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Doxorubicin and vincristine affect undifferentiated rat spermatogonia

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Abstract

Anticancer drugs, such as alkylating agents, can affect male fertility by targeting the DNA of proliferative spermatogonial stem cells (SSC). Therefore, to reduce such side effects, other chemotherapeutics are used. However, less is known about their potential genotoxicity on SSC. Moreover, DNA repair mechanisms in SSC are poorly understood. To model treatments deprived of alkylating agents that are commonly used in cancer treatment, we tested the impact of exposure to doxorubicin and vincristine, alone or in combination (MIX), on a rat spermatogonial cell line with SSC characteristics (GC-6spg). Vincristine alone induced a cell cycle arrest and cell death without genotoxic impact. On the other hand, doxorubicin and the MIX induced a dose-dependent cell death. More importantly, doxorubicin and the MIX induced DNA breaks, measured by the COMET assay, at a non-cytotoxic dose. To elucidate which DNA repair pathway is activated in spermatogonia after exposure to doxorubicin, we screened the expression of 75 genes implicated in DNA repair. Interestingly, all were expressed constitutively in GC-6spg, suggesting great potential to respond to genotoxic stress. Doxorubicin treatments affected the expression of 16 genes (>1.5 fold change; $P < 0.05$) involved in cell cycle, base/nucleotide excision repair, homologous recombination and non-homologous end joining (NHEJ). The significant increase in CDKN1A and XRCC1 suggest a cell cycle arrest and implies an alternative NHEJ pathway in response to doxorubicin-induced DNA breaks. Together, our results support the idea that undifferentiated spermatogonia have the ability to respond to DNA injury from chemotherapeutic compounds and escape DNA break accumulation.

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Introduction

Over the past 30 years, the quality of life of the growing population of cancer survivors has been an increasing healthcare issue. Indeed, among other side effects, chemotherapy treatment can affect the fertility of men, with an increased risk of azoospermia, incidence of aneuploidy and abnormal chromatin structure in sperm even two years after chemotherapy (Meistrich 1986, Tempest *et al.* 2008, O'Flaherty *et al.* 2012). This is of concern because human spermatozoa with damaged chromatin and/or DNA are linked to poor embryo development and assisted reproductive technology (ART) failure (Delbes *et al.* 2010*a*). The fact that sperm quality is impaired in cancer survivors even years after treatment suggests that these long-term side effects originate from DNA damage that occured in spermatogonial stem cells (SSC) after treatment (Marcon *et al.* 2010). In fact, one can hypothesize that long-term fertility injury comes from unrepaired DNA damage in SSC spreading in the germline through spermatogenesis cycles, and in turn affects sperm quality. As anticancer drugs target cell division, actively dividing SSCs are indeed the target of most chemotherapeutic agents (Hou *et al.* 2005, Drumond *et al.* 2011, Marcon *et al.* 2011, Zohni *et al.* 2012, Liu *et al.* 2014).

Alkylating agents are known to impair the germline through the induction of DNA crosslinks and the depletion of spermatogonia (Meistrich *et al.* 1992, Aguilar-Mahecha *et al.* 2002, Drumond *et al.* 2011, Green *et al.* 2014). Therefore, nowadays, efforts are made to decrease the use of alkylating agents in chemotherapy treatment with the aim of reducing side effects such as gonadotoxicity. Instead, cocktails of chemotherapy could include anthracyclines and alkaloids, that are less mutagenic. Nevertheless, doxorubicin (DXO), one of the most used anthracyclines, is described as medium risk for fertility by inducing only a temporary azoospermia (Howell & Shalet 2005, Wallace *et al.* 2005, Meistrich 2009). Moreover, in rat, doxorubicin-exposed SSC in prepubertal animals produced long-term damage to mature sperm DNA that might be the cause of compromised conceptus development and reduced pregnancy outcome (Hou *et al.* 2005, Vendramini *et al.* 2012). More studies are needed to better understand the mechanisms by which such compounds affect SSC.

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Maintenance of paternal genome integrity is essential to prevent the risks of paternally mediated adverse progeny outcomes later in life (Tesarik *et al.* 2004, Vaisheva *et al.* 2007). Therefore, the germline must be armed with DNA repair systems to reverse potential damage. DNA double-strand breaks (DSB), the most deleterious DNA injury, are classically repaired by the non-homologous end-joining (NHEJ) and homologous recombination (HR) pathways (Rothkamm *et al.* 2003). Components of both pathways have been shown to be expressed in the male germline suggesting repair ability throughout spermatogenesis (Ozturk & Demir 2011). However, yet, these mechanisms are still poorly understood in SSC, mainly because these cells are rare and hard to purify. In fact, it is only possible to enrich germ cell population in SSC (Yeh & Nagano 2009). As a consequence, most prior studies analyzed DNA repair in a mixed population of spermatogonia (Richardson *et al.* 2000, Intano *et al.* 2002, Hamer *et al.* 2003, Xu *et al.* 2005). Using animal knockout models or pharmacological inhibitors, some specific components of DNA repair have been shown to be necessary for the maintenance of spermatogenesis, suggesting an essential role in the maintenance of SSC. This is the case for the ATM protein that is involved in NHEJ (Takubo *et al.* 2008, Rube *et al.* 2011) or MGMT that represents an independent DNA repair pathway (Thompson *et al.* 2000). However, to our knowledge, the response of SSC to chemotherapy has not been fully characterized with respect to DNA repair capability and mechanisms.

We have previously established the first rat spermatogonial cell line, GC-6spg, with SSC characteristics (Van Pelt *et al.* 2002). This cell line can be cultured without feeder cells and is capable of homing to the basal membrane of seminiferous tubules after testicular transplantation. This, therefore, provides an excellent model to study the mechanisms by which chemotherapeutic agents affect SSC. We support the hypothesis that SSCs have the ability to trigger DNA repair. The goal of the present study was to characterize the repair gene pathways activated in SSC in response to commonly used combination of anthracyclines and alkaloids. Using DXO and VCR as model anthracyclines and alkaloids respectively, we first characterized their cytotoxicity and genotoxicity on GC-6spg. As VCR is an inhibitor of microtubule polymerization, we did not expect to measure the genotoxic effect. On the other hand, DXO is known to induce DNA breaks, and we analyzed its effect alone or in combination with VCR.

