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MITOXANTRONE ABILITY TO INDUCE PREMATURE SENESCENCE IN HUMAN DENTAL PULP STEM CELLS AND HUMAN DERMAL FIBROBLASTS

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In this study we assessed the effects of the frequently used chemotherapeutic agent mitoxantrone (MTX) on dental pulp stem cells (DPSCs) and compared it with the response of human dermal fibroblasts (HDFs). DPSCs are valuable source of mesenchymal stem cells which may be extremely useful in a number of clinical applications. It is evident that both normal and tumor cells are being affected during therapy and characterization of these cells under genotoxic stress contributes to the evaluation of their safety usage. In the experiment cells were exposed to doses 5–150 nmol/l MTX. Proliferation of cells was detected by Z2 counter and viability by Vi-Cell XR using Trypan blue exclusion staining. Cell cycle analysis was determinated by flow cytometry, induction of apoptosis by monitoring the activities of caspases. The expression of key proteins was detected by Western blotting. Senescence was analyzed by activity of β-galactosidase and by detection of persisting DSBs-associated γH2AX foci. Exposure of both cell types to lower concentrations of MTX resulted in premature senescence (SIPS), which was accompanied with typical morphological changes, increased activity of senescence-associated β-galactosidase, persisting DSBs-associated γH2AX foci and cell cycle arrest in G2 phase. MTX provokes the activation of p53-p21$^{WAF1/Cip1}$ pathway in both cell types and activates cell-cycle inhibitor p16^{INK4a} in HDFs, but not in DPSCs. Higher concentrations of MTX induced caspase-mediated apoptosis. Conclusions: MTX induces apoptosis or SIPS in both cell types in dependency on MTX doses. Both pathways prevent the proliferation of cells with damaged DNA.

Key words: *mitoxantrone, stress-induced premature senescence, apoptosis, dental pulp stem cells, dermal fibroblasts*

INTRODUCTION

Mesenchymal stem cells (MSCs) represent intensively studied field since the first description of MSCs by Friedenstein (1). This type of cells occurs practically in every tissue in the adult human body. They are a promising tool for tissue reparation, regeneration and/or reconstitution of specific tissues. One of the biggest problems of the therapeutic use of MSCs remains the accessible stem cells collecting, because the most of the tissues is not a suitable source of these cells. A lot of studies have focused on the MSCs isolated from adult bone marrow, but MSCs were obtained also from other tissues including *e.g.* liver, kidney, brain, lung, umbilical cord blood, fetal blood or adipose tissue (2). The selected tissue for MSCs collecting should contain high amount of these cells compared to the volume of the collected tissue. One of the appropriate tissues which fulfill this condition is dental pulp and thus currently it became a valuable source of autologous MSCs (3). Dental pulp is easily removable from teeth recovered by standard dental procedures and an efficiency of the extraction procedure of the stem cells from the pulp tissue is high (4). Dental pulp stem cells (DPSCs) possess the properties of self-renewal capacity, multi-lineage differentiation and high proliferative potential (5). Whereas these properties of DPSCs are relatively well known (6-8), there is not much information about their reaction to stress conditions, such as DNA damage triggered by genotoxic stress. Other important component of tissues are fibroblasts, which are involved in the regulation of epithelial differentiation and in the regulation of inflammation, and recent studies also proved their role in the process of tumourigenesis (9, 10). Response of fibroblasts to DNA damage inducing-agents also represents relatively unexplored field.

In this study we focus on the reaction of DPSCs to DNA damage caused by mitoxantrone (MTX) and compare it with the reaction of HDFs. MTX is an anthracenedione derivative with antitumor and immunosuppressive activities. MTX as a chemotherapeutic agent is used in treatment of acute nonlymphoblastic leukaemia, chronic myelogenous leukaemia, non-Hodgkin's lymphoma and other malignant diseases such as breast cancer, ovarian and hepatic cancer (11). In present, MTX is used also in treatment of multiple sclerosis (MS), since it acts an immunosuppressant that inhibits B-cell, T-cell, macrophage proliferation and promotes the maturation of natural killer cells (12). MTX has influence on enzymatic parameters of antioxidative status in patient suffering from MS and provokes the reduction of antioxidative enzymes activities (13). Despite

this fact, MTX is successfully used in clinical medicine; the drug limits the frequency of relapse and is effective in slowing the progression of several variants of MS (14).

MTX mechanisms of action involve DNA intercalation through hydrogen bonding as well as inhibition of topoisomerase II which induces double strand breaks (DSBs) of DNA (15). Central mediator in early response of cells to DSBs is ATM kinase. ATM phosphorylates and activates other target proteins such as p53, Chk1, Chk2, BRCA1 or H2AX and thus triggers the complex network of pathways (16). The networks of molecular reactions are triggered simultaneously and may lead to induction of apoptosis, or cell cycle arrest enabling reparation of DNA damage. Some cell types reacts to DSBs also by permanent cell cycle arrest known as stress-induced premature senescence (SIPS). The final reaction of cells to DSBs depends on the prevailing type of activated signaling pathways (17).

Many studies were performed to elucidate the response of topoisomerase II inhibitors in tumor cell lines, less in non-tumor cells and none in MSCs. Whereas some studies have shown that inhibitors of topoisomerase II led to induction of apoptosis (18-24), further works have shown their ability to induce SIPS (25, 26). Which pathway will prevail in MSCs after MTX exposure is currently unknown. In our previous studies, we have revealed the reaction of DPSCs to irradiation and cisplatin. We have found that irradiation provokes SIPS without any marks of apoptosis (27), whereas cisplatin treatment may lead also to induction of apoptosis (28) in DPSCs.

It is obvious that not only tumor cells, but also normal cells are exposed to DNA-damage inducing agents during anticancer treatment (29). Optimal therapeutical protocol would induce irreparable damage to cancer cells, while it should preserve physiologically functional mesenchymal cells of surrounding healthy tissues. Although many studies of reaction of tumor cells to topoisomerase II inhibitors were performed, no such studies of their effect on mesenchymal stem cells and normal fibroblasts exist.

This is the first study which reveals the response of DPSCs to a representative topoisomerase II inhibitor MTX in clinically relevant concentrations (30, 31) and thus contributes to the characterization of DPSCs response to genotoxic stress.

