims that drinking Kombucha is beneficial to health, these individual case studies are not validated scientifically (7).

Human lymphocytes cultured *in vitro* are widely used to assess the activity of various chemical compounds on human genetic material. SCEs are highly sensitive indicator of DNA damage and/or subsequent repair. SCEs represent the interchange segments of DNA, which are located on homologous loci of two chromatids of a metaphase chromosome.

For the evaluation of activity Kombucha on genetic material and induction of micronuclei (MN), the cytokinesis-block micronucleus method was used. Micronuclei are a biomarker of chromosome breakage and/or whole chromosome loss. This test system might be a useful tool for identifying protective dietary factors (8).

To our best knowledge there are no published studies referring to the cytogenetic activity of Kombucha and nothing is known about possible Kombucha genotoxic or antigenotoxic activity as well. Therefore, the aim of the present study was to investigate the activity of Kombucha on human lymphocyte, using sister chromatid exchange (SCE) and cytokinesis-blocked micronucleus (CBMN) assays.

MATERIAL AND METHODS

Chemical

The alkylating agent mitomycin C, MMC, (Bristol-Myers Squibb, USA) was used as SCE and micronuclei inducer. The solution was prepared by diluting the drug in phosphate buffer (PBS), and added at a final concentration of 0.03 μg/mL in lymphocytes cultures after 24 h of starting cultures. That concentration was defined in our previous pilot experiments. For analyses of the Kombucha activity in both assays the MMC concentration of 0.03 μg/ml was chosen because higher concentrations of MMC induced very high number of SCEs and MN that was difficult for accurate scoring (Table 1).

Kombucha samples

Substrates for Kombucha fermentation were prepared by adding 70 g/L of commercial sucrose in tap water and after boiling 5 g/L of dry crushed leaves of black tea (*Camellia sinensis* L.) was added. Kombucha from Rtanj tea (*Satureja montana* L.) was prepared in the same way. The tea leaves were steeped for 15 minutes and removed by filtration. After cooling to about 30^{0}C, the inoculum (Kombucha beverage from previous process) was added in amount of 10% (v/v). Then the 0.33L of the prepared medium was poured into small flasks (Ø=8 cm, total volume 0.72L) and incubated under aerobic conditions at 28^{0}C. Incubation period lasted 7 days, when Kombucha beverages

achieved optimal consuming acidity (3.5-4.5 g/L of acids). The solution was then filtered through a microfilter (0.22 μm) and kept in refrigerator at 4˚C.

Table 1. Frequency of SCE, MN and proliferation index after treatment with MMC on PBL

MMC μg/mL	SCE X(SD)	MN	CBPI
*0.00	6.40 (2.42)	5.94	1.74
0.01	15.96 (4.89)	27.81	1.72
0.02	33.20 (6.04)	–	–
0.03	41.64 (10.9)	49.95	1.80
0.05	71.56(14.83)	99.84	1.57
0.07	89.40(13.44)	NT	NT
0.09	110.83(18.87)	NT	NT
0.1	NT	243.3	1.69
0.2	NT	279.4	1.4
0.3	NT	422.6	1

* Control sample; SCE – Sister chromatid exchanges frequency; X(SD) – mean value(standard deviation); MN – Micronucleus frequency (‰); CBPI – Cytokinesis - Block Proliferation Index; MMC – mitomycin C (concentration in μg/mL); (NT) – not tested

Peripheral blood lymphocyte cultures

Blood sample was obtained from a healthy, nonsmoker female donor, aged 36 years, who was not undergoing any drug treatment and who had not any viral infection or X-ray exposure in the recent past. Heparinized whole blood sample was collected by venipuncture and cultured simultaneously for two different analyses: SCE and MN assay.

Briefly, 0.5 mL of whole blood was added to 5 mL of RPMI 1640 cell culture medium (Sigma) supplemented with 2 mM glutamine, 20% of heat-inactivated fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU/mL of penicillin and 100 μg/mL of streptomycin (ICN). Lymphocytes in cultures were stimulated for division with phytohemaglutinin (PHA-M, Sigma) at a final concentration of 20 μg/mL. The cultures were incubated at 37°C for 72 h in 5 % CO_2 atmosphere with 95 % humidity.

Kombucha samples from different substrates, C*amellia sinensis* (*Cs*) and *Satureja montana* (*Sm*), and substrates alone (sweetened tea) were added at a final concentration of 40 μg/mL to human lymphocytes culture 1h after starting cultures.

CBMN

CBMN assay was performed by the standard cytogenetic procedure (9) with minor modifications of cell staining (Giemsa 2% in distilled water). Cytochalasin-B (Sigma) was added to the lymphocytes cultures at a final concentration of 6 μg/mL, 44 h after stimulation with PHA.