Material and methods

Chemicals and solutions

Culture medium was modified Eagle's medium (MEM) supplemented with non-essential amino acids (1×), HEPES (15 mM), gentamicin (40 µg/mL), penicillin/streptomycin (100 U/mL), L-glutamine (4 mM), FBS Australian origin (2.5%), G418 (200 µg/mL), recombinant human basic fibroblast growth factor (bFGF; 2 ng/mL) (Thermo Fisher Scientific), sodium bicarbonate (0.12%), platelet-derived growth factor-BB (10 ng/mL), forskolin (20 µM), 17β-estradiol (1 nM) (Sigma) and recombinant human LIF (10 ng/mL) (PeproTech, Rocky Hill, USA). VCR sulfate (CAS #2068-78-2) and DXO hydrochloride (CAS #25316-40-9) were obtained from LKT Laboratories Inc (St Paul, MN, USA). Stock solutions of VCR, DXO and the MIX 1:1 were made up at 1000× in sterile water and stored at −80°C. Trypsin/EDTA 0.25% was from Thermo Fisher Scientific (#25200-056).

Cell culture and treatment

The GC-6spg cell line was maintained at 32°C and 5% CO_2, as described previously (Van Pelt *et al.* 2002). Cells were passaged once per week, and the culture medium was changed after three days. The day before treatment, GC-6spg were seeded at 19,000 cells/cm^2. 1000× stock solutions of DXO, VCR and their mixture were diluted to 1× (1 nM–10 µM) into the culture media immediately before use, and control cells received 1:1000 of the water vehicle. GC-6spg cells were exposed to 1 nM–10 µM DXO, VCR or their MIX for 24–72 h. The range of concentrations was chosen to first establish the subtoxic dose based on cell death and proliferation assays, and further select sub-lethal dose for characterizing the capacity for DNA repair in this cell line. In a recovery study, cells were exposed to 0.1 µM DXO for 24 h and further maintained in control medium for 5 days. For viability and BrdU incorporation assays, cells were proceeded directly in culture plates, whereas they were resuspended by trypsin treatment (3 min at room temperature) for apoptosis and COMET assays and for RNA or protein extractions.

Viability assay

Cell viability after treatment was investigated using the MTT (thiazolyl blue tetrazolium bromide) assay as previously described with few modifications (Riss *et al.* 2004). Briefly, GC-6spg were incubated in a 96-well plate with 0.05 mg/well of MTT (#M5655, Sigma), diluted in culture medium for 3 h at 32°C, after which half of the medium was replaced by DMSO/isopropanol (1:1). The optical density was revealed by spectrometry at 540 nm and expressed as a percentage of formazan formation in control cells treated with vehicle. Wells without cells served as a blank, and cells exposed for at least 24 h to 10% DMSO were used as positive control.

Apoptosis and cell death assay

Apoptosis and cell death were quantified by flow cytometry using the AnnexinV-FITC kit from Miltenyi Biotec (Auburn, CA, USA) as previously described (Delbes *et al.* 2013). According to the manufacturer's recommendations, resuspended GC-6spg cells were incubated with Annexin-V-FITC (10 µL per 10^5 cells) for 15 min in the dark at room temperature. After one wash, cells were resuspended in propidium iodide (PI) solution (1:60) and immediately analyzed using the FACSCalibur flow cytometer

(BD Biosciences, Mississauga, ON, Canada). Experimental gates were established using unstained GC-6spg as a negative threshold value. GC-6spg treated 16 h with 5% DMSO were used as positive control. Data were analyzed using the 3D WinList 8.0 software (Verity Software House, Topsham, USA). The Annexin V^-/PI^- represents the viable cells, PI-positive cells are dead and cells Annexin V^+/PI^- are apoptotic.

Cell proliferation assay

Proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation using the FITC BrdU kit (BD Biosciences) as previously described (Delbes *et al.* 2007*a*). Briefly, cells were fixed after a 4-h incubation at 32°C with 1 mM BrdU followed by immunofluorescent staining according to the manufacturer's recommendations (BD Biosciences, San Jose, CA, USA). Cells were visualized using the Nikon Eclipse Ti inverted fluorescent microscope equipped with a DS-Ri2 camera (Nikon). The BrdU incorporation index (percentage of labeled cells) was obtained by counting at least 100 cells spread over 4 random areas.

DNA break assay

Single- and double-strand DNA breaks were evaluated using the alkaline COMET assay, as previously described with some modifications (Delbes *et al.* 2007*b*). Briefly, 2000 cells were resuspended 1:10 in low melting point agarose and evenly spread onto slides in duplicate (Trevigen Inc., Gaithersburg, MD, USA). After the gel solidified, slides were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO and 1% Triton X-100; final pH 10) for 30 min on ice in the dark. Slides were then immersed in a freshly prepared alkaline solution (1 mM EDTA, pH 13) for 30 min at room temperature before electrophoresis was performed in the same solution at 1 V/cm for 30 min in the dark. Slides were then fixed in ice-cold 70% ethanol for 5 min and stored at room temperature. Sample IDs were hidden from the experimenter before storing images. DNA was stained using SYBR Gold solution (Life Technologies) (1:30,000 in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and immediately analyzed using the Nikon Eclipse Ti inverted fluorescent microscope equipped with a DS-Ri2 camera (Nikon). Fifty cells per replicate were randomly analyzed for a total of 100 cells per sample, and fluorescent images were scored for COMET parameters (KOMET 6.0; Kinetic Imaging Ltd, Liverpool, UK) (Andor Technology Ltd., Belfast, UK).

RNA extraction

Total RNA was extracted from GC-6spg ($0.3–1.9 \times 10^6$ cells) using the RNeasy mini kit according to the manufacturer's recommendations (Qiagen). Residual genomic DNA was degraded by deoxyribonuclease treatment for 15 min. RNA quality and quantity was evaluated by using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Gene expression analysis by PCR array

RNA (1 µg) was reverse transcribed with the RT^2 first strand kit (Qiagen). cRNA (9 ng) was amplified in the CFX96 qPCR cycler (Bio-Rad) using the DNA damage signaling PCR array RT^2 Profile (#PARN-029ZD, Qiagen), according to the manufacturer's instructions. PCR efficiency and genomic DNA contamination were evaluated using the control supplied by the manufacturer. The analysis was done using the ΔΔCt method (pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). All data were normalized using Ldha and Rplp1 housekeeping genes. Statistical significance was tested by nonparametric ANOVA using a P value of 0.05, and probe sets from that list were filtered for those that had an expression of a minimum of 1.5-fold.

