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Methamphetamine ("crystal meth") causes induction of DNA damage and chromosomal aberrations in human derived cells

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ABSTRACT

Methamphetamine (METH) is a widely consumed psychostimulant drug; its acute toxic effects in brain and liver are well known, furthermore, there is some evidence in regard to its DNA damaging properties in humans. Therefore, we studied the impact of the drug on genomic stability in human derived hepatoma (HepG2) cells, which reflect the activation/detoxification of drugs better than other cell lines. Furthermore, experiments with human buccal derived cells (TR146) were conducted as the drug is consumed orally. Induction of DNA damage in both cell types with doses reflecting the exposure in abusers was found in single cell gel electrophoresis (SCGE) assays (which detect single and double strand breaks as well as apurinic sites). Furthermore, induction of micronuclei (formed as a consequence of structural and numerical chromosomal aberrations) and formation of nuclear buds resulting from gene amplifications was detected. Additional experiments with lesion-specific enzymes showed that the drug causes oxidation of purines and pyrimidines, indicating that its genotoxic effects may be due to oxidation of the DNA. Our findings support the assumption that the drug may cause adverse health effects (such as cancer and infertility) in long-term users which are causally related to DNA damage.

1. Introduction

Methamphetamine (METH) is a psychoactive drug which is widely consumed; according to the EU drug report, 219 illegal production sites were identified in 2015 (EMCDDA, 2017). The compound is also used in small quantities to treat hyperactive children and as a short-term adjuvant in regimens of weight reduction (Courtney and Ray, 2014; DRUGS, 2018; Kish, 2008).

Only few investigations have been conducted concerning the genotoxic properties of METH, most of these studies deal with the induction of DNA base oxidation in brain cells *in vivo,* since oxidation was postulated to play a causal role in the neurodegenerative properties of the drug (Jeng et al., 2005, 2006; Tokunaga et al., 2008; Wong et al., 2008). Furthermore, it was reported by Li et al. (2003) that METH induces micronuclei (MNi), sister chromatid exchanges (SCEs) and gene mutations in Chinese hamster ovary (CHO-K1) cells and bacteria. The same authors reported that MN rates are also increased in lymphocytes of users and postulated that these effects may be due to formation of ROS (Li et al., 2003). In another study, it was found that METH causes comet formation in specific dopamine rich areas of the brain (Johnson et al., 2015). The assumption of genotoxic properties of METH is supported by the observation that amphetamine (AM), which is structurally related, is DNA reactive as well; for example, positive results were obtained in single cell gel electrophoresis (SCGE) and MN experiments with laboratory rodents (Andreazza et al., 2008; Tariq et al., 1987). Also structurally related derivatives such as fenfluramine and

Abbreviations: AM, amphetamine; BN-MNi, binucleated cells with micronuclei; CBMN assay, cytokinesis-block micronucleus assay; CBPI, cytokinesis-block proliferation index; CP, cyclophosphamide; CT, cytostasis; FCS, fetal calf serum; LOEL, lowest observed effect level; METH, methamphetamine; MN, micronucleus; MNi, micronuclei; NBUDs, nuclear buds; NPBs, nucleoplasmic bridges; SCEs, sister chromatid exchanges; SCGE, single cell gel electrophoresis

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amfepramone which are used as slimming agents were found to cause DNA damage in different test systems (Agarwal et al., 1992; Silva Nunes et al., 2013).

