Effect of cocaine and crack on the ploidy status of *Tetrahymena pyriformis*: a study using DNA image analysis

Maria E. Stefanidou · Vasiliki I. Hatzi ·
Georgia I. Terzoudi · Ariadni C. Loutsidou ·
Constantinos P. Maravelias

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Abstract The effect of cocaine and crack on the ploidy status of Feulgen-stained *Tetrahymena pyriformis* macronuclei using computerized DNA image analysis system was tested. For this purpose, selected doses of 5, 10 and 20 µg (per mL culture) of both drugs were administered for 2, 5 and 20 h to protozoa cultures and DNA image analysis of *T. pyriformis* nuclei was performed. The analysis was based on the measurement of the following parameters: *Ploidy Balance* (PB), *Degree of Aneuploidy* (DA), *skewness* and *kurtosis*. The results have shown a positive effect of both cocaine and crack on PB and on DA of *T. pyriformis* macronuclei. In particular, our results reveal that the aneugenic effect (which is expressed as a decrease in PB and an increase in DA) of cocaine on *T. pyriformis* macronuclei follows a dose-dependent

manner, while crack induces aneuploidy in a dose-independent manner. Changes in the PB and DA values would induce a disturbance in the cellular density and heterogeneity of chromatin and the increase in skewness and kurtosis values after exposure of *T. pyriformis* to both drugs, did confirm this hypothesis. These observations were further correlated with alterations in the chromosomal segregation and with damage in mitotic spindle microtubules observed previously. In this study the impact of cocaine and crack on genomic instability and carcinogenesis was further supported and *T. pyriformis* can be proposed as a model organism to test the nuclear ploidy status after exposure to harmful chemicals and drugs.

Keywords *Tetrahymena pyriformis* · Cocaine · Crack · DNA image analysis · Ploidy balance · Aneuploidy

M. E. Stefanidou (✉) · A. C. Loutsidou ·
C. P. Maravelias
Department of Forensic Medicine and Toxicology,
Medical School, University of Athens, 75 M. Asias str.,
Goudi 115.27, Athens, Greece
e-mail: mstefan@med.uoa.gr

V. I. Hatzi
Institute of Nuclear Technology & Radiation Protection,
National Centre for Scientific Research, NCSR
“Demokritos”, Ag. Paraskevi Attikis, Athens, Greece

G. I. Terzoudi
Institute of Radioisotopes & Radiodiagnostic Products,
National Centre for Scientific Research, NCSR
“Demokritos”, Ag. Paraskevi Attikis, Athens, Greece

Introduction

The protozoon *Tetrahymena pyriformis* is an established experimental model in biological studies (Sauvant et al. 1999) and has been extensively used as a model organism to test the toxicity of different substances using several endpoints, such as phagocytic activity and DNA content (Stefanidou et al. 2002, 2008). Previously, the DNA content of *T. pyriformis* macronuclei after exposure to cocaine and crack has been measured using DNA image

analysis system where it was shown that both drugs affect the DNA content of the protozoa macronuclei (Stefanidou et al. 2002). The fluctuations in the DNA content of *T. pyriformis* macronuclei after exposure to both drugs observed previously may result from a possible effect of the drugs on chromosome segregation during cellular division. In the present study, we hypothesize that this effect may potentially lead to a disturbance on the nuclear ploidy status (Terranova 2008). To test this hypothesis, the *ploidy balance* (PB) and the *degree of aneuploidy* (DOA) as well as skewness and kurtosis values of *T. pyriformis* after exposure to cocaine (benzoylecgonine) and crack (cocaine freebase) were measured using computerized DNA image analysis system (CIAS, SAMBA 2000). To our knowledge, there are no data available concerning a comparison of aneuploidic effect between cocaine and crack (cocaine freebase). Since drug-abuse has been correlated with changes at the biochemical and/or genetic level, the aim of this study was to investigate the possible differences in the nuclear ploidy status after cocaine and crack exposure in an effort to add some information and potentially explain the underlying mechanism of the differences in their signs, symptoms and addictive effects (Fattore et al. 2009).