MATERIAL AND METHODS

Cell cultures, isolation and culture conditions

Subjects or their parents/guardians gave written informed consent to use the extracted teeth in this study following ethical approval by the Ethics Committee of the Medical Faculty in Hradec Kralove. We isolated DPSCs from impacted third molars obtained from healthy donors undergoing tooth extraction for orthodontic reasons. The cells were treated with our standard operation protocols. In short, third molars were extracted under sterile conditions and dental pulp was isolated in the tissue culture laboratory. Both the tooth and dental pulp were treated by enzymes - collagenase (Sevapharma, Prague, Czech Republic) and dispase (Invitrogen, Carlsbad, CA, USA) for 70 minutes. Following centrifugation (600 g, 5 min) we obtained a cell pellet. DPSCs were cultivated in 5% CO_2 atmosphere under 37°C in low fetal calf serum cultivation media composed of alpha-minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA), heat inactivated 2% fetal calf serum (FSC; PAA, Dartmouth, USA), 10 ng/ml EGF, 10 ng/ml PDGF (both from PeproTech, Rocky Hill, NJ, USA), L-ascorbic acid (Sigma, St. Louis, MO, USA), 2% glutamine, penicillin/streptomycin, gentamycin (all from Invitrogen, Carlsbad, CA, USA) and 50 nmol/l dexamethasone (Sigma, St. Louis, MO, USA). DPSCs were initially cultivated for 5–7 days in culture flasks with Cell$^+$ surface® (Sarstedt, Newton, NC, USA). After this, small colonies were found and using trypsin-EDTA (ethylenediaminetetraacetic acid) (Invitrogen, Carlsbad, CA, USA) we dissociated them and reseeded into standard tissue culture treated flasks (NUNC, Roskilde, Denmark). Each of the following passaging was done after reaching 70% confluence. The DPSCs used in this study were characterized and their cytogenetical stability and differentiation potential tested in our previous studies (6, 32, 33). To control quality of the cell population, phenotype characteristics (CD45, CD29, CD44, CD73, CD90, CD166) were controlled every 1st, 3rd, 7th and 11th passage, morphology and viability were tested on every passage, karyotype is controlled on passage 5 and 10, and length of telomeres was measured on passage 0, 2, 5, 7 and 10. No significant changes were detected during cultivation. Early passages (3rd-9th) were used for the experiments.

Normal human dermal fibroblasts were purchased from PromoCell (Heidelberg, Germany). The cells were isolated from normal human juvenile foreskin. HDFs were cultivated in 5% CO_2 at 37°C in cultivation media composed of Dulbecco´s Modified Eagle´s medium (DMEM; Invitrogen, Carlsbad, CA, USA), 10% fetal calf serum (FSC; PAA, Dartmouth, USA), penicillin/streptomycin and glutamine (both from Invitrogen, Carlsbad, CA, USA).

Chemotherapeutic agent

The cells were exposed to chemotherapeutic agent mitoxantrone (Sigma-Aldrich, St. Louis, MO, USA). At all events, the stock solutions were prepared by dissolving 1.3 mg of MTX in 2.5 ml sterile water to reach 1 mmol/l concentration. The final concentrations to which the cells were exposed ranged from 5 to 150 nmol/l MTX in dependence on the character of experiment (described in the result section). All experiments were performed at least three times at each drug concentration per experiment.

Cytotoxicity

The WST-1 (Roche, Mannheim, Germany) reagent was used to determine the cytotoxic effect of MTX. WST-1 is designed for the spectrophotometric quantification of cell proliferation, growth, viability and chemosensitivity in cell populations using a 96-well-plate format (Sigma, St.Louis, MO, USA). The cells were seeded at a concentration 3×10^3 cells/well in 100 µl culture medium containing various concentration (10, 30, 50, 75, 100, 150, 200, 300 nmol/l) of MTX and incubated in 5% CO_2 at 37°C. WST-1 reagent (50 µl) was added 24 or 72 hours after MTX. Absorbance was measured after 3 hours incubation with WST-1 at 440 nm and a reference wavelength 690 nm. The measurements were performed in a Tecan Infinite M200 spectrometer (Tecan Group, Mannedorf, Switzerland). All experiments were performed at least three times with triplicate measurements at each drug concentration per experiment.

Activity of caspases

Induction of apoptosis was determined by monitoring the activities of caspase 3/7 by Caspase-Glo Assays (Promega, Madison, WI, USA) 72 hours after treatment with 50, 100 and 150 nmol/l MTX. A total of 1×10^4 cells were seeded per well using a 96-well-plate format (Sigma, St. Louis, MO, USA). The treated cells were cultivated in 50 µl culture medium and incubated 72 hours in 5% CO_2 at 37°C. After that, Caspase-Glo Assays reagents were added to each well (50 µl/well) and incubated for 30 minutes before measuring luminescence using

a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland).

Proliferation and viability

Cell proliferation and viability were determined 1, 3 and 6 days after treatment with 5, 20, 50, 100 and 150 nmol/l MTX. The cells were detached with 0.05% trypsin-EDTA (Invitrogen, USA) for 8 minutes. The trypsin-detached cells were pooled with medium containing floating cells. The measurement of proliferation was performed by Z2 Counter and viability by Vi-Cell XR 2.03 (both from Beckman Coulter, Miami, FL, USA) using Trypan blue exclusion staining.

Cell cycle analysis

After 1 day of incubation with 5, 20 and 50 nmol/l MTX, cells were washed with ice cold PBS and fixed with 70% ethanol. For detection of low molecular-weight fragments of DNA, the cells were incubated for 5 min at room temperature in buffer (192 ml 0.2 mol/l Na_2HPO_4 + 8 ml 0.1 mol/l citric acid, pH 7.8) and then stained with propidium iodide (PI) in Vindelov's solution for 60 minutes at 37°C. The DNA content was determined by flow cytometer BD FACS Aria III (Becton, Dickinson and Company, New Jersey, USA) using a 13 mW Coherent® Sapphire™ solid-state laser with excitation at 488 nm; total emission above 560 nm was recorded. List mode data were analyzed using BD FACS Diva 6.1.3. software (Becton, Dickinson and Company, New Jersey, USA).

Electrophoresis and Western blotting

The cells were harvested for preparation of whole-cell lysates (Cell Lysis Buffer, Cell Signaling Technology, Inc, Boston, MA, USA) 24 hours and 9 days after MTX application. The protein content was quantified using bicinchoninic acid assay - BCA assay (Sigma-Aldrich, St. Louis, MO, USA). The lysates containing an equal amount of protein (20 μg) were loaded into each lane of a polyacrylamide gel. After electrophoretic separation, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories s.r.o., Prague, Czech Republic). The membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk and then incubated with primary antibody (p53 - Exbio, Prague, Czech Republic; β-actin, p16, $p21^{WAF1/Cip1}$ - Sigma-Aldrich, St. Louis, MO, USA; p53_Ser15 - Calbiochem-Merck, Prague, Czech Republic; puma - Cell Signaling Technology, Inc, Boston, MA, USA) at 4°C overnight. After washing, the blotts were incubated with appropriate horseradish peroxidase-conjugated secondary antibody - Polyclonal Goat Anti-Mouse Immunoglobulins or Polyclonal Swine Anti-Rabbit Immunoglobulins (DakoCytomation, Brno, Czech Republic) - for one hour at room temperature. Antigen-antibody complexes were detected with a chemiluminescence detection kit (Roche, Prague, Czech Republic). The signal was quantitatively detected *via* autoradiographic film (Foma, Hradec Kralove, Czech Republic). To confirm equal protein loading each membrane was reprobed and reincubated to detect β-actin.

