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A novel micronucleus *in vitro* assay utilizing human hematopoietic stem cells

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ABSTRACT

The induction of micronucleated reticulocytes in the bone marrow is a sensitive indicator of chromosomal damage. Therefore, the micronucleus assay in rodents is widely used in genotoxicity and carcinogenicity testing. A test system based on cultured human primary cells could potentially provide better prediction compared to animal tests, increasing patient safety while also implementing the 3Rs principle, i.e. replace, reduce and refine. Hereby, we describe the development of an *in vitro* micronucleus assay based on animal-free *ex vivo* culture of human red blood cells from hematopoietic stem cells. To validate the method, five clastogens with direct action, three clastogens requiring metabolic activation, four aneugenic and three non-genotoxic compounds have been tested. Also, different metabolic systems have been applied. Flow cytometry was used for detection and enumeration of micronuclei. Altogether, the results were in agreement with the published data and indicated that a sensitive and cost effective *in vitro* assay to assess genotoxicity with a potential to high-throughput screening has been developed.

1. Introduction

Since 1959, the 3Rs rule, i.e. reduce, refine and replace, is the foundation of the ethical policy applied to animal testing in Europe and North America. Due to the vast number of chemicals and/or their mixtures to be assessed for safety, there is an increasing need to evaluate toxic effects at the cellular level as described e.g. in the term "21st century toxicology" (Berg et al., 2011). One of the challenges is to design appropriate tests which rely on a molecular and cellular mechanistic understanding of toxicants mode of action using *in vitro* methods based on human cells. Such an approach allows the evaluation of responses over a wide range of doses, providing an improved mechanistical basis for assessing human health risks associated with exposure to genotoxic and/or carcinogenic compounds. Moreover, it is well known that bioassays detecting genotoxicity in animals in some instances have limited biological relevance for humans as, for example, regarding different response to toxins in comparison to humans (Krewski et al., 2011). *In vivo* assays can also be laborious and time-consuming, as well as painful to animals.

For genotoxicity assessment, the micronucleus (MN) test in reticulocytes of bone marrow in rodents is one of the most applied tests *in vivo* (http://www.oecd-ilibrary.org). MN originate from whole chromosomes or parts of chromosomes having been separated from the main nucleus, and therefore reflect either chromosome breakage or impairment of the mitotic spindle. Mature red blood cells lack DNA and RNA while reticulocytes still contain RNA. Therefore, these cells with induced MN containing DNA fragment(s) can easily be identified by flow cytometry (FCM) (Heddle et al., 2011).

Previously, we have developed a highly sensitive method to determine the frequency of micronucleated reticulocytes *in vivo* in rodents based on dual-laser FCM (Grawe et al., 1992). Using this

Abbreviations: MN, micronuclei; FCM, flow cytometry; RBCs, red blood cells; LK, leukapheresis; CB, cord blood; IMDM, Iscove Modified Dulbecco's Medium; SCF, stem cell factor; IL-3, interleukin-3; Epo, erythropoietin; DBPD, dibenzo[a,l]pyrene-11,12-dihydrodiol; DBPDE, dibenzo[a,l]pyrene-11,12-dihydrodiol-13,14-epoxide; DMBA, 7,12-dimethylbenz(a)antracene.

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methodology, we achieved an improvement in sensitivity by a factor of about 10, as compared to manual microscopic scoring (Abramsson-Zetterberg et al., 1995; Grawe et al., 1998, 1992). The possibility to automate the evaluation of *in vivo* MN-test by FCM has further increased the application of this method in genotoxicity testing (Hayashi et al., 2000, 2007). Detailed method protocols have therefore been published (Dertinger et al., 2003; Grawe, 2005). For assessing cytogenetic damage, FCM-scoring of micronucleated erythrocytes in rodents is now a well-established and efficient platform which is highly predictive towards carcinogenicity, relatively easy to evaluate and contains improved technical advantages (Dertinger et al., 2011). The strength of this test is that it exclusively detects MN arising in the bone marrow and thus indicates that the genotoxic substance tested is effective in that tissue. Because the hematopoietic cells undergo rapid division, the test is highly sensitive to genotoxic agents as well as to aneugenic agents that produce changes in the chromosome number. The MN-test in newly-formed erythrocytes has also been applied by us in studies on humans (Abramsson-Zetterberg et al., 2000; Kotova et al., 2014; Stopper et al., 2005) as well as by others (Flanagan et al., 2010; Offer et al., 2005).

Based on our previous knowledge and experience in technical development of the MN-test, we have developed a non-animal genotoxic test *in vitro* which is analogous to micronucleus test *in vivo*. For this purpose, we have exploited the *ex vivo* generation of human erythrocytes from hematopoietic stem cells in conditions with no animal components as previously described (Giarratana et al., 2011). First, we showed that it is possible to induce and monitor MN induction by genotoxic agents in *ex vivo* generated human reticulocytes. Thereafter, in order to validate the method, a panel of clastogenic agents with direct action (γ-radiation, mitomycin C, methyl methansulfonate, etoposide, dibenzo(a,l)pyrene-11,12-di hydrodiol-13,14-epoxideand), clastogens requiring metabolic activation (7,12-dimethylbenz(a)antracene, benzo(a)pyrene, cyclophosphamide monohydrate, dibenzo(a,l)pyrene-11,12-dihy drodiol) as well as aneugens (colcemid, griseofulvin, vincristine sulfate, bisphenol A) and non-genotoxic compounds (benzyl alcohol, ethylenediaminetetraacetic acid, sodium chloride) has been tested. For metabolic activation, rat S9 mix and human S9 mix as well as co-culture with HepaRG and XEM2 cell lines have been applied.

