

Chemotherapy-induced genotoxic damage to bone marrow cells: long-term implications

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Abstract

Mesenchymal stem/stromal cells (MSC) within the bone marrow (BM) are vitally important in forming the microenvironment, supporting haematopoiesis after myeloablative chemotherapy. MSC are known to be damaged phenotypically and functionally by chemotherapy, however, to our knowledge the persistence of genotoxic effects of chemotherapy on the BM microenvironment has not been studied. We therefore aimed to evaluate genotoxic effects of chemotherapy on the BM both *in-vitro* and *in-vivo*, using the comet and micronucleus assays, focussing on the persistence of DNA lesions which may contribute to complications in the patient. The MSC cell line (HS-5) and primary cord blood mononuclear cells (CBMNC; a source of undamaged DNA) were exposed to the chemotherapeutic agent cyclophosphamide (CY) within a physiologically relevant *in vitro* model. CY treatment resulted in significant increases in CBMNC DNA damage at all time points tested (3-48hr exposure). Similarly, HS-5 cells exposed to CY exhibited significant increases in DNA damage as measured by the comet assay, with increased numbers of abnormal cells visible in the micronucleus assay. Additionally, even 48hrs after removal of 48hr CY treatment, DNA damage remains significantly increased in treated cells relative to controls. In patients treated with chemotherapy for haematological malignancy highly significant increases in damaged DNA were seen in BM cells isolated from one individual one year after completion of therapy for acute leukaemia compared with pre-treatment ($p < 0.001$). Similarly, two individuals treated 7 and 17 years previously with chemotherapy exhibited significant increases of damaged DNA in MSC compared with untreated age- and sex-matched controls ($p < 0.05$). Unlike haematopoietic cells, MSC are not replaced following a stem cell transplant. Therefore, long-term damage to MSC may impact on engraftment of either allogeneic or autologous transplants. Additionally, persistence of DNA lesions may lead to genetic instability, correlating with the significant number of chemotherapy-treated individuals who suffer with therapy-related malignancies.

Introduction

Mesenchymal stem/stromal cells (MSC) are the supportive stem cells of the bone marrow microenvironment, contributing to the bone marrow (BM) stroma and playing a vitally important role in supporting haematopoiesis, both through cell-cell contact and secretion of soluble factors (1,2). MSC have previously been shown to be phenotypically and functionally damaged by chemotherapy, particularly by high-dose chemotherapy administered prior to a stem cell transplant (SCT) as a potential cure for haematological malignancy (3,4,5,6,7). However, the exact nature of this cellular damage is poorly characterised. In addition, whilst haematopoietic cells are replaced during a SCT, MSC are not and remain of host origin (8,9). Therefore, if MSC are severely damaged by the SCT preparative regimen, this may be permanent, particularly if effected at the DNA level. Indeed, functional damage to BM stroma is thought to be slow to repair (10), and in one study was shown to be severe and irreversible, with the ability to support haematopoiesis being completely recoverable only in children under 4 years of age (11).

Clinically, damage to MSC may result in graft failure, seen following a significant number of transplants (12). Damage induced at the DNA level may also increase the risk of genetic instability and development of malignancies (13). Therapy-related primary malignancies are seen in a significant proportion of chemotherapy-treated individuals, and commonly arise within the BM, manifesting as therapy-related myelodysplastic syndrome or acute myeloid leukaemia (14,15,16).

We and others, have previously reported cellular damage to MSC from chemotherapy, both *in-vitro* and *in-vivo* (17,18), focussing on impact on differentiation, morphology, colony forming units and premature ageing (3,5,6,7,11,19,20). The genotoxic impact of chemotherapy has previously been reported in haematopoietic progenitor cells (21,22), however, this has not been studied within the MSC population and the BM microenvironment. With its critical role in supporting haematopoiesis, damage to the BM microenvironment may have significant consequences haematologically, particularly given that these cells cannot be replaced therapeutically (8,9). Furthermore, the fact that haematopoietic cells largely succumb to chemotherapy whereas MSC do not, genotoxic outcomes to the BM microenvironment arguably may have a more important impact on patient outcome.

Therefore, this study aimed to further characterise genotoxic damage to the supportive BM microenvironment from chemotherapy, including longer-term persistence of lesions, which may have considerable clinical impact.

Materials and Methods

All reagents were obtained from Sigma-Aldrich, Dorset, UK, unless otherwise specified.

Sample collection

Iliac crest BM samples were obtained from the Royal United Hospital, Bath, from patients undergoing BM aspirations for the diagnosis of haematological malignancies or for therapy monitoring purposes, with donor consent and National Research Ethics Service (NRES) approval (REC approval numbers: Southmead 048/01, BA504). BM was collected by medical staff into sterile tubes containing 1000IU heparin. Details of samples analysed in this study are shown in Table I.

We were unable to access BM MSC from healthy young donors, due to ethical considerations of the invasiveness of the procedure. As residual DNA damage amasses naturally with age (23), we therefore utilised cord blood (CB) samples as a source of MNC, of which MSC constitute a proportion. CB was obtained from healthy, full-term deliveries at Southmead Hospital, Bristol, with NRES approval and donor consent. Additionally, as a further comparison, an MSC cell line (HS-5) was also utilised to study effects of chemotherapy *in vitro*.

Untreated samples	Chemotherapy-treated samples
UPI 59 – 65yr old male. Unknown reason for BM aspirate	UPI 82 – 63yr old male, myeloma. Treated with VAD, high-dose melphalan and thalidomide (6 cycles) with autologous transplant. All 7 years prior to BM aspirate
UPI 143 – 64yr old male. Persistent thrombocytopenia	UPI 164 – 63yr old male, low grade lymphocytic lymphoma. Treated with mitoxantrone, chlorambucil and prednisolone (6 cycles). Last treatment 17 years prior to BM aspirate
UPI 150 – 50yr old female. Query acute myeloid leukaemia (AML)	UPI 193 – 51yr old female (previous sample UPI 150). AML. Treated with ADE and myelotarg (2 cycles) and MACE. Last treatment 1 year prior to BM aspirate

Table I. Clinical details of bone marrow samples used within this study. Two untreated and chemotherapy-treated samples were age- and sex-matched for analysis (UPI numbers 59, 82, 143 and 164). One individual was assessed prior to, and one-year following completion of chemotherapy (UPI numbers 150 and 193 respectively). UPI = unique patient identifier, assigned to each sample at the time the aspirate is taken to ensure anonymity within the study. VAD = vincristine, doxorubicin and dexamethasone. ADE = daunorubicin, cytosine arabinoside and etoposide. MACE = amasacrine, cytosine arabinoside and etoposide.