Aim of the present study was to investigate the impact of METH on DNA stability in human derived liver cells (HepG2) and in a buccal cell line (TR146). We used hepatoma cells as they possess active phase 1 and phase 2 enzymes and reflect the metabolism of xenobiotic drugs in humans better than other stable cell lines currently employed in genetic toxicology (Knasmuller et al., 1998, 2004). HepG2 cells are frequently used in routine genotoxicity tests of chemicals (for details see OECD guideline #487). We used these cells as there is clear evidence that the drug causes hepatotoxic effects in animals and humans (Eskandari et al., 2014; Halpin et al., 2013). Also other human derived liver cell lines such as Huh6 (Waldherr et al., 2018) and HepaRG (Le Hegarat et al., 2010) retained the activities of drug metabolizing enzymes and can be used in genotoxicity experiments. However, HepG2 were employed as the Huh6 line is not well validated and recent findings with HepaRG showed that these cells fail to detect the effects of representatives of important groups of genotoxic carcinogens (Mišík et al., 2019; Waldherr et al., 2018). TR146 cells were included, as the drug is consumed orally as tablets or smoked (https://www.verywellmind.com/how-is-methamphetamine-used-63459) and cells of the respiratory tract are exposed to relatively high concentrations. We used these cells also in previous experiments with synthetic and natural cannabinoids (Ferk et al., 2016; Russo et al., 2019).

The impact of METH was monitored in SCGE experiments under standard conditions reflecting formation of single and double strand breaks as well as apurinic sites (Azqueta and Collins, 2013). This assay is based on the determination of DNA migration in an electric field and is widely used in genetic toxicology (Neri et al., 2015). To find out if DNA damage leads to persisting chromosomal aberrations, additional experiments were conducted, in which induction of MN (formed as a consequence of numerical and structural chromosomal aberrations) was monitored. Furthermore, we evaluated the impact of the drug on the frequencies of other nuclear anomalies reflecting genomic instability, namely nuclear buds (NBUDs) which are a consequence of gene amplifications, and nucleoplasmic bridges (NPBs) formed from dicentric chromosomes (Fenech, 2007).

METH and AM were reported to cause formation of reactive oxygen species and oxidation of DNA (Jeng et al., 2005; Krasnova and Cadet, 2009; Parolini et al., 2016; Tokunaga et al., 2008; Wong et al., 2008), therefore, we used in the present study a modified version of the SCGE protocol to monitor formation of oxidized purines and pyrimidines with lesion-specific enzymes. All experiments were conducted under conditions which are relevant for users, i.e. the exposure concentrations of the cells were in the range of the blood levels found in consumers; i.e. between 1 and 8 μM (Karch et al., 1999; Kish, 2008; Logan et al., 1998; Melega et al., 2007).

2. Materials and methods

2.1. Chemicals

Low melting point agarose (LMPA) and normal melting point agarose (NMPA) were obtained from Gibco (Paisley, UK). Inorganic salts, dimethysulfoxide (DMSO), propidium iodide, hydrogen peroxide, triton X-100, trizma base, bovine serum albumine (BSA), fetal calf serum (FCS), cyclophosphamide (CP), cytochalasin-B, Dulbecco's Phosphate Buffered Saline (DPBS), Eagle's Minimal Essential Medium (EMEM) and Dulbecco's modified Eagle Medium (DMEM), trypsin-EDTA, Na_2-EDTA, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) and Entellan were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Test compounds

(+)-Methamphetamine hydrochloride (CASNr. 51-57-0, 98% purity) was purchased from Sigma-Aldrich (Steinheim, Germany).

2.3. Cultivation of human cell lines (HepG2 and TR146)

The human hepatoma cell line (HepG2) was provided by F. Darroudi (Department of Toxicogenetics, Leiden University Medical Centre, The Netherlands).

The human cell line TR146 is derived from buccal epithelial tissue (Rupniak et al., 1985) and was obtained from J.G. Rheinwald (Dermatology Institute of Boston, MA USA).

It was excluded that the cultures were infected with mycoplasms by PCR (Mycoplasma plus PCR Primer Set, Agilent Technologies, Santa Clara, CA, US).

The cells were cultivated under standard conditions (37 °C, humidified atmosphere, 5% CO_2). The media were changed every 2–3 days. When the cultures had reached confluence, the cells were washed with 1.0% DPBS, detached with trypsin/EDTA, centrifuged and sub-cultured.

The HepG2 cells were grown in Eagle's Minimal Essential Medium (EMEM, Sigma-Aldrich, Steinheim, Germany) supplemented with 1.0 mM sodium pyruvate (MNP medium) and 10% FCS. The 5th to the 8th passages from stock cultures (in liquid nitrogen) were used for the experiments.