SCE

In samples seeded for SCE test, chromatid differentiation was initiated by adding 10 μg/mL 5-bromo-2'-deoxyuridine (BrdU, Sigma) to cultures after stimulation with PHA. Differential staining of sister chromatids was performed using the fluorescence-plus-Giemsa (FPG) technique with Hoechst 33258 (Sigma) (10).

Scoring criteria

CBMN

Cytokinesis-block MN assay was performed, analyzing more than 1000 cells per each sample. Standard criteria were used for the identification of MN (11). Monitored values included: incidence of micronuclei, micronucleus distribution and cytokinesis block proliferation index (CBPI). Micronucleus incidence was presented as a number of micronuclei per 1000 examined binuclear cells.

CBPI was calculated according to formula:

$CBPI = (M_1 + 2M_2 + 3(M_3+M_4)) / N$

where M_1-M_4 represents the number of cells with 1 to 4 nuclei found, respectively, and N is the total number of scored cells (9).The CBPI is a measure of the average number of cell cycles that a cell population pass through, considering both three-nucleated and tetra-nucleated cells in the same category (12). Micronucleus distribution was acquired by scoring the binuclear cells containing 1 or more micronuclei.

SCE

Fifty complete metaphase chromosomal sets with well-differentiated sister chromatids per sample were analyzed. Counted chromatid exchanges between sister chromatids were presented as an average number per cell and the standard deviations were calculated.

Data and image processing

The prepared material was observed and analyzed by light microscopy (Olympus BX51). Specimens were coded and analyzed with no information about the origin of the material. Images were captured with a 3.2-mega pixel digital camera (Olympus CAMEDIA C3040 Zoom) attached to the computer.

SCE data were presented as mean values ±standard deviation, and MN data as number per 1000 binuclear cells. Differences between treated and control samples were analyzed by Student's t-test (SCE data) (13) and by testing of proportion (MN data) (14). A level of significance is given in the table.

RESULTS

In this pilot study mytomicin C (MMC), alkylating and interstrand cross-linking agent, was chosen as a mutagen. Frequency of SCE and MN in peripheral blood lymphocytes (PBL) increased with increasing MMC concentration. For analyses of the Kombucha activity the concentration of 0.03 μg/mL of MMC was chosen because higher concentrations of MMC induced very high number of SCEs and MN that was difficult for accurate scoring.

Table 2 shows the activity of Kombucha obtained from two different substrates, C. sinensis (KCs) and S. montana (KSm), and activity of substrates alone (Cs and Sm) in human lymphocytes cultures using the MN and the SCE assay.

The frequencies of micronuclei both in PBL after exposure to *Cs* and to Kombucha from *Cs* were higher than frequency of MN in the control culture (K*Cs* vs. Control; $p<0.001$). In contrast to *Cs*, *Sm* substrate induced slightly higher incidence of MN (8.89) compared to the control (5.94), while Kombucha from *Sm* decreased MN frequency (0.98).

The frequency of micronuclei in MMC-treated PBL was significantly higher when cells were treated with *Cs* alone or with Kombucha from *Cs*, compared to the control ($p<0.05$). Contrary to these results, *Sm* substrate and Kombucha

Table 2. Frequency of SCE, MN and proliferation index after treatment with Kombuchas or their substrates on PBL

	SCE X(SD)	MN	CBPI	Σ	BN	0	1	2	≤3
Control	6.40 (2.42)	5.94	1.74	2493	1010	1005	4	1	0
Cs	7.84 (3.88)	14.59	1.79	2397	945	930	15	0	0
K*Cs*	7.40 (3.16)	24.80**	1.77	2880	1008	985	21	2	0
Sm	8.12 (2.76)*	8.89	1.82	2199	1012	1003	9	0	0
K*Sm*	8.28 (2.89)*	0.98	1.83	2512	1027	1026	1	0	0
MMC	41.64 (10.9)	49.95	1.70	1937	1061	1011	47	3	0
MMC+*Cs*	54.83 (12.87) **	73.17*	1.75	2137	1025	953	69	3	0
MMC+K*Cs*	55.64 (10.46) **	73.86*	1.74	2111	1029	956	70	3	0
MMC+*Sm*	49.76 (8.96) *	40.99	1.79	2322	1049	1008	39	2	0
MMC+K*Sm*	53.32 (7.42) **	35.68	1.79	2346	1009	975	33	0	1

SCE – Sister chromatid exchanges frequency; X (SD) – mean value(standard deviation); MN – Micronucleus frequency (‰); CBPI – Cytokinesis - Block Proliferation Index; Σ – Total number of cells scored; BN – Binucleated cells analyzed; *Cs* – sweetened tea *C. sinensis*; *Sm* – sweetened tea *S. montana* ; *KCs* – Kombucha from *Cs*; K*Sm* – Kombucha from *Sm*; MMC – mitomycin C; $p < 0.05$ – (*); $p < 0.001$ – (**);

from *Sm* slightly reduced MN frequency ($p>0.05$), while reduced MN frequency (35.68) in MMC-treated lymphocytes compared to the control.

