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Static magnetic fields modulate X-ray-induced DNA damage in human glioblastoma primary cells

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Although static magnetic fields (SMFs) are used extensively in the occupational and medical fields, few comprehensive studies have investigated their possible genotoxic effect and the findings are controversial. With the advent of magnetic resonance imaging-guided radiation therapy, the potential effects of SMFs on ionizing radiation (IR) have become increasingly important. In this study we focused on the genotoxic effect of 80 mT SMFs, both alone and in combination with (i.e. preceding or following) X-ray (XR) irradiation, on primary glioblastoma cells in culture. The cells were exposed to: (i) SMFs alone; (ii) XRs alone; (iii) XR, with SMFs applied during recovery; (iv) SMFs both before and after XR irradiation. XR-induced DNA damage was analyzed by Single Cell Gel Electrophoresis assay (comet assay) using statistical tools designed to assess the tail DNA (TD) and tail length (TL) as indicators of DNA fragmentation. Mitochondrial membrane potential, known to be affected by IR, was assessed using the JC-1 mitochondrial probe. Our results showed that exposure of cells to 5 Gy of XR irradiation alone led to extensive DNA damage, which was significantly reduced by post-irradiation exposure to SMFs. The XR-induced loss of mitochondrial membrane potential was to a large extent averted by exposure to SMFs. These data suggest that SMFs modulate DNA damage and/or damage repair, possibly through a mechanism that affects mitochondria.

Keywords: static magnetic field; ionizing radiation; comet assay; DNA fragmentation; mitochondrial membrane potential; glioblastoma

INTRODUCTION

Whether exposure to Static Magnetic Fields (SMFs) affects DNA integrity in human cells remains a matter of debate. Many studies suggest that SMFs have no effect on cell growth rate, with results showing that cell cycle distribution is not influenced even by extremely strong SMFs (up to a maximum of 7.05 T) [1]. By contrast, we (and others) show that SMFs do have structural and functional effects according to the cell types and to the intensity, duration and orientation of the field used [2–4]. The majority of evidence suggests that SMFs do not induce genotoxic effects [5, 6], however, some studies have reported a mutagenic effect for SMFs [7, 8]. SMFs have been shown to reduce constitutive DNA damage signaling, which is considered to indicate DNA damage induced by endogenous oxidants, as has been demonstrated by analyzing oxidative phosphorylation [9]. It is of paramount importance to establish whether SMFs do cause DNA damage and, if so, to determine what doses, duration of exposure and type of SMFs (homogeneous or inhomogeneous) induce such damage, the underlying mechanisms of action, and which end-points need to be evaluated.

It has been suggested that free radicals play a key role in the mechanisms underlying DNA damage [10]. Indeed, one

of the potential mechanisms through which SMFs may interact with the living organism is via electronic interactions, in which magnetic fields influence the kinetics of the reactions with radical pair intermediates [11]. It has also been postulated that the magnetic field can increase the concentration of free radicals in living cells [11, 12]. Free radicals have, in turn, been shown to alter the mitochondrial membrane potential ($\Delta\Psi m$) and to induce genetic damage [13, 14]. At the cellular level, lipids, proteins, carbohydrates and nucleic acids may be damaged by reactions with free radicals [15, 16]. These damaging reactions give rise to functional and morphological disturbances in the cell, due to oxidative/nitrosative/carboxilative stress, which lead to reversible or irreversible DNA damage [11]. The mechanisms underlying ionizing radiation (IR)-induced DNA damage, unlike those associated with exposure to SMFs, have been thoroughly investigated in recent decades. The effects induced by IR, such as XRs, on biological tissues, have been shown to be mediated by free radicals. In recent years, IR has been used as a primary tool for studying oxidative stress responses in various organisms ranging from prokaryotes to higher eukaryotes. Molecular insights into how reactive oxygen species (ROS) generated by radiation can damage DNA have served as a basis for investigating the nature of mutagenesis, disease, aging, and innumerable biological processes ending in cell death [17, 18]. Mitochondria are assumed to be responsible for ROS production [19]. Indeed, IR has been shown to upregulate the mitochondrial electron transport chain function [20]. ROS attack DNA readily, generating a variety of DNA lesions, such as oxidized bases and strand breaks [20–22]. It has also been reported that magnetic fields interfere with the genotoxic activity of physical and chemical agents [23, 24]. However, there is a general lack of data regarding the effect of co-exposure to SMFs and XRs. The aim of this study was to investigate the genotoxicity of exposure to SMFs alone, as well as the genotoxicity of exposure to SMFs prior to and/or following XRs in a very sensitive primary line of human glioblastoma cells obtained by surgical biopsy, which in a previous study have been demonstrated to be highly responsive to SMFs [2, 24].

