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ORIGINAL PAPER

Evaluation of genotoxicity, cytotoxicity and cytostasis in human lymphocytes exposed to patulin by using the cytokinesis-block micronucleus cytome (CBMN cyt) assay

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Abstract Patulin (PAT) is a fungal secondary metabolite commonly present in apples and apple products. In the present study, PAT was evaluated for its genotoxic, cytotoxic and cytostatic effects to human peripheral blood lymphocytes by using the cytokinesis-block micronucleus cytome (CBMN Cyt) assay. Lymphocyte cultures were treated with PAT at the following concentrations, 0.1, 0.3, 0.5, 1.0, 2.5, 5.0, and 7.5 μM, as well as 0.5 μM mitomycin c (MMC) as a positive control and dimethyl sulfoxide (DMSO) as a vehicle control. PAT was found to induce nucleoplasmic bridges (NPBs) at 5.0 and 7.5 μM concentrations ($P<0.05$), apoptotic cells at 0.1, 1.0, 5.0 μM ($P<0.05$), 7.5 μM concentrations ($P<0.01$) and necrotic cells at 0.3 and 2.5 μM ($P<0.05$), 5.0 and 7.5 μM ($P<0.01$) concentrations in human lymphocytes. The 2.5, 5.0, and 7.5 μM PAT concentrations also led to a clear decrease in the nuclear division index (NDI) ($P<0.05$). PAT caused a significant dose-dependent increase in the number cells of NPBs, in the frequency of apoptotic and necrotic cells, and a significant dose-dependent decrease in the NDI values in lymphocytes. These results indicate that PAT at high concentrations is genotoxic, cytotoxic and cytostatic in cultured human lymphocytes.

Keywords Cytome assay · Cytotoxicity · Genotoxicity · Micronucleus · Nuclear division index · Patulin

Introduction

The mycotoxin patulin (PAT), 4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one, is produced by a number of species of *Penicillium, Aspergillus*, and *Byssochlamys* and is known as a common contaminant in fruits such as apples, peaches, pears, and grapes (CAST 2003; Bennett and Klich 2003; Brase et al. 2009; Moss 2008). PAT was first discovered as an antibiotic with a wide spectrum of activity. However, in addition to its antibacterial, antiviral, and antiprotozoal activity, PAT was soon proved to be too toxic to be used clinically, and was reclassified as a mycotoxin (Bennett and Klich 2003; Brase et al. 2009; Iwahashi et al. 2006; Moss 2008). Nowadays, the contamination of food and especially fruit juices with PAT is considered to be significant, and apple juice samples have been reported in a number of countries to be contaminated with PAT at different concentration ranges (Harris et al. 2009; Ito et al. 2004; Iwahashi et al. 2006; Szymczyk et al. 2004; Yurdun et al. 2001). Many countries have established a maximum level of 50 μg/L for patulin in apple juice, and the provisional maximum tolerable daily intake (PMTDI) of 0.4 μg/kg body weight has been set by the Joint Expert Committee on Food Additives and Contaminants (JECFA, 1996).

PAT is classified in group-3 by the International Agency for Research on Cancer because of inadequate evidence of carcinogenicity in humans and in experimental animals (IARC 1986).

It has been found that several organs or systems including kidney, liver, intestinal tissue, and the immune system are affected by PAT administration (Speijers et al. 1988; Wichmann et al. 2002). In addition, PAT has been reported to be a carcinogen and teratogen in some experimental animal models (Ciegler et al. 1976; Osswald et al. 1978). However, Becci et al. (1981) did not observe any tumorigenic effect of PAT in the rats after oral administration.

The effects of PAT on oxidative stress in various mammalian cell lines have been investigated. It has been found that the intracellular reactive oxygen species (ROS) levels are increased in both human embryonic kidney (HEK293) and human promyelocytic leukemia (HL-60) cells after PAT treatment (Liu et al. 2007).

