Simultaneous use of erythropoietin and prior bleeding enhances the sensitivity of the peripheral blood micronucleus assay

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Adult rats are generally not considered as a suitable model for the peripheral blood micronucleus (PBMN) assay in regulatory consideration, owing to the splenic removal of the micronucleated cells from circulation. Although prior bleeding (PrB) increases the sensitivity of the PBMN assay in young rats, the volume of bleeding and the associated stress caused are major concerns for its possible use in genotoxicity studies. The present study was aimed to overcome these limitations in using pre-bled young rats in genotoxicity studies. The bleeding volume was reduced by the simultaneous use of erythropoietin (EPO) to increase the sensitivity of PBMN assay. Young Sprague–Dawley (SD, 26 days) rats were used in the study. The kinetics of RETs-to-ERTs ratio was determined in response to EPO (10–3000 IU/kg) or PrB (0.1–1.0 ml) at different time points (0, 6, 12, 24, 36, 48, 72 and 96 h). Injection of EPO (30 IU/kg) and PrB (0.5 ml) led to a significant increase in the MN frequency in the PBMN assay in response to cyclophosphamide and zidovudine. The effect of EPO treatment and/or PrB on cell viability and proliferation in the bone marrow (BM) was examined. The results of the present study clearly demonstrate that the simultaneous use of both EPO and PrB enhances the sensitivity of the PBMN assay in young rats due to increased cellular proliferation in the BM. This may provide a useful experimental model for the evaluation of marginally active genotoxicants.

Introduction

The in vivo micronucleus assay is an integral part of regulatory studies to determine the genotoxic potential of different chemicals. In response to the genotoxicant exposure, micronucleated erythrocytes (MNERTs) are formed in the bone marrow (BM), released into the peripheral blood (PB) circulation and selectively removed by the spleen in case of rats. Due to efficient splenic removal, adult rats are generally not considered as a suitable model for the conduct of PB micronucleus (PBMN) assay. However, rats are the preferred model for general toxicity and toxicokinetics studies (1,2). The PBMN assay has several advantages over the BM micronucleus assay such as (i) rapid detection, (ii) assessment of the kinetics of MN formation using the same animal and (iii) evaluation of cumulative damage (3). Previously, young rats have been suggested as an alternative model for the PBMN assay (4). However, increased sensitivity is advantageous for the successful detection of marginally active genotoxicants and hence, several strategies have been employed in the past to improve the sensitivity of the PBMN assay in rats. Flow cytometry is a validated method for improving the sensitivity of PBMN assay (5,6) and has a good correlation with manual microscopic scoring (7,8). Furthermore, Dertinger et al. (9) reported the suitability of flow cytometry for MN frequency determination using a low dose of zidovudine (AZT) that was previously reported as non-genotoxic (10). Although the Organization of Economic Cooperation and Development and the International Committee on Harmonisation guidelines indicate automated scoring as an alternative to manual microscopy, no clear recommendation for validation of automated scoring has been given (11). In the past few years, reports have suggested the use of a specific cell surface marker of the transferrin receptor (CD71) to differentially label reticulocytes (RETs) and normochromatic erythrocytes (NCEs) in flow cytometry analysis (6,8,12). However, several recent studies have used manual microscopic scoring for the determination of PBMN frequency (13–15). Furthermore, it has been reported that acridine orange supravital staining is able to discriminate between RETs and ERTs on the basis of their RNA content (16,17). With the use of acridine orange, the MN frequencies in the RETs obtained by flow cytometry and microscopic scoring are similar, although the degree of scoring error differs (12). Considering all these facts, the present study was aimed to improve the sensitivity of PBMN assay using manual microscopic scoring. Theoretically, the sensitivity of the assay can be improved either by (i) stimulating the erythropoiesis process or (ii) decreasing the removal of micronucleated cells from the circulation. Splenectomy has been used in various experimental studies to diminish splenic removal of micronucleated cells (17–20). However, splenectomy causes loss of hematopoietic activity and depletion of ERTs reserves (21). Owing to these drawbacks, effort has been made to strategies that stimulate the formation and release of ERTs, such as recombinant erythropoietin (EPO) (22,23), prior bleeding (PrB) (3,21,24–26), hypoxia (27), cyclic adenosine monophosphate (28), protein deprivation (29) and hypothermia (30) (Figure 1).

