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Genomic DNA damage induced by co-exposure to DNA damaging agents and pulsed magnetic field

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RUNNING HEAD: DNA damage by MMS, bleomycin and pulsed MF

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Abstract

Purpose: Many articles describe the effects of extremely low-frequency magnetic fields (MF) on DNA damage induction. However, the mechanism of MF interaction with living matter is not yet known with certainty. Some works suggest that MF could induce an increase in the efficacy of Reactive Oxygen Species (ROS) production. This work investigates whether pulsed MF exposure produces alterations in genomic DNA damage induced by co-exposure to DNA damaging agents (bleomycin and methyl methanesulfonate (MMS)).

Materials and methods: Genomic DNA, prepared from *S. cerevisiae* cultures, was exposed to pulsed MF (1.5 mT peak, 25 Hz) and MMS (0-1 %) (15-60 minutes), and to MF and bleomycin (0-0.6 IU/ml) (24-72 hours). The damage induced to DNA was evaluated by electrophoresis and image analysis.

Results: Pulsed MF induced an increment in the level of DNA damage produced by MMS and bleomycin in all groups at the exposure conditions assayed.

Conclusions: Pulsed MF could modulate the cytotoxic action of MMS and bleomycin. The observed effect could be the result of a multifactorial process influenced by the type of agent that damages DNA, the dose, and the duration of the exposure to the pulsed MF.

Keywords: yeast; DNA damage; methyl methanesulfonate; bleomycin; pulsed magnetic field.

Introduction

The possible effect of electromagnetic fields (EMF) on the health of human beings lead researchers to study the possible harmful effects, especially those related to cancer induction. Many laboratory studies have been published using different types of magnetic fields (MF) (continuous, sinusoidal, intermittent and pulsed) and exposure protocols in terms of frequency, magnetic flux density, exposure time as well as different cell types. Despite the large number of effects and data published, and although the International Agency for Research on Cancer (IARC) has concluded that extremely low-frequency EMF are "possible carcinogenic" (IARC 2002), to date, the intimate mechanism of interaction between the MF and living matter is still unknown. Some works suggest that MF could induce an increment in the generation of Reactive Oxygen Species (ROS) (Sherrad et al. 2018; Solek et al. 2017; Mercado-Sáenz et al. 2021).

Two types of effects on DNA induced by damage-causing agents are distinguished. On the one hand, the genotoxic effect in which direct or indirect damage to DNA is observed after exposure only to the toxic agent. On the other hand, the additive effect consists of combining a known toxic agent with MF (co-exposure), and whose combination increases the toxic activity of the agent on DNA. Numerous articles describe the genotoxic damage that extremely low-frequency MF produce on DNA. However, when co-exposure effects are analyzed, it has been observed that they predominate over those. In this sense, the co-exposure to MF and toxic agents has led to the publication of more results that observe an increase in cytotoxic effects than when exposures are made to MF alone (Ruiz-Gómez and Martínez-Morillo, 2009). Among the additive effects observed on DNA, an increase in single and double DNA strand breaks has been described mainly by co-exposure to MF with $SnCl_2$ (Lourencini da Silva et al. 2000), X-ray (Miyakoshi et al. 2000), H_2O_2 (Li and Chow, 2001), benzenetriol (Moretti et al. 2005) or nitroquinodine N-oxide (Villarini et al. 2006); as an examples among other physical and chemical agents. Therefore, MF could act as a co-inductor of DNA damage rather than a toxic agent.

A causal relationship between exposure to MF and cancer has been suggested (IARC, 2002). DNA strand breaks constitute a type of damage closely related to the appearance of mutations due to defects in DNA repair, with carcinogenesis and in cases in which the number of breaks is high with cell death and therefore with a significant cytotoxic effect.

On the other hand, it is important to note that living systems have very effective DNA damage repair mechanisms. Therefore, when a level of damage is observed and measured, the global balance between the damage produced and the repair carried out is quantified. The work presented in this article has been performed using isolated DNA molecules as a biological model to evaluate the damage produced without the intervention of repair mechanisms. A previous report from our lab demonstrated that the exposure to a pulsed MF (25 Hz, 1.5 mT, 8 h/day, 16 days) increases

the spontaneous DNA degradation with time when DNA molecules are isolated and maintained in a buffer (López-Díaz et al. 2014).

In this work we have used bleomycin (Figure 1a) and methyl methanesulfonate (MMS) (Figure 1b) as genotoxic agents in co-exposure to pulsed MF. Bleomycin is an antineoplastic agent that belongs to the group of antitumoral antibiotics used in the treatment of squamous cell carcinoma, Hodgkin disease and non-Hodgkin lymphoma, among others. It is a radiomimetic agent whose mechanism of action consists in the generation of ROS which react with the DNA molecules causing their fragmentation (Obe et al. 2010). MMS is a genotoxic alkylating agent that exhibits activity on the nitrogenous rings of puric bases, producing the methylation of adenine and guanine bases. It is used as a model drug, causing poorly paired bases that lead to DNA fragmentation. It could be also considered a radiomimetic agent (Lundin et al. 2005; Ovejero et al. 2021).