MATERIALS AND METHODS

Cell culture

Cells were taken from a patient with Stage III multiform glioblastoma undergoing a brain tumor resection for therapeutic purposes. These cells were selected from one particular patient out of four under study, since they showed the highest sensitivity to chemical and physical agents [24]. The secondary use of the human specimens was approved by the hospital ethical board, and the patients' data were adequately anonymized. Primary cultures were grown in RPMI medium supplemented with 5% fetal calf serum and used in their early passages. Cells in exponential growth, in triplicate Petri dishes for each time-point under investigation, were exposed to SMFs and/or XRs, as reported below and illustrated in the scheme in Table 1. In order to submit all the samples to the same environmental conditions (i.e. light and temperature), the control cells underwent sham procedures (e.g. transportation to the XR generator, with a delay of return to the incubator equal to XR irradiation time) and are referred to as 'sham' throughout the paper. In the case of simple exposure to SMF, sham-treated cells were submitted to the same environmental conditions as SMF-treated cells, except for the lack of exposure to SMFs.

SMF exposure

The exposure system has been previously described in detail [3] and is briefly described here. SMFs of known flux densities were produced by custom-made 4 cm × 4 cm Neodymium magnetic plaques. These magnetic plaques were placed underneath the Petri dish at a 1-mm distance from the cell monolayer in a custom-made device, which kept them suspended in the incubator at least 10 cm from any metallic surface (to avoid distortion of the field). Samples were also placed > 10 cm from each other (at which distance the magnetic field of the plaque magnets is undetectable). The position of the samples inside the incubator was randomly chosen. The fields were axial, with the magnetic North (the pole of the magnet which would be repulsed by the Earth's North magnetic pole) vector crossing the cells.

The magnitude of the magnetic field of each plaque was 80 ± 5 mT (1 mT = 10 G) and was checked by means of a gaussmeter (Hall-effect Gaussmeter, GM04 Hirst Magnetic Instruments, UK). The gaussmeter was also used to measure the magnetic field inside the incubator (0.5 ± 0.02 mT), which must be considered as background for all the cell cultures used in this study, including sham cells, and added to the 80 mT magnetic field for the exposed cells. Magnets, in contrast with other exposure devices, do not produce temperature variation. For schematic protocols of treatments and sampling times see Table 1.

For the comet assay, experiments were repeated twice on each of three independent culture dishes for each experimental

Table 1. Scheme of treatments, sampling times and assays performed

Treatment					
SHAM	#	° #	°	° #	°
XR	#	° #		° #	
SMF	#	° #	°	° #	°
XR + SMF	#	° #		° #	
SMF + XR + SMF				°	
Sampling time (h)	3	6	12	20	24

° = comet assay, # = JC-1.

point. For JC-1 analysis, experiments were repeated twice with a single measure within each experiment for each time-point.

XR irradiation

Cells in triplicate Petri dishes for each time-point, were irradiated with 5 Gy XR generated by a CHF 320 G generator (Gilardoni, Mandello del Lario, LC, Italy) equipped with a Cu filter of 0.5 mm, operating at 250 keV, 15 mA, delivering a dose rate of 1.1 Gy/min. Cells underwent 6 or 20 h of recovery in the incubator before the comet assay. For ΔΨm investigation, cells were analyzed after 3, 6 and 20 h of recovery (Table 1).

XR irradiation and SMF

Cell cultures were exposed to 5 Gy XRs, and during their recovery continuously exposed to SMF for 6 or 20 h. In addition, an experimental group was exposed to 6 h of SMF before XR irradiation, followed by recovery under SMF for 6 and 20 h (Table 1).

Comet assay

The alkaline comet assay was performed on viable cells as previously described [25]. Dead and apoptotic cells were removed during rinsing. Trypan blue staining demonstrated that adherent cells contained no more than 2% and 4% of dead cells in sham cells and 5-Gy-irradiated cells, respectively. It has been reported that detaching cells with trypsin may increase the cells' ROS production [26]; however, scraping is considered worse; thus we used trypsin, so as to minimize cellular stress. Cells were thoroughly rinsed three times with 37°C, Ca- and Mg-free, sterile PBS, then incubated at 37°C with 1 ml 0.25% trypsin/EDTA solution for ~ 4 min, checking during this period the numbers of detached cells. Trypsinization was stopped by adding complete medium, and after this step cells were maintained on ice. Cells were gently resuspended, centrifuged at 200 G, then 20 µl of the cell pellet was mixed into 180 µl of 0.7% low-melting-point agarose in PBS (Ca- and Mg-free) at 38°C, and immediately pipetted onto a frosted glass microscope slide precoated with a layer of 1% normal-melting-point agarose, in PBS. Slides were covered with coverslips, set at 4°C for solidifying the agarose, then coverslips were removed and slides were incubated in a lysis solution (2.5 M NaCl, 10 mM Tris–HCl, 100 mM Na_2EDTA, NaOH to pH = 10, 1% Triton X-100, 10% dimethyl sulfoxide) for 45 min; after this step all the operations were performed at 4°C under dim light. After lysis, slides were rinsed for 10 min with electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH = 13) and placed for 20 min onto a horizontal electrophoresis unit containing the same electrophoresis buffer to allow DNA unwinding. Electrophoresis was conducted with the Sub-Cell GT System (15 × 25 cm) equipped with Power Pack 300 (Bio-Rad Laboratories Inc., Hercules, CA, USA) for 15 min (25 V, 300 mA). Subsequently, slides were gently washed in neutralization buffer solution for 5 min (0.4 M Tris–HCl, pH = 7.5), dehydrated with an ethanol series (70, 85 and 100%), dried at room temperature and stored. For microscopy analysis, slides were stained with 2 mg/ml distilled water ethidium bromide. Where not differently indicated, all the chemicals were purchased from Sigma (St Louis, MO).