Materials and methods

Culture conditions and drug exposures

T. pyriformis protozoa were grown in a proteose-peptone broth at 25 °C, until reaching late log phase. Cocaine hydrochloride or crack was added each separately to a 100-mL culture, as aqueous solutions, at doses of 0.5, 1 and 2 mg (5, 10 and 20 µg per mL culture). These doses were selected based on previous studies carried out in our laboratory and are considered as acutely non-toxic (Stefanidou et al. 1999, 2002). For each drug and dose selected one protozoa culture was tested and another culture served as control. The cultures were allowed to stand for 2, 5 or 20 h after administration of drug and Feulgen-stained followed.

Feulgen staining

One drop (50 µL) of each protozoa culture, was pipetted onto a microscope slide, impregnated with

the adhesive agent poly-L-lysine hydrobromide (Sigma) 0.1% in water for injection, and was allowed to stand until drying (Jacobson and Branton 1977). The DNA of the protozoa nuclei was stained red to purple with the Feulgen reagent to stain DNA specifically and quantitatively, according to the static cytophotometric procedure.

DNA image analysis

A computerized image analysis system (CIAS), based on high resolution scanning, has proven to be an effective approach to examine and classify stained cells spread on a glass slide in the form of smears (Stefanidou et al. 2002, 2008). This technique is based on a conversion of optical images of cells into arbitrary numerical values (digitization) based on light absorbance or transmission. The quantitative analysis of the Ploidy Balance (PB), the Degree of Aneuploidy (DA), skewness and kurtosis values of the macronuclei of the protozoa was performed by means of the digital DNA image analysis system (SAMBA 2000). A total number of 50 nuclei of *T. pyriformis* from each culture were selected randomly from each sample (i.e. microscopic slide) and the quantification of morphometric and densitometric parameters (PB, DA, skewness and kurtosis) were performed at the wavelength of 560 nm. For each tested substance an experimental series consisting of three independent cultures of the protozoa was used and another experimental series served as control. The statistical analysis of the results was carried out using the Student's *t* test. All *p*-values were considered statistically significant at $p < 0.05$.

Results and discussion

The quantification of nuclear DNA is increasingly coming into practice in both research and clinical application (Cohen 1996; Roels et al. 1996; Stefanidou et al. 2002). Among the various stains and chromogenic reagents for quantitative staining of DNA have been recommended in literature, Feulgen reaction is one of the most widely accepted staining method for DNA cytometry (Cohen 1996; Roels et al. 2000). DNA image analysis, results in two types of parameters: the *distributional* and *interpretative* ones (Cohen 1996). Among the distributional parameters the main and most popular one is the DNA content

(index). Previous studies have shown that cocaine and crack induce fluctuations in *Tetrahymena* macronuclei DNA content (Stefanidou et al. 2002), which may result from a possible effect of cocaine and crack on chromosome segregation during cellular division. This effect may lead potentially to a disturbance on the nuclear ploidy status (Terranova 2008). To test this hypothesis, the ploidy balance (PB), the degree of aneuploidy (DA) as well as skewness and kurtosis values of Feulgen-stained *T. pyriformis* macronuclei previously exposed to 0, 5, 10 and 20 μg (per mL culture) of cocaine and crack for 2, 5 and 20 h were measured by means of DNA image analysis. The selection of the drug-doses was based on previous studies carried out in our laboratory with doses ranging from 0.15 to 20 μg of both cocaine and crack per mL of protozoan cultures. The doses ranging from 0.15 to 0.60 μg per mL of protozoan cultures did not induce any effects (Stefanidou et al. 1999; 2002). Therefore, the doses selected were 5, 10 and 20 μg of cocaine and crack per mL of protozoan cultures. Approximately 50 nuclei of *T. pyriformis* from each sample (i.e. microscopic slide) were measured for each experimental point and each experiment was repeated three times. The viability and the motility of the protozoa were checked under a light microscope. The protozoa population was counted by using the haematocytometer under the microscope at a magnification of 80 $\times$ (Fig. 1).