Microscopy

The cells were exposed to MTX in chamber slides (Thermo Fisher Scientific). Activity of β-galactosidase was detected by senescence β-galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA) according to manufactures instructions. For detection of γH2AX 1×10^4 cells were treated with MTX, centrifugated, washed in PBS and fixed with paraformaldehyde for 10 min at room temperature, then washed in PBS. Cells were blocked in solution with 7% FCS and 2% BSA and immunostained with appropriate primary antibody (γH2AX - Cell Signaling Technology, Inc, Boston, MA, USA) overnight at 4°C. The secondary antibody was applied to each slide (after their pre-incubation with 5.5% donkey serum in PBS for 30 min at room temperature) and incubation in the dark was succeeded by washing (3 × 5 min) in PBS. The nuclei were counterstained by DAPI solution (Sigma, St. Louis, MO, USA), incubated 8 minutes, washed and fixed. Images were obtained by Nikon Eclipse fluorescence microscope; the exposition time and dynamic range of camera in all channels were adjusted to the same values for all slides to obtain quantitatively comparable images.

Statistical analysis

The descriptive statistics of the results was calculated and the charts were made in the Microsoft Office Excel 2003 (Microsoft Inc., Redmond, WA, USA). In this study, all the values were expressed as arithmetic means with standard deviation (S.D.) unless otherwise indicated. The significant differences between the groups were analyzed by the Student's t-test and P values ≤0.05 were considered significant.

RESULTS

Cytotoxicity of mitoxantrone

The cytotoxicity of MTX was determinated by cell proliferation reagent WST-1. DPSCs and HDFs exposed to the range of MTX concentrations (10–300 nmol/l) showed a time- and dose-dependent decrease in cell viability. The cytotoxic effect of the drug was more noticeable after 72 hours, we observed steep decrease of viable cells up to 100 nmol/l MTX. From concentration of 150 nmol/l MTX and above approximately 30% of HDFs and 40% of DPSCs remained viable (*Fig. 1*). The results indicated the concentrations for further experiments: in subsequent experiments we studied the effects of lower concentrations of MTX - 5, 20 and 50 nmol/l, and higher concentrations - 100 and 150 nmol/l MTX. The WST-1 assay does not discern the induction of cell death from the inhibition of proliferation, therefore additional tests for proliferation, viability and apoptosis were performed.

Effects of mitoxantrone on proliferation and viability

We examined the effects of MTX on proliferation and viability using Trypan blue exclusion staining. At first, we measured proliferation of the cells during 6 days after treatment of both cell types with lower concentration of MTX (5, 20 and 50 nmol/l). We observed that the proliferation of treated DPSCs and HDFs was almost completely inhibited, the number of affected cells remained constant during 6 days after application of MTX (*Fig. 2A*). Moreover, concentration of 5, 20 and 50 nmol/l MTX did not cause significant decrease in viability during the whole interval (6 days). Viability of DPSCs and HDFs varied about 87–95% in control as well as in treated cells (*Fig. 2B*).

Further we focused on the effect of higher concentration of MTX. We measured the viability of cells 3 days after application of 100 and 150 nmol/l MTX. The application of MTX in these concentrations led to a significant decrease in viability of DPSCs and HDFs after 3 days. Exposure of DPSCs to 100 and 150 nmol/l MTX resulted in decrease in cellular viability to 78% and 70%, respectively, when compared with control (90%). The

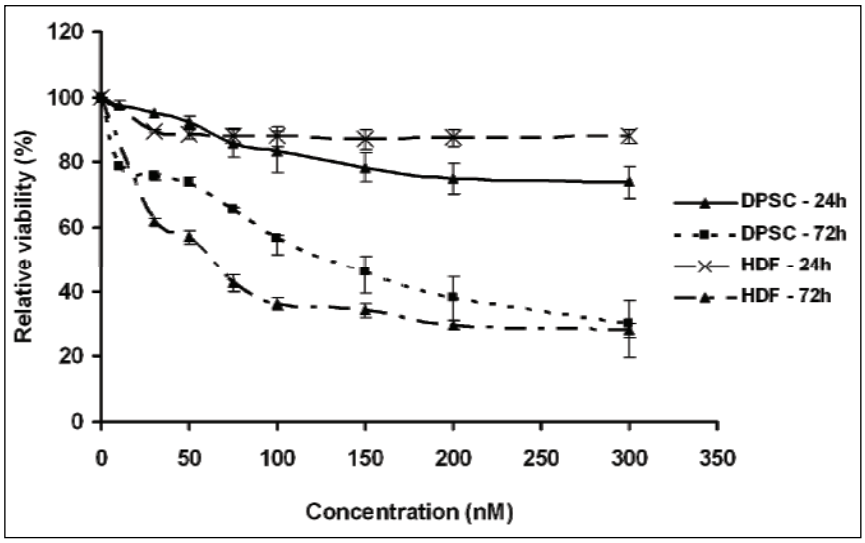

Fig. 1. The effect of MTX on the cell viability and proliferation of dental pulp stem cells (DPSCs) and human dermal fibroblasts (HDFs) performed by cell proliferation reagent WST-1. The relative viability was determined 24 and 72 hours after the application of MTX in concentration range from 10 to 300 nmol/l. To calculation of cell viability, the value of the signal from the treated culture well was expressed as a percentage of that of the control well. Results are shown as mean ±S.D. from three independent experiments.

