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Static electric fields interfere in the viability of cells exposed to ionising radiation

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Abstract

Purpose: The interference of electric fields (EF) with biological processes is an issue of considerable interest. No studies have as yet been reported on the combined effect of EF plus ionising radiation. Here we report studies on this combined effect using the prokaryote Microcystis panniformis, the eukaryote Candida albicans and human cells.

Materials and methods: Cultures of Microcystis panniformis (Cyanobacteria) in glass tubes were irradiated with doses in the interval 0.5–5 kGy, using a 60Co gamma source facility. Samples irradiated with 3 kGy were exposed for 2 h to a 20 V/cm static electric field and viable cells were enumerated. Cultures of Microcystis panniformis (Cyanobacteria) in glass tubes were irradiated with doses from 1–4 kGy, and submitted to an electric field of 180 V/cm. Samples were examined under a fluorescence microscope and the number of unviable (red) and viable (apple green fluorescence) cells was determined. For crossing-check purposes, MRC5 strain of lung cells were irradiated with 2 Gy, exposed to an electric field of 1250 V/cm, incubated overnight with the antibody anti-phospho-histone H2AX and examined under a fluorescence microscope to quantify nuclei with γ-H2AX foci.

Results: In cells exposed to EF, death increased substantially compared to irradiation alone. In C. albicans we observed suppression of the DNA repair shoulder. The effect of EF in growth of M. panniformis was substantial; the number of surviving cells on day-2 after irradiation was 12 times greater than when an EF was applied. By the action of a static electric field on the irradiated MRC5 cells the number of nuclei with γ-H2AX foci increased 40%, approximately.

Conclusions: Application of an EF following irradiation greatly increases cell death. The observation that the DNA repair shoulder in the survival curve of C. albicans is suppressed when cells are exposed to irradiation + EF suggests that EF likely inactivate cellular recovering processes. The result for the number of nuclei with γ-H2AX foci in MRC5 cells indicates that an EF interferes mostly in the DNA repair mechanisms. A molecular ad-hoc model is proposed.

Keywords: Cellular radiobiology, radiation, radiosensitivity, bacteria

Introduction

At the cellular level, phenomena such as transport processes are induced by local gradients of electric fields, temperature, or chemical potential (Beckerman 2005). Endogenous fields are also important in development (Levin et al. 2002) and wound healing (Song et al. 2002). In particular, electric field (EF) effects in biological systems are of long-standing interest. For example, small exogenous fields, static or pulsed (up to 1 GHz) are of interest with respect to sensory systems, medical applications, and...
possible human health hazards (Gowrishankar and Weaver 2003). Indeed, high-intensity external pulsed fields have been used to stimulate excitable cells (Aidley 1998, Hille 2001), for electroporation and for heating tissues in vivo (Prausnitz et al. 1993, Jaroszkesi et al. 2000). Furthermore, the orientation of cell division is influenced by small static electric fields (Zhao et al. 1999).

Over two decades ago Goodman et al. (1983) demonstrated that very weakly pulsing exogenous electromagnetic fields (pulse amplitude around 0.15 V/m) induce the transcription of genes in cultured salivary gland cells of the fruitfly. Likewise, basic cellular phenomena such as growth, differentiation, dedifferentiation, and repair have been reported to be modified by weak electromagnetic fields (Crombie et al. 1990, Panagopoulos et al. 2002, Dini and Abbro 2004). We note too that the potential for genotoxicity of electric and magnetic fields has been discussed by McCann et al. (1998) in the context of a significant body of genotoxicity data. Finally, we draw attention to the fact that exogenous EF interference is apparently high on the agenda of biophysical and medical physics research groups, as recently evidenced by the seminal studies of Kirson et al. (2004, 2007), demonstrating that low-intensity alternating fields can kill dividing cells and slow the growth of brain tumors in cancer patients.

To our knowledge there are no reported studies on the combined effect of static EF and ionising radiation. Here we report the results of studies using both prokaryote and eukaryote organisms, with an emphasis on possible synergistic roles played by these two physical stresses at the cellular level. The examination of both prokaryotic and eukaryotic models was driven by several considerations:

1. Many DNA repair mechanisms in bacteria are conserved in eukaryotes. Hence, a comparative study of their responses to combined ionising radiation and static EF exposure is both relevant and opportune.