Protein extraction and Western blotting

Protein was extracted from GC-6spg ($0.5–3 \times 10^6$ cells) in RIPA buffer (150 mM NaCl; 1% Triton; 0.5% deoxycholate; 0.1% SDS and 50 mM Tris pH 8) containing protease inhibitor cocktail (Roche) using a needle (Gauge 20) and/or sonication (Q125, QSonica, Newtown, CT, USA) and centrifuged at 10,000 ***g*** for 8 min at 4°C. The remaining supernatant from each sample was aliquoted and stored at −80°C for protein assay (Pierce BCA, Thermo Fisher Scientific) and Western blotting. 10–15 µg protein of each sample were separated with 10% SDS-PAGE and immediately transferred onto PVDF membrane (Trans-Blot Turbo RTA transfer kit, Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). Total lane protein was stained with reversible MemCode protein stain PVDF (Thermo Scientific, Pierce) for normalization of the signal blotting. Membranes were blocked in 5% milk diluted in Tris buffer containing 0.1% Tween and further probed overnight with primary antibodies against P21 (1:2500; Abcam ab109199) or XRCC1 (1:200; Abcam ab1838) diluted in 3% non-fat milk. Horseradish peroxidase-coupled anti-rabbit or anti-mouse antibodies (Cell Signaling Technology) were used to detect antigen–antibody interactions by enhanced chemiluminescence (Bio-Rad). Capturing the signal was performed with Chemidoc MP Imaging Systems and quantified by densitometric analysis using Image Lab 5.2.1 software (Bio-Rad).

Statistical analysis

All experiments were repeated at least three times in duplicate. Statistical analyses were done using GraphPad Prism, version 5.01 (GraphPad Software). Dose- and time-dependent cytotoxicity due to the treatments was determined using a 2-way analysis of variance followed by the Bonferroni test ($P < 0.05$). Dose-dependent effects of the treatments on apoptosis, DNA breaks and protein expression were analyzed using a 1-way analysis of variance followed by the Dunn's *post hoc* tests ($P < 0.05$). Time-dependent effects after treatment arrest compared to the time-matched control group on cytotoxicity, proliferation and DNA breaks were analyzed using a 2-way analysis of variance followed by the Bonferroni test ($P < 0.05$).

Results

Cytotoxicity of VCR, DXO and the MIX

The cytotoxicity of VCR (1 nM–10 µM), DXO (1 nM–10 µM) and their MIX (1 nM–1 µM) was tested on the GC-6spg cell line using the MTT assay, after 24, 48 and 72 h of exposure (Fig. 1). A dose- and time-dependent cytotoxicity was observed for VCR (Fig. 1A) and DXO (Fig. 1B), with a lowest observed adverse effect level (LOAEL) of 0.1 µM at 72 h for both compounds (Table 1). Importantly, the effect of the MIX was more cytotoxic than each compound alone as we measured an LOAEL of 0.01 µM at 72 h (Fig. 1C and Table 1), suggesting a synergic effect of the mixture.

Interestingly, the dose–response curves in response to the two compounds alone showed very different shapes. At each time, the maximum effect of VCR was obtained with 0.1 µM. Moreover, although the maximum effect of exposure to VCR was a significant reduction of half of the MTT signal (Fig. 1A), exposure to DXO drastically shut off the MTT signals (Fig. 1B). On the other hand, the MIX showed similar response curves to DXO alone. To further understand the differential cytotoxicity response curves in response to VCR, DXO or their MIX, the Annexin V /propidium iodide assay was done to quantify apoptosis and cell death in GC-6spg (Fig. 2). After 48 h of treatment, VCR alone induced a significant increase in apoptosis and cell death from 0.1 µM (Fig. 2A), suggesting that the maximum of cytotoxicity could be due to a balance between cell proliferation and cell death. On the other hand, DXO and the MIX did not induce apoptosis but cell death significantly at 1 µM and 0.1 µM respectively (Fig. 2B and C), corresponding to the doses when the MTT signal shut off at that time.

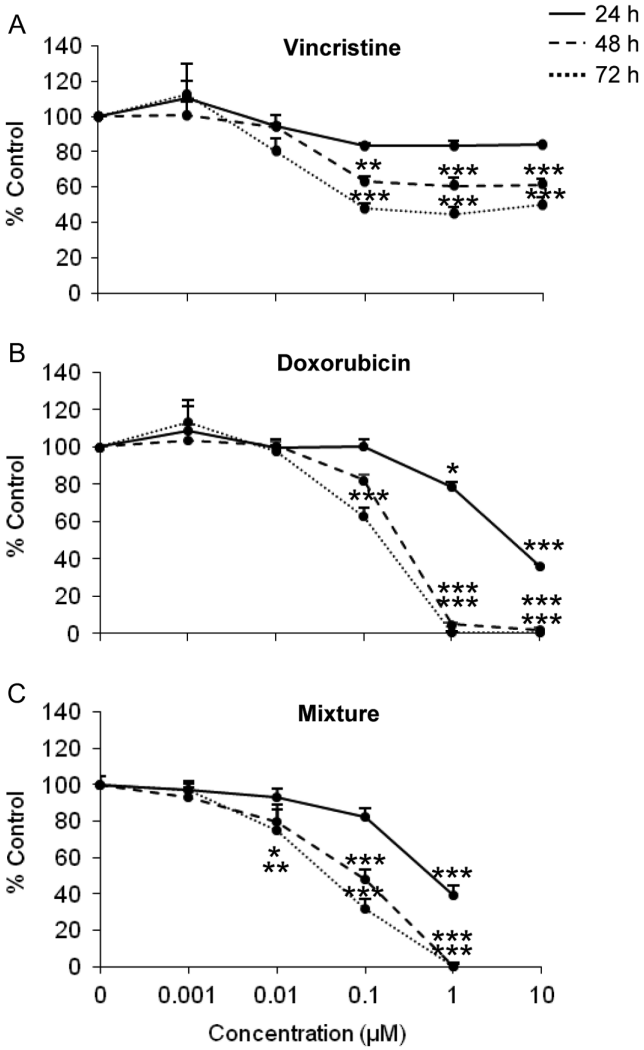

Figure 1 Cytotoxicity of vincristine, doxorubicin and their mixture on GC-6spg viability. GC-6spg cells were exposed to vehicle (control) or to different concentrations of vincristine (A), doxorubicin (B) or their mixture 1:1 (C). Cytotoxicity was evaluated using the MTT assay after 24, 48 and 72 h (A, B and C). Data are expressed as the % of control and represent the mean ± S.E.M. ($n = 4$). Time ($P < 0.0001$) and dose effects were analyzed by a two-way analysis of variance followed by the Bonferroni test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to the time-matched control group.