The TR146 cells were cultivated in Dulbecco's modified Eagle Medium (DMEM, Sigma-Aldrich, Steinheim, Germany) with 10% FCS and stored in liquid nitrogen. The 4th to the 6th passages from stock culture were used for the experiments.

2.4. Cytotoxicity measurements

The viability of the cells was determined with a CASY® cell counter (Schärfe-System GmbH, Reutlingen, Germany). This method is based on the determination of electric potential differences (Lindl et al., 2005). 2.0 x 10^5 cells/well were seeded in 24-well plates (Becton, Dickinson and Company, NJ, USA) in serum-free media containing different concentrations of METH (1.0 μM, 8.0 μM, 2.0 mM, 4.0 mM, 8.0 mM) or the solvent control for 24h. The cells were detached with trypsin–EDTA, centrifuged (200 *g*, 5 min, 21 °C) and suspended in 1.0 mL medium. 50 μL of the suspensions were transferred to CASY-cups (OLS OMNI Life Science GmbH & Co. KG, Bremen, Germany). For each experimental point, two independent experiments (three measurements/concentration/experiment) were performed and means ± standard deviations were calculated.

2.5. Single cell gel electrophoresis (SCGE) assays under standard conditions

The experiments were conducted according to the protocol of Tice et al. (2000) under alkaline conditions. Only cultures with a viability ≥70% were evaluated in SCGE assays, since acute toxic effects may cause positive results (Henderson et al., 1998; Koppen et al., 2017).

The cells (2.0 x 10^5 cells/well) were transferred into 24-well plates which contained medium with different concentrations of METH (1.0 μM-4.0 mM) for 24h. H_2O_2 (30 μM) was used as a positive control in all experiments. After the treatment, the cells were detached with trypsin–EDTA, centrifuged (200 g, 5 min, 21 °C) and suspended in 0.5 mL medium. 80 μL of the cell suspensions were mixed with low melting point agarose (0.5% LMPA). Subsequently, they were spread on pre-coated agarose slides (1.5% NMPA) and lysed in the dark at 4 °C for at least 60 min. After unwinding under alkaline conditions (pH > 13, 30 min), electrophoresis was carried out for 30 min (300 mA, 0.8 V/cm, at 4 °C) followed by neutralization for 8 min (2x).

Air-dried slides were stained with propidium iodide (10 μg/mL), the %age of DNA in the comet tails was determined with an automated image analysis system (Comet IV, Perceptive Instruments Ltd., Burry St.

Edmunds', UK). Two independent experiments were performed (3 slides/experiment, n = 6/concentration, 50 nuclei were randomly selected and evaluated).

2.6. Cytokinesis-block micronucleus (CBMN) experiments with HepG2

The experiments were conducted as described by Koller et al. (2014). HepG2 cells (5.0 x 10^5/well) were seeded to 6-well plates with 3.0 mL medium and allowed to attach overnight.

Subsequently, the medium was removed. After washing with DPBS, the cells were treated with different concentrations (0.5–8.0 μM and 2.0–4.0 mM) of the test compound in serum-free medium for 24 h. CP (final concentration 500 μg/mL) was used as a positive control.

After treatment of the cells with the drug, they were washed with DPBS. Subsequently, they were incubated with cytochalasin B (3.0 μg/mL) to block cytokinesis and EMEM (with 10% FCS) for 27–28 h. Finally, the cells were washed, trypsinized and harvested. Slides were prepared with the cyto-centrifugation method of Fenech (2007). After drying, they were stained with Diff Quik (Dade Behring, Deerfield, IL, USA) and fixed with Entellan (Sigma-Aldrich, Steinheim, Germany).