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PAT is regarded as a possible mutagen and carcinogen. With PAT, negative results were obtained in the Ames test using several strains of *Salmonella typhimurium* (IARC 1986; Wurgler et al. 1991), but PAT caused DNA single- and double-strand breaks in living cells of *E.coli* and DNA repair synthesis in permeabilized *E.coli* cells (Lee and Roschenthaler 1986). Moreover, there are a number of in vitro studies that have shown genotoxicity of PAT in mammalian cells, although the mutagenicity of PAT is a matter of debate. PAT caused a significant increase in the sister chromatid exchange (SCE) frequency in both Chinese hamster ovary cells (CHO-K1) and human lymphocytes (Cooray et al. 1982; Liu et al. 2003), but did not increase the SCE rate within a dose range permitting survival of Chinese hamster V79-E cells (Thust et al. 1982). PAT did not cause DNA strand breaks or oxidative DNA modifications at non- or low cytotoxic concentrations in Chinese hamster V79 cells (Schumacher et al. 2006). However, PAT induced DNA breaks and inhibited DNA synthesis in CHO and transformed rat fibroblasts (AWRF) cells at cytotoxic concentrations (Stetina and Votava 1986). Liu et al. (2003) also showed that PAT induced oxidative DNA damage in HEK293 cells by using the single cell gel electrophoresis assay (SCGE; comet assay).

PAT was shown to inhibit microtubule polymerization in a concentration-dependent manner, to cause cytotoxic effects and to induce CREST-positive micronuclei (MNi) containing whole chromosomes/chromatids in Chinese hamster V79 cells (Pfeiffer et al. 1998). In addition, an induction of MNi by PAT in cytokinesis-blocked human lymphocytes, and the cytotoxicity and induction of both chromatid and chromosomal aberrations in Chinese hamster V79 cells has been shown (Alves et al. 2000). PAT was found to increase the frequency of MNi, induce chromosomal aberrations, and decrease cell viability in human hepatoma HepG2 cells (Ayed-Boussema et al. 2011; Zhou et al. 2009; Yang et al. 2011). PAT also increased the formation of MNi and nucleoplasmic bridges (NPBs), while cell proliferation was reduced dose-dependently with the toxin in Chinese hamster V79 cells (Glaser and Stopper 2012).

The micronucleus (MN) assay and cytokinesis-block micronucleus (CBMN) assay have been proposed as a method for assessing chromosome damage in cultured human lymphocytes because they provide a convenient and reliable index of both chromosome breakage and chromosome loss (Fenech 1993, 2000). An important further development is the cytokinesis-block micronucleus cytome (CBMN cyt) assay approach that not only scores MN but also includes other nuclear abnormalities such as NPBs and nuclear buds (NBUDs) and provides measures of necrotic and apoptotic cells as well as the proportion of cells undergoing cell division (Fenech et al. 1999, 2003, 2011; Fenech 2006, 2007). So, this method allows the measuring of MNi as a biomarker of chromosome breakage and/or whole chromosome loss, NPBs as a biomarker of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair, NBUDs as a biomarker of gene amplification, necrosis and apoptosis as a viability status of cells, and mononucleated, metaphase, anaphase, binucleated (BN), multinucleated cells as a mitotic status of cells (Fenech et al. 1999, 2003, 2011; Fenech 2006, 2007). Also, CBMN cyt assay can be used as a comprehensive technique to measure the genetic impacts of a physical or chemical agent both in vitro and in vivo in different tissue types including lymphocytes (Fenech 2007; Fenech et al. 2011).

In a literature review, it has been possible to find some studies on the genotoxic and cytotoxic effects of PAT in mammalian cells. However, there is no study that has simultaneiously evaluated the effects of PAT on CBMN Cyt assay parameters in the lymphocytes of healthy subjects. Thus, in this study, we aimed to assess all the CBMN Cyt assay parameters such as NBUDs, necrotic cells, apoptotic cells, cells in metaphase and the percentage of nuclear division index (NDI) as well as MN and NPBs parameters on the genotoxic, cytotoxic and cytostatic effects of PAT in cultured human peripheral blood lymphocytes of healthy subjects.

Materials and methods

Chemicals

PAT, cytochalasin B (cyt-B) and mitomycin c (MMC) were obtained from Sigma-Aldrich (St. Louis, MO, USA); peripheral blood karyotyping medium and phytohemagglutinin-M were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). PAT was dissolved in dimethyl sulfoxide (DMSO) and this stock solution was further diluted with DMSO at required concentrations, i.e. 0.1 μM, 0.3 μM, 0.5 μM, 1 μM, 2.5 μM, 5 μM, and 7.5 μM. DMSO concentration in the cultures containing PAT was between 0.1 and 0.4 % (v/v). MMC was dissolved in distilled water at a concentration of 0.5 μM. Cyt-B was prepared as a stock solution in DMSO at a concentration of 1 mg in 1 ml and the stock solution was diluted in culture medium. These solutions were kept at −20 °C. All the other chemicals and solvents used were of the highest purity commercially available.