2. Two of the three organisms selected for study are both endowed with high levels of radioresistance. Using genetically well defined radiosensitive organisms such as E. coli and the yeast S. cerevisiae would likely introduce complexities associated with additive cytotoxicity.

Finally, we decided to add more evidences on the possible role played by an exogenous electric field on DNA repair mechanisms, vis-a-vis the results obtained with these prokaryotic and eukaryotic models, by carrying out an experiment with human cells where the amount of nuclei with γ-H2AX foci were quantified.

Materials and methods

Cell culture and density counting

(A) Microcystis panniformis BCCUSP100 strain (Cyano bacteria) was grown in BG-11 medium (after a prescription found in Rippka et al. 1979) for a 14:10 hours (light:dark) photoperiod, at 22 ± 0.5°C with an intensity of 30 ± 2 μmol photons · m−2 · s−1. Prior to the beginning of the experiments, the precultures (exponential growth phase) were divided in 10 ml samples and inoculated in 30 ml of new medium in triplicate. Each total volume of 40 ml was housed in a glass tube. The cultures were initiated with 2.6 × 10⁶ cells.ml⁻¹ and incubated as described above. The irradiations were carried out with a ⁶⁰Co gamma source facility (Gammabeam, model 650 from MSD Nordion, Otawa, Canada). All cell samples in glass tubes were irradiated with doses in the interval 0.5–5 kGy at a rate of 0.94 kGy/h. Sets of three glass tubes previously irradiated were exposed for 2 h to a 20 V·cm⁻¹ static electric field between the plates of a capacitor, immediately after irradiation. The control tubes were exposed either only to irradiation or only to the static electric field.

Total cells were enumerated by microscopic counts of culture samples stained with Lugol’s 4% solution in a Fuchs Rosenthal haemacytometer (Optik Labor, Friedrichsdorf, Germany).

The average number of counted cells ranged from 600–1000 in the first and second days after irradiation. It should be noticed that a total of 400 counted cells is required to obtain an error of ~10% at a confidence level of 95% (Guillard 1973). In this sense, errors obtained in this work were considerably better than 10% for each single sample. All measurements were performed in triplicate. The results were averaged, and it was found that their dispersion did not exceed 10% (the same was verified in the case of C. albicans).

(B) Candida albicans (strain ICB-12-A) was inoculated in a tube containing Sabouraud agar, incubated at 36°C for 20 h, gamma irradiated with doses from 1–4 kGy, and then submitted to a static electric field with net intensity (inside the medium) equal to 180 V·cm⁻¹ for 1 h and 30 min. A total of 1 μl of the culture was diluted in 200 μl of PBS (Phosphate Buffered Saline), to which 200 μl of ethidium bromide (Sigma, St Louis, MO, EUA) and 200 μl of fluorescein diacetate (Sigma) were added. A drop of the suspension was set between slide and cover slip and examined under a fluorescence microscope (Leika, DMLB, Wetzlar, Germany), coupled to a digital camera (Hitachi, KCP-D581, color, Tokyo, Japan). The number of unviable (red) and viable (apple green fluorescence) cells was counted by means of the fungal-cell viability method in a solution of fluorescein diacetate (Calich et al.
One hundred fluorescent cells were counted on each prepared slide, three counts were performed for every sample, and the results are presented as averages of these measurements.

**Peculiarities of the elected counting techniques**

We used microscopy techniques to count the number of total cells, which include those that might divide and those that will die after the treatment.

In the case of *M. panniformis*, the counting was carried out by direct optical recognition of total cells in the sample solutions. In order to discriminate those that might divide from those that will die, cell growth was monitored during 10 days as a criterion, since in the 10th day the cell titer decreased by three orders of magnitude (fraction $\approx 10^{-3}$).

With *C. albicans*, intact cells were identified by their green color, since those with red color present membrane rupture. However, some of the cells turning green could have already suffered clonogenic death.

Therefore, in both cases our results represent lower limits of the observed effect, that is, the effect should be more intense. However, lower limits are appropriate for the purposes of the present work.