For each experimental point, two experiments were performed, each with two cultures. From each culture two slides were prepared. Different endpoints were scored on each slide, namely mono-nucleated, binucleated (BN) and multi-nucleated cells as well as the rates of binucleated cells with MN (BN-MN), the total number of MN in binucleated cells (MNi), nuclear buds (NBUDs) and nucleoplasmic bridges (NPBs). The cytokinesis-block proliferation indices (CBPIs) were calculated with 500 cells according to the formula CBPI = [M1 + 2M2 + 3M3]/N (N is the total number of scored cells, M1 refers to the number of mono-nucleated cells, M2 number of binucleated cells, M3 number of multi-nucleated cells). The toxicity of the compounds was indirectly assessed by calculation of the %age of cytostasis (% Cytostasis = 100-100{($CBPI_T$ - 1) ÷ ($CBPI_C$ - 1)}, T = test chemical treatment culture, C = control culture) according to the OECD guideline (OECD, 2014). Five concentrations of the drug were used to determine the CBPI values. According to OECD guideline #487, 2000 cells were evaluated per experimental point (OECD, 2014). Only doses causing less than 60% cytotoxicity were analyzed in regard to formation of nuclear anomalies (OECD, 2014).

2.7. Single cell gel electrophoresis (SCGE) experiments with lesion-specific enzymes

The impact of METH on formation of oxidatively damaged purines and pyrimidines was monitored in additional experiments with lesion-specific enzymes. The measurements with formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (Endo III) were conducted according to the protocol of Collins and Dusinska (2002). HepG2 cells were exposed to the test compounds as described above. The optimal amounts of the enzymes were established in calibration experiments according to the protocol of Azqueta et al. (2017) before the main experiments (results not shown).

After lysis, the slides were washed twice with enzyme reaction buffer (pH 8.0) for 8 min. Subsequently, the nuclei were treated either with 50 μL of the enzyme solutions or with the corresponding enzyme buffers. The incubation time in experiments with FPG was 30 min and with ENDO III 45 min at 37 °C, respectively. After the treatment, electrophoresis was carried out under standard conditions (30 min, 300 mA, 0.8 V/cm, at 4 °C, pH > 13). Subsequently, the slides were processed and evaluated as described above. For evaluation, %age DNA in tail obtained after treatment with the enzyme buffer was subtracted from the corresponding %age DNA in tail measured in lesion-specific enzyme measurements. For each concentration results obtained with six slides are available.

2.8. Statistical analyses

For each experimental point in the SCGE experiments results obtained with six slides are available. The overall experimental design was based on suggestions made in papers concerning the statistical analysis of comet experiments. Lovell and Omori (2008) provide in their publication a description of the design of SCGE experiments and state that replicate cultures should be made and provide a measure for inter-culture variability. Wiklund and Agurell (2003) recommend using the slide as the unit of measurement and the use of 50 cells from 3 slides/culture and 2 to 3 cultures per group.

On each slide 50 cells were randomly selected and the tail intensity (%age DNA in tail) measured by a specialized image analysis software (Comet IV, Perceptive Instruments Ltd., Burry St. Edmunds', UK). The median tail intensity from each slide served as starting point of the statistical analysis. In the experiments with lesion-specific enzymes, the tail intensity of the cells with buffer only was measured and subtracted from the tail intensity of the respective slides with the lesion-specific enzyme. The resulting tail intensities were arcsine transformed to stabilize the variances and normalize the distribution. Statistical analysis for each experiment was performed by application of the general linear model with experiment and dose as factors. Homogeneity of variances was tested by Levene's tests and normality was assessed by Kolmogorov-Smirnov tests of residuals with Lilliefors' corrected p-values. Comparisons of the different doses against controls were done by linear contrasts.

Micronucleus assays were done in two independent experiments each with three doses (two higher doses were omitted due to acute toxicity) and a negative and a positive control. Two cultures were treated in each experiment per dose and two slides with 500 cells were evaluated (2000 cells per dose and experiment, n(2 experiments x 2 cultures x 2 slides) = 8). This scheme is in agreement with the OECD guideline #487 which suggests the use of duplicate cultures and the testing of three concentrations per experiment. In the present study the experiment was repeated. Statistical evaluation was done applying the generalized linear model for Poisson counts and the number of counted cells for each slide was used as offset value. No significant over-dispersion was detected for any endpoint. Parameters were estimated with negative controls as reference and tested for deviation from zero by Wald tests. Trend was tested with doses as continuous variable.