Donors and collection of blood samples

Approximately 10 ml of peripheral blood samples were collected from 10 healthy non-smoking individuals, of both genders, aged 20–35 years, with no history of exposure to any genotoxic agent. The local ethics committee approved the study protocol (No. 2008/248). The study was conducted in accordance with the Helsinki Declaration and local laws. Each blood donor completed a questionnaire, designed to obtain their exposure history and current health

status; and informed consent forms were signed by each of them. The study excluded volunteers who reported alcohol, tea or/and coffee consumption, treatment for acute infections and/or chronic non-infectious diseases different dietary habits, intake of vitamin preparations, or intensive sport activities a week before the beginning of the study. No subject reported medicinal treatment over the 6 months before blood sampling, had a history of occupational and environmental exposure to known genotoxic chemicals/radiation, or had specific diseases such as hypertension, diabetes mellitus, hypercholesterolaemia, heart diseases or cancer.

Whole-blood cultures for human lymphocytes

Heparinized blood samples (~0.4 ml) were incubated for 72 h at 37 °C in 5 ml of the peripheral blood karyotyping medium supplemented with 2 % phytohemagglutinin. Two parallel cultures of each person were made for each concentration of PAT. At 24 h of incubation, PAT was added to cultures. Thus, the cultures were treated for 48 h with PAT at seven concentrations from 0.1 to 7.5 μM. In all experiments, doses were chosen from the range of non-cytotoxic concentrations as assessed by the percentages of BN cells and the NDI values. Additional cultures were incubated with MMC as a positive control and DMSO as a vehicle control; these cultures contained 0.84 % of the MMC and 0.4 % DMSO.

The cytokinesis-block micronucleus cytome assay

CBMN Cyt assay was performed as described by Fenech (2000, 2006, 2007) with some modifications. After 44 h of incubation, cyt-B at the final concentration of 3 μg/ml was added to cultures to block cytokinesis. The cultures were stopped at 72 h, treated with hypotonic solution (0.1 M KCl) for 4 min and fixed in two changes of methanol:acetic acid (3:1, v/v) (Donmez-Altuntas et al. 2003, 2007; Hamurcu et al. 2011). The fixed cells were spread onto clean glass slides and stained with 5 % Giemsa for 10 min. All slides were coded and read blind. To determine the intra-individual differences, the different slides of two parallel cultures of one person at each concentration were made and evaluated. Microscopic analysis was done under a light microscope with a ×400 magnification, and CBMN Cyt assay parameters such as MN, NBUDs and NPBs was additionally verified under ×1,000 magnification. A score was obtained for slides from each duplicate culture from two different scorers using identical microscopes.

The CBMN Cyt assay was described in detail and the criteria for scoring MNi, NPBs and NBUDs in BN cells, mononucleated cells, BN cells and multinucleated cells and necrotic and apoptotic cells are also published (Fenech 2000, 2007; Fenech et al. 2003, 2011). We followed these criteria for the selection of BN cells and identification of CBMN Cyt assay parameters. For each concentration and control, every subject was analyzed for the total number of MNi, NBPs and NBUDs per 1,000 BN cells to determine DNA damage effects, when 1,000 BN cells with two macronuclei surrounded by cytoplasm and a cell membrane obtained from whole-blood cultures were scored. The frequency of BN cells containing one or more MN was also determined. In addition, the cells in metaphase were scored when BN cells were scored.

The number of necrotic and apoptotic cells were scored in 1,000 mononucleated cells to determine cytotoxicity and the criterion for the identification of necrotic and apoptotic cells was according to Fenech (2007).

The number of mono-, bi- and multinucleated cells per 1,000 viable mononucleated cells to determine cytostatic effects was scored in peripheral blood lymphocytes of all individuals. The nuclear division index (NDI) has been calculated by using the formula

$$\mathrm{NDI} = (\mathrm{M1} + 2\ \mathrm{M2} + 3\ \mathrm{M3} + 4\ \mathrm{M4})/\mathrm{N},$$

where M1–M4 represent the number of cells with 1–4 nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells) (Eastmond and Tucker 1989; Fenech 2007).