Cell capture and analysis

As previously indicated we analyzed six Petri dishes for each experimental point (2 × 3 replicas). Randomly captured cells (150 cells) for each Petri dish (obtaining a total of 6 slides for each time-point) were examined at × 400 magnification using a fluorescent Axiolab Zeiss microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a Coolsnap cooled digital (CCD) camera (Roper Scientific, Princeton, NJ, USA). DNA migration was measured using the freely available CASP comet assay software program (http://www.casp.of.pl/). DNA migration was measured by analyzing the percentage of DNA in Tail (TD) and tail length (TL) [27].

Data analysis

The comparison between sham and exposed samples (SMF and/or XR) was carried out by applying the analysis of variance (ANOVA) with Scheffé's multiple comparisons test. In all cases, P-values < 0.05 were considered statistically significant. The statistical tests were performed using MATLAB software.

ΔΨm

Depolarization of ΔΨm was evaluated by the incorporation of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanin iodide (JC-1) (Molecular Probe, Invitrogen, Milan Italy). Cells, harvested as previously described, were stained at 3, 6 and 20 h of recovery after XR irradiation. Cells at the concentration of 10^6/ml in PBS were incubated with 3 µg/ml (final concentration) JC-1 for 15 min in the dark at 37°C, from a stock solution of JC-1 at 1mg/ml in DMSO. Then, cells were washed once in PBS. Green vs red fluorescence was determined with a PASIII PARTEC flow cytometer (Partec, Muenster, Germany). At least 20 000 cells were counted for each cytogram. Estimation of emitted fluorescence was performed on forward/side scatter-gated cytograms.

RESULTS

SMF effects on glioblastoma cells

No significant difference was observed in either the TD or TL between sham-treated cells and cells exposed to 80 mT SMF (for either 6 or 12 h). By contrast, a significant difference emerged between the two groups of cells in both the TD and TL at 24 h exposure, as shown in Fig. 1. It is noteworthy that as the culture times grew longer, the sham cells also exhibited a significant increase in the comet parameters,