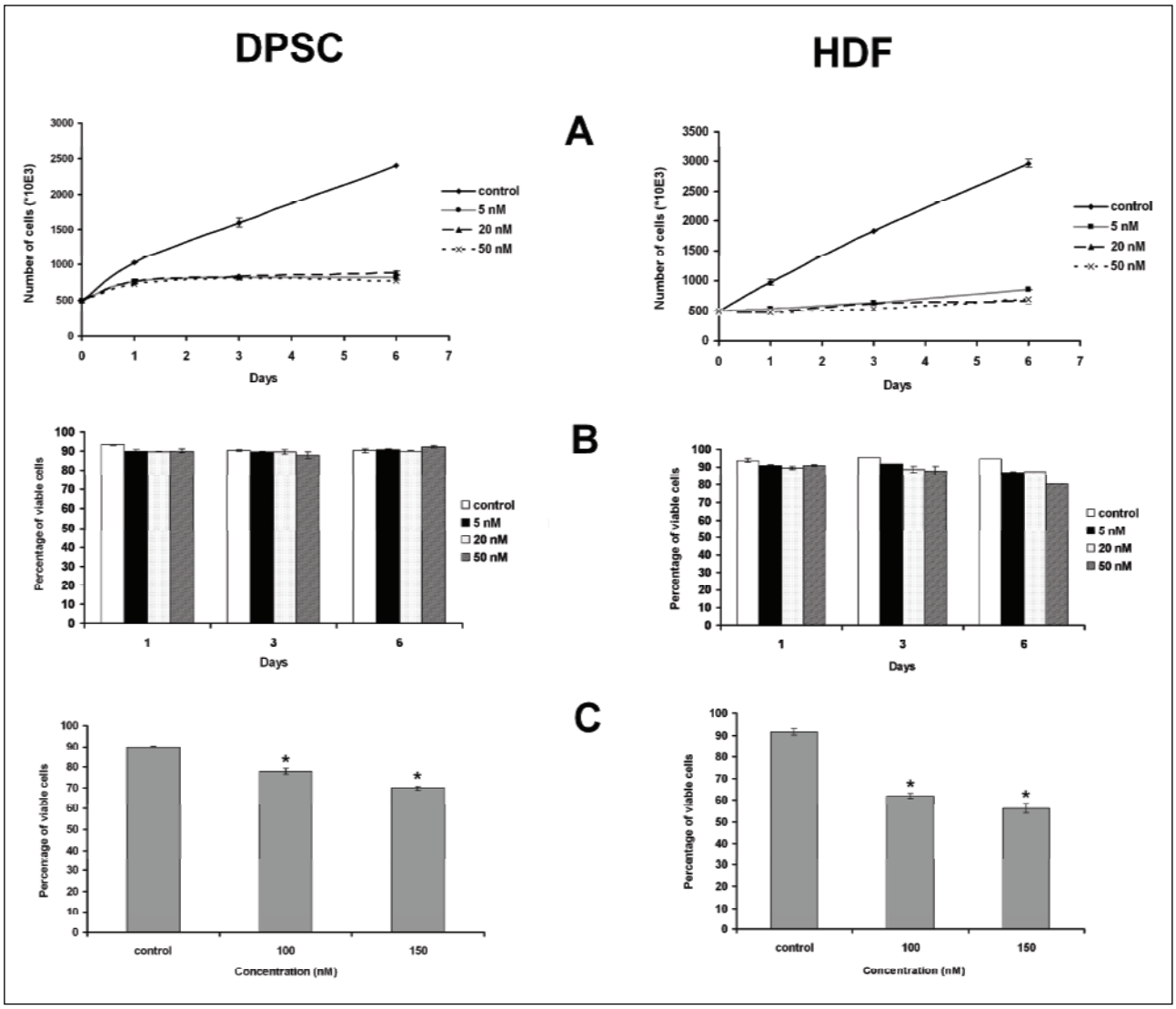

Fig. 2. The effect of MTX on the proliferation and viability of dental pulp stem cells (DPSCs) and human dermal fibroblasts (HDFs). (**A**), (**B**) Both cell types were exposed to 5, 20 and 50 nmol/l MTX. The effect on the proliferation and viability was measured during the interval of 6 days after MTX application. (**C**) Cells were treated with 100 and 150 nmol/l MTX and the viability was evaluated 3 days after MTX administration. The analysis of cell number was performed by Z2 Counter (A) and the percentage of viable cells (B, C) was determined by Vi-Cell using Trypan blue exclusion staining. Results are shown as mean ±S.D. from three experiments. * - significantly different to control (P≤0.05).

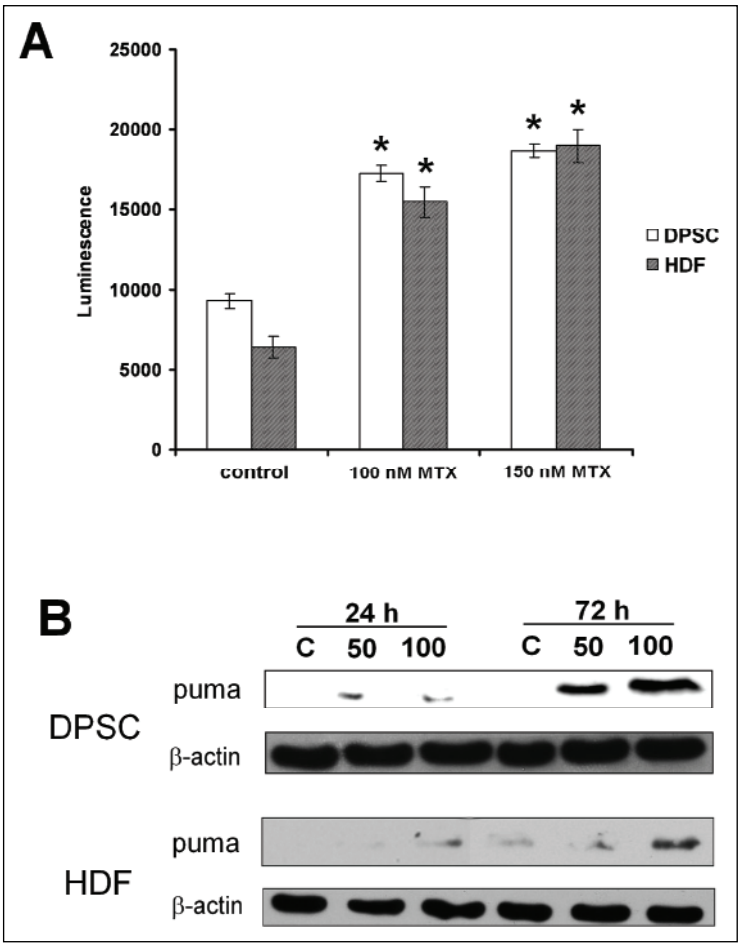

Fig. 3. The markers of apoptotic process in MTX-treated dental pulp stem cells (DPSCs) and human dermal fibroblasts (HDFs).
(**A**) Activity of caspases 3/7 in DPSCs and HDFs exposed to 50, 100 and 150 nmol/l MTX. Using Caspase-Glo assays, the activity of caspases 3/7 was measured 3 days after MTX administration. Results are shown as mean ±S.D. from three experiments. * - significantly different to control ($P \leq 0.05$).
(**B**) Expression of pro-apoptotic protein puma in DPSCs and HDFs exposed to 50 and 100 nmol/l MTX 24 and 72 hours after treatment with MTX. To confirm equal protein loading, membranes were reincubated with β-actin. Representative results of one of two experiments.