Statistical analysis

Statistical comparisons on CBMN Cyt assay parameters between treated and control groups were made using Friedman and Wilcoxon nonparametric tests for K-related samples and two-related samples, respectively. Differences between treated and control groups were considered significant at $p < 0.05$ and $p<0.01$. The relationship of CBMN Cyt assay parameters with concentrations of PAT was evaluated by Spearman's rho correlation analysis. Significant levels were set at $p<0.05$ and $p<0.01$. All analyses were conducted using the SPSS for Windows statistical package, version 11.0.

Results

The effects of PAT on MN frequency and other CBMN cyt assay parameters were evaluated for seven different concentrations of PAT (0.1, 0.3, 0.5, 1, 2.5, 5, and 7.5 μM), MMC (positive control), DMSO (vehicle control) and negative control in human peripheral blood lymphocytes. Human lymphocyte cultures were treated for the last 48 h with PAT at the each concentration, MMC and DMSO. Negative control contains neither PAT nor DMSO or MMC.

For the mycotoxin PAT tested, the dose range used was chosen according to our preliminary experiments where 10 μM PAT concentration caused an important reduction of cell

viability that made an evaluation of BN cells for CBMN cyt assay parameters such as MNi, NPBs, NBUDs and NDI impossible (data not shown). Thus, the human peripheral blood lymphocytes were exposed to concentrations of PAT ranging from 0.1 to 7.5 μM.

The effects of PAT on DNA damage parameters in BN cells of peripheral blood lymphocytes

The effects of PAT on DNA damage parameters including the number of BN cells with MN, total number of MN in BN cells, distribution of BN cells with MN, MN frequency (%) in BN cells and number of BN cells with NPBs and NBUDs in the human lymphocytes are summarized in Table 1. Not all DNA damage parameters in the vehicle (DMSO) control group were significantly different from the negative control group ($p>0.05$). The number of BN cells with NPBs was significantly increased at PAT concentrations of 5 and 7.5 μM in comparison with the negative control group ($p<0.05$), but the other DNA damage parameters were not significantly different from the negative control group ($p>0.05$). Also, at the different PAT concentrations, except for NPBs, all other DNA damage parameters were not significantly different from the DMSO control group ($p>0.05$). The positive control (MMC 0.5 μM) was shown to significantly induce all the DNA damage parameters, except the number of BN cells with 2 MN, in comparison with the negative control group ($p<0.05$ and $p<0.01$). With respect to DNA damage effects of PAT on human lymphocytes, PAT induced a statistically significant concentration-dependent increase in the number of BN cells with NPBs in human lymphocytes, at least at between 7.5 and 5 μM (r 0.771; $p<0.01$), 7.5 and 2.5 μM (r 0.810; $p<0.01$), 5 and 2.5 μM (r 0.883; $p<0.05$), 2.5 and 1 μM (r 0.765; $p<0.05$), 1 and 0.1 μM (r 0.900; $p<0.01$), 0.5 and 0.1 μM (r 0.713; $p<0.05$), and 0.3 and 0.1 μM (r 0.789; $p<0.01$) concentrations tested.

The effects of PAT on cytotoxicity in peripheral blood lymphocytes

Table 2 shows the results of cytotoxicity (the frequencies of apoptotic and necrotic cells) in different concentrations of PAT, negative control, vehicle (DMSO) and positive controls in peripheral blood lymphocytes.

When compared to the negative control group, statistically significant increases in the frequency of apoptotic cells were observed in the 0.1 μM ($p<0.05$), 1.0 μM ($p<0.05$), 5.0 μM ($p<0.05$) and 7.5 μM ($p<0.01$) concentrations in human lymphocytes treated with PAT, while the increases in the frequency of necrotic cells at the 0.3 μM and 2.5 μM ($p<0.05$), 5.0 μM and 7.5 μM ($p<0.01$) PAT concentrations were found to be statistically significant. Statistically significant increases were found in the frequency of necrotic cells in the 5.0 μM and 7.5 μM ($p<0.01$) and 2.5 μM ($p<0.05$) concentrations of PAT, while statistically significant increases in the frequency of apoptotic cells were observed