2. Materials and methods

2.1. Cells and culture conditions

2.1.1. Cultured red blood cells (RBCs)

The culture conditions described here allow highly selective differentiation of CD34+ hematopoietic stem cells into fully matured RBCs. CD34+ cells have been isolated either from cord blood (CB) cells or leukapheresis (LK) from healthy individuals once informed consent obtained.

CD34+ cells were isolated by supermagnetic microbead selection by the use of Mini-MACS columns (Miltenyi Biotec; 94% ± 3% purity). The cells were cultured in the presence of Iscove Modified Dulbecco's Medium (IMDM) supplemented with stabilized glutamine (Biochrom) containing 330 µg/mL human holo-transferrin (Scipac), 10 µg/mL recombinant human insulin (Incelligent SG; CellGen), 2 IU/mL heparin (Choay), 5% solvent/detergent virus-inactivated human plasma (Etablissement Français du Sang) and with sequential addition of 100 ng/mL stem cell factor (SCF; PeproTech), 5 ng/mL interleukin-3 (IL-3; PeproTech) and 3 IU/mL erythropoietin (Epo; Eprex). On day 4, one volume of cell culture was diluted in four volumes of fresh medium containing SCF, IL-3 and Epo. On day 8, the cells were resuspended at 10^5 cells/mL in fresh medium supplemented with SCF and Epo. In the second phase (day 11–day 18), the cells were adjusted to 1×10^6 cells/mL and cultured in fresh medium supplemented with Epo alone. By day 15, the mean cell amplification of CD34+ cells reached a plateau of approximately 30000-fold for LK and 100000-fold for CB cells.

2.1.2. Metabolic activation

For metabolic activation, one part of rat S9 liver postmitochondrial fraction (S9) (MOLTOX, Trinova Biochem, Germany) and 1.5 parts cofactor mixture were used. The cofactor mixture was ß-nicotinamid adenine dinucleotide phosphate sodium salt and glucose-6-phosphate sodium salt dissolved in IMDM (final concentration in cell culture was 0.6 mmol/L and 3.75 mmol/L respectively). As an alternative, human S9 (XenoTech, USA) with the same cofactor mixture as for rat S9 was applied. XEM2 cells were grown in Dulbeccos modified Eagle's media (DMEM) containing 9% serum and 0.9% penicillin/streptomycin, in 37 °C at 5% CO_2. The medium for HepaRG cells (Biopredic International, France) was prepared according to user guide from a supplier. For co-culture with HepaRG or XEM2 cells in inserts, HTS Transwell-24 well plates with 0.4 µm polycarbonate membrane were used (Corning Incorporated, USA).

2.2. Compounds and γ-radiation source

The compounds used were paraformaldehyde (Sigma–Aldrich, Germany, CAS no 30525-89-4), May-Grünwald solution (Merck, Germany), Giemsa solution (Merck, Germany), phosphate-buffered saline (PBS; Sigma–Aldrich, Germany), glucose-6-phosphate sodium salt (Sigma–Aldrich, Germany, CAS no 54010-71-8), ß-nicotinamid adenine dinucleotide phosphate sodium salt (Sigma–Aldrich. Germany, CAS no 698999-85-8), GlutaMax (LifeTechnologies, USA), Iscove's Modified Dulbecco's Medium (IMDM, Sigma–Aldrich, Germany), dimethylsulfoxide (DMSO, Scharlau, CAS no 67-68-5), Hoechst 33342 (Sigma–Aldrich, Germany, CAS no 23491-52-3), Thiazole orange (Sigma–Aldrich, Germany, CAS no 107091-89-4). Compounds tested are listed in Table 1. The final concentration of DMSO or water in complete medium did not exceed 0.1% (v/v). In all cases of γ-radiation, the cells were irradiated with a single acute dose by ^{137}Cs source at the dose-rate of 0.37 Gy/min.

2.3. Treatment schedule

2.3.1. Compounds with direct action

During the initial experiments, the cells were treated on days 11–15 in order to test different culture conditions. The cells were transferred from T-flasks into 24-wells plates in concentration 1×10^6 cells per mL per well in a final volume of 1 mL per well. For testing in 96-wells plates, a final volume was 0.2 mL per well. To test a short-term or prolonged treatment to genotoxic agents with a direct mechanism of action, i.e. without metabolic activation, cells were treated for 1 h or 24 h and had a recovery time of 24-, 48-, 72- or 120 h. After the treatment, the cells were washed twice, first time with 9 ml and then with 7 ml PBS.

2.3.2. Compounds that require metabolic activation

For testing of genotoxic agents which require metabolic activation, cells were treated with a respective chemical for 3 h (short-term exposure) or 24 h (prolonged treatment) with final concentration of 1% or 2% microsomal liver cell fraction from rat (S9 mix) followed by 69- or 48-h recovery time, respectively. For tests with human S9 mix, cells were treated with a respective chemical for 3 h with final concentration of 1%, 2%, 5% or 10% mix followed by 69-h recovery time. XEM2 and HepaRG cells were

Table 1
Tested compounds.

Category	Chemical	CAS No.	Supplier
1. Clastogens active without metabolic activation			
	Mitomycin C	50-07-7	Sigma–Aldrich
	Methyl methansulfonate	66-27-3	Fluka
	Etoposide	33419-42-0	Sigma–Aldrich
	Dibenzo(*a*,*l*)pyrene-11,12-dihydrodiol-13,14-epoxide		The Biochemical Institute for Environmental Carcinogens
2. Clastogens requiring metabolic activation			
	Benzo(*a*)pyrene	50-32-8	Sigma–Aldrich
	Cyclophosphamide monohydrate	6055-19-2	Sigma–Aldrich
	Dibenzo(*a*,*l*)pyrene-11,12-dihydrodiol		The Biochemical Institute for Environmental Carcinogens
	7,12-Dimethylbenz(a)antracene	57-97-6	Sigma–Aldrich
3. Aneugens			
	Bisphenol A	80-05-07	Sigma–Aldrich
	Colcemid	477-30-5	Gibco
	Griseofulvin	126-07-8	Sigma–Aldrich
	Vincristine sulfate	2068-78-2	Sigma–Aldrich
4. Negative substances			
	Benzyl alcohol	100-51-6	Sigma–Aldrich
	Ethylenediaminetetraacetic acid	6381-92-6	Scharlau
	Sodium chloride	7647-14-5	Merck

used for co-cultivation in inserts or directly in wells. All types of co-culture were applied just before the treatment and directly afterwards the cells were washed by PBS twice as described above.