Previously, we have reported that PrB (~20% of total volume) of young rats enhances the sensitivity of the PBMN assay and this has been validated with several chemicals (3,26). However, the physiological stress associated with the volume of bleeding was a major concern to its use as a possible model in genotoxicity testing. The 5th International Workshop on Genotoxicity Testing (Basel, Switzerland, August 17–19, 2009) also suggested the inclusion of the pre-bled young-rat model in genotoxicity testing. To minimise the stress associated with the
previous model, an attempt has been made to reduce the volume of PrB by the simultaneous use of the low dose of EPO. The results of the present study clearly demonstrate that the simultaneous use of a low dose of EPO (30 IU/kg) with the minimal volume of PrB (0.5 ml) further increases the sensitivity of the PBMN assay. Our results highlight that the simultaneous use of EPO and PrB increases cellular proliferation in the BM and thereby the sensitivity of the PBMN assay. This may be considered as a useful experimental model for the evaluation of the marginally active clastogens.

### Materials and methods

#### Animals

All the animal experiments were approved by the Institutional Animal Ethics Committee. Experiments were performed on young male Sprague–Dawley (SD, 26 days, 50–60 g) rats that were procured from the Institute’s Central Animal Facility. Treatment and experiments were performed in National Toxicology Centre. The animals were kept under controlled environmental conditions at room temperature (22°C–2°C), humidity (50±10%) and automatically controlled 12 h light and dark cycles (0600–1800 h). Standard laboratory animal feed (purchased from Tetragon Chemi Pvt. Ltd Bangaluru, India) and water were provided ad libitum. Animals were acclimatized to the experimental conditions prior to the start of dosing for a period of 2–3 days.

#### Chemicals

Cyclophosphamide (CP, CAS no. 6055-19-2), SYBR Green I (CAS no. 163795-75-3), acridine orange (AO, CAS no. 10127-02-3), bovine serum albumin (BSA) and 3,3′-diaminobenzidine tetrachloride (DAB) were purchased from Sigma Aldrich Chemicals (Saint Louis, MO, USA). Zidovudine (AZT, CAS no. 30516-87-1) was a generous gift from APL Research Centre, Aurobindo Pharma Ltd (Hyderabad, India). Recombinant human (Zyrop 4000 IU) was purchased from Zydus Cadila (Biogen Division). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), HAM 12 media, foetal calf serum (FCS), dimethylsulphoxide (DMSO), normal melting agarose (NMA), low melting agarose (LMA), triton X-100, ethylenediaminetetraacetic acid (EDTA) and Hank’s balanced salt solution (HBSS) were obtained from Hi-media Laboratories Ltd (Mumbai, India).

#### Experimental design and animal dosing

The experimental design describing the timing of chemical administration and sample collection is shown in Figure 2. Five animals were included in each group. For kinetics of MN formation in PB, blood was removed with the help of heparanized glass capillary from the retro-orbital plexus under anaesthesia. EPO (10–3000 IU/kg), CP (50 mg/kg) and AZT (400 mg/kg) were given intraperitoneally. EPO was reconstituted in 0.25% BSA in normal saline. CP was dissolved in distilled water. AZT was first dissolved in the minimal amount of absolute ethanol and then the volume was adjusted with distilled water. Control animals were injected with the appropriate vehicle.

#### PBMN assay

The PBMN assay was performed as described by Vikram et al. (3) with some modifications. Briefly, smears were prepared on clean grease-free slides. The smears were allowed to dry at room temperature and fixed in absolute methanol for 5 min. After fixation, slides were stained with acridine orange solution and washed three times with phosphate buffer (pH 6.8).