Induction of DNA breaks by DXO and the MIX but not VCR

To assess the potential of VCR, DXO and the MIX to induce DNA strand breaks on GC-6spg, we measured the % tail DNA using the COMET assay in alkaline conditions after 24 h of exposure to increasing doses (Fig. 3). Although we did not observe any effect of VCR at the doses tested, exposure to DXO and the MIX from 0.1 µM significantly increased the % tail DNA in GC-6spg. These effects were observed on GC-6spg at non-cytotoxic doses of DXO and the MIX (0.1 µM; Fig. 1B and C). In addition, no difference was observed between exposure to DXO alone or the MIX, suggesting that there are no synergistic effects on DNA breaks between the two compounds. Because we aimed to determine the capacity of spermatogonia to respond to genotoxic stress in a non-cytotoxic condition, we restricted further analysis to the exposure to DXO or MIX at 0.1 µM for 24 h.

DNA break recovery after DXO treatment

To determine the ability of spermatogonia to recover from exposure to 0.1 µM DXO for 24 h, we maintained the cells after treatment for up to 5 days in control

Table 1 Determination of the cytotoxicity lowest observed adverse effect level (LOAEL) of doxorubicin, vincristine and their mixture 1:1 on GC-6spg (calculated based on Fig. 1).

LOAEL (µM)	**24 h**	**48 h**	**72 h**
Vincristine	–	0.1	0.1
Doxorubicin	1	1	0.1
Mixture	1	0.01	0.01

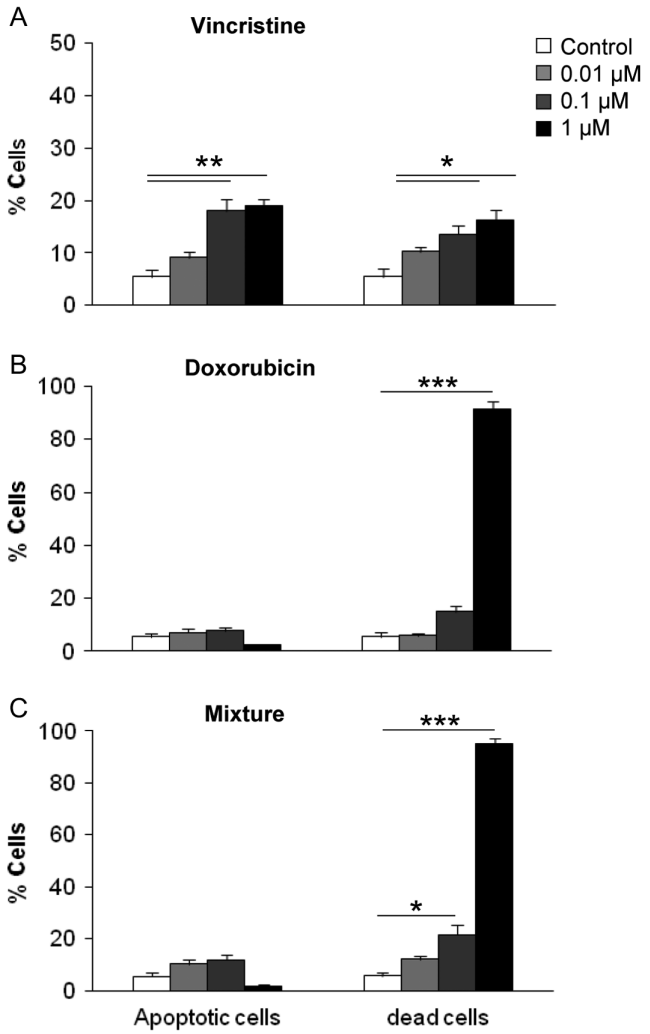

Figure 2 Impact of vincristine, doxorubicin and their mixture on apoptosis and cell death. GC-6spg cells were exposed to vehicle (control) or to different doses of vincristine (A), doxorubicin (B) and their mixture 1:1 (C) for 48 h. Percentages of apoptosis and cell death were quantified by annexin V and propidium iodide staining respectively. Data are expressed as mean ± S.E.M. ($n = 4–6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ using a Kruskal–Wallis test followed by a Dunn's multiple comparison test to control group.

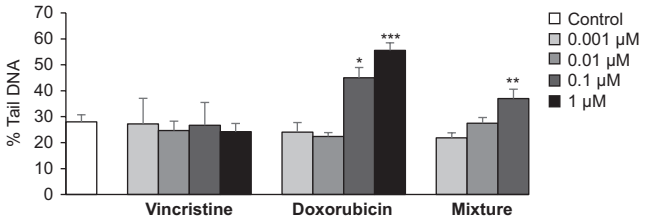

Figure 3 Impact of vincristine, doxorucibin and their mixture on DNA breaks in GC-6spg. DNA breaks were quantified using the COMET assay after 24 h of exposure to vehicle (control), or different doses of vincristine, doxorubicin or their mixture 1:1. Data are expressed as the mean percentage of tail DNA ± S.E.M. ($n = 5–13$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, using a one-way analysis of variance followed by a Dunnett's multiple comparison test to control group.

conditions and assessed cell viability, proliferation and the amount of DNA breaks (Fig. 4A). From day 0 (R0) to day 5 (R5) of the recovery period, cell number and viability were indirectly assessed using the MTT assay (Fig. 4B), and proliferation was evaluated by the BrdU incorporation index (Fig. 4C). In control conditions, the regular increasing MTT signal from R0 to R5 indicated a significant increase in cell number (Fig. 4B). This was correlated with the active proliferation rate at R0 (Fig. 4C). However, we observed a decrease in the percentage of proliferating cells over time in control cells up to R5, which may be due to over confluence in culture wells at that stage (Fig. 4C). In parallel, DXO-treated cells did not show any change in the MTT signal, which became significantly different from controls from R2 to R5 (Fig. 4B). The proliferation rate was also significantly lower in DXO-treated cells at R0 compared to that in controls and remained low throughout the recovery period. Interestingly, in three prolonged experiments, we observed an increasing number of cells in DXO-treated wells after about 13 days of recovery (data not shown). More importantly, using the COMET assay, we observed that the significant increase of DNA breaks induced by the treatment recovered with time (Fig. 4D). Indeed, although in control cells, we did not observe any change in the % tail DNA measured by the COMET assay from R0 to R5, the significant increase in the percentage of tail DNA measured in DXO-treated cells at R0 significantly decreased to become similar to the controls from R2 to R5.