2.4. Cell harvesting and fixation

After the treatment, the cells were harvested by collection into 15 ml FALCON-type tubes, and washed twice in 7 ml PBS. The tubes were centrifuged at 275 $*$ *g* for 8 min at room temperature. Afterwards, pellets were re-suspended in 1 mL of fresh medium pre-warmed to 37 °C and transferred to new 24-wells plate for recovery incubation. After the recovery time, the cells were again collected into 15 mL tubes and washed twice as described above. The pellets were re-suspended and the cells were fixed by addition of 4 ml 2% paraformaldehyde in PBS (warmed at max 80 °C and cooled down to room temperature), added drop wise. Thereafter the cells were transferred to 5 ml polystyrene round-bottom FALCON-type tubes (12 × 75 mm) which were tightly capped and stored in the dark at room temperature for at least 3 days. Thereafter, the fixed cells were transferred to +4 °C for further storage up to 3 months until FCM analysis.

2.5. Flow cytometry based MN detection

2.5.1. Staining of cells

The cells were stained according to Abramsson-Zetterberg et al. (2000). In brief, the coded FCM-tubes with the fixed cells were centrifuged at 400 $*$ *g* for 5 min at room temperature. The pellets were resuspended in 0.5 mL staining solution prepared by mixing 10 mL PBS with 7.5 μL Hoechst 33342 (500 μM in purified water) for DNA-staining and 2 μL Thiazole orange (1 mg/mL in methanol) for RNA-staining. The tubes were incubated at 37 °C for 60 min and kept at +4 °C until FCM analysis the same day.

2.5.2. FCM analysis

The coded stained samples were analyzed by FCM at a rate of 1000–2500 cells per second with a threshold in forward scatter (FSC) set to include all intact cells. FACSVantage SE or LSRII flow cytometers (BD Biosciences, CA, USA), both equipped with UV excitation capability and available at SciLifeLab BioVis platform at Uppsala University in Sweden, were used. Using BD FACSDiva software (BD Biosciences, CA, USA) peak values for FSC, SSC, Thiazole orange fluorescence and Hoechst 33342 fluorescence signals were collected. The FSC signals were acquired using a linear scale and the SSC, Thiazole orange and Hoechst 33342 signals were acquired using a log scale. From each sample, two separate FCM-analyses were done, generally at least of 200 000 events each were collected resulting in about 400 000–800 000 cells analyzed per treatment. FCM analysis was applied to obtain all data on MN measurements presented in this paper.

2.6. Morphology and viability

For microscopic analyses, samples were taken when harvesting the cells. The sampled cells were diluted with PBS and spun onto slides by cytocentrifugation (Shandon Cytospin 2, 700 rpm for 3 min). The next day, cells were first stained with May-Grünwald for 3 min, rinsed in tap water for 1 min and thereafter stained with Giemsa (1:10 in tap water) for 15 min. The slides were thereafter washed, dried and mounted. The percentage of enucleated reticulocytes in total cells on stained slides was counted as enucleation rate. Viable cell counting was based on trypan blue exclusion and conducted by microscope scoring.

2.7. Statistical analysis and positivity criteria for MN test

Regression analysis was performed in order to test for a trend in the increase of micronucleated cells across the treatment range (dose-related). A compound was considered positive if it induced the frequency of MN (fMN) according to the following criteria: (a) the induced fMN was above the maximum value of the negative control; (b) the fMN showed at least twofold increase; and (c) the data analysis was statistically significant, i.e. $p < 0.05$ (regression analysis).

3. Results

3.1. Cultivation of RBCs in 24-well plates can be utilized for genotoxicity testing in terms of MN-induction

The present protocol allows *ex vivo* erythroid differentiation under animal-free culture conditions and was optimized for massive expansion and terminal differentiation of erythroblasts starting from human hematopoietic stem cells isolated from bone marrow or cord blood (Giarratana et al., 2011). Following this protocol, we have enhanced the method to cultivate erythroblasts in 24-well plates for genotoxicity testing in terms of MN-induction. The cultured human CD34+ cells followed a two-phase large-scale amplification toward erythrocytes production, i.e. expansion (day 0–day 10) and differentiation (day 11–day 18) (Fig. 1). The immunophenotypic characterization of erythroid commitment and maturation in terms of expression of the relevant erythroid markers CD71, CD36 and CD235a was previously reported