**Human cells – MRC5 strain of lung cells**

Plated cultures of these cells were irradiated with 2 Gy and immediately exposed to a static electric field of 1250 V/cm. Besides all the conventional procedures, the cells were incubated overnight with the anti-body anti-phospho-histone H2AX (Billerica, MA, USA) – dilution 1: 200. The cell plates were covered with 1 mg/ml of DAPI (4', 6'-diamidino-2-fenilindol-Invitrogen), examined under a fluorescence microscope (Zeiss – model Axiovert 200 – 1000 X. Göttlingen, Niedersachsen, Germany), and nuclei with $\gamma$-H2AX foci were quantified using the program IMAGEJ 1.38. It should be noted that given both the much higher complexity and the huge genome size of the mammalian cells, a more intense electric field was used.

**Results**

**Cellular survival in Candida albicans and Microcystis panniformis**

The survival of *C. albicans* cells following exposure to ionising radiation with and without an exogenous EF is shown in Figure 1, in which the logarithm of the viable cells fraction (S) is plotted as a function of gamma ray dose (D). The survival curve following irradiation exhibits the well-known initial DNA repair shoulder, characterised by a slow decrease of viable cells, followed by a faster decrease at doses greater than $\approx 2$ kGy. However, when irradiated cells were exposed to static EF the shoulder in the survival curve disappeared (Figure 1). The number of viable cells in non-irradiated control samples was

![Figure 1. Survival curves of *C. albicans* as a function of $\gamma$-rays doses in the range 0–4 kGy. Irradiated samples were also submitted to a static electric field of 180 V · cm$^{-1}$ (net intensity inside the medium) for 1 h and 30 min. Open circles – irradiation only. Full circles – irradiation plus application of the electric field (EF). The ordinate axis provides the logarithm of the viable cells fraction (S). This fraction is given by $S = N/N_0$, where N represents the number of cells surviving after irradiation (or after irradiation plus electric field), and $N_0$ is the number of cells before irradiation. Results for control samples (zero dose) are represented by an open square (without EF application) and by a full square (with EF application). The error bars indicate the standard error of the mean (SEM) for three independent experiments.](image-url)
unchanged following the application of a static EF (Figure 1).

Similar to the results observed with \textit{C. albicans}, the survival of \textit{M. panniformis} following exposure to irradiation alone also exhibits an initial shoulder and a rapid decrease above \textasciitilde 2 kGy (Figure 2). However, in contrast to \textit{C. albicans}, application of a static EF to the irradiated cells reduced but did not completely abolish the DNA repair shoulder component of the survival curves (Figure 2).

\textbf{Growth kinetics of \textit{M. panniformis}}

In addition to the studies of survival in \textit{C. albicans} and \textit{M. panniformis} as a function of dose, we examined the effects of EF interference on the growth of \textit{M. panniformis} as a function of incubation times up to several days, at a fixed dose of 3 kGy. This was facilitated by the availability of a unique method to measure cell growth in this organism (see \textit{Materials and methods}). As shown in Figure 3A, the application of an EF (20 V/cm) did not affect cell growth in control experiments. The average of the relative differences between the

![Figure 2. Survival curves of \textit{M. panniformis} as a function of \textgamma-rays doses in the range 0–5 kGy. Irradiated samples were also submitted to a static electric field of 20 V · cm$^{-1}$ (net intensity inside the medium) for 2 h. Full squares – irradiation only. Full circles – irradiation plus application of the electric field (EF). Results for control samples (zero dose) are in Figure 4A. The error bars are of the size of the data points, and they indicate the SEM for three independent experiments.](image1)

![Figure 3. Growth curves of \textit{M. panniformis}. (A) Growth curves for the control and control plus the application of an electric field, as function of the incubation time. (B) Growth curves following \textgamma-rays irradiation with a single dose of 3 kGy, with and without the subsequent application of an electric field. (C) Ratio between the two data sets shown in (B). The ordinate axis of Figure 2A (and in Figure 2B) provides the fraction of the cells. The 0th day corresponds to the beginning of the experiment. The lines connecting the data points are only to guide the eyes. The error bars are of the size of the data points, and they indicate the SEM for three independent experiments.](image2)
two sets of data points shown is negligible. However, when the same EF was applied for 2 h immediately following radiation exposure, there was a significant decrease in cell growth (Figure 3B). This result is better appreciated in Figure 3C, where the ratio of the two data sets in Figure 3B is plotted.

**Cell death rate of M. panniformis**

The cell death rate, the number of killed cells per unit of time, can be defined as $r_{de}(t) = \frac{D N_{de}(t)}{D t}$, where $D N_{de}(t) = N(t) - N(t + \Delta t)$ is the number of cells killed in the time interval $\Delta t$, and $N(t)$ is the number of cells of *M. panniformis* measured at time $t$ (in days) – Figure 3. Considering $\Delta t = 1$ day, we have $r_{de}(t) = D N_{de}(t)$ – Figure 4.