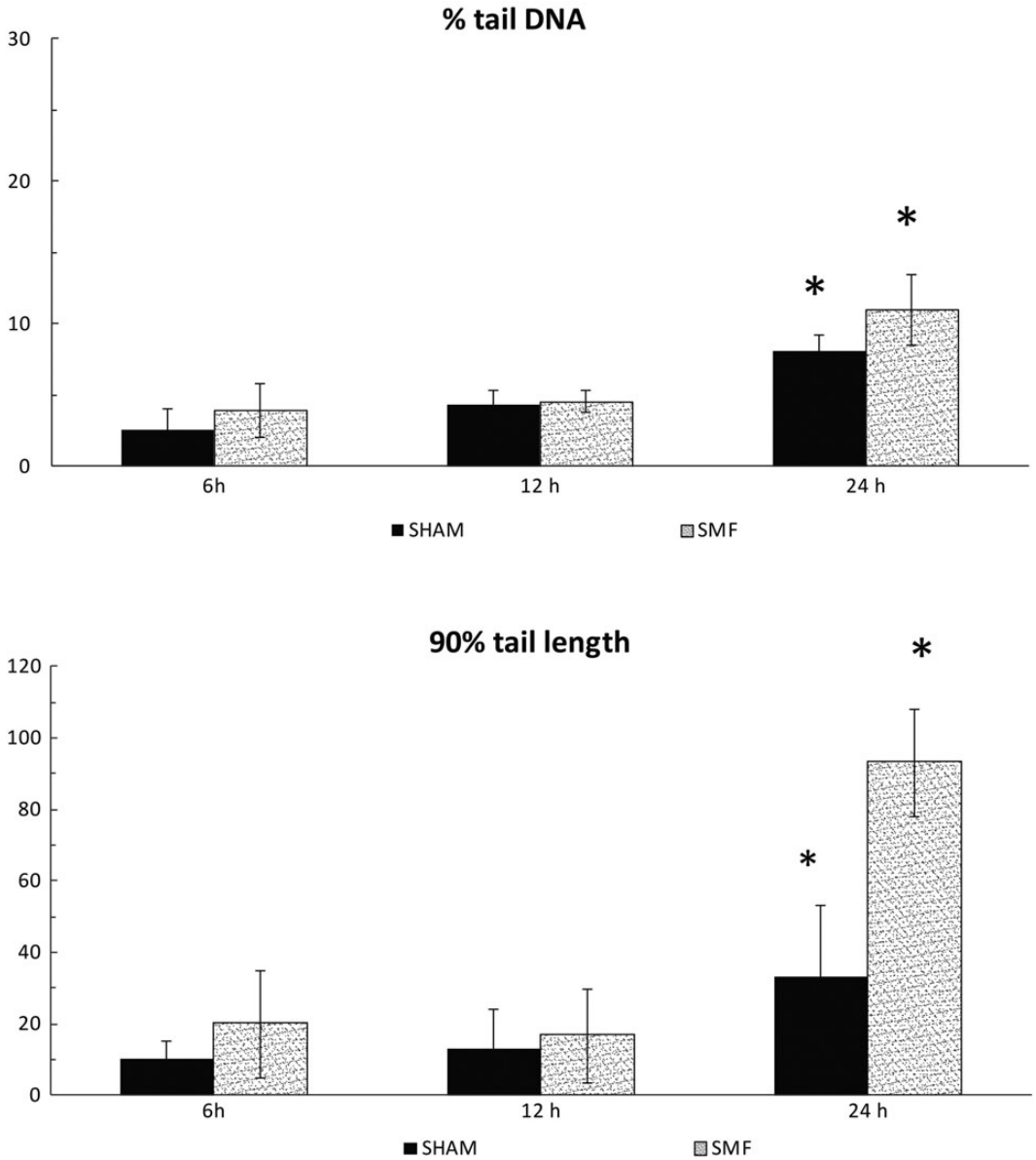

Fig. 1. Comet assay results of cells exposed to SMFs. The percentage of DNA in tail (TD) and 90% of tail length (TL) are shown in the upper and lower panel, respectively. The results of triplicate samples of two independent experiments are reported together with the SD for each time-point. Cells were exposed to an 80 ± 5 mT SMF in exponential growth for 6, 12 and 24 h. A statistically significant difference was observed (asterisk) between sham- and SMF-exposed cells after 24 h, and within both sham and exposed groups between 6 and 24 h of treatment (both TD and TL parameters) ($P < 0.01$).

indicating that a certain amount of DNA fragmentation may have occurred as a result of active metabolic endogenous activity in these cells.

XR and SMF interactions on glioblastoma cells

As expected, the exposure of cells to 5 Gy XRs induced a high degree of DNA fragmentation, as demonstrated by the consistent increase in the TD and TL, at both 6 h and 20 h following exposure (Figs 2 and 3), though it should be borne in mind that the standard deviation was quite high (see *Discussion*). The results for SMFs applied after XR irradiation are also shown in Figs 2 and 3. They reveal a dramatic reduction in DNA fragmentation when recovery was performed under SMF exposure ($P < 0.01$). No major change was observed when 6 h SMF pretreatment was performed before XR irradiation.

ΔΨm

In order to assess the ΔΨm, known to be affected by XR irradiation, we investigated the green vs red fluorescence JC-1