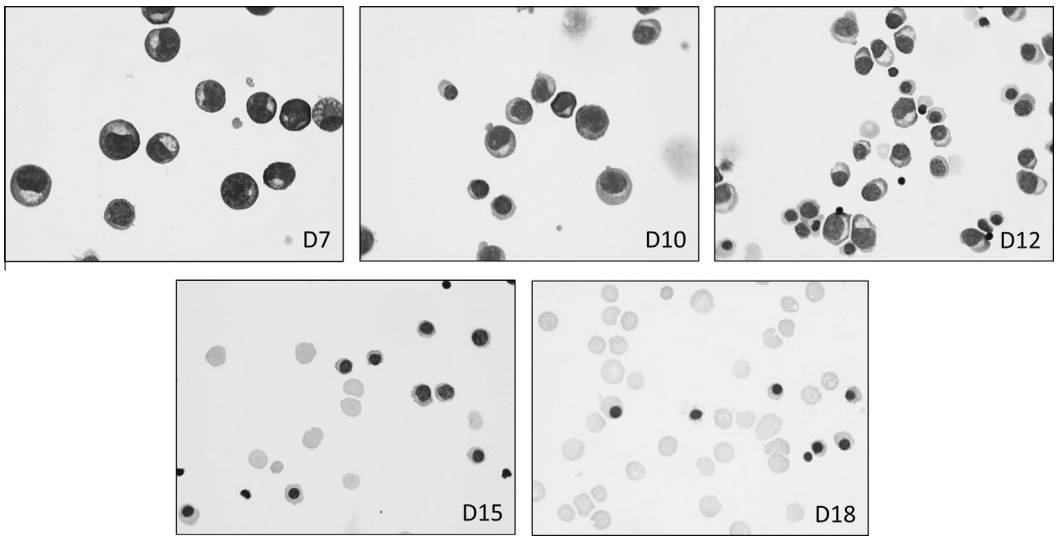

Fig. 1. Representative pictures of cells undergoing erythroid differentiation on days 7, 10, 12, 15 and 18 of culture after May-Gründwald-Giemsa staining. Enucleation rates are respectively 0%, 1%, 5%, 53% and 82% (magnification 60×).

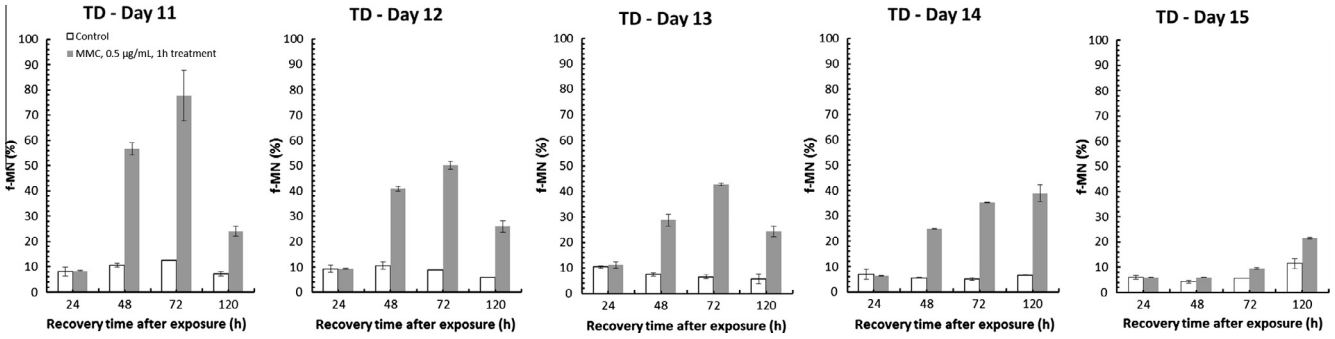

Fig. 2. Induction of MN in human erythroblasts detected by flow cytometry after 1 h treatment with MMC on different days of terminal differentiation (TD) followed by cell harvest on day 12 up to day 19 of cell culture. The enucleation rate at day 11 was up to 5%, day 12 – about 20%, day 15 – up to 80%.

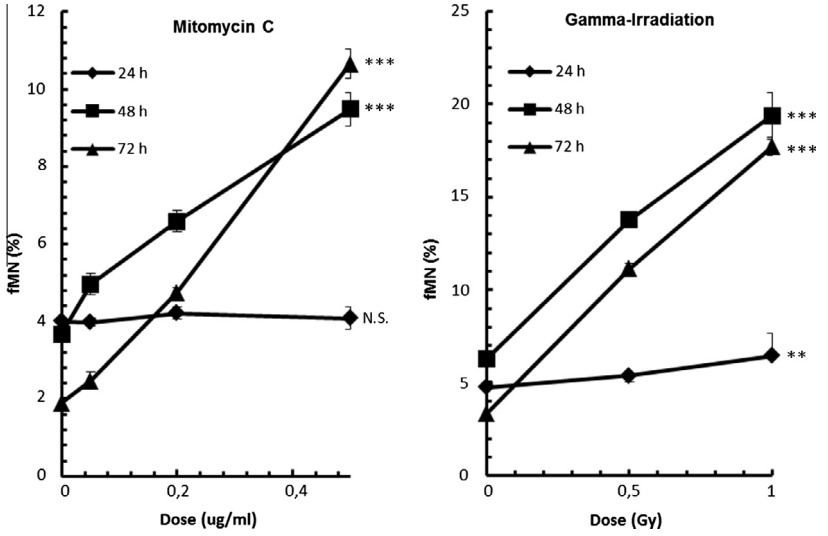

Fig. 3. Induction of MN in human erythroblasts detected by flow cytometry after 1 h treatment with Mitomycin C and acute γ-radiation. The cells were recovered for 24-, 48- and 72-h after exposure. ***P-value < 0.001, **P-value < 0.01, N.S. = not significant.

(Giarratana et al., 2011). Furthermore, we have studied the optimal time frame for treatment, which is crucial since the exposure to genotoxic agents must be conducted at the beginning of differentiation in order to be able to study the endpoint. As a result, it was concluded that the most optimal time point to conduct the treatment is immediately after switching to differentiation phase,

i.e. at day 11, to get the optimal MN induction (Fig. 2). Since the recovery time might be crucial while studying genotoxic response, different time schedules were tested. As a result, 48- or 72-h recovery time was found to be the most optimal in order to detect genotoxic response in terms of induced fMN (Figs. 2 and 3). Therefore, harvesting of cells only 48- or 72-h after the initiated treatment was applied afterwards. At time of harvesting, a slight difference in the enucleation rate of CB (59% ± 12%) and LK (42% ± 16%) was found. This is related to the source of cells as usually observed in our previous experiments (Giarratana et al., 2005).