In order to gain an insight into the biological effects behind the effect of cocaine and crack on *T. pyriformis* DNA content, we first used one of the interpretative parameters of the DNA image analysis technology, the ploidy balance (PB). The PB better indicates the occurrence of macronuclei having abnormal DNA content. It represents the sum of the percentage of euploid cells minus the sum of the percentage of aneuploid cells and indicates the proportion of euploid cells versus aneuploid cells within one sample and can vary from +100% (all cells are euploid) to -100% (all cells are aneuploid). Therefore, the more negative the aneuploidy value is, the more aneuploid the cells are. The percentages of PB obtained after exposure of *T. pyriformis* cells to (0, 5, 10 and 20 $\mu\text{g}/\text{mL}$ -culture) of cocaine and crack at various exposure times (2, 5 and 20 h) are shown in Table 1. The results indicate a positive effect of both drugs in the PB parameter which is expressed as an increase in aneuploidy after drug exposure. In

Fig. 1 Microscopic pictures of protozoa *Tetrahymena pyriformis*. (A) Unstained macronuclei, (B) Feulgen-stained DNA macronuclei

particular, Table 1, shows that cocaine after 2, 5 and 20 h exposure causes a dose dependent statistically significant decrease in percentages of PB that ranges from -19.2 to -36%, from -27.5 to -53.8% and from -31.1 to -37.1% after exposure of *T. pyriformis* macronuclei to 5, 10 and 20 $\mu\text{g}/\text{mL}$ -culture, respectively. Interestingly, although crack after the same exposure times and doses, causes a decrease in the PB percentages of *T. pyriformis* macronuclei, the observed decrease does not follow a dose-dependent manner.

The observed statistically significant fluctuation in the PB after exposure of *T. pyriformis* to cocaine and crack triggered us to measure the effect of both drugs in the Degree of Aneuploidy (DA) parameter. For this purpose, using the DNA image analysis, 2, 5 and 20 h exposure of *T. pyriformis* macronuclei to cocaine and crack (at concentrations of 0, 5, 10 and 20 $\mu\text{g}/\text{mL}$ culture), a positive effect of both drugs on the DA

Table 1 The percentages of Ploidy Balance (PB) after exposure of *T. pyriformis* cells to cocaine and crack at various exposure times (2, 5 and 20 h) and (concentrations of 0, 5, 10 and 20 µg/mL culture) indicated a positive effect of both drugs to induce aneuploidy. Cocaine affected the PB of *T. pyriformis* macronuclei in a dose-dependent manner, while crack affected

the PB in a dose-independent manner. Mean values percentages and standard deviations arise from three independent experiments. From each experimental point, the control value was subtracted in order to obtain comparative results between the different exposure times. The statistical analysis was performed using the Student's *t* test ($p < 0.05$)

% Ploidy balance after exposure of *T. pyriformis* to cocaine and crack

µg/mL culture	2 h		5 h		20 h	
	Cocaine	Crack	Cocaine	Crack	Cocaine	Crack
5	-19.2 ± 2.1	8.3 ± 4.3	-27.5 ± 3.9	-44 ± 3.9	-31.1 ± 4.1	-51.5 ± 8.9
10	-24.4 ± 2.2	-40 ± 5.2	-37.1 ± 4.4	-36 ± 2.7	-44.6 ± 4.1	-23 ± 8.3
	$t = 2.86$	$t = 12.38$	$t = 2.80$	$t = 2.92$	$t = 4.03$	$t = 4.06$
	$p < 0.05$	$p < 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.02$	$p < 0.02$
20	-36 ± 4.2	-22.2 ± 6.7	-53.8 ± 6.3	-58 ± 4.1	-37.1 ± 2.2	-55 ± 6.4
	$t = 4.23$	$t = 3.63$	$t = 3.56$	$t = 7.76$	$t = 2.79$	$t = 5.29$
	$p < 0.02$	$p < 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.05$	$p < 0.01$