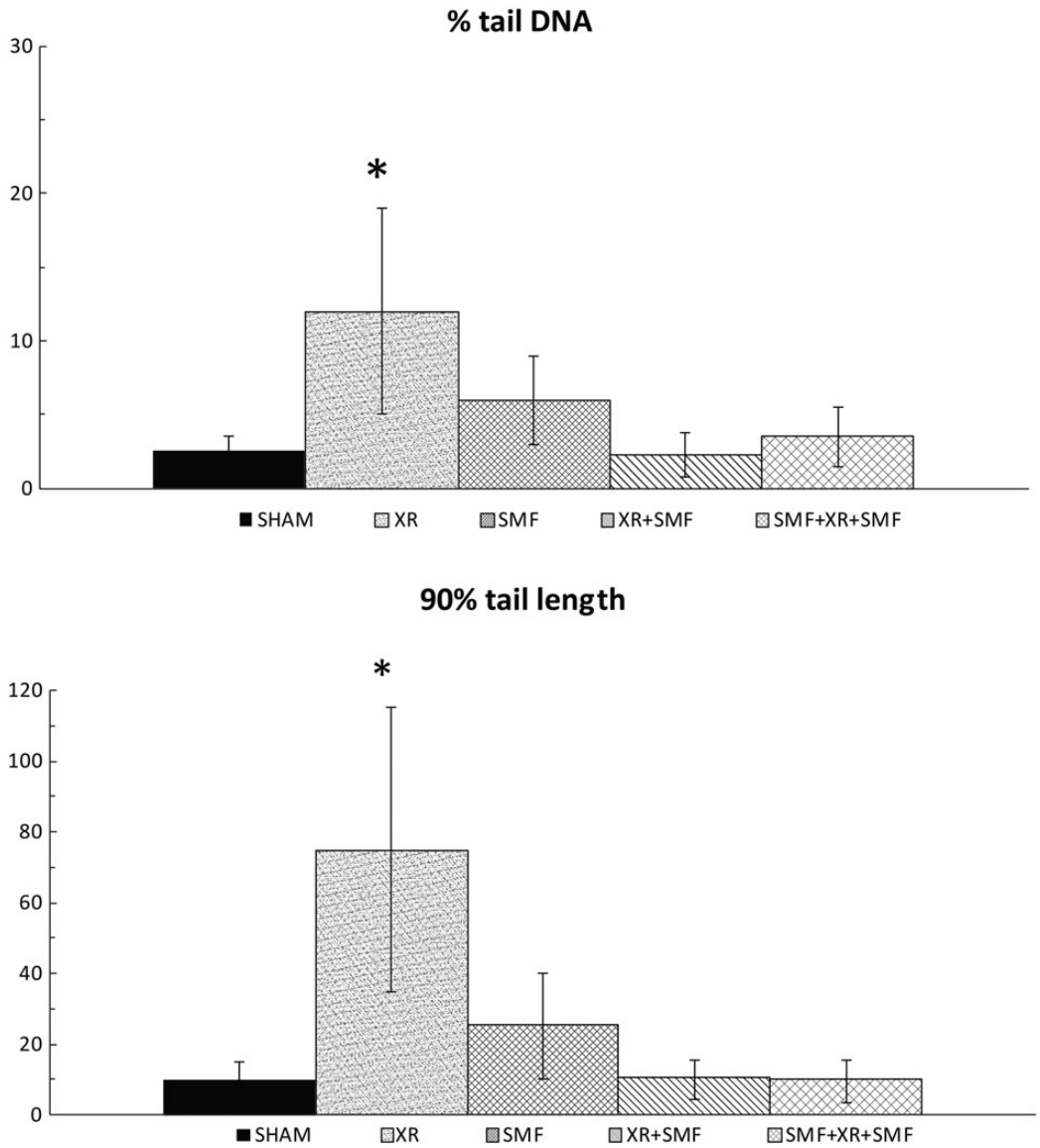

Fig. 2. Comet assay results of cells exposed to XR irradiation that underwent 6 h of recovery in the absence and in the presence of SMFs. TD and TL are shown in the upper and lower panels, respectively. The results of triplicate samples of two independent experiments are reported together with the SD. Five groups are reported in the figure. These groups are (i) sham: cells not exposed to any treatment but submitted to the same environmental conditions; (ii) XR: cells exposed to XR irradiation only; (iii) SMF: cells exposed to SMFs only; (iv) XR + SMF: cells exposed to SMFs during 6 h of recovery following XR irradiation; (v) SMF + XR + SMF: cells exposed to SMFs prior to XR irradiation and during 6 h of recovery. Recovery under SMFs significantly (asterisk) reduced the TD and TL ($P < 0.001$) compared with XR irradiation alone.

staining using flow cytometry. The results demonstrated a consistent shift of the red fluorescence according to treatment. A representative histogram with the cumulative results of the four groups analyzed after 6 h of recovery is shown in Fig. 4. As expected, and as emerges in the figure, a dramatic decrease in 590 nm fluorescence, indicating $\Delta\Psi$m, is observed in the XR samples (green line) when compared with the sham samples (blue line). A less marked shift in 590 nm fluorescence was observed in the SMF-treated cells. What is very interesting is that the samples exposed to SMFs after XR irradiation unexpectedly displayed a significant recovery in the amount of 590 nm fluorescence, thereby pointing to rescue from $\Delta\Psi$m, which is likely due to the presence of SMF during recovery.

The time-course of $\Delta\Psi$m during recovery is illustrated in Fig. 5. These results highlight the ratios between the 590 nm fluorescence-positive cells and the 590 nm fluorescence-negative cells (i.e. RN2 vs RN1 of Fig. 5) analyzed after 3, 6