Table 2
MN induction after 24 h treatment with direct acting clastogens.

Cell culture	Treatment	%Viability[a]	%ER[b]	%MN[c]	Fold[d]	Statistical significance
Mitomycin C (μg/mL)						
LK	0	92.1	31	2.9	1.0	***
	0.025	92.1	31	5.5	1.9	
	0.050	90.1	21	7.9	2.7	
	0.10	88.2	34	10.4	3.6	
CB	0	99.1	63	2.8	1.0	**
	0.025	100	52	13.4	4.8	
	0.050	99.5	60	19.9	7.2	
	0.10	96.8	49	23.0	8.3	
Methyl methanesulfonate (μg/mL)						
LK	0	93.7	23	2.4	1.0	**
	1.3	93.1	28	3.5	1.5	
	2.6	91.3	27	3.4	1.4	
	5.0	92.7	ND	5.9	2.5	
CB	0	95.1	67	2.6	1.0	***
	1.3	95.4	73	3.0	1.2	
	5.1	96.0	56	8.5	3.3	
	13	92.3	40	13.0	5.0	
Dibenzo[a,l]pyrene-11,12-dihydrodiol-13,14-epoxide (nM)						
LK	0	ND	ND	1.5	1.0	***
	1	ND	ND	4.0	2.7	
	3	ND	ND	6.5	4.5	
	5	ND	ND	7.7	5.3	
Etoposide (μg/mL)						
LK	0	ND	ND	1.5	1.0	***
	0.05	ND	ND	3.7	2.5	
	0.10	ND	ND	5.4	3.7	
	0.15	ND	ND	8.3	5.7	
	0.20	ND	ND	12.4	8.5	
	0.30	ND	ND	14.5	9.9	
CB	0	96.1	67	2.1	1.0	***
	0.05	97.9	67	5.7	2.7	
	0.15	96.7	62	13.2	6.2	
	0.30	94.3	72	20.7	9.7	
Gamma irradiation (Gy)						
LK	0	94.4	45	3.1	1.0	***
	0.1	92.2	24	3.8	1.3	
	0.5	91.5	46	11.7	3.8	
CB	0	96.3	66	2.5	1.0	***
	0.1	94.0	63	2.4	1.0	
	0.25	93.2	64	7.8	3.2	
	0.5	96.0	65	12.4	5.0	

LK – human red blood cells from leukapheresis.
CB – human red blood cells from cord blood.
ND – not determined.
[a] Viability based on trypan blue exclusion.
[b] Enucleation rate (ER) = % of enucleated reticulocytes in total cells.
[c] %MN = % micronucleated erythrocytes.
[d] Fold = %MN of the treated cultures/%MN of the negative control cultures.
** *P*-value < 0.01.
*** *P*-value < 0.001.

3.2. Directly acting genotoxic agents induced MN levels

To test chemicals with different mechanisms of action such as known clastogens and aneugens, the cells were treated in 24-well plates for 24 h with 48-h recovery time.

3.2.1. Clastogenic agents

The validation set of agents included γ-radiation, etoposide, methyl methanesulfonate (MMS), mitomycin C and dibenzo(a,l)pyrene-11,12-dihydrodiol-13,14-epoxide (DBPDE) (Table 2). According to criteria, positive response in terms of increased fMN was observed for all treatments. Typical FCM-plots for respective treated and untreated cells stained with Hoechst 33342 and Thiazole Orange are presented in Fig. 4A. Representative images of MN in erythrocytes treated with γ-irradiation (0.5 Gy) and MMC (0.1 μg/ml) are shown in Fig. 4B.

3.2.2. Aneugenic agents

The validation set of agents included bisphenol A, colcemid, griseofulvin and vincristine sulfate (Table 3). Colcemid and vincristine sulfate induced positive response in terms of MN induction. A threshold in MN induction was observed for these agents. Treatment with bisphenol A and griseofulvin showed no MN induction in the present test system.

3.2.3. Negative compounds

The validation set of known non-genotoxic agents included benzyl alcohol, ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) (Table 4). According to the chosen positivity criteria for MN induction, none of the negative compounds resulted in positive responce.

To further improve high-throughput feature of the test, the treatments with MMC, MMS, colcemide and γ-irradiation were conducted in 96-well plates giving responses comparable to 24-well plate cultures in terms of MN induction (data not shown).

3.3. Induction of MN was achieved after treatments with genotoxic agents requiring metabolic activation

Since there are no well-known aneugenic agents requiring metabolic activation, only agents with clastogenic action were tested.

The 24-h treatment with rat S9 resulted in high toxicity which was in agreement with the published data (Ku et al., 2007), and was therefore excluded for further testing. Further, compared to 1% rat S9 mix, 2% rat S9 mix resulted in a decrease in enucleation rate (data not shown) and therefore was not applied for further investigation. Since the recommended short-time exposure with S9 mix is limited to 2–4 h (Ku et al., 2007), the cells were treated with a respective chemical with 1% rat S9 mix for 3 h followed by 69-h recovery time. The initial validation set of agents included 3-h treatment with cyclophosphamide (CP), benzo(a)pyrene (B(a)P) and dimethylbenz(a)antracene (DMBA) metabolized by 1% rat S9 (Table 5). The obtained results from all three compounds indicated a significant induction of fMN.