was observed. The DA values (%) obtained after exposure of *T. pyriformis* cells to (0, 0.5, 1 and 2 mg/100 mL-culture) of cocaine and crack at various exposure times (2, 5 and 20 h) are shown in Table 2. In particular, cocaine increases the DA of *T. pyriformis* macronuclei in a statistically significant dose-dependent manner ranging from 66 to 68.5% (after 2 h exposure), 62.8–76.9% (after 5 h exposure) and 59.6–68% (after 20 h exposure), while crack increased the DA in a dose-independent manner.

Briefly, in this study, DNA image analysis revealed that cocaine and crack affect the PB and the DA of Feulgen-stained macronuclei of *T. pyriformis*. In particular, both drugs induce a decrease in the PB parameter and an increase in the DA of *T. pyriformis* macronuclei in all concentrations and time intervals tested. Since aneuploidy disrupts the natural balance of chromosomal segregation during cellular division (Duesberg et al. 2000), as well as the overall content of the macronuclear genetic material, the observed disturbances in the nuclear ploidy status of *T. pyriformis* after exposure both to cocaine and crack may result from an effect of the drugs on chromosome segregation during cellular division (Terranova 2008). Furthermore, any observed effect on cellular segregation mechanisms (such as aneuploidy) could induce disturbances in the DNA content of *T. pyriformis* macronuclei. Indeed, such fluctuations in the DNA content which were observed after exposure to cocaine and crack in previous studies

(Stefanidou et al. 2002), justify partly the observed changes in the ploidy status.

The aneugenic effects of cocaine and crack observed here are in consistence with data from previous studies in other experimental systems. Aneuploidy, accompanied with a dose dependent increase in the DNA content, was observed after exposure of murine thymocytes to cocaine (Wu et al. 2003). Moreover, the effect of cocaine has been linked with alterations at gene level, RNA level and protein level (Freeman et al. 2002). Furthermore, in other studies, cocaine has been shown to induce chromosome segregation alterations, changes in the spindle apparatus and premature centromere separation in mouse oocytes (Combelles et al. 2000) as well as damage in meiotic and mitotic spindle microtubules in embryonic mammalian cultured mouse oocytes (Combelles et al. 2000) and cardiac cells (Meyer and Zhang 2009), respectively. These alterations have been correlated with genomic instability that may result in aneuploidy and carcinogenesis (Brinkley et al. 1985; Duesberg and Rasnick 2000; Duesberg et al. 2000). Indeed, previous studies have been reported where it was shown that cocaine stimulates tumor growth in mice (Grufferman et al. 1993) and these results are justified from the observations reported here.

Exposure of *Tetrahymena* macronuclei to 5, 10 and 20 µg cocaine and crack (per mL of culture) for 2 and 5 h, induces an increase in skewness and kurtosis values. In both parameters, the observed increase has

Table 2 Effect of cocaine and crack on *T. pyriformis* on the Degree of Aneuploidy (DA). After 2, 5 and 20 h exposure of *T. pyriformis* cells to cocaine and crack (at concentrations of 0, 5, 10 and 20 µg/mL culture) a positive effect of both drugs on the DA was observed. Cocaine increased the DA of *T. pyriformis* macronuclei in a dose-dependent manner, while crack

increased the DA in a dose-independent manner. Mean values percentages and standard deviations arise from three independent experiments. From each experimental point, the control value was subtracted in order to obtain comparative results between the different exposure times. The statistical analysis was performed using the Student's *t* test ($p < 0.05$)