Isolation of mononuclear cells

Mononuclear cells (MNC) were isolated from BM and CB samples by lymphoprep separation, as described previously (5). Samples were diluted 1:1 with low glucose Dulbecco's Modified Eagle's Medium (DMEM) and layered onto lymphoprep (Axis Shield, Cambridgeshire, UK; density 1.077+/-0.001g/ml). Following centrifugation (600 x g for 30mins), the buffy coat layer was harvested, washed in DMEM and then cultured in MSC medium (DMEM containing 10% foetal calf serum (FCS) selected for optimal MSC growth (Stem Cell Technologies, Grenoble, France) containing 100U penicillin and 0.1mg streptomycin/ml). MNC from BM samples were seeded in primary culture to enable expansion of the MSC population (see 'MSC culture'). MNC from CB were used immediately in *in-vitro* chemotherapy treatment experiments (see 'chemotherapeutic treatment of CB MNC and HS-5 cells')

MSC culture

MSC were cultured from bone marrow aspirates as described by (24). Briefly, primary cultures (1x10⁷ MNC per T-25 flask in MSC medium) were incubated at 37°C in 5% CO₂ and maintained by weekly demi-depletion of medium until 70% confluent. Cultures were then passaged using 0.25% trypsin and reseeded at

first passage at 1×10^5 cells per flask. Weekly demi-depletion of medium and passaging enables isolation of MSC utilising plastic adherence (25).

MSC were immunophenotyped at the end of second passage (see 'immunophenotyping of MSC') before being cryopreserved in DMEM, containing 25% FCS and 10% DMSO in the vapour phase of liquid nitrogen until required for comet assay.

HS-5 culture

HS-5 cells were obtained from American Type Culture Collection (Middlesex, UK) and cultured in high glucose DMEM, supplemented with 10% FCS. Cultures were maintained by sub cultivation at a ratio of 1:3, with medium replaced every 2-3 days. Experiments were performed on low passage number cells (<10 passages).

Immunophenotyping of MSC

Immunophenotyping was performed at the end of second passage to confirm presence of a pure population of MSC, according to International guidelines (26). Cells (1×10^5) were stained for surface markers by labelling with anti-CD105 PE/anti-CD90 FITC (AbD Serotec, Oxford, UK and BD Biosciences Oxford, UK respectively), anti-CD73 PE/anti-CD45 FITC (BD Biosciences and Dako, Cambridgeshire, UK, respectively), anti-CD44 PE/anti-CD19 FITC (BD Biosciences), anti-CD14 PE/anti-CD34 FITC (BD Biosciences and AbD serotec respectively), anti-CD29 PE and anti-CD166 PE, and isotype controls (all BD Biosciences) for 45mins on ice, followed by flow cytometry analysis (FACS Vantage SE using Cell Quest™ software, Becton Dickinson, Oxford, UK). Cells were gated to exclude debris and dead cells, with 10000 gated events analysed. All further experiments using MSC were performed immediately after immunophenotyping at the end of second passage.

Chemotherapeutic Treatment of CB MNC and HS-5 cells

Cells were seeded in 6-well plates (2×10^6 cells per well for CB MNC) or in 24-well plates (2.5×10^4 cells per well for HS-5) and exposed to $500 \mu\text{M}$ cyclophosphamide (CY) either in the presence of filtered S9 extract (0.4 mg/ml) (27), or HepG2 liver spheroids (17) to enable conversion of CY to active metabolites. Control wells containing cells only, cells + S9, cells + CY, cells + HepG2 spheroids (SPH) were also utilised. Treatments were added for 3-48 hours, mimicking the treatment time *in vivo* when CY is given 2 days prior to SCT (28). A shorter period of 3hrs was also used as S9 is known to be toxic over extended periods in culture (29). Comet

assays were performed immediately after removal of CY at 3hrs and 48hrs exposure, as well as up to 48hrs after removal of 48hr CY treatment, to investigate the persistence of genotoxicity after removal of the agent. At all time points where genotoxicity assays were performed, corresponding cytotoxicity was determined by the calculating relative increase in cell count (RICC) as detailed by Fellows *et al.*, (30).

HepG2 spheroid culture

HepG2 hepatoma cells (European Collection of Authenticated Cell cultures, Porton Down, Salisbury, UK) were cultured in high glucose DMEM, with 10% FCS, 100U penicillin and 0.1mg streptomycin/ml, 2mM L-glutamine and 1% non-essential amino acids, at 37°C with 5% CO₂. Confluent cells were trypsinised, seeded into a 6-well plate (3x10⁶ cells per well) and incubated on a gyrotatory shaker at 83rpm for 24hrs and 79rpm thereafter to enable the formation of spheroids. Medium was demi-depleted every second day and maturity reached after 6 days of culture (31).

Alkaline comet assay

To determine levels of DNA damage, alkaline comet assays were performed following the manufacturer's instructions (AMSBIO, Abingdon, UK). Briefly, cells were harvested by gentle scraping, washed twice with cold PBS and enumerated. Samples were kept on ice at all times and protected from light to prevent further DNA damage. Untreated cells and positive controls (100µM H₂O₂ for 20-30mins on ice) were always processed simultaneously. Cells were resuspended in ice cold PBS (4x10⁵/ml), of which 25µl was then combined with 250µl molten agarose and 50µl spread onto the comet slide. Lysis and alkali unwinding was performed for 1hr each, with electrophoresis similarly performed for 1hr (1V/cm); ensuring the tank remained on ice and protected from light. Slides were stained with SYBR green at 4°C in the dark for 5 minutes, and visualised using a Nikon TE300 inverted microscope with images taken using a Roper Scientific camera and Image-Pro Plus software. Comet assay images were analysed for % DNA in the tail, using Comet Assay V software (Perceptive Instruments, Bury St Edmunds, UK).

Micronucleus Assay

Immediately after 48hr *in-vitro* chemotherapy treatment and again 48hrs later, CBMNC and HS-5 cells were analysed using the micronucleus assay. Cells (2x10⁴) were harvested, washed in PBS and cytospun for 10mins at 300g. Slides were air dried, fixed in 100% methanol for 8mins, then dipped in fresh phosphate buffer (0.66% w/v K₂HPO₄ and 0.32% w/v NaH₂PO₄, pH6.4-6.5), before being stained in acridine orange solution

(12mgs in 100mls of the previous phosphate buffer). After staining for 1min, slides were transferred back into the initial phosphate buffer for 10mins, followed by transfer into a new batch of the same buffer for a further 15mins. Following air drying, slides were protected from light and examined using a Nikon TE300 inverted microscope with images taken using a Roper Scientific camera and Image-Pro Plus software. Fifty images were taken for each treatment, and all cells within each field of view scored for morphology, including identification of 1, 2 or 3 micronuclei, bi-nucleated, multinucleated, lobed, blebbed or apoptotic cells.

Statistical analysis

A minimum of 30 comets were scored in the initial time course assay and a minimum of 50 comets scored thereafter, with a minimum of 1000 cells scored for each treatment in the micronucleus assay as recommended by genotoxicology testing guidelines in place when relevant experimental work was undertaken (32).