#### BM micronucleus assay

BM slides were prepared as described by Vikram et al. (26). Femur bones were isolated and BM was homogenised with foetal bovine serum (FBS). After centrifugation, the supernatant was discarded and the pellet was re-suspended with the residual FBS. From this suspension, smears were prepared on clean grease-free slides and fixed in absolute methanol for 5 min. After fixation, slides were stained with acridine orange and washed three times with phosphate buffer (pH 6.8).

#### BM sample preparations for comet assay

Femurs were removed and the contents were flushed out with a 30-gauge needle into a microcentrifuge tube using 100 μl of HBSS containing 20 mM EDTA and 1% DMSO. The cell population was counted and adjusted to (2–5) × 10⁶ cells/ml and 5 μl of cell suspension was mixed with 95 μl of 0.5% LMA.

#### Comet assay

The comet assay was performed as described by Singh et al. (31) with some modifications. The entire procedure was conducted in the dark to avoid possible photo-induced DNA damage. From the final cell-agarose suspension, 100 μl...
were spread over a microscope slide (75 × 25 mm glass slides with 19 mm frosted ends, Axiva Sichem Pvt. Ltd, Delhi, India) and pre-coated with 1% NMA. The cells were then lysed in a buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0) with freshly prepared 1% Triton X-100 and 10% DMSO for 24 h at 4°C. After lysis, the slides were rinsed three times in de-ionised water to remove the salt and detergent. The slides were then coded and placed in a specifically designed horizontal electrophoresis tank (Model, CSLCOM20, Cleaver Scientific Ltd, Warwickshire, UK) and the DNA was allowed to unwind for 20 min in an alkaline solution containing 300 mM NaOH and 1 mM EDTA (pH > 13) and then electrophoresis was performed at 300 mA and 38 V (0.90 V/cm) for 30 min. The slides were then neutralised with 0.4 M Tris (pH 7.5) for 15 min and stained with SYBR Green 1 (1/10000) for 1 h and covered with coverslips (32).

Cell proliferation determination using MTT assay
Immediately after sacrifice of animals, femurs were removed and were flushed with HAM 12 media (containing 10% FCS) using 30-gauge needle into a microcentrifuge tube and centrifuged at 1000 r.p.m. for 1 min. Pellet was resuspended in the 1 ml of fresh media and the cell population was counted. Cells of 1 × 10⁵ were added to each well of 96 wells plate. Hundred microlitres of MTT solution (0.5 mg/ml) was added to each well and incubated for 4 h at 37°C and 5% CO₂. The formazan crystals were dissolved using dimethylsulfoxide and absorbance was taken at 550 nm.

Tissue processing for histology and immunohistochemistry
Femurs were isolated, washed with 0.1 mol/l phosphate-buffered saline (PBS) (pH 7.4; 20–50 ml) and fixed in 10% formal saline overnight. After fixation, decalcifications were performed. Paraffin blocks were prepared after completing the routine processing. Sections (5 µm) of femur have been taken from paraffin blocks.

Histological and immunohistochemical examination
Histological examination was performed according to the standardised protocol in our laboratory (33). Briefly, Sections were deparaffinised with xylene, rehydrated with alcohol and water and stained with eosin-Harris hematoxylin mounted using DPX. Immunohistochemical staining was performed as described by Tripathi et al. (34). Briefly, tissue sections were incubated in citrate buffer at 95–100°C for antigen retrieval. This was followed by endogenous peroxidase blocking in 3% H₂O₂ for 10 min. After cooling, non-specific binding was blocked by incubating with non-fat dried milk. Tissue sections were incubated with primary Ki-67 antibody (Rabbit polyclonal IgG FL-393; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight in a humidified chamber. After washing the slides with PBS, sections were incubated with biotinylated secondary antibody (anti-rabbit IgG; Medical and Biological Laboratories Co. Ltd, Nagoya, Japan) at room temperature for 1 h. After washing the slides, sections were incubated with streptavidin peroxidase (Medical and Biological Laboratories Co. Ltd) for 1 h at room temperature. Antibody binding was visualised with DAB. This was followed by counterstaining with Mayer’s Hematoxylin and the subsequent dehydration and mounting.