% Degree of aneuploidy after exposure of *T. pyriformis* to cocaine and crack

µg/mL culture	2 h		5 h		20 h	
	Cocaine	Crack	Cocaine	Crack	Cocaine	Crack
5	66 ± 0.5	45.8 ± 0.46	62.8 ± 0.74	72 ± 1.4	59.6 ± 0.8	75.5 ± 1.89
10	67.3 ± 0.61	70 ± 0.62	68.5 ± 0.8	68 ± 1.6	62.2 ± 1.02	61.5 ± 2.53
	$t = 2.85$	$t = 54.29$	$t = 9.06$	$t = 3.26$	$t = 3.47$	$t = 7.68$
	$p < 0.05$	$p < 0.001$	$p < 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.01$
20	68.5 ± 0.41	61.1 ± 0.7	76.9 ± 0.84	76.9 ± 2.01	68 ± 1.56	77.5 ± 2.31
	$t = 2.82$	$t = 16.48$	$t = 12.54$	$t = 6$	$t = 5.39$	$t = 8.09$
	$p < 0.05$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.01$	$p < 0.01$

been shown to be statistically significant after 5 h of exposure in all concentrations of cocaine and crack tested while after 2 h of exposure a statistically difference observed only at the highest concentration tested in both drugs.

In particular, it is observed that skewness and kurtosis values after crack exposure is more potent compared with the values obtained after cocaine exposure; a result which can be explained from the fact that freebase cocaine (crack) is much more potent and rapid in action than the salt form (cocaine) (Stefanidou et al. 2002). It is known, that the densitometric parameters: skewness and kurtosis examine the deviation of the distribution from normality (i.e. the global repartition of chromatin). In particular, skewness indicates the distribution of pixel densities relative to the median of all possible optical densities in the analyzed nuclei (Werness et al. 1999). In general, higher skewness values indicate more globally dense nuclei and chromatin. Kurtosis, measures the range of pixel densities in the analyzed nuclei with greater values corresponding to a more heterogeneous distribution of chromatin (Werness et al. 1999). The larger value for kurtosis reflects a more heterogeneous chromatin distribution. Based on these, our results indicate that both drugs, under the experimental conditions tested, affect both the cellular density and the heterogeneity of *Tetrahymena* cells chromatin structure. A denser chromatin folding in the genome, would hypothetically affect the access of

these sites by the multiprotein transcription machinery. Our results reveal that both cocaine and crack affect the overall chromatin condensation/decondensation level when protozoa cells are exposed for more than 2 h to the drug. However, further studies are needed to evaluate the role of cocaine and crack on chromatin condensation levels as well as its effect on proteins considered to be involved in the dynamic condensation/decondensation, structure such as phosphorylases and histones.

In conclusion, the results of the present study reveal that the aneugenic effect of cocaine (which is expressed as a decrease in the PB and an increase in the DA) on *T. pyriformis* macronuclei follows a dose-dependent manner, while crack induces aneuploidy in a dose-independent manner. Furthermore, the statistically significant increase in skewness and kurtosis values after exposure of *T. pyriformis* macronuclei to both drugs highlight their affect in the cellular density and the heterogeneity of chromatin. These observations may partly explain the underlying mechanism for the highly addictive effect of crack (Fattore et al. 2009) as well as its intensity and short lasting signs and symptoms with respect to cocaine. Since the aneugenic effect of both drugs has been shown in other organisms in previous studies (Combelles et al. 2000; Wu et al. 2003), *T. pyriformis* could be proposed not only as a biomarker for different toxic agents (Stefanidou et al. 2008), but also as a model organism to test the ploidy status after exposure to harmful chemicals and drugs.