All statistical analysis was performed in GraphPad Prism 7, using Mann-Whitney test to analyse differences between two groups/samples, and one-way ANOVA performed for comparison of 3 or more groups, with significance considered to be $p < 0.05$.

Results

Genotoxicity from *in-vitro* treatment

Following 3hr or 48hr *in-vitro* CY treatment, CB samples exhibited significantly increased percentages of damaged DNA measured by the comet assay compared with the untreated control (Figure 1), whilst cell viability remained above 70% (figure 1C). Treatment with CY alone over 3hrs did not result in a significant difference compared with untreated controls (Figure 1A), due to CY itself being largely inactive and requiring hepatic metabolism. In contrast, in the presence of either S9 liver extract or HepG2 liver spheroids, a significant increase in DNA within the comet tail was seen compared to both untreated cells and those exposed to CY alone ($p < 0.001$) (Figure 1A). It is also worth noting the increase in DNA damage seen following exposure of cells to S9 extract alone. S9 is known to be toxic over extended periods in culture (29), but these data indicate genotoxic effects within even 3hrs exposure (Figure 1A).

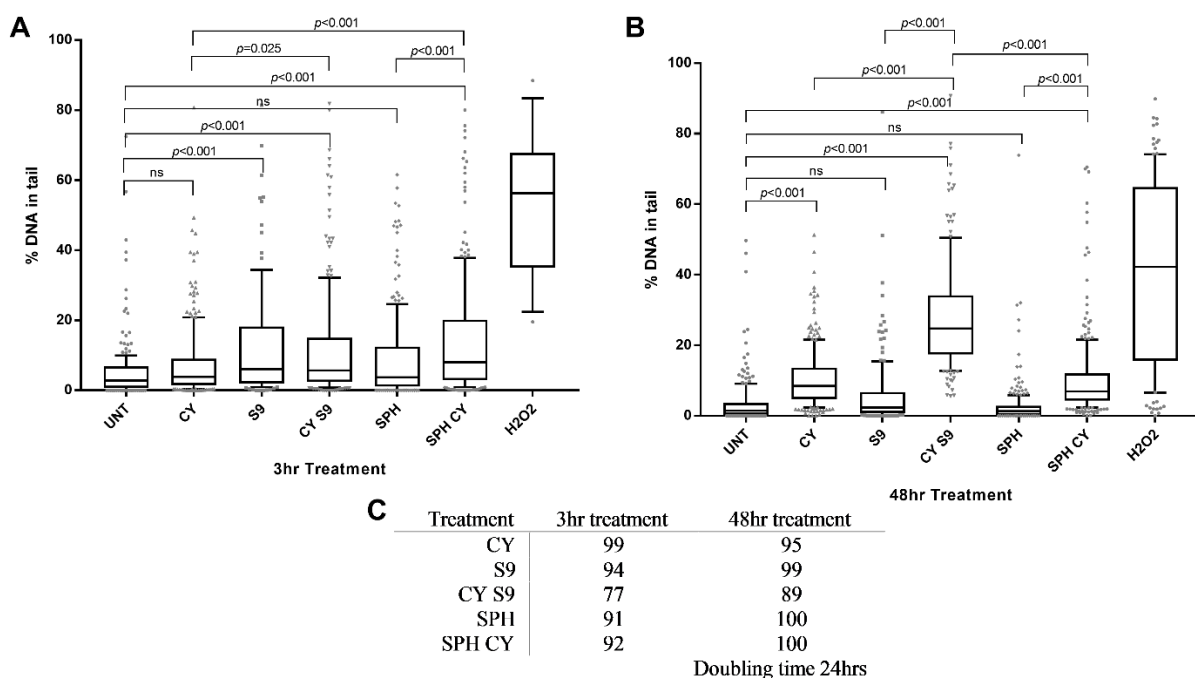


Fig 1. Assessment of DNA damage in CB MNC following *in-vitro* CY treatment. Percentage of DNA within the comet tail following 3hr (A) or 48hr (B) CY treatment in the presence of S9 extract or HepG2 liver spheroids (SPH) as a source of metabolism. Corresponding calculated relative increase in cell count for each treatment as an indication of cytotoxicity (C). A minimum of 50 comets were analysed for each treatment, with the combined data from two CB samples shown for each time period. All samples at both time points differ significantly from untreated cells ($p < 0.001$) except S9 and SPH alone at 48hr. Boxes represent the middle 50% of data, with 10-90% of the population then encompassed by the extended lines, and outliers indicated by symbols. H_2O_2 treatment for 20-30 minutes was used as a positive control.

Significant increases in numbers of DNA strand breaks are also seen after 48hr treatment of CB with CY in the presence of either S9 or liver spheroids, in comparison to untreated cells ($p < 0.001$) (Figure 1B). A significant increase above that of cells exposed to CY alone is only seen with S9 and CY, although the distribution of percentage of damaged DNA is skewed towards greater damage when CY is combined with SPH. This is partially due to increased damage seen in cells exposed to CY alone as a result of spontaneous decomposition of the parent drug in aqueous conditions during extended culture periods (33). Additionally, DNA crosslinking by nitrogen mustards can retard comet tails (34), and thus spheroids may be more efficient than S9 at metabolizing CY, resulting in greater crosslinks. A time-course assay investigating extent of genotoxic damage to MNC from CY *in vitro* in combination with liver spheroids also demonstrated significant increases in DNA damage at all time points examined beyond 3hrs exposure ($p < 0.001$), even up until 48hrs after removal of the drug (Figure 2A), whilst cell viability does not fall below 86% (Figure 2B). Even 48hrs after cessation of treatment genotoxicity is not statistically different to levels seen 24hrs into the 48hr treatment period ($p > 0.999$).

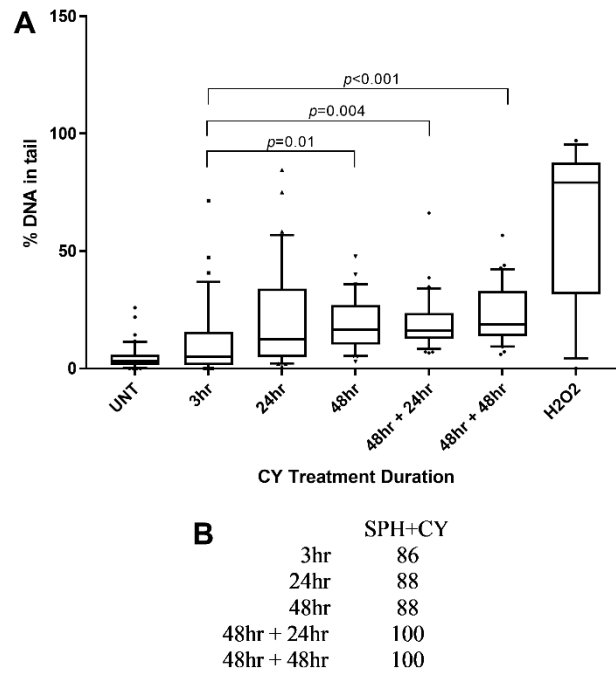


Fig 2. CB MNC were exposed to CY in the presence of liver spheroids for 3hrs, 24hrs or 48hrs after which they were immediately analysed (A). Additionally, some cells were exposed to this treatment for 48hrs after which they were maintained in normal medium and genotoxic damage assessed 24hrs and 48hrs later (A). Corresponding calculated relative increase in cell count for each treatment as an indication of cytotoxicity (B). A minimum of 30 comets were analysed at each time point, with the combined data from two cord blood samples shown. All samples differ significantly from the untreated control ($p < 0.001$) except cells treated for 3 hours only. Boxes represent the middle 50% of data, with 10-90% of the population then encompassed by the extended lines, and outliers indicated by symbols. H_2O_2 treatment for 20-30 minutes was used as a positive control.