Table 1 The results of DNA damage in PAT concentrations, negative control, vehicle (DMSO) control and positive control (MMC; 0.5 μM) in peripheral blood lymphocytes of 10 healthy individuals (mean ± SD)

Group	No. of BN cells with MN	Total no. of MN in BN cells[a]	Distribution of BN cells with		MN Frequency in BN cells (%)	No. of BN cells with NPBs	No. of BN cells with NBUDs
			1MN	2MN			
Negative control	3.60±2.22	3.80±2.39	3.40±2.12	0.20±0.42	0.38±0.24	5.50±5.21	4.10±3.90
Vehicle control	3.90±3.73	4.00±3.83	3.80±3.65	0.10±0.32	0.40±0.38	8.60±6.15	6.60±7.65
0.1 μM PAT	4.10±3.11	4.40±3.60	3.80±2.70	0.30±0.67	0.44±0.36	7.60±6.43	4.80±3.88
0.3 μM PAT	4.20±3.80	4.50±4.14	3.80±3.39	0.40±0.70	0.46±0.43	7.00±6.07	4.00±4.90
0.5 μM PAT	3.50±4.20	3.60±4.48	3.40±3.92	0.10±0.32	0.32±0.45	7.70±9.60	4.30±7.17
1 μM PAT	2.80±3.12	2.90±3.41	2.70±2.83	0.10±0.32	0.29±0.34	7.10±7.59	6.20±8.82
2.5 μM PAT	3.40±3.20	4.00±3.65	2.90±3.00	0.40±0.97	0.40±0.37	11.20±12.27	6.20±5.92
5 μM PAT	3.50±2.68	3.70±3.02	3.20±2.25	0.30±0.68	0.37±0.33	16.70±20.28*,#	5.70±6.60
7.5 μM PAT	5.60±5.27	6.10±6.19	5.10±4.43	0.50±1.08	0.61±0.62	13.40±12.13*,#	5.90±6.86
Positive control	17.40±12.91*,#	18.60±13.35*,#	16.90±12.32*,#	0.60±0.70	1.68±1.48*,#	13.50±15.75*,#	11.20±7.94*,#
Friedman p	0.002	0.001	0.002	0.289	0.012	0.036	0.014
Wilcoxon p[b]	0.007*	0.007*	0.007*	>0.05	0.017*	<0.05*	0.008*
Wilcoxon p[c]	0.007#	0.007#	0.007#	>0.05	0.014#	<0.05#	0.032#

MN Micronucleus, *BN cells* binucleated cells, *NPBs* nucleoplasmic bridges, *NBUDs* nuclear buds; *SD* standard deviation

[a] Total number of MN : (1MNX1)+(2MNX2)

[b] Statistically significant when compared to the negative control.*<0.05 and <0.01

[c] Statistically significant when compared to the vehicle control.#<0.05 and <0.01

in the 5.0 μM and 7.5 μM concentrations with PAT ($p<0.05$) when compared to the DMSO control group. No statistically significant increases in the frequency of apoptotic and necrotic cells in the positive control group were found in comparison with both negative and DMSO control groups ($p>0.05$). In respect of the cytotoxic effects of the PAT on human lymphocytes, PAT caused a significant concentration-dependent increase in the frequency of apoptotic cells, at least at between 7.5 and 5 μM (r 0.872; $p<0.01$), 5 and 0.3 μM (r 0.776; $p<0.01$), 2.5 and 0.5 μM (r 0.724; $p<0.05$) concentrations tested and in the frequency of necrotic cells, at least at between 7.5 and 0.3 μM (r 0.821; $p<0.01$), and 5 and 2.5 μM (r 0.806; $p<0.01$) concentrations tested.

The effects of PAT on cytostasis in peripheral blood lymphocytes

The results of cytostasis [the number of metaphase, the frequencies of BN cells (%), numbers of 2, 3, and 4 nucleated cells and NDI values] for the different concentrations of PAT, negative control, vehicle (DMSO) and positive controls in peripheral blood lymphocytes are given in Table 2.