References

- Brinkley BR, Tousson A, Valdivia MM (1985) The kinetochore of mammalian chromosomes: structure and function in normal mitosis and aneuploidy. *Basic Life Sci* 36:243–267
- Cohen C (1996) Image cytometric analysis in pathology. *Hum Pathol* 27:482–493
- Combelles CM, Carabatsos MJ, London SN, Mailhes JB, Albertini DF (2000) Centrosome-specific perturbations during in vitro maturation of mouse oocytes exposed to cocaine. *Exp Cell Res* 260:116–126
- Duesberg P, Rasnick D (2000) Aneuploidy, the somatic mutation that makes cancer a species of its own. *Cell Motil Cytoskeleton* 47:81–107
- Duesberg P, Li R, Rasnick D, Rausch C, Willer A, Kraemer A, Yerganian G, Hehlmann R (2000) Aneuploidy precedes and segregates with chemical carcinogenesis. *Cancer Genet Cytogenet* 119:83–93
- Fattore L, Piras G, Corda MG, Giorgi O (2009) The Roman high- and low-avoidance rat lines differ in the acquisition, maintenance, extinction, and reinstatement of intravenous cocaine self-administration. *Neuropsychopharmacology* 34:1091–1101
- Freeman WM, Dougherty KE, Vacca SE, Vrana KE (2002) An interactive database of cocaine-responsive gene expression. *Sci World J* 2:701–706
- Grufferman S, Schwartz AG, Ruymann FB, Maurer HM (1993) Parents' use of cocaine and marijuana and increased risk of rhabdomyosarcoma in their children. *Cancer Causes Control* 4:217–224
- Jacobson BS, Branton D (1977) Plasma membrane: rapid isolation and exposure of the cytoplasmic surface by use of positively charged beads. *Science* 195:302–304
- Meyer KD, Zhang L (2009) Short- and long-term adverse effects of cocaine abuse during pregnancy on the heart development. *Ther Adv Cardiovasc Dis* 3:7–16
- Roels S, Ducatelle R, Cornelissen H (1996) Quantitative image analysis as an alternative to chemical analysis for follow-up of liver biopsies from a toucan with hemochromatosis. A technique with potential value for the follow-up of hemochromatosis in humans. *Anal Quant Cytol Histol* 18:221–224
- Roels SL, Van Daele AJ, Van Marck EA, Ducatelle RV (2000) DNA ploidy and nuclear morphometric variables for the evaluation of melanocytic tumors in dogs and cats. *Am J Vet Res* 61:1074–1079
- Sauvant MP, Pepin D, Piccinni E (1999) *Tetrahymena pyriformis*: a tool for toxicological studies. A review. *Chemosphere* 38:1631–1669
- Stefanidou M, Alevisopoulos G, Maravelias C, Loutsidis C, Koutselinis A (1999) Phagocytosis of the protozoan *Tetrahymena pyriformis* as an endpoint in the estimation of cocaine salt and cocaine freebase toxicity. *Addict Biol* 4:449–452
- Stefanidou M, Chatziioannou A, Livaditou A, Rellaki A, Alevisopoulos G, Spiliopoulou H, Koutselinis A (2002) DNA toxicity of cocaine hydrochloride and cocaine freebase by means of DNA image analysis on *Tetrahymena pyriformis*. *Biol Pharm Bull* 25:332–334
- Stefanidou M, Alevizopoulos G, Spiliopoulou C (2008) DNA content of *Tetrahymena pyriformis* as a biomarker for different toxic agents. *Chemosphere* 74:178–180
- Terranova R (2008) The many faces of constitutive heterochromatin. *Med Sci (Paris)* 24:720–724
- Werness BA, Afify AM, Bielat KL, Eltabbakh GH, Piver MS, Paterson JM (1999) Altered surface and cyst epithelium of ovaries removed prophylactically from women with a family history of ovarian cancer. *Hum Pathol* 30:151–157
- Wu YB, Hagen KL, Shen ML, Ou DW (2003) Evidence of increased DNA content of murine thymocytes caused by cocaine. *Immunopharmacol Immunotoxicol* 25:53–64