Similarly, following exposure of HS-5 stromal cells to CY *in vitro*, significant increases in DNA damage were seen, when treated in the presence of both S9 liver extract ($p < 0.001$) and HepG2 liver spheroids ($p = 0.003$) as a source of metabolism (Figure 3A). As with CB MNC, HS-5 DNA damage increased with exposure time (Figure 3B), gradually decreasing after CY was removed, but remaining higher than in untreated cells even 48 hours after removal of the chemotherapy. Whilst corresponding cell viability remained above 75% for all shorter 3hr treatments (Figure 3C), higher levels of cytotoxicity were seen over prolonged treatment periods beyond 24hrs (Figure 3C-D).

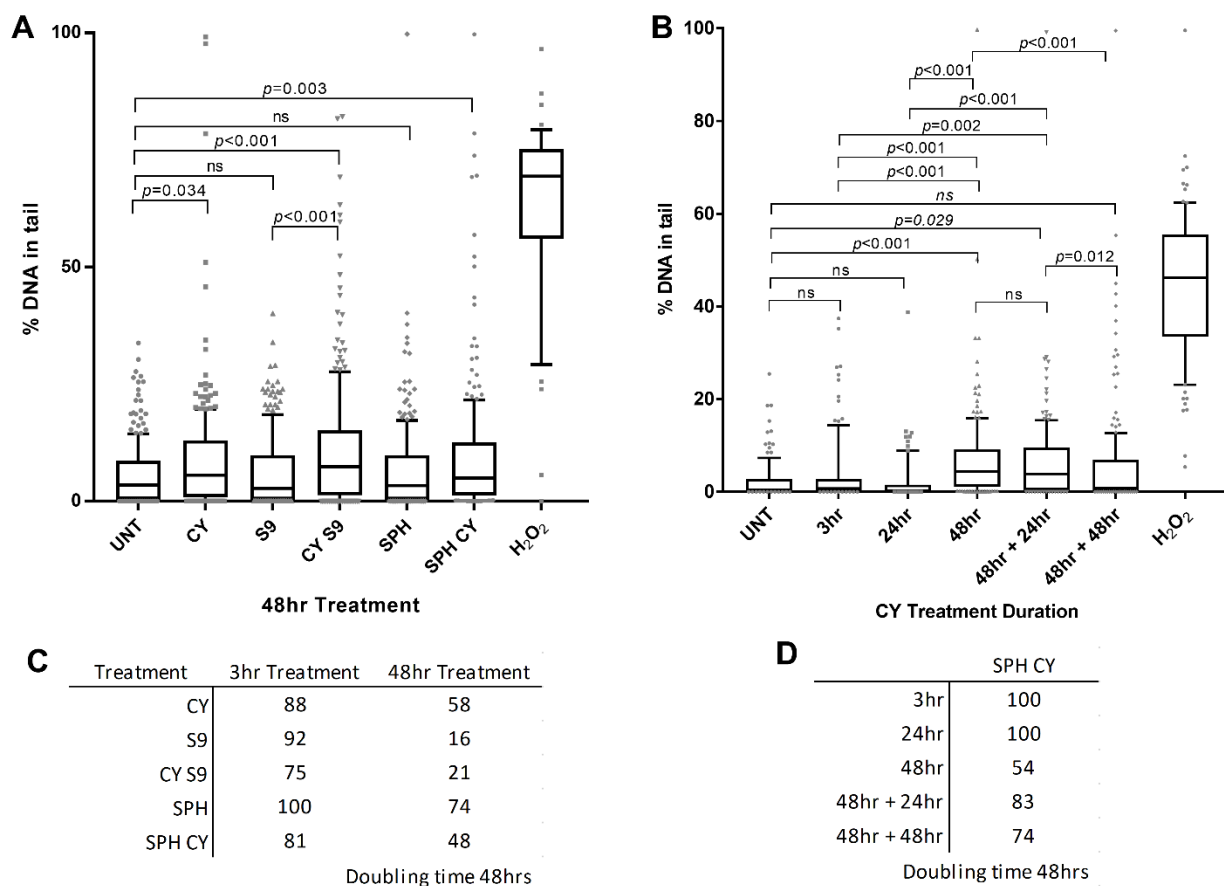


Fig 3. HS-5 bone marrow stromal cells were exposed to CY in the presence of S9 extract or HepG2 liver spheroids as a source of metabolism. Percentage of DNA within the comet tail analysed immediately after 48hr treatment (A) or treated for up to 48hrs in the presence of HepG2 liver spheroids (SPH), with cells analysed immediately, or maintained in normal medium and genotoxic damage assessed 24hrs and 48hrs later (B). Corresponding calculated relative increase in cell count for each treatment as an indication of cytotoxicity (C and D). A minimum of 50 comets were analysed for each treatment, with the combined data from three independent repeats shown. Boxes represent the middle 50% of data, with 10-90% of the population then encompassed by the extended lines, and outliers indicated by symbols. H₂O₂ treatment for 20-30 minutes was used as a positive control.

A MN assay was initially performed on HS-5 immediately after removal of 48hr chemotherapy treatment (Figure 4A, B, E). An increase in MN were seen in cells exposed to chemotherapy either in the presence of S9 liver extract or HepG2 liver spheroids (data not shown). These increases then became statistically significant 48hrs after removal of the chemotherapy (figure 4C), for cells exposed to CY in the presence of liver spheroids ($p=0.037$). As was seen in the comet assay, S9 itself also resulted in as many abnormal cells as CY combined with S9, whereas HepG2 liver spheroids themselves had no effect on numbers of abnormal cells seen ($p=0.9996$).