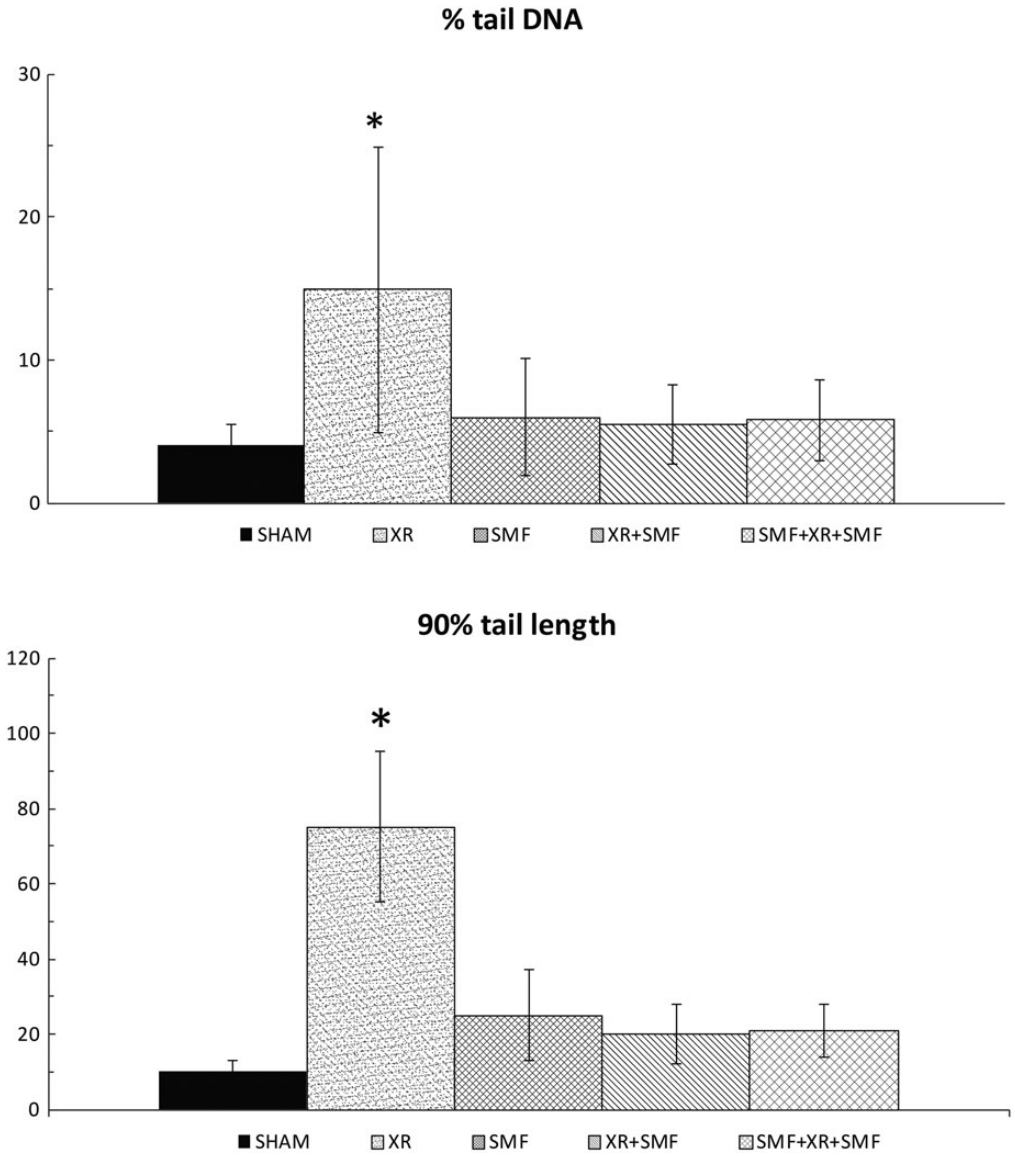

Fig. 3. Comet test results for cells exposed to 5 Gy XR irradiation that subsequently underwent 20 h of recovery with or without SMFs. TD and TL are shown in the upper and lower panels, respectively. The results of triplicate samples of two independent experiments are reported together with the SD. Five groups are reported in the figure. These groups are (i) sham: cells not exposed to any treatment but submitted to the same environmental conditions; (ii) XR: cells exposed to XR irradiation only; (iii) SMF: cells exposed to SMF only; (iv) XR + SMF: cells exposed to SMF during 20 h of recovery following XR irradiation; (v) SMF + XR + SMF: cells exposed to SMFs prior to XR irradiation and during 20 h of recovery. Recovery under SMFs significantly (asterisk) reduced the MDL and TL ($P < 0.001$) compared with XR irradiation alone.

and 20 h of recovery following XR irradiation. The two-way Anova test demonstrated an effect of both time ($P < 0.001$) and treatment ($P = 0.004$), as well as an interaction effect (of time and treatment) ($P = 0.002$). Using Scheffé's multiple comparisons test, a significantly lower $\Delta\Psi$m was observed at 3 h in the XR and XR + SMF groups, than in the sham cells. However, the presence of SMFs for 3 h during recovery yielded a significantly higher $\Delta\Psi$m compared with XR irradiation alone, thereby demonstrating the ability of SMFs to modulate the $\Delta\Psi$m. A significant difference was observed in all the groups at 6 h, whereas at 20 h the $\Delta\Psi$m was restored and no statistical significance was observed between the treatments. It is noteworthy, however, that both XR and XR + SMF groups displayed a trend toward an increased $\Delta\Psi$m when compared with sham-treated cells.