Our definition for $r_{de}$ refers to the mean cell death rate in the time interval of $t$ to $t + \Delta t$, yielding an observation time of $t + \Delta t/2$. The cell death rate is a useful quantity to identify since it provides the rate at which cells are killed daily.

**Quantification of nuclei with γ-H2AX foci in MRC5 strain of lung cells**

Fluorescence results for the MRC5 cells are shown in Figure 5. We observe that the non-subjective quantification of the γ-H2AX foci, carried out by means of the routine IMAGEJ 1.38, provided statistical counting error equal or smaller than 10%.

We note in Figure 5 that the number of γ-H2AX foci counted in cells growing under normal conditions (37°C, 5% CO$_2$) was equal to the one when these cells were submitted to a static electric field of 1250 V/cm by 1 h, within the statistical error.

On the other hand, in cells irradiated with 2 Gy and submitted to a static electric field of 1250 V/cm by 1 h, the number of γ-H2AX foci was nearly 40% higher than in cells only irradiated with 2 Gy.

**Discussion and conclusion**

**Choice of dose and electric field exposure time**

Figure 3B reveals that for 3 kGy of ionising radiation exposure, about 80% of cells of the bacterium *M. panniformis* counted at $t = 24$ h have disappeared by the following day ($t = 48$ h). We conclude that 3 kGy is an appropriate dose to consider because the $1/e$ ($= 0.37$) fraction lies between the two most important observation days (the first and the second days). It is also relevant to point out that the dose corresponding to 37% of survivors is equal to $(1/ z) \times 100$, where $z$ is the inactivation factor, since this represents the dose required to deliver one inactivating event per cell (Alpen 1990). Regarding the optimal duration of exposure to a static EF, we determined that 2 h was sufficient to hinder cell recovery processes, since longer exposure time did not increase cell death.

**Puzzling results and questions**

The data presented in Figures 1 and 2 reveals the following relevant and surprising results: (a) Depletion of the so-called repair shoulder in the survival curves of cells exposed to both irradiation and an EF demonstrates that the EF substantially increased the radiosensitivity of the organisms examined. For *C. albicans*, in particular, this effect is similar to that resulting from exposure to dense ionising radiations such as neutrons and alpha particles, which generate clusters of double strand breaks in DNA (Alpen 1990). In contrast, for *M. panniformis* the shoulder is depleted but not suppressed; and (b) For the prokaryote *M. panniformis* the effect of EF on cell growth is profound.
the number of surviving cells on the second day after irradiation was more than 12 times greater than when an EF was applied.

These observations pose the following relevant questions:

1. Why does a static EF have no cytotoxic effect on cells, but when associated with exposure to radiation cytotoxicity increases substantially?
2. What is the mechanism by which DNA repair (reflected in the shoulder of the survival curves for both *C. albicans* and *M. panniformis*) is depleted when cells are exposed to a non-cytotoxic agent, like a static electric field?

**Answers and a model proposition**

With respect to question 1, first phrase, it is well-known that static EF of the intensities used in this study are not cytotoxic. As noted in the literature for example, electroporation is only achieved with high-intensity external pulsed fields (from several hundreds to thousands of V/cm (see e.g., Prausnitz et al. 1993, Jaroszeski et al. 2000). The answer to question 2 (and question 1, second phrase) on the other hand, led us to the proposition of a model, as depicted below.

Double strand breaks (DSBs) generate quadrupole-like static electric fields, as revealed by experiments of perturbed angular correlations of gamma-rays (PAC), an alternative and elegant experimental technique to study the molecular dynamics of DNA (Kalfas et al. 1984, 1994). These static electric (quadrupole) fields persist until completion of repair. Because of e.g. the presence of salt ions in the cytoplasm, one would argue that the net charge at each DSB site is gradually screened during the signaling-repairing time interval. However, although undergoing reorientation and attraction by the electric field toward the DSB site, ion movement is not propelled as in a repair protein. Therefore, while the former moves diffusively, the latter moves processively (Okada and Hirokawa 1999).