Expression of genes involved in cell cycle and DNA damage signaling

To further assess which DNA repair pathway is activated in spermatogonia after exposure to DXO, we screened the expression of 75 genes implicated in DNA repair using the DNA damage signaling PCR array RT^2 (Supplementary Table 1). In control GC-6spg, we determined the level of expression of the 75 genes involved in DNA repair according to their C_T (Table 2). Most of the DNA repair genes studied are moderately to highly expressed in GC-6spg. We further showed that 0.1 µM DXO and the MIX significantly affected the expression of 16 genes (14 and 13 genes respectively) with a fold-change at least 1.5-fold in GC-6spg (Fig. 5A, B and C). Out of the 16 deregulated genes, 11 were commonly affected by both treatments, 3 genes were exclusively activated by DXO (*Bard1*, *Cdc25c* and *Terf1*), and 2 others were exclusively changed by the MIX (*Gadd45g* and *Ung*). However, the genes that are significantly affected exclusively by one treatment are in fact also affected by the other but with lower significance or fold-change (Fig. 5C).

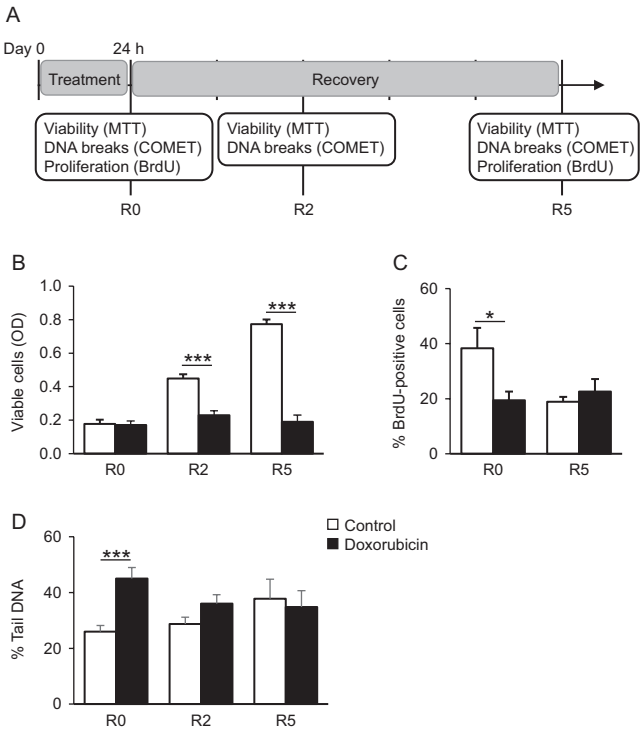

Figure 4 Survival, proliferation and DNA breaks in GC-6spg after 24-h exposure to 0.1 µM doxorubicin and after a recovery period. GC-6spg cells were exposed to vehicle (control) or 0.1 µM doxorubicin for 24 h. Viability, proliferation and DNA breaks were measured at the end of treatment (R0) or after a recovery period of 2 and 5 days (R2 and R5) (A). Optical density (OD) measured after the MTT assay ($n = 3–5$) (B), percentage BrdU-positive cells ($n = 3$) (C) and percentage of tail DNA measured by the COMET assay ($n = 4–16$) (D) are expressed as the mean ± S.E.M. and analysed by a two-way ANOVA followed by Bonferroni post-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to the time-matched control group.

Affected genes were further subdivided based on their known function. Out of the 16 significantly affected genes, 8 are linked to cell cycle and apoptosis and 8 others are actors of different DNA repair pathways (Fig. 5C). Interestingly, *Cdk1a* (cyclin-dependent kinase inhibitor 1A) was the most upregulated gene with a fold-change of 11.17 after DXO treatment. Further analysis of the level of CDKN1A protein in GC-6spg after 24-h exposure to 0.1 µM DXO or MIX was done by Western blot. Although this protein was very weakly expressed in controls GC-6spg, CDKN1A protein level was significantly increased by each treatment (Fig. 6A).

Most importantly, as shown in Fig. 5C, the expression of 8 genes implicated in DNA repair was affected by at least one of the treatments: 7 were upregulated (*Mgmt*, *Polh*, *Xrcc1*, *Rev1*, *Gadd45a/g* and *Bard1*) and 1 was downregulated (*Ung*). The expression of four of these genes (*Mgmt*, *Xrcc1*, *Gadd45a* and *Polh*) was similar to the control after exposure to the non-genotoxic dose of 0.01 µM (Supplementary Fig. 1), showing that the DNA repair gene activation is DNA break dependent. Interestingly, these 8 genes cover different DNA repair pathways such as base/nucleotide excision repair (BER/NER), homologous recombination (HR), or non-homologous end-joining (NHEJ) (Supplementary Table 1), suggesting that no specific pathway is activated in response to exposure to DXO or the MIX. Because *Xrcc1* is known to play a key role in the BER and NHEJ pathways, we further quantified its protein level by Western blot. XRCC1 protein level was not affected by the exposure to DXO or MIX after 24 h but was significantly increased after 48-h exposure (Fig. 6B). Together, these data demonstrated the ability of GC-6spg to activate DNA repair pathways after exposure to DXO.

Table 2 Expression levels of DNA repair genes in GC-6spg.