The first tested metabolizing system not containing any animal components was human S9 mix at 1%, 2%, 5% or 10% concentration. As for the experiments using rat S9 mix, the cells were treated with a respective chemical with human S9 mix for 3 h followed by 69-h recovery time. None of the applied human S9 mix concentrations introduced an adverse effect in enucleation rate which stably ranged between approximately 50% and 60%. B(a)P and CP (2, 4, 6 μg/mL and 1, 2.5, 5 μg/mL respectively) were tested in different series of experiments, but none of them showed an induction in fMN (data not shown).

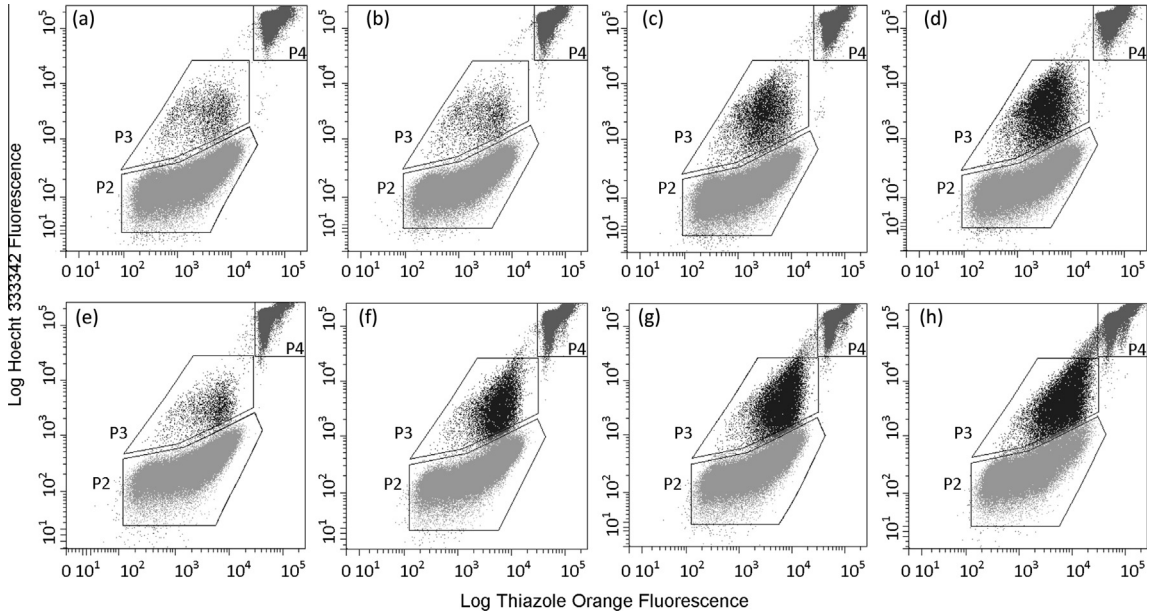

Fig. 4A. Representative plots of flow cytometric data for: (a and e) negative control; (b–d) cell culture treated with γ-radiation; (f–h) cell culture treated with MMC. Gate P2 represents population of erythrocytes, P3 – micronucleated erythrocytes, P4 – nucleated erythroblasts.

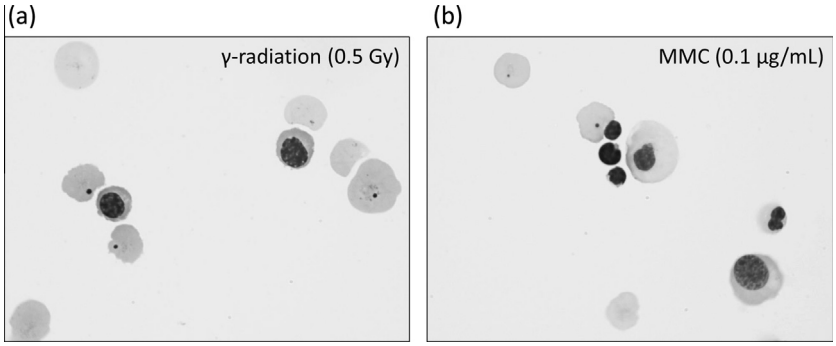

Fig. 4B. Pictures of representative slides showing micronucleated human reticulocytes after treatment with (a) 0.5 Gy γ-radiation and (b) 0.1 μg/mL MMC (magnification 100×).

In case of co-culture with metabolizing cell lines, 24 h treatment followed by 48-h recovery was applied. XEM2 cells were used to evaluate the metabolic activation through co-cultivation in inserts or directly in wells. It was clear that co-cultivation in inserts did not provide metabolic activation (data not shown) and therefore only co-cultivation directly in wells was thereafter conducted for XEM2 and HepaRG cells. Significant inductions of MN by DMBA with HepaRG co-culture as well as by dibenzo[a,l]pyrene-11,12-dihydrodiol (DBPD) with XEM2 and HepaRG co-culture were obtained (Table 6). B(a)P induced fMN almost 2-fold with HepaRG co-culture, however the trend was not significant (Table 6). CP showed no induction of fMN with neither HepaRG nor XEM2 co-culture.

Except for vincristine sulfate treatments (Table 3), no differences in terms of positive MN induction was observed between cultured RBCs generated from LK or CB cells. This exception remains unclear and needs further investigation. Cell viability after recovery time was generally >85%. The viable cell yields were mostly found to be lower for treated cells vs. control cells (data not shown). Cell proliferation rate and cell death were not explicitly measured, thus reductions in viable cell yields cannot be specifically attributed to cytotoxicity or cytotoxic mechanisms. However, we believe that decreased enucleation rate is an indicator of cytotoxic exposure in this erythropoetic culture system.

4. Conclusions

According to the developed protocol, the most appropriate time point to conduct the treatment with genotoxic agents is on day 11 of cell culture in order to study the endpoint. This obtained time point is in agreement with previously described timetable of erythropoiesis *in vivo* in humans (Grawe et al., 2005). It was also observed that day 15 of cell culture is the most appropriate for harvesting of cells to detect the induced fMN. As a result of high expansion rate and complete differentiation process of cultured RBCs, the samples can easily be analyzed within a rather short time.