No statistically significant increases in the number of metaphase in the all PAT concentrations were observed in comparison with both negative and DMSO control groups ($p>0.05$). Statistically significant decreases in the frequencies of BN cells and/or numbers of 2 nucleated cells (M2) were observed in the 2.5, 5.0, and 7.5 μM ($p<0.05$) concentrations in human lymphocytes treated with PAT when compared to the negative control group and in the 2.5 μM ($p<0.05$), 5.0 and 7.5 μM ($p<0.01$) concentrations with PAT in comparison with the DMSO control group. The decreases in the numbers of 3 nucleated cells (M3) in the PAT concentrations of 2.5, 5.0 and 7.5 μM were found statistically significant when compared to both the negative and DMSO control groups ($p<0.05$, $p<0.01$, $p<0.01$, respectively), while the decreases in the numbers of 4 nucleated cells (M4) in the PAT concentrations of 5.0 and 7.5 μM were found statistically significant when compared to both the negative and DMSO control groups ($p<0.01$).

NDI values at the of 2.5, 5.0 and 7.5 μM PAT concentrations were significantly decreased in comparison with both the negative control ($p<0.05$) and DMSO control groups ($p<0.05$, $p<0.01$, $p<0.01$, respectively).

In addition, statistically significant decreases in the numbers of M3 and M4 ($p<0.01$) cells and NDI values ($p<0.05$) in the positive control group were found in comparison with negative control group, while statistically significant decreases in the frequency of BN cells ($p<0.05$), numbers of M2 ($p<0.05$), M3 ($p<0.01$), M4 ($p<0.01$) cells and NDI values ($p<0.05$) in the positive control group were found in comparison with the DMSO control group.

The NDI values were decreased concentration-dependently in human lymphocytes, at least at between 5 and 2.5 μM (r 0.887; $p<0.01$), 5 and 1 μM (r 0.716; $p<0.05$), 2.5 and 1 μM (r 0.844; $p<0.01$), 1 and 0.3 μM (r 0.707; $p<0.05$), 0.5 and 0.3 μM (r 0.817; $p<0.01$), 0.5 and 0.1 μM (r 0.701; $p<0.05$), 0.3 and 0.1 μM (r 0.705; $p<0.05$) PAT concentrations tested (Fig. 1).

Discussion

There have been some studies on the in vitro genotoxic and cytotoxic effects of PAT in mammalian cells. However, there is no study on the combined evaluation of the effects of PAT on all CBMN cyt assay parameters such as MN, NPBs, NBUDs, necrotic cells, apoptotic cells, cells in metaphase, and NDI in mitogen-stimulated human lymphocytes. This is the first study using the CBMN cyt assay to examine in parallel the genotoxic, cytotoxic, and cytostatic potential of PAT in human lymphocytes.

Our results for several parameters of DNA damage demonstrated that PAT increased the number of BN cells with NPBs while it did not cause a significant increase in MN in human lymphocytes. The latter result differs from a dose-dependent increase in MN frequency at three PAT concentrations (2.5, 5.0, and 7.5 μM; 48 h treatment) in human lymphocytes reported by Alves et al. (2000), although PAT concentrations and exposure times used in their study are similar to those in our study. Possibly, differences in the scoring/evaluation procedure (one parameter in the Alves et al. 2000 study, and MN and NBUDs in BN cells in our study) may explain this. However, our results showed that the increase in the number of BN cells with NPBs from other DNA damage data confirm the findings of increasing in the MN frequency (Alves et al. 2000). On the other hand, induction of NPBs and increases in MN frequency by PAT were also found in other cells such as Chinese hamster V79 cells treated at even lower concentrations (0.1, 0.25, and 0.5 μM, 4 h exposure, Glaser and Stopper 2012; or 0.1 and 0.5 μM, 6 h exposure, Pfeiffer et al. 1998), human hepatoma HepG2 cells (0.38 and 0.75 μM, 24 h exposure; Zhou et al. 2009), and also much stronger the frequency of MN and NPBs induced by PAT in buthionine sulfoximine (BSO)-pretreated cells (Zhou et al. 2009; Glaser and Stopper 2012). In addition, after treatment with non- or low cytotoxic (0.4–2.4 μM, 1 or 4 h exposure) concentrations of PAT no increase in DNA strand breaks or oxidative DNA modifications has been observed in Chinese hamster V79 cells (Schumacher et al. 2006). But PAT increased DNA breaks at highly cytotoxic concentrations (20–50 μM) in Chinese hamster V79 cells (Schumacher et al. 2006) and at concentrations greater than 2 μg/ml (>10 μM) in CHO and AWRF cells (Stetina and Votava 1986). These results obtained (by more or less the same assay) in different

Table 2 The results of cytotoxicity and cytostasis in PAT concentrations, negative control, vehicle (DMSO) control and positive control (MMC; 0.5 μM) in peripheral blood lymphocytes of 10 healthy individuals (mean±S.D.)