Data scoring and imaging
MN assay: Micronucleus scoring was performed under oil immersion objective (×100) using an Olympus fluorescent microscope (Model BX 51) connected to digital photomicrograph software (OLYSIA BioReport). For the PBMN assay, a total of 1000 ERTs that include a NCEs (green coloured) and RETs (orange coloured) were examined for the determination of total MN frequency in peripheral blood erythrocytes (MNERTs) and represented as MNERTs/1000 ERTs. Further, 1000 RETs were examined to determine the frequency of micronucleated reticulocytes (MNRETs) and represented as MNRETs/1000 RETs. For the bone marrow micronucleus (BMMN) assay, a total of 1000 cells that included polychromatemic erythrocytes (PCEs, orange coloured) and NCEs (green coloured) were examined to determine the total MN frequency (total MN/1000 cells). Further, MN frequency has been determined in PCEs and represented as MNPCES/1000 PCEs.

Comet assay: The comet DNA damage was visualised under objective (×20) using an AXIO Imager M1 fluorescence microscope (Carl Zeiss, Altussheim, Germany) and the images were captured with image analysis software (Comet Imager V.2.0.0). The parameters for DNA damage analysis includes tail length (TL, µmeters), tail moment (TM) and olive tail moment (OTM) and % DNA in comet tail (% DNA). A total of 50 comets per slide were scored from each animal for the quantification of DNA damage. The edges of the slides, any other damaged part of the gel, any debris, superimposed comets and comets without distinct head (‘hedgehogs’ or ‘ghost’ or ‘clouds’) were not considered for the analysis.

Histology and immunohistochemistry: Slides were examined under objective (×2, ×10, ×40 and ×100) using Olympus fluorescent microscope (Model BX 51) connected to digital photomicrograph software (OLYSIA BioReport). Immunohistochemical localisation of Ki-67-positive cells (brown coloured) has been determined as compared to Ki-67-negative cells (blue coloured).

Statistical analysis
Results are shown as mean ± standard error of the mean for each group. Statistical analysis was performed using Jandel Sigma Stat (Version 2.03) software. For multiple comparisons, one-way analysis of variance (ANOVA) was used. Where ANOVA showed significant differences, post-hoc analysis was performed with Tukey’s test. P <0.05 was considered to be statistically significant.

Results
Effect of EPO and PrB on the RETs-to-ERTs ratio
The RETs-to-ERTs ratio was determined with different doses of EPO (10–3000 IU/kg) and volume of PrB (0.1–1.0 ml) for the selection of a suitable dose of EPO and volume of PrB for the subsequent experiments. In both the cases (EPO and PrB), the peak of RETs-to-ERTs ratio was observed at 36 h post-treatment. However with the EPO treatment, an early rise was observed that persisted for a longer time. Furthermore, a significant rise in RETs-to-ERTs was observed at 36 h. Post treatment. However with the EPO treatment, an early rise was observed that persisted for a longer time. Furthermore, a significant rise in RETs-to-ERTs was observed at a low dose of EPO (30 IU/kg) and small volume of PrB (0.5 ml) that was selected for the MN assessment (Figure 3A and B). Representative photomicrographs showed increased incidence of RETs (orange colour) in the PB smear stained with acridine orange (supplementary Figure 1, available at Mutagenesis Online).
Effect of EPO and PrB treatment on the kinetics of MN formation using PBMN assay

The effect of EPO (30 IU/kg) and/or PrB (0.5 ml) on the kinetics of MN formation was determined with CP (50 mg/kg) and AZT (400 mg/kg).

Cyclophosphamide

The peak of MN (MNERTs and MNRETs) frequency with CP treatment was observed at 36 h post-treatment. MNERTs frequency increased with EPO or PrB treatment and combined treatment of EPO and PrB caused further amplification of the peak. Furthermore, MNRETs frequency increased with EPO and/or PrB treatment; however, an appreciable difference was not observed between EPO and/or PrB treatment groups. RETs-to-ERTs ratio was shown to decrease in CP-treated groups with or without EPO and/or PrB. However, the EPO and/or PrB treatment group with CP started to recover after 72 h post-treatment, while only the CP-treated group did not start to recover until 96 h (Figure 4A).