The conducted experiments have proved that RBCs culture was stable under the presence of tested chemicals for both short-term exposure (1 h or 3 h) and prolonged treatment (24 h). In this study we showed that genotoxicity testing of both clastogenic and aneugenic compounds by this method are in agreement with published data. It was indicated that this method has the potential to be sensitive enough for induction of MN by 0.025 μg/mL MMC treatment (Table 2). This is comparable to the lowest dose applied in other studies with MN-test *in vitro* (Hashimoto et al., 2010; Sobol et al., 2012). Moreover, the dose response was detectible down to 0.25 Gy of γ-radiation (Table 2). Interestingly, an approximately sixfold of MN induction by 1.0 Gy γ-radiation (Fig. 3) is in

Table 3
MN induction after 24 h treatment with aneugens.

Cell culture	Treatment	%Viability[a]	%ER[b]	%MN[c]	Fold[d]	Statistical significance
Vincristine sulfate (μg/mL)						
LK	0	ND	ND	1.5	1.0	NS
	0.025	ND	ND	4.3	2.9	
	0.050	ND	ND	4.5	3.1	
	0.075	ND	ND	3.4	2.3	
	0.100	ND	ND	4.3	2.9	
CB	0	94.6	78	2.4	1.0	***
	0.003	94.4	43	5.3	2.2	
	0.005	93.2	54	5.3	2.2	
	0.007	91.6	54	6.6	2.7	
	0.0125	91.6	39	7.2	3.0	
	0.0250	92.4	33	7.1	2.9	
Bisphenol A (μg/mL)						
LK	0	ND	ND	1.5	1.0	NS
	20	ND	ND	0.8	0.6	
	30	ND	ND	1.5	1.0	
	40	ND	ND	1.5	1.0	
CB	0	96.0	63	2.6	1.0	NS
	5	97.0	63	2.0	0.8	
	10	96.4	69	1.5	0.6	
	20	95.0	67	1.3	0.5	
	40	90.0	47	1.7	0.7	
Griseofulvin (μg/mL)						
LK	0	ND	ND	1.5	1.0	NS
	4	ND	ND	1.4	1.0	
	7	ND	ND	1.5	1.0	
	14	ND	ND	1.1	0.8	
	21	ND	ND	1.2	0.8	
CB	0	94.8	63	2.0	1.0	NS
	20	91.6	70	1.4	0.7	
	36	94.4	59	1.2	0.6	
Colcemid (μg/mL)						
CB	0	95.8	72	3.2	1.0	***
	0.01	96.0	65	2.6	0.8	
	0.02	94.8	69	5.1	1.6	
	0.04	91.8	61	6.9	2.2	
	0.06	90.4	64	7.6	2.4	
	0.08	89.2	57	7.8	2.4	

LK – human red blood cells from leukapheresis.
CB – human red blood cells from cord blood.
NS – not significant; ND – not determined.
[a–d] See footnotes of Table 2.
*** *P*-value < 0.001.

Table 4
MN induction after 24 h treatment with negative and false positive compounds in cord blood cells culture.

Treatment	%Viability[a]	%ER[b]	%MN[c]	Fold[d]	Statistical significance
Benzyl alcohol (μM)					
0	96.0	73	2.2	1.0	NS
10	94.4	71	2.2	1.0	
100	98.0	67	2.2	1.0	
1000	96.5	69	2.0	0.9	
4825	97.8	79	1.8	0.8	
Sodium chloride (μg/mL)					
0	95.6	64	2.7	1.0	***
500	97.1	71	2.9	1.1	
1000	93.7	64	3.2	1.2	
2000	94.1	65	3.6	1.3	
Ethylenediaminetetraacetic acid (μg/mL)					
0	96.0	64	2.7	1.0	NS
500	95.8	79	1.8	0.7	
1000	88.5	52	1.3	0.5	
2000	87.3	49	2.2	0.8	

NS – not significant.
[a–d] See footnotes of Table 2.
*** *P*-value < 0.001.

Table 5
MN induction with clastogens requiring metabolic activation (1% rat S9 mix) after 3 h treatment followed by 69 h recovery.

Cell culture	Treatment	%Viability[a]	%ER[b]	%MN[c]	Fold[d]	Statistical significance
Benzo(a)pyrene (μg/mL)						
CB	0	97.2	ND	2.6	1.0	*
	1.0	94.8	66	3.5	1.4	
	2.0	94.4	80	6.3	2.5	
	4.0	92.4	49	6.4	2.5	
Cyclophosphamide (μg/mL)						
LK	0	94.8	54	2.9	1.0	***
	0.1	91.6	60	3.6	1.2	
	0.3	90.8	69	4.7	1.6	
	1.0	90.8	70	11.3	3.9	
	2.5	91.2	51	22.4	7.8	
CB	0	96.4	64	2.2	1.0	***
	0.3	94.6	57	2.7	1.3	
	2.5	95.9	62	17.3	8.0	
	5.0	96.0	36	23.8	10.9	
7,12-Dimethylbenz(a)antracene (μg/mL)						
LK	0	94.8	54	2.9	1.0	*
	0.5	93.6	43	5.6	1.9	
	1.0	94.0	ND	6.9	2.4	
CB	0	96.7	61	2.2	1.0	***
	0.5	95.6	ND	3.6	1.6	
	1.0	96.4	35	4.8	2.1	
	1.5	92.2	53	5.1	2.3	
	2.0	92.4	21	5.5	2.5	

LK – human red blood cells from leukapheresis.
CB – human red blood cells from cord blood.
ND – not determined.
[a–d] See footnotes of Table 2.
* *P*-value < 0.05.
*** *P*-value < 0.001.

agreement with results reported by (Sun et al., 2009) in their model of erythropoiesis. Concerning aneugenic agents, colcemid and vincristine sulfate induced positive response in terms of MN induction containing a threshold (Table 3) which is in agreement with data reported for MN test *in vivo* (Cammerer et al., 2010). Although, bisphenol A and griseofulvin have previously been shown to give a positive effect in other types of MN test *in vitro* (Hashimoto et al., 2010; Johnson and Parry, 2008), no effect in terms of MN induction have been reported here, which is in agreement with findings using MN test *in vivo* (Labay et al., 2001; NTP/NIH/USA).