All analyses were performed by Stata 13.1 (StataCorp, TX, USA). P-values below 0.05 were considered significant.

3. Results

3.1. Acute toxicity tests

To exclude the possibility that acute toxic effects lead to false positive results in SCGE assays, experiments were conducted in which the impact of METH on the viability of the indicator cells was monitored. The results are summarized in Fig. 1A–B. It can be seen that a significant decrease of the viability was observed at the highest concentration (8.0 mM).

3.2. SCGE assay (standard conditions)

Induction of DNA damage was monitored in experiments with concentrations ≤ 4.0 mM. The drug caused in both cell types induction of comets (2A-B). A more pronounced effect was seen in the buccal derived cells. At the highest dose, the extent of DNA migration was in these cells approximately 2.5-fold higher than the extent of DNA migration in the liver derived cells. The lowest observed effect level (LOEL) was in both cell types 8.0 μM.

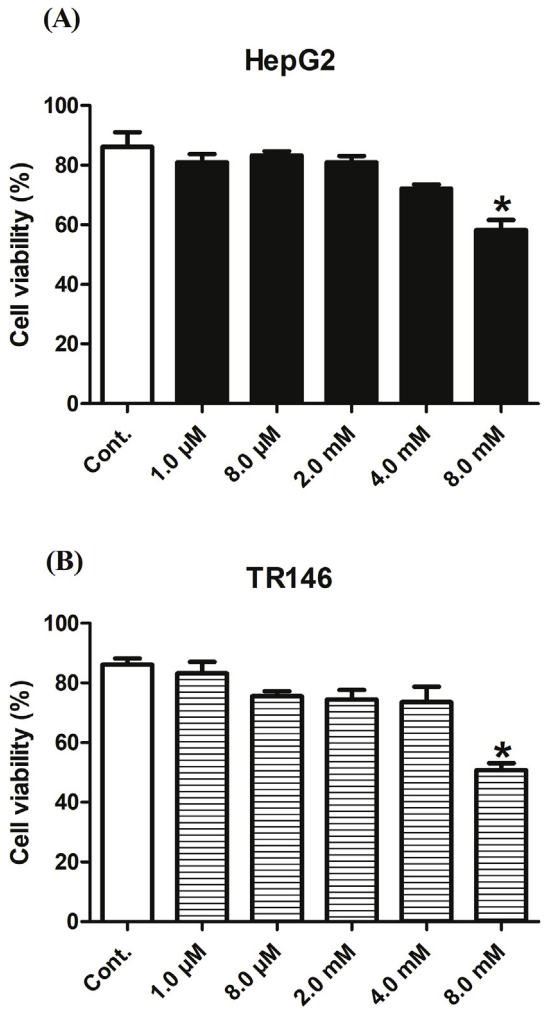

Fig. 1. Impact of METH on the viability of (A) HepG2 and (B) TR146 cells. The cells were exposed to the drug for 24h, subsequently their viability was determined by use of a CASY-counter. Bars indicate mean ± SD of results from two independent experiments. Stars indicate statistical significance ($p \leq 0.05$).