DISCUSSION

Over the past decade, the possible biological effects (including genotoxicity) of SMFs have been investigated in a wide

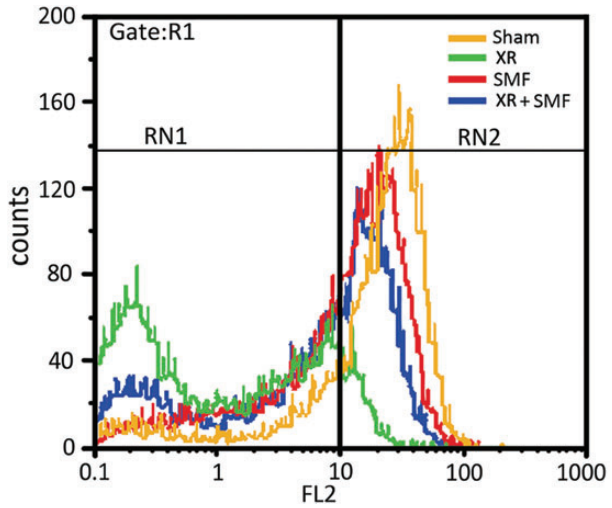

Fig. 4. Representative histogram of JC-1 590 nm fluorescence, indicative of the ΔΨm induced by the treatment, analyzed after 6 h of recovery following XR irradiation. Each fluorescence group is highlighted with a different color. A shift toward a reduced 590 fluorescence is evident in the XR-irradiated cells (green curve) compared with the sham cells (yellow curve). The presence of SMFs during the recovery phase following XR irradiation, instead, reduced the ΔΨm (blue curve).

range of biological systems; for a comprehensive overview see Leszczynski [28] and the ICNIRP guidelines [29, 30]. Since the application of SMFs in industry, medicine and the environment (e.g. transportation) is growing, the possibility of occupational and general exposure is increasing, raising the need for more investigations on SMFs alone, as well as in combination with, other physical/chemical agents. SMFs are also considered to be a very promising tool for modulating cell orientation and differentiation in regenerative medicine and tissue engineering [3], and it is essential to be aware of any possible genotoxic or cytotoxic effects of SMFs. There only a few studies designed to investigate the interaction between non-IR and IR at the cellular level [31–33]. It should be noted also that, for some tumors, magnetic resonance imaging (MRI)-guided radiation therapy is showing promise when compared with traditional therapies [34], thus it is increasingly important to consider the potential influence of SMFs on IR.

Perusal of the literature reveals that the effects of SMFs vary according to the intensity of the magnetic force, the duration of the treatment and cellular systems [4, 35]. In particular, gene expression, including that of potentially oncogenic genes, seems to be modulated by SMFs [36]. Although the paucity and the heterogeneity of the studies on weak vs strong flux density SMFs makes it difficult to draw any clear-cut conclusion, the evidence suggests that both flux densities may have considerable biological outcomes. In our experimental procedure and in the cellular system used in the present study, SMFs were found to induce a time-dependent increase in DNA fragmentation. This result is in accordance with other studies that have reported time- and dose-dependent genetic effects [35, 37].

Very surprisingly, SMFs modulated the XR response, reducing the degree of DNA fragmentation. It should be borne in mind that our findings were characterized by a high standard deviation, which may be ascribable to several factors: the intrinsic heterogeneity of the primary culture cells, reflecting the *in vivo* situation, might not contain a homogeneous cell genotype; differences in cell cycle phase sensitivity may also account for a range in the response. The difference that emerged from our experiments was, however, highly significant, despite dispersion of the data.

A recent study has also revealed a 'beneficial' effect of an homogeneous SMF on the repair of radiation-damaged DNA in leukocytes, though only after 4 h of recovery [38]. What explanation may be provided for our results showing that SMFs attenuate the level of DNA fragmentation and reduce the two comet parameters? It should be noted that SMFs prolongs the lifespan of reactive oxidants through the 'radical pair mechanism', which facilitates the interconversion of the singlet to the triplet excitation state (intersystem crossing) and would be expected to enhance rather than reduce DNA damage [10]. Yet another mechanism that might explain our findings is the interference by SMFs in the electron transport chain in mitochondria; cell respiration, which is the process that generates reactive oxidants, may be reduced by this interference. Surprisingly little information is available in the literature on the effect of SMFs on this process. If the process of electron transport, and consequently of oxidative phosphorylation, is perturbed by the SMF, oxidative DNA damage would be reduced. Our results demonstrate that a decreased mitochondrial depolarization occurs after XR irradiation in the presence of an SMF during recovery. Our previous observations on the same cells [24] demonstrated that the SMF rescues cells from apoptosis after they have been exposed to chemical agents. This result is in accordance with the attenuation of the loss of the ΔΨm induced by SMF exposure during XR irradiation recovery reported here. The present data shed light on the mechanism of interaction during combined treatment with IR and non-IR by pointing to a possible key role for mitochondria. The attenuation of the loss of the ΔΨm following exposure to an SMF as observed by us may impair the release of ROS from the mitochondrial interior, their direct transfer to nuclei, and also the induction of secondary radicals, thereby preventing oxidative DNA damage.