In addition to considering MN alone, the total number of cells exhibiting altered morphologies (including blebbed, multinucleated, lobed, apoptotic and binucleate with MN) was also significantly increased 48hr after chemotherapy removal, in both those cells treated in the presence of S9 or liver spheroids ($p<0.001$

and $p=0.021$ respectively) (Figure 4D). In particular, blebbed cells were much more prominent following chemotherapy treatment, although not significantly ($p>0.05$). Additionally, whilst apoptosis was initially diminished in cells exposed to S9 for 48hrs either with or without CY (Figure 4E), it was significantly increased 48hrs later ($p=0.005$ and $p=0.032$ respectively), whilst all other treatments remained comparable to the untreated control (Figure 4F).

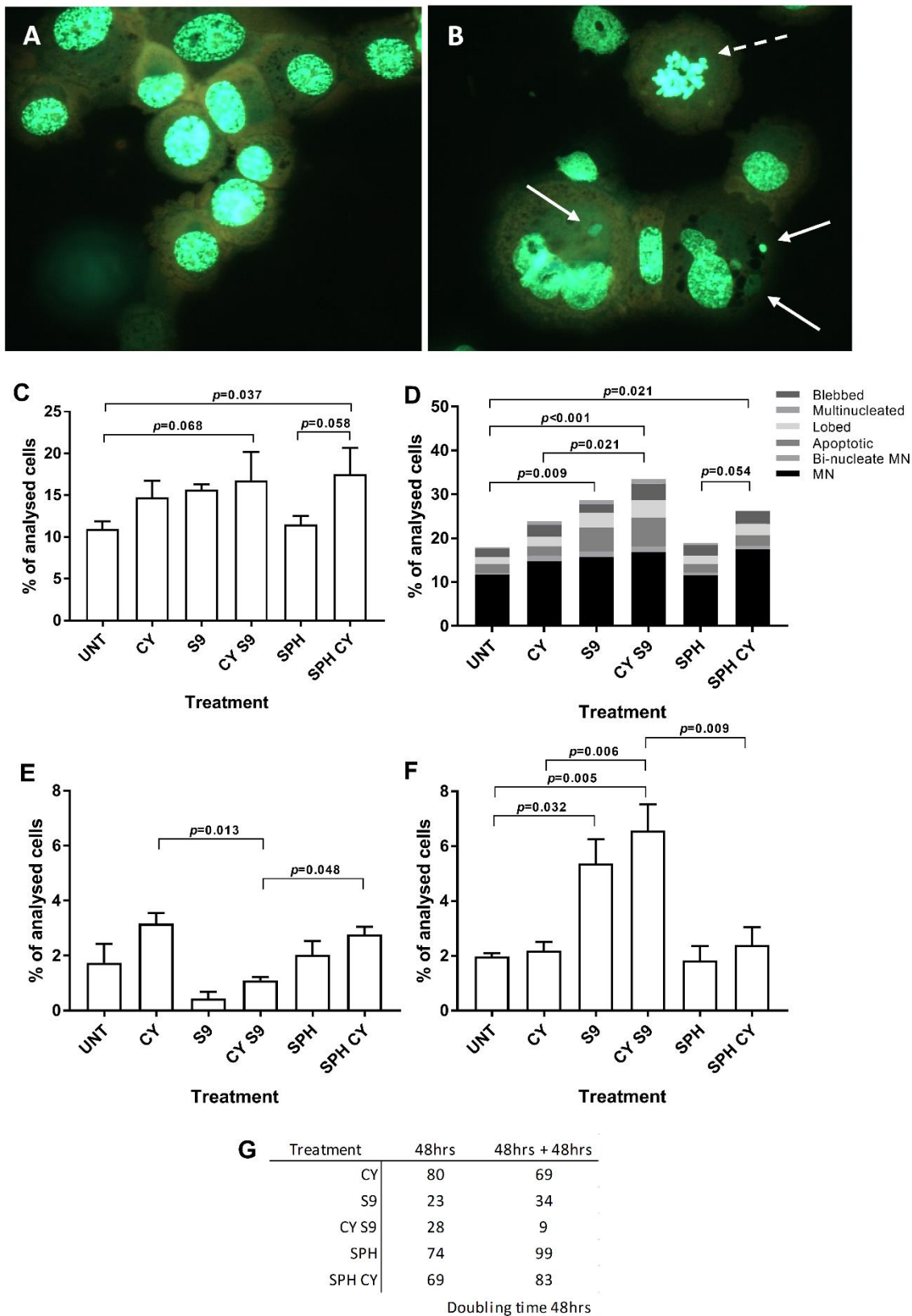


Fig 4. Following 48hr treatment with CY in the presence of S9 extract or HepG2 liver spheroids, a micronucleus assay was performed on HS-5 bone marrow stromal cells both at 48hrs when treatment was removed (A, B and E), and again 48hrs after removal of treatment (C, D and F). Examples of typical mononucleated cells seen in untreated slides are shown (A), together with examples of altered morphologies in cells exposed to CY and liver spheroids (B). An apoptotic cell is highlighted with a dashed arrow, and micronuclei highlighted by solid arrows (B). Percentage of cells with micronuclei are shown as a total of all cells analysed within each population (C) Total abnormal cells in each population, showing percentages of each type of morphology (D). Percentage of apoptotic cells at the time of treatment removal (E) and 48hrs later (F) are shown as a total of all cells analysed within each population. Corresponding calculated relative increase in cell count for each treatment as an indication of cytotoxicity (G). A minimum of 1000 cells were analysed per treatment. Data shown as mean +/- SD, combined data from three separate experiments at each time point tested.

Genotoxicity from *in-vivo* treatment

All BM MSC were plastic adherent and immunophenotyped according to ISCT guidelines (26) and purity found to be greater than 95% (data not shown). Unfortunately, it was not possible to also perform differentiation studies on patient samples due to inadequate cell numbers.

Following identification of increased DNA damage in CB cells exposed to *in-vitro* chemotherapeutic treatment, BM cells from small numbers of individuals treated previously with chemotherapy were assessed for genotoxicity. Particularly striking is the highly significant increase in damaged DNA seen in BM MNC from one individual post-treatment ($p < 0.001$) (Figure 5). It is also noteworthy that this effect is not recent, with chemotherapy completed more than 1 year prior to analysis.