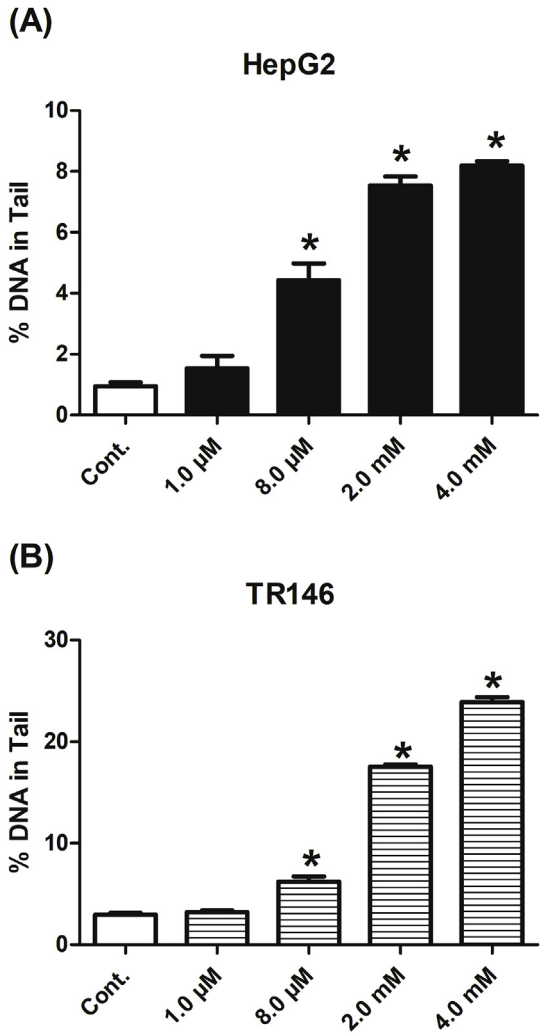

Fig. 2. Induction of DNA damage by METH in (A) HepG2 and (B) TR146 cells. The cells were exposed to different concentrations of the drug for 24h, subsequently the extent of DNA damage was monitored as %age DNA in tail in comet assays under standard conditions (for details see Materials and Methods). H_2O_2 was used as a positive control (30 µM, 5 min on ice). Bars indicate means of medians ± SD of results obtained from scores of N = 6 slides from two independent experiments (50 cells/slide). Stars indicate statistical significance ($p \leq 0.05$, linear contrasts from general linear model).

Table 1
Impact of (+) methamphetamine hydrochloride on MN formation (MNi) and on the frequencies of nucleoplasmic bridges (NPBs) and buds (NBUDs) in HepG2 cells.[1].

Compound	Concentration	CPBI	CT	BN-MNi[3]		NBUDs		NPBs	
	µM		%	Mean ± SD (‰)	p-value[4]	Mean ± SD (‰)	p-value[4]	Mean ± SD (‰)	p-value[4]
Neg. Control	0.0	1.53	0.00	8.5 ± 5.0		56.8 ± 13.0		4.8 ± 3.8	
Methamphetamine hydrochloride	0.5	1.49	3.54	9.1 ± 4.2	0.698	67.0 ± 16.4	0.127	6.0 ± 4.9	0.526
	1.0	1.51	3.28	12.8 ± 6.2	0.067	52.0 ± 12.5	0.362	6.5 ± 4.4	0.299
	8.0	1.47	11.34	**17.3 ± 7.2**	< 0.001	**75.3 ± 15.0**	0.001	8.0 ± 4.9	0.072
Pos. Control[2]	500 µg/mL	1.47	12.46	**29.3 ± 9.4**	< 0.001	**124.3 ± 19.3**	< 0.001	**11.5 ± 5.9**	0.001
Test for trend	p-value				< 0.001		0.003		0.069

Abbreviations: CBPI – Cytokinesis-Block Proliferation Indices; CT – Cytostasis (%); BN-MNi – binucleated cells with micronuclei; MNi – micronuclei, NBUDs – nuclear buds; NPBs – nucleoplasmic bridges.

[1] HepG2 cells were treated with different concentrations of the test compound for 24 h. Numbers represent means ± SD from N = 8 scores from two independent experiments with two cultures per experimental point. Overall, 2000 cells were evaluated. Statistically significant results ($p \leq 0.05$) generalized linear model with Poisson counts Wald test are printed in bold.

[2] Cyclophosphamide (500 µg/mL).

[3] Number of binucleated cells with MNi.

[4] generalized linear model with Poisson counts Wald test against negative control.