The fact that XR-induced DNA damage is still marked after 20 h of recovery, while the ΔΨm is completely restored is, despite not being the primary aim of this study, of considerable interest. A possible explanation for the persistent DNA damage might be the occurrence of the second wave of ROS, which occurs after XR irradiation [20, 39, 40]. Indeed, whereas IR instantaneously induces the formation of water

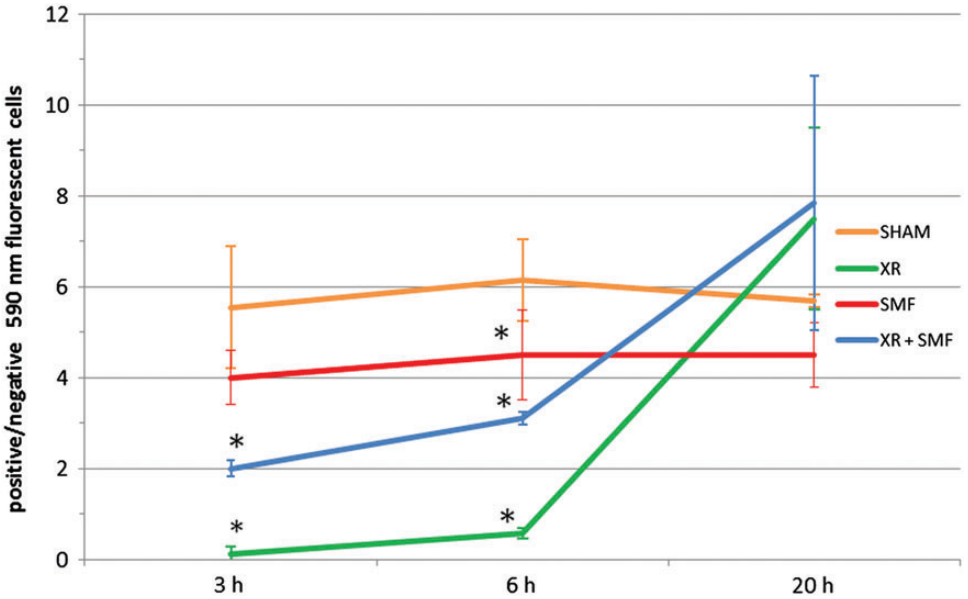

Fig. 5. Time-course of ΔΨm variation as determined by JC-1 staining. The figure shows the ratios between the positive and negative 590 nm fluorescent cells measured after 3, 6 and 20 h of recovery following XR irradiation, in the presence of SMFs (XR + SMF) or absence of SMFs (XR). The above ratios of cells exposed to SMFs alone (SMF) and of sham cells (SHAM) are also reported.

radiolysis products that contain some ROS, the latter are also believed to be released secondarily by biological sources in irradiated cells. Mitochondria are assumed to be responsible for this secondarily IR-induced ROS production [20], though they may not be the only source. The type of ROS produced following radiation might vary depending on the time of recovery. In this regard, following XR irradiation, the results we obtained with 2′-7′ dichlorofluorescin, which labels hydrogen peroxide, differed from those we obtained with dihydroethidium, which labels superoxide anions (data not shown), thus pointing to the existence of a highly complex network involving ROS, DNA damage-triggering, and the recovery time-scale. The uncoupled results we observed between DNA repair and ΔΨm 20 h after XR irradiation may also be due to the fact that DNA repair and recovery of ΔΨm follow different kinetic patterns.

The role of SMFs in protecting mitochondria, and consequently of modulating the genotoxic effects of IR, is clearly of considerable importance and warrants further investigation. The results of the present study represent a proof of principle that SMFs can interfere with IR and raise the very important question of whether the reduced XR-induced DNA damage caused by SMFs results in a reduced effectiveness of radiotherapy when guided by MRI. The magnetic field used in this study is much weaker than that used in MRI (which are in the order of magnitude of milliTesla and Tesla, respectively). However, remarkable biological effects have been shown for both weak and strong SMFs [24, 41, 42]. Notwithstanding, there is lack of exhaustive studies showing the intensity-dependence of these phenomena in the very same experimental model, thus making it hard to extrapolate conclusions from weak to strong SMFs and vice versa. Since our study was limited to low intensity SMFs, the issue of comparing weak SMFs with strong SMFs, such as that used in MRI, warrants further investigation. Nonetheless, since the combined use of SMFs and IR is usually related to diagnostic applications, based on animal studies the antagonistic effects of this combination on biological structures might benefit patient health [43].

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