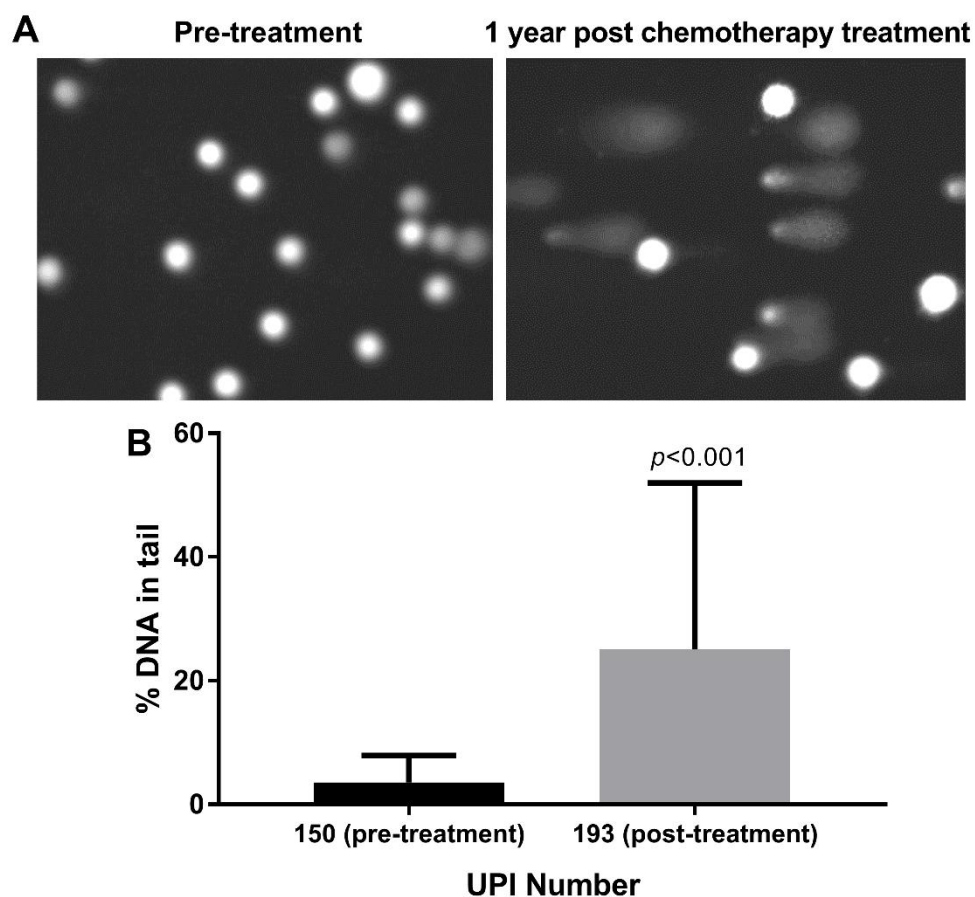


Fig 5. Assessment of DNA damage in BM cells following *in-vivo* chemotherapeutic treatment. Alkaline comet assays were performed to assess genotoxic damage, with damaged DNA represented by the tail portion of the comet. Images (A) from a comet assay performed on cells isolated from samples from the same individual pre- and post- chemotherapy treatment *in vivo* (treatment details shown in Table I), with percentages of damaged DNA (B). A minimum of 50 comets were analysed for each sample. Columns represent the lowest 50% of cells, with the highest 50% shown by the extended line.

A significant increase in DNA damage is also seen in BM MSC from chemotherapy-treated patients compared with age- and sex-matched untreated individuals ($p=0.037$) (Figure 6). Similarly, this appears to be a long-term effect, with last treatment received 7 and 17 years prior to analysis.

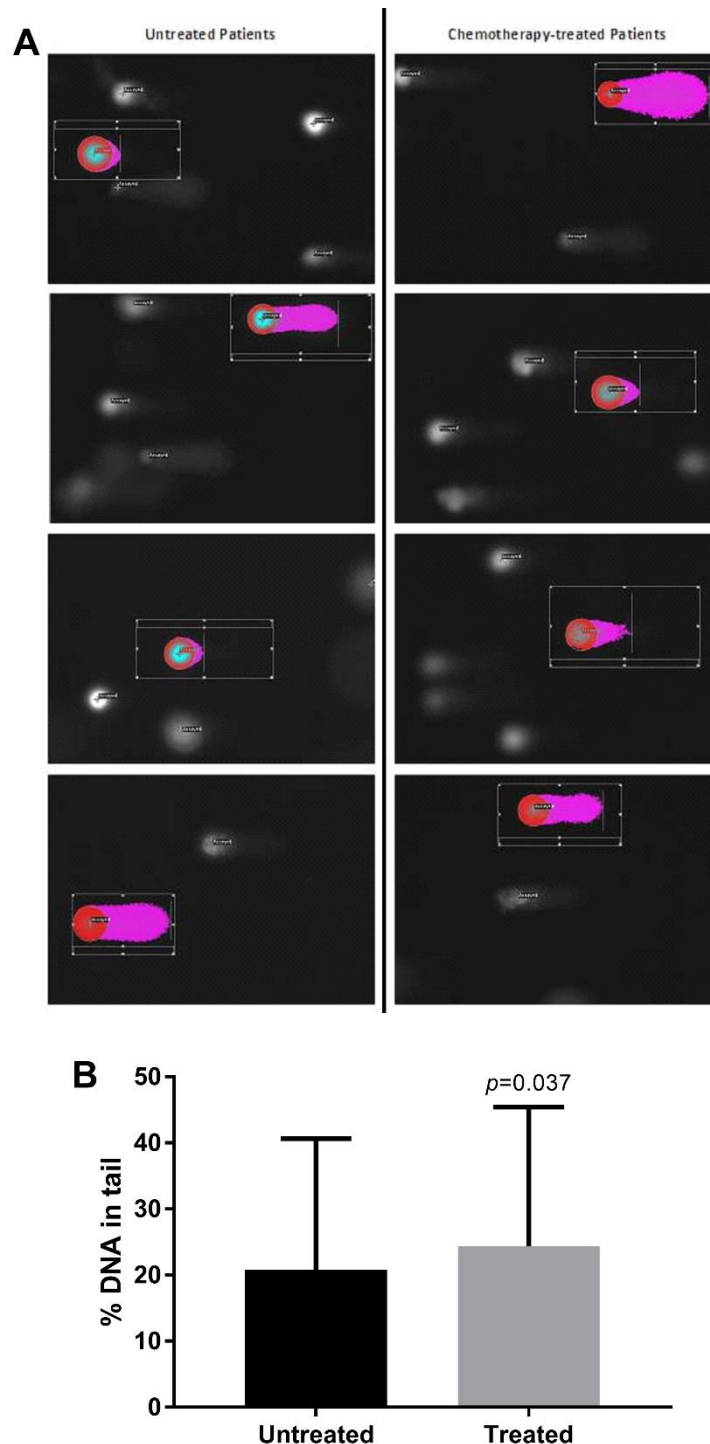


Fig 6. Assessment of DNA damage in BM cells following *in-vivo* chemotherapeutic treatment. Alkaline comet assays were performed to assess genotoxic damage, with damaged DNA represented by the tail portion of the comet. Representative images (A) from a comet assay performed on BM MSC from two age- and sex-matched untreated and chemotherapy-treated individuals (treatment details shown in Table I). A minimum of 50 comets were analysed for each sample, with combined data from 2 samples per group shown in (B), indicating percentage of DNA within the comet in untreated and treated patients. Columns represent the lowest 50% of cells within each population, with the highest 50% shown by the extended line.