Level of expression	DNA repair pathway	Gene name
21 > Ct < 25	ATM	*Csnk2a2*
	Multiple	*Tp53, Parp1, Pcna, Smc1a*
	Other	*Pttg1, Rad21, Smc3, Sumo1, Wrnip1*
25 > Ct < 30	ATM	*Cdc25a*
	BER	*Ung, Apex1, Fen1, Xrcc1, Mpg, Wrn*
	DSB	*Rad51, Rad52, Xrcc2, Xrcc6, Tp53bp1, Mre11a, Blm, Nbn, Prkdc*
	NER	*Dclre1a, Ddb2, Ercc2, Pold3, Sirt3, Xpc*
	MMR	*Msh2, Msh3, Abl1, Exo1, Pms1, Pms2, Mlh1*
	Multiple	*Atm, Bard1, Brca1, Brca2, Chek1, Chek2, Fancd2, Hus1, Lig1, Nthl1, Ogg1, Parp2, Pole, Rad9, Rad17, Rad50, Rnf8, Rpa1, Topbp1*
	Other	*Mgmt, Atrx, Fanca, Fancg, Gadd45a, Gadd45b, Polh, Poli, Rad1, Rad18, Rad51b, Rad51c, Rev1*
30 > Ct < 32	BER	*Mbd4*
	NER	*Ercc1, Fancc*
	MMR	*Mlh3*

Expressed genes are presented according to their level of expression: high (21 > Ct < 25), moderate (25 > Ct < 30), and low (30 > Ct < 32); and their associated repair pathway.
ATM, ataxia telangiectasia mutated; BER/NER, base/nucleotide excision repair; DSB, double strand break repair; MMR, mismatch repair.

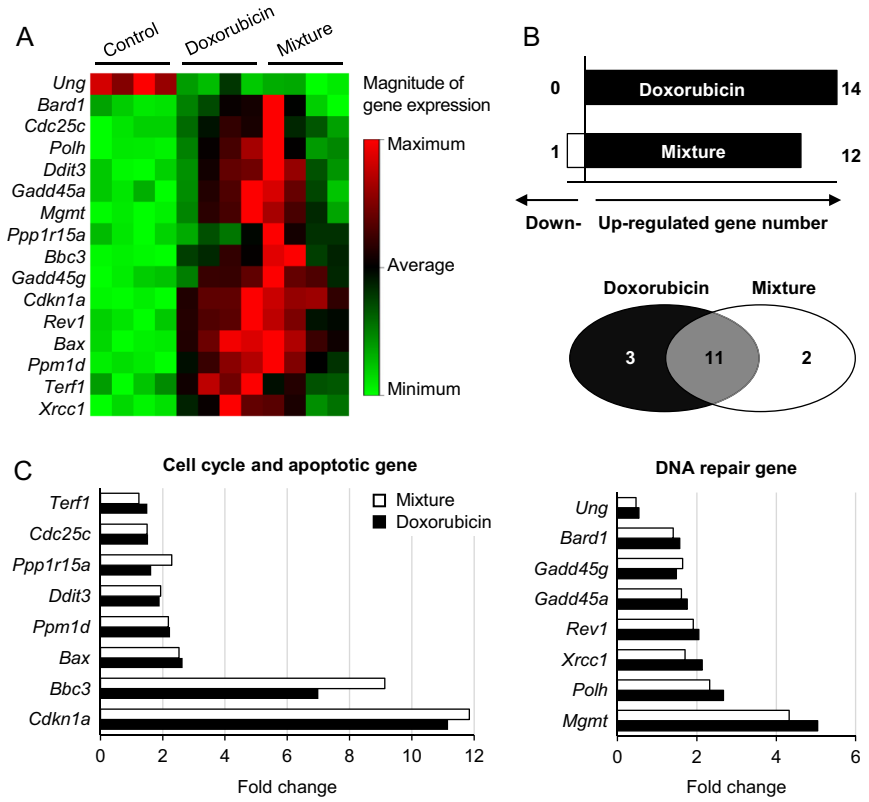

Figure 5 Impact of DXO alone or in mixture with vincristine on gene expression after 24 h of exposure to 0.1 µM. GC-6spg cells were exposed for 24 h to the vehicle (control) or to 0.1 µM doxorubicin alone or in mixture 1:1 with vincristine (Mixture). Gene expression was evaluated by RT-qPCR using the PCR array RT2 kit from Qiagen in GC-6spg ($n = 4$). Significantly affected genes are represented according to their magnitude of gene expression in each biological replicate by row clustering (A), the number of upregulated or downregulated genes in each with the corresponding Venn diagram (B) and their fold-change among the cell cycle/apoptotic genes and DNA repair genes (C).

Discussion

In this study, we evaluated the toxicity of DXO and VCR, two commonly used anticancer drugs, on a rat spermatogonial cell line with SSC characteristics (Van Pelt *et al.* 2002). It is well established that most chemotherapeutic agents can target the dividing male germ cells (Meistrich 2013) and very recently, Habas and coworkers have shown that DXO induces apoptosis and DNA breaks in male germ cells, with spermatogonia being the most sensitive type of germ cell (Habas *et al.* 2016). Indeed, diploid cells in proliferation are the main target of the chemotherapeutic compounds, which explains the high sensitivity of the precursors and pre-meiotic germ cells compared to the non-replicative meiotic germ cells (Jahnukainen *et al.* 2000). SSCs are at the origin of spermatogenesis and any damage in these cells can have consequences on the fertility of the individual. Evidence of a direct negative impact specifically on SSC comes from a small number of studies using transplantation assays or *in vitro* models (Marcon *et al.* 2010, 2011, Liu *et al.* 2014). However, to our knowledge, the direct effect of VCR and DXO had never been tested before using an *in vitro* model of a spermatogonial cell line. We have demonstrated that both compounds are cytotoxic to GC-6spg with synergistic effects. Interestingly, their profiles of cytotoxicity are very different; although VCR reduces the MTT signal by half with a maximum effect that plateaus at 0.1 µM, DXO completely shuts the signal off in a dose-dependent manner. This is in line with their mechanisms of action. In fact, as VCR classically inhibits microtubule polymerization (Jordan & Wilson 2004), our results suggest that GC-6spg are mitotically arrested with few dying by apoptosis. On the other hand, DXO is primarily used as a topoisomerase II inhibitor in many types of cancer, but it has been shown to induce DNA crosslinks also, DNA double-strand breaks (DSB) and produce oxidative stress inducing high cytotoxicity and many side effects (Rochette *et al.* 2015). In GC-6spg, DXO induced a dose-dependent cell death.