Zidovudine

The peak MN (MNERTs and MNRETs) frequency was observed at 36 h post-treatment with AZT treatment that was further increased with EPO and/or PrB treatment. The RETs-to-ERTs ratio decreased slightly until 48 h followed by recovery in all treatment groups (Figure 4B).

Effect of EPO and PrB treatment on the kinetics of MN formation using BMMN assay

The effect of EPO (30 IU/kg) and/or PrB (0.5 ml) on the kinetics of MN formation was determined with CP (50 mg/kg) at different time-points (0, 12, 24, 36 and 48 h). The peak MN frequency was observed at 24 h post-treatment that was further amplified with EPO and/or PrB treatment (Figure 5a and b). In PCEs-to-Total cells, ratio significantly increased with EPO and/or PrB treatment showing peak at 24 h post-treatment. However, CP treatment with or without EPO and/or PrB caused significant reduction of PCEs-to-Total cell ratio (Figure 5c). A representative photomicrograph of MN-containing cells with acridine orange stain is shown (Figure 5d).
Effect EPO and PrB on BM cell proliferation

BM histological examination shows increased cellularity with the EPO and/or PrB treatment. However, a massive decrease in the cellularity and deposition of adipocytes was observed with CP treatment that was exaggerated with the EPO treatment. In contrast, PrB alone or its combination with EPO improved BM cellularity as compared to the CP control (Figure 6).

MTT reduction assay showed higher proliferation with the simultaneous use of EPO and PrB treatment as compared to EPO or PrB alone. In the EPO + CP-50 group, a significant decrease in the cell proliferation was observed as compared to PrB + CP-50 and EPO + PrB + CP-50 treatment groups (Figure 7). Increased expression of Ki-67 (a cell proliferation marker) in EPO and/or PrB treated groups was observed as compared to the respective control (supplementary Figure 2, available at Mutagenesis Online).

Effect of EPO and PrB on DNA damage in BM

BM DNA damage was analysed with comet assay parameters (TL, TM, OTM and % DNA). A significant increase in the background level of DNA damage was observed with the EPO and/or PrB treatment. Further, CP and AZT treatment with EPO and/or PrB showed a significant increase in the DNA damage in comparison to the respective control (Figure 8).

Discussion

EPO and PrB are known to increase the sensitivity of PBMN assay in rats (3,23,26,35). However, the dose of EPO used in these studies was high (~50 000 IU/kg). Previously, we...
reported an alternative model using PrB in young rats for the PBMN assay (3,26), as bleeding has more pleiotropic affects on the proliferation and differentiation of hematopoietic cells than EPO alone (24). However, the physiological stress due to volume of blood withdrawal was a major scientific concern for its regulatory use. Therefore, the present study aimed to reduce the stress associated with the PrB in young rats by reducing the volume of bleeding and the simultaneous use of EPO. The results suggest the use of a low dose of EPO (30 IU/kg) with minimal volume of PrB (0.5 ml) increases the sensitivity of the PBMN assay.

To standardise the suitable dose and volume of EPO and PrB, RETs-to-ERTs ratio were determined and, based on these results, the dose of EPO (30 IU/kg) and the volume of PrB (0.5 ml) have been determined for the present study (Figure 3A and B). To validate the suitability of the model, the known mutagens cyclophosphamide (an alkylating chemotherapeutic agent) and zidovudine (an antiretroviral agent) were included in the study. The peak MN frequency in the ERTs and RETs (MNERTs and MNRETs) was observed at 36 h post-treatment of mutagens, which is consistent with our previous reports (3,26,36). Furthermore, the incidence of MN was significantly amplified with the EPO and/or PrB treatment. CP in combination with EPO and/or PrB has not shown significant difference in the MN frequency in RETs (MNRETs) that can be attributed to the cytoreductive effects of CP in BM (37). However, in AZT-treated groups with EPO and/or PrB, a significant difference in MNRETs frequency was observed.