Discussion

Genotoxicity from *in-vitro* treatment

Results seen in this study are comparable with data generated in other similar *in vitro* studies using other cell types. Oshida *et al.*, (35), reported genotoxic effects following exposure of the murine BM cell line, FDC-P2 to CY *in vitro* in the presence of S9 preparation, where slightly higher proportions of damaged DNA were seen, although damage in untreated cells was also considerably higher. However, S9 was used at a higher concentration, and genotoxicity of S9 itself is not mentioned. Additionally, Oshida's study (35) utilised S9 from rat liver, which has been shown to have much higher metabolic activity than human-derived S9 and is thus more likely to produce mutagenic effects (36).

In the current study, genotoxic effects of *in vitro* chemotherapeutic exposure have been examined in CB MNC rather than BM MSC. Ideally, this should also be examined in MSC, as different cell types may differ in terms of sensitivity, repair capacity etc. However, due to ethical problems with obtaining BM from healthy, young individuals, CB MNC were used as a 'clean' source of DNA. Attempts were made to isolate MSC from CB, however, we, as others have encountered previously, found it unrealistic to isolate and culture sufficient cell numbers for analysis (24,37). Cord blood and haematopoietic cells are much more sensitive to chemotherapy than MSC, indeed with haematopoietic cells being the intended target for cytotoxic and genotoxic effects in the cases of haematological malignancy therapy. MSC are known to be much more resistant to the effects of chemotherapy, with some studies showing them to be relatively resistant to certain agents (38), however, functional damage has been shown with other agents (5,7). In this work, we show high viability following chemotherapeutic treatment and the ability to remain present both *in vitro* and *in vivo*, yet with considerable genotoxicity. Clinically, it is essential that these cells remain viable with their critical function to support haematopoiesis; nevertheless, any genotoxicity that persists may have important clinical considerations.

As an additional comparison, the MSC cell line HS-5 was also treated with CY *in vitro* in the same way as the CB MNC cells, to enable study of a uniform population of specifically BM stromal cells. Similar trends were seen in both cell types following *in vitro* exposure, with a peak in damage seen as treatment progresses, and levels then decreasing once the drug was removed, although remaining higher than untreated cells at all time points tested. The point at which maximal damage was seen differed between the two cell types, however, seen at 48hrs in HS-5 ($p < 0.001$ compared to the untreated control), and at 24hrs in CBMNC ($p < 0.001$ compared

to the untreated control). This may be due to differing cell turnover times, with HS-5 doubling time calculated as approximately 48hrs within this study, although reported by others to take up to 72hrs (39), whilst CB MNC doubling time may vary widely between different donors and within different cell populations within each sample.

Initially MN assays were performed on HS-5 cells immediately after removal of 48hr chemotherapy treatment. Subsequently, these were repeated 48hrs later after 2 calculated population doublings, as recommended by Kirsch-Volder *et al.*, (27) who report optimum results if cells are harvested after 1.5-2 cell cycles. Data from both time points followed the same trend, with increases in MN in treated cells becoming statistically significant after 2 population doublings. In addition to MN, other abnormal morphologies were also increased in treated cells, although not significantly. Blebbed cells in particular, were increased following treatment, and it has been suggested that these, together with MN are important indicators for genome instability (40).

Comet assay and MN are acknowledged to be robust and sensitive assays to evaluate DNA breaks, linked to mutagenicity. The alkaline comet assay in particular is able to detect a broad spectrum of DNA damage and is recommended as a first line assay due to higher sensitivity than the MN assay (41). However, the MN assay has the advantage of greater ease of controlling conditions and thus is a regulatory genotoxicity test, hence both assays were performed in conjunction in this study, with complementary results obtained.

Whilst the HS-5 cell line represents a readily available source of relevant bone marrow stromal cells for this study, these cells were originally isolated from a 30-year old individual (42) and hence it is important to acknowledge that these will also have residual DNA damage due to unknown exposures throughout the individuals' lifetime.

Early genotoxic damage tends to arise from single strand breaks (SSB), which are short-lived lesions and rapidly repaired (43). SSB are the primary lesion detected by the alkaline comet assay, with virtually all genotoxicants producing far more SSB than double strand breaks or alkali labile sites (32). Therefore, following 3hr incubation with CY *in vitro*, it is likely that some of the initial genotoxic effects seen are predominantly SSB, possibly caused by rapid depletion of cellular glutathione and oxidative damage induced by the metabolite acrolein (44).

Conversely, damage seen later (24hrs onwards), is likely due to repair of DNA adducts resulting in strand breaks as an intermediate process in base excision repair or nucleotide excision repair. Therefore, slightly decreased genotoxic effects seen at 48hrs onwards may be as a result of some damage having been repaired, although considerable quantities of DNA remain within the comet tail, even 2 days after removal of the agent, suggesting persistence of some lesions. Clinically, patients are routinely treated with myeloablative chemotherapy for 48 hours prior to a stem cell transplant (28), therefore genotoxic damage persisting beyond this point, as seen in this work for up to a further 48 hours, may have implications for support of incoming donor haematopoietic stem cells and therefore patient outcome.

It is also possible that some heavily damaged cells have undergone apoptosis prior to analysis at later time points in this study, with only less extensively damaged cells undergoing repair. Indeed, >2.5% of HS-5 cells exposed to CY alone or in the presence of liver spheroids were found to be apoptotic in the MN assay at 48hrs, compared with only 1.7% of untreated cells. Additionally, scoring of heavily damaged cells within the comet assay is challenging, with some debate about how hedgehog comets should be scored and documented (54).

As inferred earlier, the apparent “loss” of DNA in the comet tail can also occur where crosslinking agents retard the movement of DNA (46). It was evident that S9 produced larger comet tails in comparison to spheroids, despite our functional data favouring spheroids as a model of physiological metabolism (17,18). This could suggest that either spheroids balance the generation of genotoxicity by activation/deactivation of the parent compound whereas S9 over-estimates damage, or spheroids are more capable of the metabolic processes leading to crosslink formation. Additionally, S9 is known to be cytotoxic over extended periods of time (29), which may have negatively impacted on results from the comet assay. It is recommended that cell viability should be above 70% for the assay, although there is some discrepancy over acceptable cytotoxicity levels, with some experts in the field allowing as low as 50% cell viability (32,45). Within this work, relative increase in cell count (RICC) has been used as a measure to estimate cytotoxicity, as this has been shown to underestimate cytotoxicity less than some other measures such as relative cell counts (30). All CB samples exposed to all agents, had a calculated viability of greater than 75% at all time points analysed. HS-5 RICC were greater than 75% following exposure to all agents over a 3hr period (Figure 3C). RICC above 70% was maintained after 48hr exposure except where cells were exposed to S9 and S9/CY, with values of 24% and 31% respectively

(Figure 3C and 3D). Even after removal of this treatment, cell recovery following exposure to S9 alone was poor, and viability of cells exposed to a combination of CY and S9 decreased further over the next 48hrs (Figure 4G). Therefore, it should be acknowledged that genotoxicity tests performed on HS-5 after 48hr treatment with S9 alone or in combination, will have been compounded by these high levels of cell death.