Group	Frequency of apoptotic cells[a] (%)	Frequency of necrotic cells[a] (%)	No. of Metaphase	Frequency of BN cells (%)	Number of multinucleated cells			NDI
					M2	M3	M4	
Negative control	0.32±0.20	1.01±0.35	75.70±24.79	68.37±23.73	683.70±237.30	48.10±23.44	26.90±17.70	1.47±0.12
Vehicle control	0.40±0.22	1.23±0.64	67.70±21.26	65.12±20.69	651.20±206.86	40.20±19.34	26.70±17.59	1.46±0.11
0.1 μM PAT	0.55±0.32*	1.02±0.34	73.10±30.81	68.42±22.32	684.20±223.22	46.80±24.64	29.00±22.80	1.48±0.12
0.3 μM PAT	0.48±0.25	1.37±0.47*	75.30±30.92	60.98±21.60	609.80±215.95	42.50±26.00	22.40±13.39	1.44±0.12
0.5 μM PAT	1.12±2.08	1.34±0.64	77.20±46.39	58.66±24.19	586.60±241.86	37.30±20.20	24.20±20.14	1.43±0.13
1 μM PAT	0.52±0.19*	1.49±0.84	69.50±30.87	55.05±21.43	550.50±214.27	32.20±16.09	19.20±11.45	1.41±0.10
2.5 μM PAT	0.56±0.44	1.64±0.87*,#	79.20±38.71	45.35±16.55*,#	453.50±165.53*,#	26.10±13.37*,#	14.20±7.89	1.36±0.10*,#
5 μM PAT	0.83±0.51*,#	2.18±1.01**,##	74.10±35.92	36.53±13.88*,##	365.30±138.76*,##	15.90±10.08**,##	7.70±5.12**,##	1.29±0.08*,##
7.5 μM PAT	1.23±0.82**,#	3.32±1.88**,##	59.00±36.26	33.44±10.57*,##	334.40±105.73*,##	11.20±9.77**,##	6.60±6.02**,##	1.27±0.07*,##
Positive control	0.56±0.31	1.68±1.45	64.10±30.12	45.73±19.20#	457.30±191.99#	11.60±9.95**,##	6.50±6.17**,##	1.33±0.10*,#
Friedman *p*	0.005	0.001	0.716	0.001	0.001	0.001	0.001	0.001
Wilcoxon *p*[b]	<0.05*	<0.05*	>0.05	<0.05*	<0.05*	<0.05*	<0.01**	<0.05*
	<0.01**	<0.01**				<0.01**		
Wilcoxon *p*[c]	<0.05#	<0.05#	>0.05	<0.05#	<0.05#	<0.05#		<0.05#
		<0.01##		<0.01##	<0.01##	<0.01##	<0.01##	<0.01##

vBN cells Binucleated cells, *SD* standard deviation

[a] The frequency of necrotic and apoptotic cells scored on 1,000 cells

[b] Statistically significant when compared to the negative control.*<0.05 and **<0.01

[c] Statistically significant when compared to the vehicle control.#<0.05 and ##<0.01

Nuclear Division Index (NDI)=[M1+2(M2)+3(M3)+4(M4)]/N

Where M1-M4 represent the number of cells with one to four nuclei scored on 1,000 viable cells (excluding necrotic and apoptotic cells; M: The number of nuclei; N: The total number of viable cells scored)

mammalian cell types, point to differences in the susceptibility of the target cells. This may be related to differences in cellular glutathione levels, lengths of cell cycle, and/or repair capacities. Nonetheless, together the in vitro results demonstrate a genotoxic potential of PAT.