According to cytotoxicity assay, we observed a synergistic effect on cell viability when GC-6spgs were exposed to a mixture of both compounds. Indeed, the LOAEL of the MIX was lower than the one of each compound alone. These results underline the importance of considering the impact of each compound alone and in combination to evaluate the chemotherapy damage on the germline. This is important to consider as one strategy to improve the efficacy of chemotherapy is to give combinations of drugs. Such mixtures allow each drug to be given at a lower dose with the hope of reducing toxic side effects. Our present data, in accordance with others, suggest that such combinations even at lower doses are cytotoxic to SSC (Marcon *et al.* 2010).

Chemotherapeutic compounds are known to induce DNA damage in the male germline of cancer survivors (O'Flaherty *et al.* 2010, 2012). *In vitro* studies have demonstrated the genotoxic potential of some chemotherapeutic compounds using spermatogonial

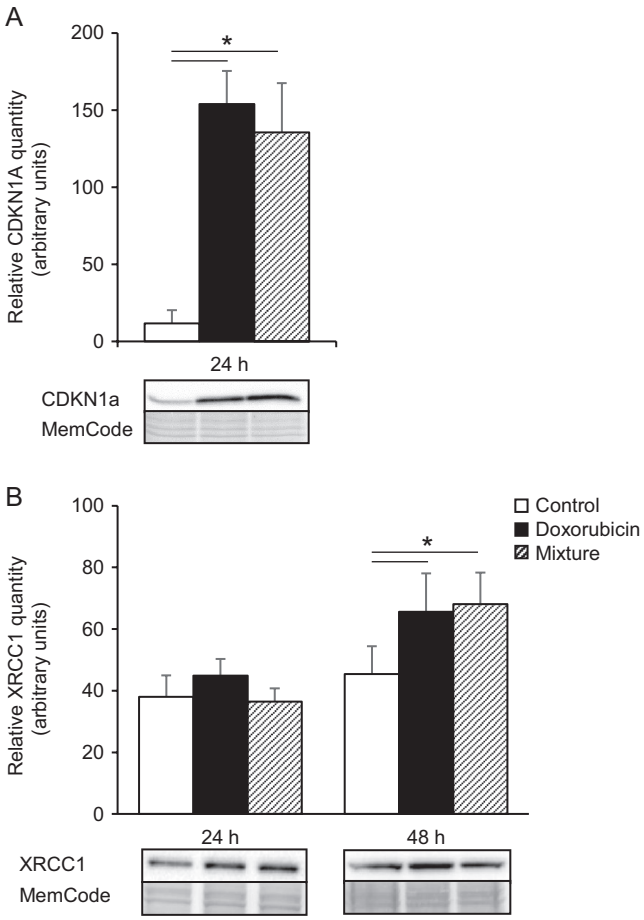

Figure 6 Protein quantification of CDKN1a and XRCC1 in GC-6spg cells after 24 h or 48 h of treatment with doxorubicin alone or the mixture 1:1. After 24-h or 48-h exposure to vehicle (Control), 0.1 µM of doxorubicin alone or in mixture 1:1 with vincristine (Mixture), proteins were extracted from GC-6spg cells and used to quantify CDKN1a (A) or XRCC1 (B) by Western blot. For each protein, representative blots are shown and their quantification by densitometry represent the mean ± S.E.M. ($n = 4–7$). *$P < 0.05$ compared with the corresponding control group using a paired *t*-test.

cell lines (Liu *et al.* 2014) or primary culture of germ cells (Habas *et al.* 2016) and SSC (Marcon *et al.* 2010). To test the mutagenic potential of VCR and DXO alone or in mixture, we used the COMET assay to reveal double- and single-strand breaks. We showed that DXO but not VCR induced DNA breaks in GC-6spg. This is in accordance with the DXO mode of action that intercalates into DNA and binds the topoisomerase II to block its progression leading to DNA DSB (Rochette *et al.* 2015). Importantly, DXO-induced DNA breaks at a dose below its cytotoxic LOAEL. Interestingly, genotoxicity at a non-cytotoxic dose had already been demonstrated for etoposide, another topoisomerase II inhibitor in a mouse spermatogonial cell line (Liu *et al.* 2014). The MIX induced comparable effect as DXO alone which is in accordance with the fact that VCR alone had no effect on DNA breaks. *In vivo*, it was shown that doxorubicin-exposed SSC in prepubertal animals resulted in long-term damage to mature sperm DNA (Hou *et al.* 2005, Vendramini *et al.* 2012). Moreover, we have shown that male rats exposed to DXO in combination with other anticancer drugs (CHOP regimen) had significantly increased the amount of DNA breaks in spermatozoa even after a recovery period (Delbes *et al.* 2010*b*, Vendramini *et al.* 2012). Such persistent DNA damage in spermatozoa after a recovery period suggests impaired DNA repair ability in male germ cells. Our model of DNA break induction at a non-cytotoxic dose of DXO or MIX is therefore a unique opportunity to study the defense mechanisms existing in precursor male germ cells against genotoxic stress.

Classically, the response of mammalian cells to DNA damage is an arrest of the cell cycle and DNA repair, which if deficient is followed by cell death. Our data strongly suggest such a cell cycle arrest in GC-6spg in response to 0.1 µM of DXO for 24 h. Indeed, GC-6spg proliferation measured by BrdU incorporation was significantly decreased for up to 5 days after the end of treatment. Interestingly, we observed that GC-6spg re-enters mitosis about 13 days after treatment arrest (data not shown). In addition, the demonstration of the increased expression of *Cdkn1a* mRNA and protein in GC-6spg in response to DXO strengthens the hypothesis of a cell cycle arrest. Interestingly, in most adult stem cells, such as hematopoietic stem cells and mammary stem cells, the cell cycle arrest by CDKN1a was shown to induce a reduction of DNA damage accumulation (Insinga *et al.* 2014). This temporary cell cycle arrest observed in our *in vitro* model nicely mimics the common *in vivo* kinetics of oligospermia during chemotherapy followed by spermatogenesis recovery after treatment arrest (Vendramini *et al.* 2010, Bujan *et al.* 2014) and suggests that our *in vitro* model is very relevant to investigate germ cell response to chemotherapeutic insult. In the recovery study, we have further demonstrated that GC-6spg can recuperate from the DNA breaks induced by the treatment. It is important to consider that the level of DNA damage could have decreased over time in treated cells because GC-6spg with slight DNA damage had taken over highly damaged cells. Nevertheless, assessing the DNA repair pathways at the time of treatment arrest would help better understand the mechanism by which spermatogonial stem cells respond to DNA injury.