Confirmation of the nature of DNA lesions present in these cells could be achieved by a larger study utilising the FLARE assay, and methods developed by Spanswick and colleagues for crosslink quantitation (34,46). Clinically, persisting DNA lesions may have important ramifications, therefore, preliminary data was gathered concerning genotoxic effects after *in vivo* chemotherapeutic treatment.

Genotoxicity from *in-vivo* treatment

Considerable increases in genotoxicity were seen following exposure to chemotherapeutic agents *in vivo*. This is particularly evident in samples from the same individual pre- and post-chemotherapy ($p < 0.001$), treated with only 2 cycles of chemotherapy, the last of which was received one year prior to harvesting BM from which these cells were isolated (UPI 150/193). The vast majority of DNA damage is detected and repaired within 24 hours; therefore, if damage is still present one year after ceasing treatment, this would suggest it is highly likely to be long-term if not permanent, with damage tolerance pathways playing a role in maintaining the cellular population.

Similarly, two patients who completed their chemotherapy 7 and 17 years prior to this analysis were compared with age- and sex-matched untreated individuals. Greater damage was seen in treated individuals ($p < 0.05$), although inter-individual variation was also seen. The differences observed in these patients were less marked than the individual assessed pre- and post-chemotherapy, possibly due to inability to test pre- vs. post-treatment samples limiting direct comparison and exacerbating inter-individual differences. Also, the extended timescale may reflect gradual loss of lesions, or through “dilution” due to cellular turnover. Finally, crosslinks may represent long-term persistent lesions and can retard DNA tails (46).

Nevertheless, most recent treatment in all of these individuals was measured in years, strongly indicating the long-term nature and persistence of these genotoxic lesions. It is therefore likely that they are tolerated by the cell and are compatible with survival, but may increase risk of mutations and future malignancies, concurring with the increased risk of secondary malignancy seen in patients following chemotherapy (14,15,16,47).

Other than confirmation of no prior chemotherapy, it was not possible to obtain other clinical details from any of the individuals analysed. Clearly a diverse range of factors, including lifestyle and occupation will greatly impact on baseline levels of DNA damage, and will therefore contribute to inter-individual variation. Additionally, the chemotherapy treatment administered to treated patients varied, both in terms of agents used and numbers of cycles, and this will influence genotoxicity (see Table I).

Haematopoietic cells in neonates and children have also been shown to have increased sensitivity to genotoxicity (48), with children treated with chemotherapy shown to have significantly increased MN frequencies in peripheral blood (49). Haematopoietic cells subsequently become less sensitive to MN formation with increasing age of the individual (48). Samples tested in this study were all from older adults, therefore there may be even more significant effects seen in individuals treated with chemotherapy at a young age, and consequently potentially a longer lifespan in which carcinogenesis can occur.

Higher levels of genotoxicity in peripheral blood lymphocytes have previously been shown to be predictive of cancer risk, with MN formation thought to be associated with early carcinogenic events (50). Ideally, the patient samples in this study analysed by the comet assay would have also been assessed by MN assay, however, this was not possible due to low cell numbers obtained from chemotherapy-treated individuals.

Clinical implication within the bone marrow

For the first time, we show long-term genotoxic effects following chemotherapy in bone marrow stromal cells. Persisting aberrations have previously been detected in other haematological cell types following chemotherapy, with increased sister chromatid exchanges seen in peripheral blood lymphocytes up to 9 months after completion of therapy for breast cancer (21,22). Similarly, Lambert *et al.*, (51) detected chromosomal aberrations in lymphocytes from ovarian cancer patients up to 10 years later, with 14% of individuals developing second primary tumours. However, another study reported that the majority of DNA base modifications in lymphocytes were repaired within 24 hours, although the study examined oxidative damage primarily (52). Genotoxicity can also vary between tissues, with persisting chromosomal aberrations detected in rat lymphocytes for up to 20 weeks after CY treatment, whereas clastogenic effects in BM were repaired within 72hrs (53). A study by Yeh *et al.*, (4) reported cytogenetic changes in BM MSC, but only in patients who were treated with both chemotherapy and radiotherapy, with no cytogenetic changes detected in patients treated with chemotherapy only.

Clinically, significant damage to the BM microenvironment may have a number of ramifications. Severe damage may result in increased apoptosis or senescence and consequently depletion of the stem cell pool. In haematopoietic stem cells this can manifest as clonal haematopoiesis (54,55), identified as a predictor of development of second primary malignancies (56,57). Potentially, this effect could also occur in the mesenchymal compartment.

Surviving cells containing genetic lesions may also be affected functionally, which if occurring within MSC might significantly impact on haematopoiesis and ability to support a transplant. Failure to engraft occurs in up to 25% of SCTs, depending on stem cell source, conditioning regimen and recipient-donor disparity (12). Graft failure (GF) carries a dismal prognosis, with survival remaining very low in patients who undergo a second transplant. In a large study investigating 122 patients experiencing primary GF, following a second transplant overall one-year survival was only 11% (58). If links between engraftment and MSC health can be further studied, it may be possible to identify individuals most at risk of MSC damage and possible graft failure.

Persistence of genetic aberrations/lesions can also lead to genetic instability and increased risk of developing a subsequent malignancy. This is currently a complication in up to 15% of chemotherapy-treated individuals, with many of these malignancies arising within the BM (15,59). Analysis of damage to the marrow microenvironment may allow identification of those at greatest risk, enabling dose-stratification or treatment modification to improve outcome and quality of life.

The results presented here are only preliminary data gained on very small patient numbers; however, they provide strong indication of genotoxic lesions persisting within the BM following chemotherapy and warrant a large future study to confirm these effects.

In conclusion, significant increases in genotoxicity were seen in supportive bone marrow cells following both *in-vitro* and *in-vivo* chemotherapeutic treatment. *In vitro* treatment at a clinically relevant dose induced genotoxicity that persisted for 48 hours after treatment removal, significantly, this is later than the point at which a stem cell transplant would be administered clinically, and may have important clinical implications. Genotoxic damage to BM MSC following *in-vivo* treatment appears to be a long-term effect, with significant damage persisting even 17 years after completion of therapy. Clinically, genotoxic damage to the BM microenvironment may have significant impacts on haematopoiesis, including ability to support a SCT, and increasing the risk of developing second primary malignancies.

Funding

This work was supported by an Early Career Pilot Study Grant from the UK Environmental Mutagenesis Society [JM] and a PhD studentship at the University of the West of England [JM].

Acknowledgements

The authors would like to thank patients and staff at the Royal United Hospital, Bath, for donation and collection of bone marrow, and mothers and midwives at Southmead Hospital, Bristol for cord blood donation. Thank you also to Professor Myra Conway, for helpful discussions in preparation of the manuscript.

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