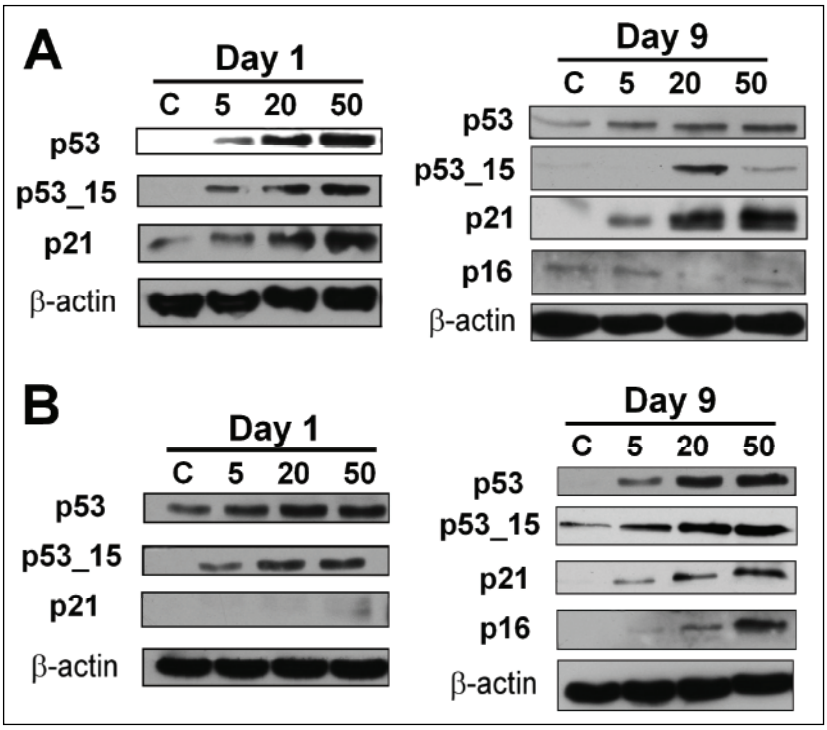

Fig. 4. Induction and activation of protein p53 and its downstream effectors in MTX-treated dental pulp stem cells (DPSCs) and human dermal fibroblasts (HDFs).
Activation of p53, p53 phosphorylated on serine 15 (p53_15), $p21^{WAF1/Cip1}$ and $p16^{INK4a}$ in DPSCs (**A**) and HDFs (**B**) exposed to 5, 20 and 50 nmol/l MTX 1 and 9 days after drug application. Protein expression was detected by electrophoresis and Western blotting as described in the text. To confirm equal protein loading, membranes were reincubated with β-actin. Representative results of one of two experiments.

percentage of viable HDFs was reduced to 62% and 50% in comparison to control untreated cells (91%) (*Fig. 2C*).

Whereas application of lower MTX concentrations (5, 20 and 50 nmol/l) did not change the cell viability, the higher MTX concentrations (100 and 150 nmol/l) led to significant decrease in viability of DPSCs. Therefore, we focused on the effects of these two groups of concentrations in further experiments.

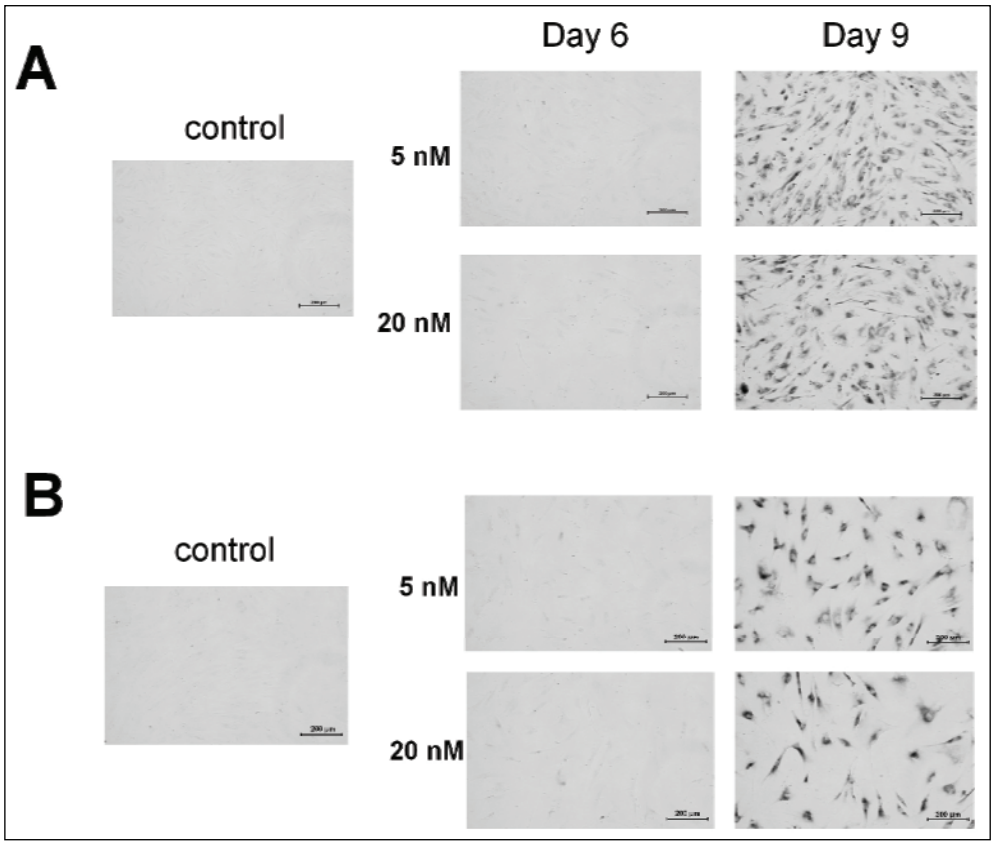

Fig. 5. Activity of β-galactosidase in dental pulp stem cells (DPSCs) and human dermal fibroblasts (HDFs) exposed to MTX.
DPSCs (**A**) and HDFs (**B**) were exposed to 5 and 20 nmol/l MTX in chamber slides. The activity of β-galactosidase was detected histochemically by senescence β-galactosidase staining kit at intervals 6 and 9 days. Activity of β-galactosidase was increased from day 9 after MTX exposition.