We also investigated possible effects of Kombucha activity on PBL DNA using SCE test. The results showed that substrates *Cs* and *Sm* given alone, and Kombucha from these substrates, increased SCE values in both MMC-treated and MMC-untreated PBL compared to control values ($p<0.001$; $p<0.05$) (Table 2).

Regarding CBPI values, results showed that treatments with Kombucha in different substrates and substrates alone did not alter CBPI values compared to the control.

DISCUSSION

Kombucha is a health beverage which is consumed due to its potential beneficial effects on human health, but an extensive literature search did not show human or animal studies of Kombucha health benefits. To our best knowledge, this pilot study represents the first results about Kombucha activity in cultured human PBL by MN and SCE assays.

Our results showed that Kombucha from *Cs* and *Cs* alone (concentration 40μg/ml) increased MN and SCE frequency on MMC-damaged and -undamaged PBL. On the other site, Kombucha from *Sm* reduced MN frequency on MMC-treated and -untreated PBL, but a SCE frequency remained higher compared to control values. Micronuclei are biomarker of chromosome damage. Increased number of micronuclei is evidence of prior induction of structural chromosome damage or changes in chromosome number.

Results of literature search (15,16) showed that Kombucha, acetic acid solution and heat-denatured kombucha samples showed significant antimicrobial activity against gram negative and gram positive bacteria. It is also known that antioxidant activity of Kombucha is increased during fermentation (17,18). The beneficial effects of Kombucha to human health are attributed, among other compounds, to tea polyphenols. The probable mechanisms by which *Cs* polyphenols act might include inhibition of promutagen activation; the inactivation or detoxification of reactive forms of mutagens and carcinogens; induction of DNA repair; and inhibition of promotion, invasion and metastasis of tumor cells (19).

Cs polyphenols coincubated with benzo(a)pyrene (BP) and cyclophosphamide (CP) induced a dose-dependent reduction in BP- and CP-induced chromosomal aberrations, micronuclei and sister chromatid exchanges (20,19). Study of *Cs* consummation *in vivo* using MN assay showed that *Cs* polyphenols did not induce micronuclei in mouse bone marrow erythrocytes (21). However, there are differences in antimutagenic/antigenotoxic effects of various teas against diverse types of mutagens. This may be attributed to the variable composition and contents of major components formed during the manufacturing process (22). Goldbohm and Ohe (23,24) suggested that since in *Cs* most of the catechins have been oxidized, they might have reduced anticarcinogenic properties.

Lack of MN and SCE frequency reduction induced by Kombucha in *Cs* and *Cs* alone in our investigation, might be due to variable composition and contents of components in Kombucha in *Cs* and *Cs* alone. Gupta (24) concluded that more studies on *Cs* and its polyphenols are needed before a final conclusion can be made.

Our results also showed that Kombucha from *Sm* reduced incidence of MN on MMC-damaged and -undamaged PBL, while SCE frequency was higher than control value. There is a correlation between the SCEs frequency and DNA damage. It is also known that exchanges of DNA segments between sister chromatids take place during S phase of cell cycle. During this cell cycle phase post-replicative repair system repairs errors using undamaged chromatid. One of the principal mechanisms responsible for SCE in vertebrate cells is homologous recombination (26) and therefore SCE have been related to post-replication repair (27). According to this, increased SCE frequency caused by Kombucha in *Sm* might be indication of intensive reparation of primary DNA damage and therefore the incidence of MN on MMC-treated and -untreated PBL was reduced. Based on the fact that cytotoxic compounds are able to delay the cell cycle progression in cultured cells, the cytokinesis-block proliferation index (CBPI) can be considered as an index of cell kinetics or average cell division (12). Our data suggested that Kombucha from *Cs* and *Sm* , and *Cs* or *Sm* alone, did not disturb cell-proliferation kinetics and suggest lack of cytotoxicity.

Increased number of blood donors, wide concentration range of Kombucha and different time treatment would probably highlight the effects of examined substances on human genetic material.

In our pilot study we showed for the first time that Kombucha from different substrates induced different effects on MMC-treated and -untreated PBL. Further research is needed to clarify the effects by which Kombucha in different substrates induced changes in MN and SCE frequency in damaged and normal PBL. This would help to resolve whether Kombucha has genotoxic or antigenotoxic effects on cultured PBL.

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Conflict of interest

We declare no conflict of interest.

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