Fig. 7. Effect of EPO and PrB on BM cell proliferation. MTT assay; the open bars indicate control group with or without EPO or PrB treatment represented as ‘+’ and ‘−’ and filled bar indicates simultaneous treatment of CP-50; all the values are shown as mean ± standard error of the mean (n = 5), *P < 0.05, versus (a) Control, (b) EPO, (c) PrB, (d) EPO + PrB.

Fig. 8. Comet DNA damage analysis in BM cells post-mutagen exposure. DNA damage represented by various comet assay parameters (tail length, tail moment, olive tail moment and % DNA of tail) after CP-50 (A) and AZT-400 (B) treatment with or without EPO and/or PrB. All the values are shown as mean ± standard error of the mean (n = 5), ***P < 0.001, **P < 0.01, *P < 0.05 versus control. (C) Representative photomicrographs indicating DNA migration pattern. Symbol ‘−’ and ‘+’ indicates cathode and anode, respectively, during electrophoresis of negatively charged DNA (stain: SYBR Green I; magnification: ×40).
and this can be attributed to the minimal changes in RETs-to-ERTs ratio (Figure 4A and B). The results are consistent with the previous published reports showing minimal effect of AZT on erythropoiesis and stem cell (9).

The results of the BMMN assay show a significant increase in the MN frequency with CP treatment that was further amplified with EPO and/or PrB treatment. The peak MN frequency with or without EPO and/or PrB treatment observed at 24 h post-treatment that are in concordance with previous published reports (26,38,39). The peak of MN frequency in BM (~24 h) was observed at 12 h prior to PB (~36 h) due to a transition time of PCEs in the BM of ~10 h (39).

Micronucleated cells are formed in the BM and subsequently released into the peripheral circulation in response to genotoxic insult. By analysing different parameters in the BM (histology, immunohistochemistry and MTT assay), the possible mechanisms by which EPO and PrB increases the MN frequency has been demonstrated. Histological examination of the BM revealed the effect of EPO and/or PrB on the cellularity (Figure 6). Further, increased cellular proliferation was confirmed by the MTT assay and by determining Ki-67 expression. The MTT assay is widely used for the determination of cell viability and proliferation (40–44). Furthermore, Ki-67 protein expressions are strictly correlated to cell proliferation and to the active phases of the cell cycle (45). In the present study, the results of MTT assay indicated a higher rate of BM cellular proliferation with the simultaneous use of EPO and/or PrB as compared to respective control (Figure 7) that were further confirmed with immunohistochemical determination of Ki-67 (supplementary Figure 2, available at Mutagenesis Online). The results indicate that increase in the BM cellular proliferation may be attributed towards the enhanced MN frequency in the PB.

In addition, the results suggest that CP treatment with or without EPO in the BM causes a significant decrease in the proliferation and cellularity while there is an increase in the adipocytes deposition (46). However, the effect was minimal with PrB and the EPO + PrB combination (Figure 6). The results of the present study are in accordance with previous reports showing that CP induced a decrease in the BM cell numbers due to stem cell mobilisation (47,48) that was further augmented with the EPO treatment (37). Furthermore, an in vitro study also reported the inadequacy of EPO against cytotoxic and antiproliferative properties of anticancer drugs (49). The effect of cell proliferation on DNA damage (DNA strand breaks) as determined by comet assay parameters with CP and AZT indicates that DNA damage was increased with the proliferation (Figure 8). The present model was found more suitable for the screening of genotoxicity than the previous pre-bled young-rat model. Furthermore, the volume of blood withdrawal was less and was found equivalent to the volume previously used in mice to increase the sensitivity of PBMN assay (21,25). This can further help to mitigate concerns due to the stress to experimental animals associated with prior blood removal and lead to its possible acceptance in regulatory toxicity studies. The simultaneous use of EPO and PrB further increases the sensitivity and reliability of PBMN assay, although further studies using other mutagens with different mechanisms of action are required to validate the model.

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References

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