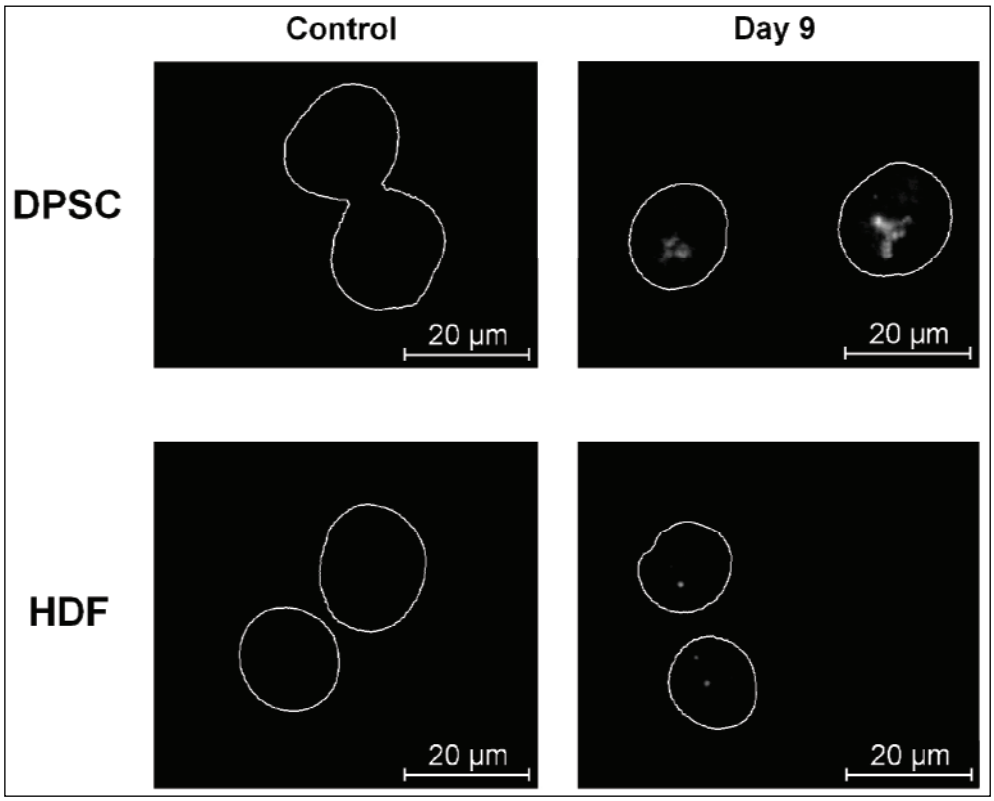

Fig. 6. Phosporylation of histone H2AX in MTX-treated dental pulp stem cells (DPSCs) and human dermal fibroblasts (HDFs).
Using immunocytochemistry, the persisting DSBs-associated γH2AX foci were observed 9 days after treatment with 5 nmol/l MTX.

Mitoxantrone in higher concentrations (>50 nmol/l) induces apoptosis

As the concentrations 100 and 150 nmol/l led to the significant decrease of viable cells, we evaluated activity of effector caspase 3/7 as the marker of apoptosis. The analysis was performed after application of 100 and 150 nmol/l MTX in interval 3 days. The activity of caspases 3/7 significantly increased in MTX-treated cells in comparison to control and the increase was dose-dependent (*Fig. 3A*). Moreover we detected one of the

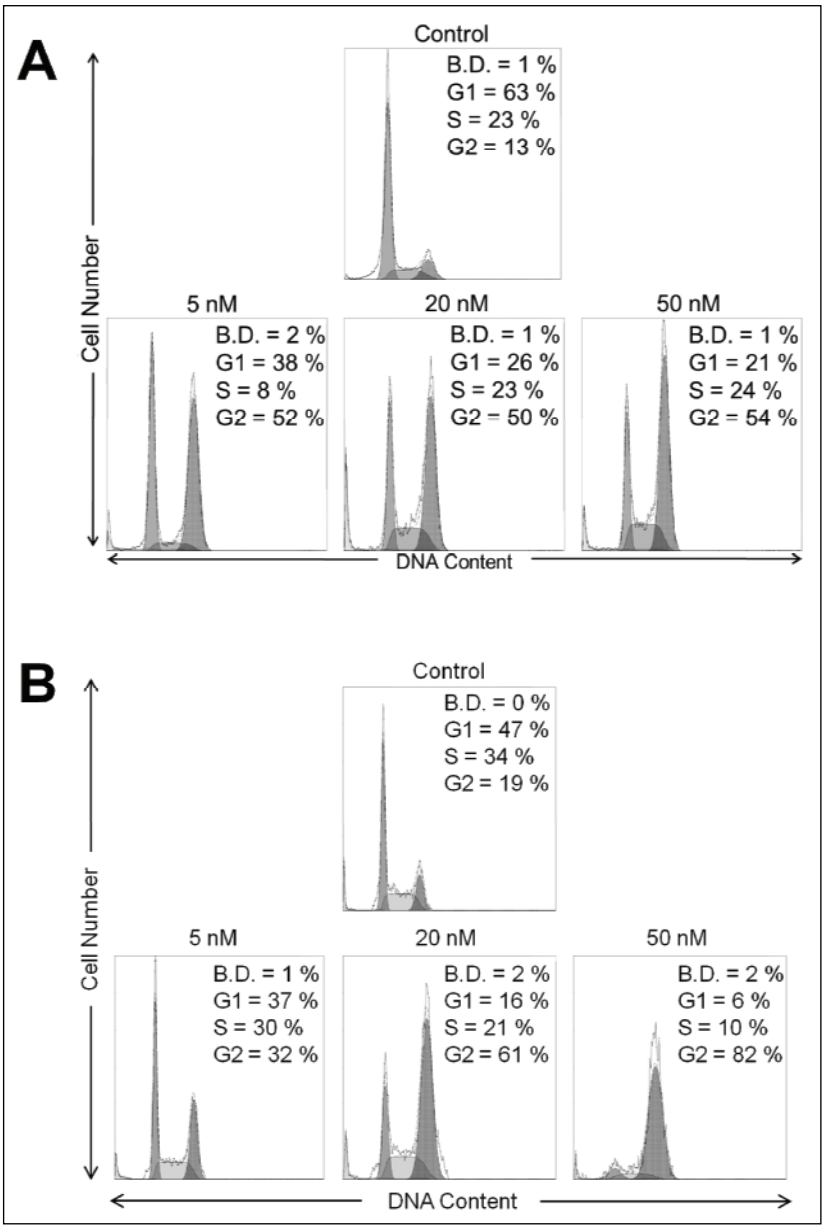

Fig. 7. Analysis of cell cycle in MTX-affected dental pulp stem cells (DPSCs) and human dermal fibroblasts (HDFs).
Using flow cytometry, cell cycle distribution was measured after treatment with 5, 20 and 50 nmol/l MTX in DPSCs (**A**) and HDFs (**B**). Representative results of DNA content 24 hours after MTX application.

proapoptotic members of Bcl-2 family - puma (p53 upregulated modulator of apoptosis) (*Fig. 3B*). The increase in puma level and activation of caspases 3/7 suggest that DPSCs as well as HDFs react to MTX (>50 nmol/l) by induction of apoptosis.