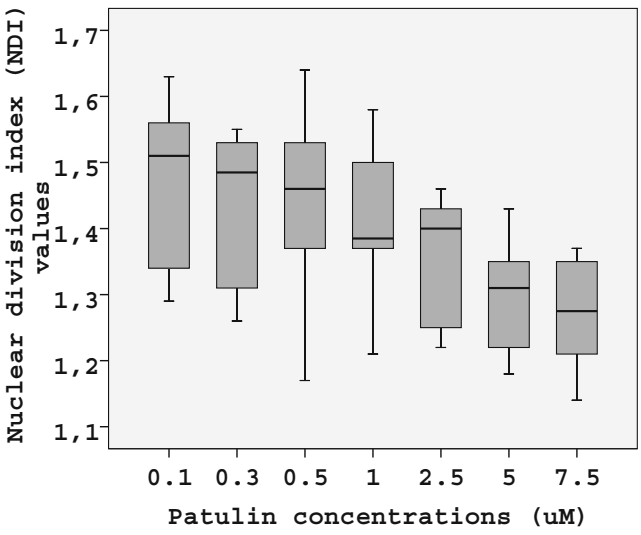

Fig. 1 NDI values decreased concentration-dependently in human lymphocytes after 48 h of exposure to PAT at concentrations of 0.1, 0.3, 0.5, 1, 2.5, 5, and 7.5 μM ($p<0.05$ and $p<0.01$)

The results of our study showed that PAT caused a significant concentration-dependent increase in the frequency of both apoptotic cells (at 0.3, 0.5, 2.5, 5.0, and 7.5 μM concentrations≈0.046, 0.077, 0.39, 0.77, 1.16 μg/ml; 48 h exposure times) and necrotic cells (at 0.3, 2.5, 5.0, and 7.5 μM concentrations; 48 h exposure times) in human lymphocytes. Although it is difficult to compare directly, the PAT concentrations used in present study (0.1–7.5 μM≈0.0154–1.1558 μg/ml) correspond to the higher concentrations for limit values of 0.05 μg/ml of PAT in apple juice. In agreement with our results, PAT has been shown to increase in the number of dead cells in V79 cells as determined with trypan blue assay after 6 h incubation with PAT at 0.5, 1, and 5 μM concentrations (Pfeiffer et al. 1998). Also, our cytotoxicity findings in lymphocytes are in accord with results of previous studies that showed decreases in cell viality as measured by the tetrazolium dye-based MTT assay in several other cell types. In these previous studies, PAT caused a decrease in the cell viability upon treatment of HEK 293 cells with 1 and 2 μM for 48 h (Liu et al. 2003), of HL-60 cells with 1 and 2.5 μM for 6 h (Wu et al. 2008), of HepG2 cells with concentrations ranging from 5 to 100 μM for 24 h (Ayed-Boussema et al. 2011), and of V79 cells with >1 μM for 20 h (Alves et al. 2000).

The significant decreases in the numbers of 2 and 3 nucleated cells (M2 and M3) and NDI values at PAT of the 2.5, 5.0,

and 7.5 μM concentrations and in the numbers of 4 nucleated cells (M4) at PAT of the 5.0 and 7.5 μM concentrations were found in human lymphocytes for cytostasis effect after 48 h treatment with PAT. Inversely to our cytostasis results, in one study that proliferation index is used as a measure of cytotoxicity, and the frequency of BN cells (%) and the cytokinesis-block proliferating index (CBPI) at similar PAT concentrations (2.5, 5.0, and 7.5 μM) as in our study were not significantly different from those observed in control cells for human lymphocytes (Alves et al. 2000). Similarly, in another study, for parameters of the frequency of BN cells and CBPI at the different PAT concentrations and cell type, cytotoxicity was not present at 0.19, 0.38, and 0.75 μM PAT concentrations with 24 h exposure times in human hepatoma HepG2 cells without BSO pre-treatment (Zhou et al. 2009). However, CBPI was reduced with increasing concentrations of PAT in BSO-pretreated (glutathione-depleted) Chinese hamster V79 cells, while it was reduced slightly without BSO pre-treatment (Glaser and Stopper 2012).

In conclusion, our results demonstrated that PAT at the 2.5, 5.0, and 7.5 μM concentrations caused a significant dose-dependent increase in the frequency of apoptotic cells and in the frequency of necrotic cells, an increase in the number cells of NPBs, and a significant dose-dependent decrease in the NDI rates in human lymphocytes. Our data indicate that PAT has the ability to induce DNA damage (at 5 μM or more), but the cytotoxic and cytostatic effects were more pronounced in human lymphocytes.

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Conflict of interest The authors declare that there are no conflicts of interest.

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