Our model states that the electric dipole of the repair proteins senses the endogenous static electric field at the damage and uses it as a navigation cue. This is consistent with the fact that repair proteins can locate damaged sites within seconds after double strand break (DSB) formation (Jakob et al. 2005), although those sites are constituted by only a few base pairs in the entire genome. Also, our premise provides answer to the long-standing question of Bartek and Lukas: ‘How do proteins throughout the cell nucleus respond in a coordinated way, and how are a few DSBs within three billion base pairs recognised?’ (Bartek and Lukas 2003).

When irradiated cells are exposed to an exogenous EF stronger than the endogenous EF, repair proteins would tend gradually to align their electric dipoles with the direction of this exogenous EF, as pictorially represented in Figure 6. Consequently, the majority of these proteins would be unable to reach damaged sites in DNA. Such a possibility is corroborated by our results for the H2AX histones (Figure 5). In fact, these results show that by the action of a static electric field on the irradiated cells the number of nuclei with γ-H2AX foci increased 40%, approximately. This indicated that the static electric field impeded the action of 40% of the H2AX histones.

![Figure 6](image-url)

**Figure 6.** (a) Pictorial representation of DSB recognition by repair proteins (our hypothesis). These proteins usually have huge electric dipole momenta \( \mathbf{p} \) (represented in [b] with more details), which are oriented toward the damaged site by the static electric field \( \mathbf{E}_{ds} \) produced by the electric imbalance at the strand breaks. (c) An external static electric field \( \mathbf{E}_{ext} \) stronger than \( \mathbf{E}_{ds} \) would reorient the repair proteins displacement all along its direction.
pool initially recruited for the DNA damage sites. In fact, they were still phosphorilating, indicating that they were not used in the repair final process. Also, since 2 Gy is a dose near the repairing shoulder of the MRC5 cells, this result is an additional evidence that the electric field action is more effective at doses where cells are much more repair efficient.

Thus, many of the damages were not repaired because the H2AX histones are crucial players of the 'repairing cascade'. Since a single DSB is lethal if unrepaired (Friedberg 2003), the predictable final consequence for a persistent high-level of unrepaired DSBs would be cell death, as suggested by the experimental results obtained in this study.

The increasing cytotoxicity in C. albicans and M. panniformis, when irradiation is combined with exposure to a static EF, is expected since the fundamental mechanisms of DNA repair are similar in both microorganisms. However, the most striking observation is shoulder suppression in C. albicans compared to shoulder depletion M. panniformis. From a radiological point of view, suppression of the DNA repair shoulder component of the survival curve reflects the persistence of DSBs. At the lower-dose regime (shoulder region of the survival curve) the concentration of damage inflicted to the DNA, for a given dose, is proportional to the amount of targeting material (base pairs) exposed to the radiation – more precisely, the effective radiation interaction cross section. In this sense, our results suggest that damage clustering may be higher in C. albicans compared to M. panniformis. This is consistent with the fact that in prokaryotes the genome is constituted by a single circular DNA molecule, about a millimeter in length, while in eukaryotes genomic DNA is densely wrapped as chromatin.

Our ability to monitor cell death rate for M. panniformis was facilitated by the availability of appropriate methodology (see Materials and methods). While following irradiation alone the average cell death rate from the 0th to the first day is roughly \( r_{de}(0.5) = 0.35 \times N(0) \), i.e., the initial cell populations \( N(0) \) was killed at a rate of about 35% per day. In the case of irradiation plus EF we obtained \( r_{de}(0.5) = 0.75 \times N(0) \), i.e., 75% of the total \( N(0) \) is killed per day. Besides its massive killing rate, the combination of irradiation plus EF is considerably faster, exhibiting a peak at \( t = 0.5d \). Actually, 3/4 of the entire cell population is killed in the first 12 h (\( t = 0.5d \)), explaining why the rate drops so abruptly in subsequent days. In contrast, when only irradiation is applied, the death rate peaks at about 24–36 h (\( t = 1–1.5d \)).

In conclusion, the results of these studies demonstrate that the application a static EF following exposure of both radioresistant prokaryotic and eukaryotic cells to ionising radiation greatly increases cell death. The effect is similar to that observed when a highly ionising radiation is used. Our results also suggest that EF likely act by inactivating cellular DNA repair processes. In fact, the observation of a 40% surplus of nuclei with \( \gamma \)-H2AX foci in MRC5 strain of lung cells, when irradiated and exposed to an intense EF, points into this direction.

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