Mitoxantrone in lower concentrations (<50 nmol/l) induces stress-induced premature senescence

To further clarify mechanisms underlying total inhibition of proliferation without any significant decrease in viability by lower MTX concetrations we focused on the activation of DNA-damage response - up-regulation and activation of tumor suppressor protein p53 and its downstream effector $p21^{WAF1/Cip1}$ and changes in cell cycle inhibitor $p16^{INK4a}$, which is considered to be one of the markers of senescence. We analyzed the expression of proteins at day 1 and 9 after treatment with 5, 20 and 50 nmol/l MTX.

MTX caused up-regulation of p53 and its phosphorylation on serine 15 (p53_15) in dose-dependent manner. Also the level of downstream effector of p53, protein $p21^{WAF1/Cip1}$, was increased with ascending concentration of MTX in DPSCs (*Fig. 4A*) as well as in HDFs (*Fig. 4B*). Levels of p53, p53_15 and $p21^{WAF1/Cip1}$ remained elevated during whole experiment (9 days). Further we determined changes in cell cycle inhibitor $p16^{INK4a}$. Up-regulation of $p16^{INK4a}$ was not observed at day 1, 3 and 6 after MTX treatment in both cell types (data not shown). The increased level of the protein $p16^{INK4a}$ was markedly elevated 9 days after the application of the drug in HDFs (*Fig. 4B* - Day 9), but not in DPSCs (*Fig. 4A* - Day 9).

To confirm SIPS in MTX-treated cells, the markers of senescence were evaluated. As shown in *Fig. 5*, increased activity of β-galactosidase did not occur at day 6, but it perceptibly increased 9 days after the treatment with 5 and 20 nmol/l MTX. This result corresponds with the expression of $p16^{INK4a}$ in HDFs, which also occurred 9 days after MTX application. *Fig. 6* illustrates presense of persisting DNA-damage foci containing phosphorylated histone H2AX. Persisting DNA-damage foci of γH2AX were detected 9 days after treatment with 5 nmol/l MTX in both cell types.

To elucidate of cell cycle distribution, we analyzed possible cell cycle arrest by flow cytometry first day after MTX exposition. The application of 5, 20 and 50 nmol/l MTX led to the accumulation of both DPSCs and HDFs in the G2 phase. In

control, 13% of DPSCs and 19% of HDFs occurred in G2 phase, whereas MTX application led to increase of cells in the G2 phase of the cell cycle. In DPSCs, approximately 50% of cells were arrested in G2 phase (5 nmol/l - 52%; 20 nmol/l - 50%; 50 nmol/l - 54%) (*Fig. 7A*). In HDFs, MTX caused dose-dependent increase of cells in G2 phase (5 nmol/l - 32%; 20 nmol/l - 61%; 50 nmol/l - 82%) (*Fig. 7B*).

DISCUSSION

Apoptosis has been suggested as common mechanism by which the cells are eradicated during chemotherapy. However, there are other possible ways how the cells react to DNA damage, which strongly depends on the cell type and extent of DNA damage. In the case of reparable DNA damage, the cells stop division and repair DNA. In the case of irreparable damage, the cells undergo apoptosis or mitotic catastrophe or enter permanent cell cycle arrest called stress-induced premature senescence (SIPS) (34).

The chemotherapy and radiotherapy is consider to be the "gold standard" for the treatment of cancer, thus most of studies have focused on the reaction to DNA damage in tumor cell lines. Despite this fact, non-tumor cells are also exposed to DNA-damaging agents and not only their failure to maintain intact genome is the initiator of tumorigenesis, but also the response of tumor tissue to anticancer therapy is modulated by the surrounding non-tumor cells reaction. While apoptosis removes the damaged cell definitely, senescence is a double-edge sword: it functions as an important natural barrier to tumorigenesis, but in some settings senescent cells promote tumor progression by secretion of matrix metalloproteases, growth factors, and cytokines (29, 35). Within this study we focus on the reaction of normal mesenchymal stem cells isolated from dental pulp and compared it with the reaction of normal dermal fibroblasts. Adult MSCs in their niches are responsible for tissue homeostasis and could be extremely useful in a number of clinical applications, if their reactions are fully understood. Dental pulp stem cells (DPSCs) become of significant interest nowadays because of their ability to differentiate into a variety of cell types and convenient way of collecting. DPSCs have potential to differentiate into odontoblasts, osteoblasts, chondrocytes, adipocytes and myoblasts under specific stimuli (36). Moreover, DPSCs can differentiate into neural lineages under special culture conditions (37). Several other types of stem cells isolated from different tissues represent potential source for regeneration of the central nervous system (38) and DPSCs have high potential to serve as source for neuroregenerative treatments.

In this work, DNA-damage was caused by mitoxantrone, an inhibitor of topoisomerase II, which induces probably the most harmful type of DNA damage DNA-double strand breaks (DSBs). MTX triggers apoptotic process in human leukaemic cells MOLT-4 and Jurkat (24), in B-chronic lymphatic leukaemia cells (19), in human myeloid leukeamia cells HL-60 (18, 20, 21) as well as in mammary adenocarcinoma MTLn3 cells (22) and in LNCaP human prostate cancer cells (23). In the study of Tanaka *et al.* (39) another inhibitor of topoisomerase II, etoposide, triggered apoptosis accompanied with the presence of activated caspases-3 in human B cell lymphoblastoid TK6 cells.

On the other hand, some cancer cell lines, predominantly isolated from solid tumours, react to DSBs also by induction of SIPS. Permanent cell cycle arrest was observed after treatment with inhibitor of topoisomerase II doxorubicin in MCF-7 breast cancer cells and HCT-116 colon cancer cells (40). Similarly, topoisomerase II inhibitor etoposide caused SIPS in LS174T, HCT116 colon, MCF-7 breast, and in A2780 ovarian carcinoma cells (26). In the study of McKenna *et al.* (41), human non-small cell lung carcinoma A549 cells underwent SIPS after treatment with lower doses of mitomycin C, whereas higher concentration of the drug triggered apoptosis.