To elucidate the ability of GC-6spg to repair DXO-induced DNA damage, we screened many DNA repair genes using the DNA damage signaling PCR array RT^2. Our data showed that all the 75 DNA repair genes tested were expressed, suggesting the high ability to respond to genotoxic stress. To the best of our knowledge, this is the first demonstration of the constitutive expression of a large library of DNA genes in spermatogonia with SSC characteristics. In GC-6spg, we showed that DXO treatment alone or in mixture affected the expression

of 16 genes playing roles in cell cycle regulation and DNA repair. This high overlap of effects between the two treatments strongly suggests that in the MIX, DXO is responsible for all gene expression changes observed. Importantly, DXO treatments activated genes related to various DNA repair pathways. Genotoxic stress activates specific DNA repair pathways depending on the nature of the injury (Genois *et al.* 2014). DXO toxicity is mediated by different modes of action as described previously, and as a consequence, multiple DNA repair pathways could be triggered. Surprisingly, the *Mgmt* gene (O^6–methylguanine–DNA methyltransferase) was the most activated DNA repair gene with a fold-change of 5.04 after exposure to DXO alone. MGMT protein is involved in direct damage reversal as it catalyzes the repair of alkylated guanines by stoichiometrically and irreversibly transferring O^6-alkyl adducts in a suicide reaction (Tubbs *et al.* 2007). Thompson and coworkers have already demonstrated the role of MGMT in the survival of SSC after alkylating treatment (Thompson *et al.* 2000). It was shown that DXO-induced oxidative stress can lead to the alkylation of DNA (Taatjes *et al.* 1997), but to our knowledge, this is the first demonstration of *Mgmt* mRNA expression activation in response to DXO.

DXO-induced DSBs, the most deleterious DNA damage, could be processed by two main DNA repair pathways: the error-free HR pathway that happens during the S/G2 phase of the cell cycle and the NHEJ pathway that proceeds by default through the error-prone ligation of broken DNA ends without sequence homology (Rothkamm *et al.* 2003). The NHEJ pathway is presumed to be the main repair mechanism of DSBs in undifferentiated spermatogonia (Rube *et al.* 2011). Indeed, SSCs are mostly in the G0/G1 phase and components of the canonic NHEJ pathway, including DNA-PKcs (encoded by the *Prkdc* gene), Ku70 (encoded by the *Xrcc6* gene) and Ku80 (encoded by *Xrcc5* gene) are known to be expressed by undifferentiated spermatogonia (Hamer *et al.* 2003, Rube *et al.* 2011). In agreement with these previous studies, the expression of *Prkdc* and *Xrcc6* were detected in GC-6spg. Unfortunately, we could not test the expression of *Xrcc5* as it was not included in the PCR array design. The NHEJ pathway can be further subdivided in the canonic and alternative pathways. Both use the MRE11/RAD50/NBS1 protein complex to detect DSBs and activate the ATM protein (Lavin 2007). The activation of ATM then triggers a cascade of reactions indirectly triggering the cell cycle regulator CDKN1a and the components of apoptosis, BBC3 and BAX, by the activation of P53 (Insinga *et al.* 2014). In our model, the activation of *Cdkn1a*, *Bbc3* (Bcl-2 binding component 3) and *Bax* (Bcl2-associated X), therefore suggests that the NHEJ pathway can be involved in the GC-6spg response to DXO-induced DNA breaks. On the other hand, the alternative NHEJ that is DNA-PK independent was also suggested to be involved in the repair of DSBs in SSC (Rube *et al.* 2011). Indeed, a deficiency of the canonic NHEJ could be compensated by an alternative NHEJ pathway involving XRCC1 (Audebert *et al.* 2004, Velic *et al.* 2015). Interestingly, in our model, *Prkdc* and *Xrcc6*, coding for proteins involved in the DNA–PK complex, were not affected after DXO treatment, whereas *Xrcc1* was significantly activated. According to these results, we hypothesize that the alternative NHEJ pathway is activated in undifferentiated spermatogonia in response to DXO. This hypothesis is supported by data showing the complete absence of components of the canonic NHEJ pathway such as γH2AX and MDC1 (Rube *et al.* 2011). However, our data show that DXO induced the expression of genes related to various other DNA repair pathways such as BER and NER, which are known to be active in spermatogonia (Intano *et al.* 2002, Xu *et al.* 2005).

In the present study, we originally tested a range of concentrations of DXO and VCR alone or in mixture to establish the sub-toxic dose based on cell viability, and further select sub-lethal dose for characterizing the capacity for DNA repair. However, the concentrations tested also included the reported plasmatic concentrations of DXO and VCR after clinical i.v. injections in children with acute lymphoblastic leukemia or non-Hodgkin lymphoma (Sethi & Kimball 1981, Hempel *et al.* 2002). Plasma levels may not reflect the levels of exposure of spermatogonia but intra-testicular levels of chemotherapeutic compounds after treatment in human is unknown. On the other hand, pharmacokinetic studies in rats suggest that testicular levels of DXO are higher than plasma levels after acute exposure (Arakawa *et al.* 1991, Nwankwoala *et al.* 2008). In addition, testicular levels appear to remain stable 48 h after treatment, whereas plasma levels decrease. This suggests that testicular cells are exposed to doses equivalent to or greater than plasma concentrations. Even though it is difficult to extrapolate *in vivo* exposure and *in vitro* experiments, together, these data suggest that the cellular outcomes quantified in this study are relevant to human exposure.

Considering the imperative use of chemotherapy to combat malignant cells, the improvement of anticancer clinical protocols to decrease side effects and provide protection of healthy cells is urgently needed. Toward this objective, decreasing doses and combining drugs have been successful. As well, co-treatment with cytoprotective compounds are now included in oncology clinical guidelines to reduce neurotoxicity (Hershman *et al.* 2014). Here, we show that a common combination of DXO and VCR can be toxic to rat GC-6spg. However, more importantly, we have established a model to study DNA repair in undifferentiated spermatogonia. Our results support the idea that undifferentiated spermatogonia have the ability to respond to DNA injury and escape from DNA break accumulation. Further studies will be needed to

elucidate which pathway is essential for the maintenance of DNA integrity in SSC. To open up fertility protection strategies, understanding the mechanism by which anticancer drugs affect human SSC is essential.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0005.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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