While treated to aneugenic agents, the viability of cultured RBCs was rather stable. However, FCM plots indicated that the treated cells may undergo defragmentation which can result in decreased enucleation rate. The presented assay allows size discrimination of smaller MN induced by clastogens from larger ones induced by aneugens, a feature that has previously been applied by others (Matsuoka et al., 1999; Torous et al., 1998). Application of flow sorting is another advantage of the method allowing enrichment of only MN-cells. It would therefore allow possibility to study mechanisms of MN induction by FISH-analysis or similar techniques as being previously described (Grawe et al., 1997).

As a reference to the tests of animal-free metabolizing systems, the commonly used rat S9 mix was applied (Table 5). Also, a co-culture with XEM2 cell line, previously genetically engineered derivative of the V79 Chinese hamster lung fibroblasts to stably express active cytochrome CYP1A1 (Ellard et al., 1991) was used to evaluate the metabolic activation through co-cultivation in

Table 6
MN induction with clastogens requiring metabolic activation (co-cultivation directly in wells) after 24 h treatment followed by 48 h recovery in cord blood cell culture.

Treatment	XEM2				HepaRG			
	%ER[b]	%MN[c]	Fold[d]	Statistical significance	%ER[b]	%MN[c]	Fold[d]	Statistical significance
7,12-Dimethylbenz(a)antracene (μg/mL)								
0	57	2.0	1.0	NS	68	2.1	1.0	***
0.5	ND	ND	ND		69	4.3	2.1	
1.0	ND	ND	ND		69	4.1	1.9	
1.5	60	3.7	1.8		64	4.3	2.0	
2.0	ND	ND	ND		63	3.7	1.8	
Cyclophosphamide (μg/mL)								
0	54	1.9	1.0	NS	68	2.1	1.0	NS
2.5	57	1.5	0.8		69	1.8	0.9	
5.0	45	1.5	0.8		ND	ND	ND	
Benzo(a)pyrene (μg/mL)								
0	ND	ND	ND		77	1.8	1.0	NS
2.0	ND	ND	ND		81	2.9	1.6	
4.0	ND	ND	ND		62	3.4	1.9	
Dibenzo[a,l]pyrene-11,12-dihydrodiol (nM)								
0	55	1.9	1.0	***	65	2.2	1.0	***
1.5	62	3.6	1.9		63	4.6	2.0	
3.3	53	4.4	2.2		62	3.1	1.4	
5.0	59	6.9	3.5		61	5.6	2.5	
10	60	8.1	4.2		63	8.3	3.7	
15	49	8.2	4.2		ND	ND	ND	
25	ND	8.4	4.3		ND	ND	ND	

NS – not significant; ND – not determined.
[b–d] See footnotes of Table 2.
*** *P*-value < 0.001.

inserts or directly in wells. Since co-cultivation in inserts did not provide metabolic activation, only co-cultivation directly in wells was thereafter conducted. As previously proposed candidates, two animal-free metabolizing systems, human S9 mix (Kirkland et al., 2007; Ku et al., 2007) and HepaRG cell line (Lubberstedt et al., 2011), were applied. Our results preliminary indicate that there is no common animal-free metabolic system which has potential efficiency similar to rat S9 applicable to RBCs cultures (Tables 5 and 6). Therefore, for the present method to be used for alternative genotoxic testing of a wide range of agents, metabolic activation by rat S9 mix is required. However, we believe that an efficient animal-free metabolic system would also work as soon as such a system is available.

The present method provides a robust *in vitro* tool to assess the potential risk of chemicals completely avoiding animal components and/or decreasing animal testing. It has high sensitivity particularly due to implementation of unmodified primary human stem cells but also due to high speed analysis (up to 300 000 cells in approximately 2 min) and potential robot application towards high throughput application. Moreover, the source of CB cells is unlimited and easily available. As a putative extrapolation of the data reported, an average cord blood sample of approximately 50 ml could generate enough cells to screen up to 30 000 different genotoxic compounds which is far more compared to the current test in rodents. In addition to the potential to be used as a genotoxicity screening assay, the present *in vitro* test has a high relevance to human health assessment. This is due to the fact that the methodology of RBCs culture is adaptable to CD34+ cells of diverse origins such as peripheral blood, bone marrow or cord blood, i.e. any source of hematopoietic stem cells of an individual. It opens a potential for clinical applications and might be used in individualized medicine to predict genotoxic outcome induced by certain medical treatments, i.e. cytostatics or radiotherapy.

In conclusion, an alternative *in vitro* test for detection of genotoxic damage entirely based on human stem cells according to 3Rs principle is hereby described. By using this method, human erythroblasts differentiated *in vitro* into mature erythrocytes exhibit the same stages as defined *in vivo*. The method allows a cost-effective production of high relevance data and the capture of genotoxic damage induced at very early stages of development. The obtained results indicate that this developed *in vitro* genotoxic test is relevant, sensitive, highly reproducible, easy to perform and evaluate, enables high-throughput application, and last but not least has the potential to reduce animal testing while retaining, or even improving, the quality and applicability of generated data.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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