What is known about the reaction of normal human cells to DNA damage caused by various stimuli? DPSCs and HDFs are cells of mesenchymal origin and accumulating evidence shows that cells derived from mesenchym generally prefer stress-induced premature senescence (SIPS) as a reaction to DNA damage. Most of studies were performed on human fibroblasts which represent an easily available model for cell research. It was found that human fibroblasts exposed to oxidative stress (42), ultraviolet B radiation (43), hydrogen peroxide (44, 45), tert-butylhydroperoxide (t-BHP), ethanol (46), busulfan (47), copper sulfate and cytokine stimulation (48) undergo SIPS. In minority of studies, human fibroblasts responded to genotoxic stress by induction of apoptosis in specific conditions. Human fibroblasts are indeed capable of apoptosis induction, as it was demonstrated *e.g.* after cisplatin treatment (28). Similarly, MTX can induce caspase-3 mediated apoptosis in immortalized mouse embryonic NIH 3T3 fibroblasts, and, at higher concentration (1 μM), also necrotic death (49). Recent studies on gamma-irradiated human embryonic fibroblasts WI-38 (50) and zinc pyrithione treated human skin fibroblasts (51) show that while some of the cells died by apoptosis, in the remaining cells also SIPS is induced. In another study, normal colonic epithelial cells (NCM) and normal colonic fibroblasts (NCF) reacted to inhibitor of topoisomerase I irinotecan by induction of apoptosis as well as SIPS, but in NCM the induction of apoptosis prevailed whereas in NCF the induction of SIPS was dominant (52).

In MSCs, apoptosis as a prevailing reaction to DNA damage was observed rarely. However, Lan *et al.* (53) have shown that human embryonic stem cells react to irradiation with dose 5 Gy by increasing number of apoptotic cells 1 and 2 days after exposure. In our work, higher MTX concentrations (>50 nmol/l) led to apoptotic process in DPSCs. The viability of MTX-treated cells significantly decreased, activity of caspases 3/7 increased in dose-dependent manner and pro-apoptotic protein from Bcl-2 family puma was up-regulated. The result is in agreement with the reaction of DPSCs to cisplatin (28). On the other hand, the reaction of DPSCs to irradiation does not include apoptosis. Muthna *et al.* (27) did not observe any marks of apoptosis in irradiated DPSCs even in doses 20 Gy 13 days after irradiation, while marks of SIPS were present. Similarly, SIPS was detected in irradiated mesenchymal stem cells isolated from the bone marrow (54).

In summary, whereas MTX in doses 50 nmol/l and higher triggered apoptosis, exposure of both DPSCs and HDFs to lower concentrations of MTX (<50 nmol/l) led to induction of SIPS. After treatment with lower MTX doses, the proliferation of DPSCs and HDFs was completely inhibited while the cell viability remains unchanged. We have observed typical marks of SIPS such as increased activity of senescence-associated β-galactosidase, persisting DNA damage and cell cycle arrest in G2 phase.

Further, MTX provoked an increase in the amount of p53 and its phosphorylation on serine 15 as well as an increase in the expression of cell cycle inhibitor $p21^{WAF1/Cip1}$ in both cell types. The up-regulation of cell cycle inhibitor $p16^{INK4a}$ was observed in HDFs, but not in DPSCs. Depending on the severity of DNA damage, enforced expression of p53 and the p53 effector molecule $p21^{WAF1/Cip1}$ as well as $p16^{INK4a}$ in certain cell types can induce senescence-like phenotype. It is supposed that p53 initiates the senescence growth arrest at least in part by inducing $p21^{WAF1/Cip1}$. The subsequent rise in $p16^{INK4a}$ then acts to maintain irreversible cell cycle arrest (55). We have detected increased expression of cell cycle regulator protein $p16^{INK4a}$ 9 days after exposition to MTX in HDFs. Our findings correlate with the study of Robles and Adami (56) who have shown that exposure of HDFs to DNA strand breaking agents, bleomycin and

actinomycin D, results in increased levels of $p16^{INK4a}$ and in SIPS 8 days after application of these agents. In DPSCs, activation of $p16^{INK4a}$ was not detected during the whole experiment (1, 3, 6 and 9 days after MTX application). In most of studies the induction of SIPS is associated with the increase in $p16^{INK4a}$, including SIPS induced in stem cells - irradiation of hematopoietic stem cells as well as DPSCs induced SIPS by activation of both p53-$p21^{WAF1/Cip1}$ and $p16^{INK4a}$ pathways (27, 57). Despite these facts, in some studies SIPS was not associated with up-regulation of $p16^{INK4a}$ (58). In the study of Chang *et al.* (59) induction of SIPS was observed after irradiation in human cancer cell lines (*e.g.* fibrosarcoma) that do not express $p16^{INK4a}$. Mirzayans *et al.* (58) have discussed the reciprocal relationship between p53 and $p16^{INK4a}$. It was also found that protein p53 contributes to the down-regulation of $p16^{INK4a}$ (60). In our study, p53 is still up-regulated 9 days after MTX application in DPSCs.

Further, we observed that DPSCs as well as HDFs are preferentially arrested in G2 phase of the cell cycle after treatment with conventional doses of MTX (<50 nmol/l). This finding is in agreement with the results of Zhao *et al.* (15), who detected mainly G2 or S phase cell cycle arrest in MTX-treated human lung adenocarcinoma cells. Similarly, cell cycle arrest in G2 phase was also observed in human embryonic stem cells (61) and in DPSCs (27) in reaction to irradiation. Bleomycin and actinomycin D caused cell cycle arrest in both G1 and G2 phases in HDFs (56) and also γ-irradiation-treated fibroblasts were permanently arrested in both G1 and G2 phases of cell cycle (62). In our previous study, an alkylation agent cisplatin induced cell cycle arrest in late S and G2 phase in DPSCs and HDFs (28). We have also detected persisting γH2AX foci in nuclei 9 days after the treatment with MTX in both cell types. This reaction is similar to reaction of MTX-treated human lung adenocarcinoma cells (15). It has been shown that γH2AX phosphoprotein increases during cellular senescence in culture and is considered to be one of the markers of persisting DNA damage (63).

In conclusion, our study reveals molecular mechanisms of DPSCs in response to DNA damage caused by MTX and compares it with the response of HDFs. Our data indicate that both cell types treated with clinical relevant concentrations of MTX underwent SIPS, which was proved by increased activity of senescence-associated β-galactosidase, persisting DSBs-associated γH2AX foci and cell cycle arrest in G2 phase. The reaction of DPSCs differs from the reaction of HDFs only in the activation of $p16^{INK4a}$ pathway. In DPSCs, cell cycle inhibitor $p16^{INK4a}$ was not activated. Whereas lower concentration of MTX induce SIPS, higher concentration of MTX induced programmed cell death, which was accompanied with increased expression of proapoptotic protein puma and activation of effector caspases 3/7 in both cell types. Both pathways prevent the proliferation of stem cells and fibroblasts with damaged DNA and thus help to maintain tissues homeostasis in the organisms.

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