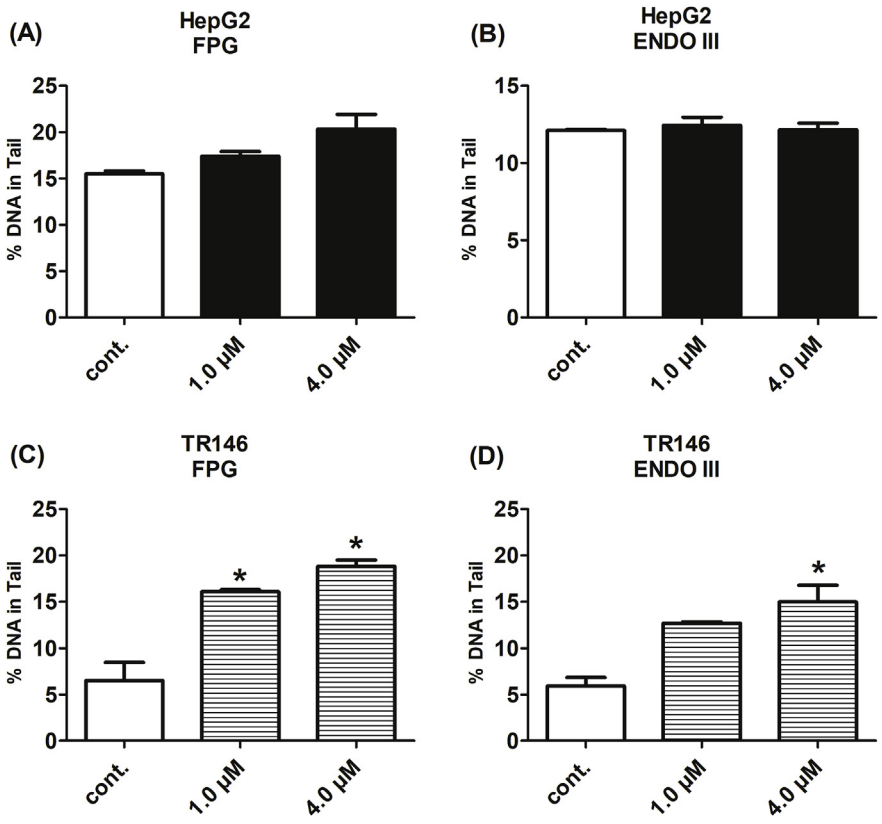

Fig. 3. Impact of METH on formation of oxidized purines and pyrimidines in (A–B) HepG2 and (C–D) TR146 cells. The cells were lysed after treatment with METH. Subsequently the nuclei were treated with solutions of FPG or ENDO III or with the corresponding reaction buffers (for details see Materials and Methods). Then, the extent of DNA migration from buffer treated cells was subtracted from that obtained with the enzyme treated cells. Bars indicate means of medians ± SD of results from scores of N = 6 slides from two independent experiments (50 cells/slide). For each dose, 300 nuclei were evaluated. Stars indicate statistical significance ($p \leq 0.05$, linear contrasts from general linear model).

3.3. CBMN-MN assays with HepG2 cells

The results of cytome MN experiments with HepG2 cells are summarized in Table 1. The drug caused significant formation of MNi and NBUDs at 8.0 µM. Higher concentrations could not be evaluated due to inhibition of cell division, i.e. the CPBI values decreased strongly after exposure of the cells to concentrations ≥ 2 mM (data not shown).

3.4. SCGE experiments with lesion-specific enzymes

To find out if oxidative damage of DNA bases is involved in the genotoxic effects, additional SCGE experiments were conducted with lesion-specific enzymes (FPG and ENDO III). The results are shown in Fig. 3A–D. With FPG (which detects oxidized purines) increased comet formation was observed in the buccal derived cells with concentrations ≥ 1.0 µM (Fig. 3C). ENDO III-sensitive sites (indicative for oxidized pyrimidines) were increased in these cells after treatment with 4.0 mM of the drug (Fig. 3D).

4. Discussion

The results of the present study show that low concentrations of METH cause induction of comets which reflect single and double strand breaks as well as apurinic sites in human derived liver and buccal cells (Fig. 2A–B). We found also clear induction of MNi which indicate that the DNA damage observed in the SCGE experiments leads to persisting mutations at the chromosomal level (Table 1).

The genotoxic effects were seen with concentrations which are relevant for users; i.e. the blood concentrations which were measured in several investigations in consumers were in the same range as the LOEL values of the present SCGE and cytome MN experiments (Karch et al., 1999; Logan et al., 1998; Melega et al., 2007).

The observation of MN induction is in agreement with earlier findings of Li et al. (2003) who obtained positive results in experiments with Chinese hamster ovary (CHO-K1) cells. However, clear induction of MNi was seen in this study only with concentrations which were substantially (ca. 250-fold) higher than the concentrations which were effective in the present experiment. This difference may be due to the fact that a different (rodent derived) cell line was used. Notably, we found also evidence for induction of nuclear anomalies other than MNi, namely an increase of NBUDs, which reflect gene amplifications (Fenech, 2007). Both types of anomalies are induced by genotoxic carcinogens.

As shown in Fig. 3C, we found induction of FPG sensitive sites which are indicative for formation of oxidized purines when the cells were treated with METH (1.0 and 4.0 µM). These findings are in agreement with results of several studies which found formation of 8-OH-dG and 8-oxo-dG in neuronal cells of METH treated rodents (Jeng et al., 2005; Tokunaga et al., 2008; Wong et al., 2008). Notably, DNA-damage attributable to ENDO III sensitive sites (which reflect oxidized pyrimidines) was also increased in buccal derived cells (Fig. 3D) at the highest dose. In this context, it is notable that there is some evidence that induction of chromosomal damage by METH and the structurally related compound AM is due to formation of reactive oxygen species. Li et al. (2003) found in their high dose *in vitro* MN experiments (see above) protective effects of antioxidant enzymes (catalase and superoxide dismutase) and Andreazza et al. (2008) reported that induction of MN in lymphoblastoid cells of rats was strongly reduced after co-administration of drugs with antioxidant properties. Furthermore, *in vitro* experiments demonstrated that METH causes formation of 8-OH-dG in specific regions of the brains of rodents (Jeng et al., 2005, 2006; Tokunaga et al., 2008; Wells et al., 2010). It is also notable that Li et al. (2003), found inhibition of the genotoxic properties of the drug in experiments with bacteria and CHO-K1 cells after addition of a hepatic enzyme mix (S9). This phenomenon may be due to direct binding of ROS to proteins in the activation mix. The positive effects which we detected in the human cell lines show that the drug or DNA reactive products enter the cells; obviously they are only partly detoxified and cause damage of the genetic material.

The results of the present investigation suggest that METH may

cause cancer and other adverse health effects associated with damage to the genetic material in users. It is well documented that DNA damage plays a key role in the etiology of malignant diseases but the carcinogenic properties of MN have not been studied so far in long-term studies. With AM, negative results were obtained in a NTP cancer study with mice and rats (National Toxicology, 1991), but several investigations found acceleration of the growth of experimentally induced tumors in rodents (Freire-Garabal, 1996; Freire-Garabal et al., 1992; National Toxicology, 1991). On the contrary, induction of hepatocellular adenomas and hepatoblastomas was observed with methylphenidate, a piperidine derivative of METH, in male mice (Amerio et al., 2015). Another relevant issue is the impact of METH on fertility and DNA integrity in sperm. In this context it is notable that it was found in a recent study that METH has a detrimental effect on sperm parameters and DNA integrity in mice (Sabour et al., 2017).

Taken together, our findings indicate that METH may cause damage of the genetic material in users. This assumption is supported by findings of Li et al. (2003) who found evidence for induction of MN in lymphocytes of consumers, i.e. the frequencies were in this group about 2-fold higher as in non-users and increased with the dose. Since METH is consumed worldwide by a large number of humans, further investigations concerning its adverse health effects as a consequence of its DNA damaging properties are warranted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.035.

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