Other authors reported that sinusoidal MF (5 mT) enhances the apurinic/apyrimidinic site number in human glioma cells exposed to MMS, suggesting an increment in the activity or lengthen of lifetime of radical pairs as a mechanism of action (Koyama et al. 2008). Moreover, Luukkonen et al. (2011) found that 100 μT sinusoidal 50 Hz MF alters the cellular response of neuroblastoma cells to MMS, i.e., increasing its sensitivity to MMS-induced damage. On the other hand, Cho et al. (2007) found that exposure of fibroblasts to 0.8 mT, sinusoidal 60 Hz, MF increases the damage induced by bleomycin to DNA. In addition, Miyakoshi et al. (2012) indicated that the possible mechanism responsible for DNA damage induced by co-exposure of rats to bleomycin and 10 mT 50 Hz MF is related to ROS. Recently, Sanie-Jahromi and Saadat (2017) published that exposures of MCF-7 breast cancer cells to bleomycin in combination with MF (50 Hz, 0.5 mT, intermittent 15/15 min. on/off, 30 min. total exposure) increases the levels of expression of some genes related to DNA repair mechanisms, suggesting an increase in the damage produced by the MF exposure.

Very few articles have been published using pulsed MF in co-exposure to antineoplastic drugs and practically no articles using isolated DNA molecules as a biological model.

The aim of this study is to investigate if the exposure to pulsed MF (1.5 mT peak, 25 Hz) produces alterations in genomic DNA damage induced by co-exposure to DNA damaging agents (methyl methanesulfonate and bleomycin).

Materials and Methods

Yeast and DNA source

The yeast strain used as a source of genomic DNA was the wild-type haploid WS8105-1C *Saccharomyces cerevisiae* (ATCC® 200383™) strain (Genotype: *MATalpha RAD HDF ade2 arg4-17 trp1-289 ura3-52*). A rich medium (Yeast extract–Peptone–Dextrose (YPD) 1:2:2 (%) ± 2 % Bacto-agar), under constant agitation (300 rpm) at 30°C, was used for vegetative growth (Friedl et al. 1998; Siede et al. 1996; Ruiz-Gómez et al. 2010). The reagents were purchased from Difco, BD, Co. Sparks, MD, USA.

The DNA used for DNA damaging agents and pulsed MF exposures was genomic DNA prepared from overnight cultures.

DNA damaging agents

Methyl methanesulfonate (MMS) (purchased from Sigma-Aldrich Inc. St. Louis-MO, USA) and bleomycin (Mylan pharmaceuticals SL, Barcelona, Spain) were used as DNA damaging agents. MMS doses were 0, 0.2, 0.5, 0.8 and 1 % and the time of exposures: 15, 30, 45 and 60 minutes. Bleomycin doses were 0, 0.015, 0.03, 0.045, 0.06, 0.15, 0.3, 0.45 and 0.6 IU/ml and the time of exposures: 24, 48 and 72 hours. These concentration ranges and the exposure times have been chosen after previously evaluation of the effect of these agents on DNA. It was found that MMS has a higher activity because shorter exposure times were required. Bleomycin did not induce DNA breaks at times below 24 h.

The different concentrations of damaging agents were prepared from a stock diluted in distilled water. The sample volume (25 µl) contained 12 µl of isolated DNA plus the appropriate volume of damaging agent from the stock so that an additional 13 µl, in water, would obtain the desired concentration. The correct volumes for each concentration of DNA damaging agent were obtained from dilutions in water of the concentrated stock. The entire volume of sample (25 µl) was exposed to MF and subsequently included in each well of the electrophoresis gels.

Magnetic Field exposure system

The exposure equipment was described in previous papers by Ruiz-Gómez and Martínez-Morillo (2005), López-Díaz et al. (2014) and González-Vidal et al. (2021). As indicated in these articles, the pulsed MF equipment (Pulsatrón, CEM – 84/J; J&J Electromédica; Málaga, Spain)

produces rectangular voltage pulses (25 Hz) that feed 2 coils (15x10.5 cm) in air connected like Helmholtz type (in series) (Figure 2). This equipment is used in rehabilitation clinics for magnetotherapy purposes. The voltage waveform (Figure 3) consists in 15 rectangular pulses repeated at the frequency of 25 Hz. The associated MF according to Biot-Savart Law is equivalent to the current intensity generated in the circuit and therefore, its time derivative according to Faraday's Law of electromagnetic induction has a similar waveform as the electromotive force when a pickup coil in air is used. This waveform was studied with the use of a pickup coil in air and checked in an inductive circuit. As shown in figure 3, the generated pulsed MF consisted in trains of 15 square pulses (180 µs width, 20 µs gap, 1.5 mT peak). There were 25 wave-trains/second, equally-spaced.

The frequency and the intensity (25 Hz, 1.5 mT peak) used were the highest values that can be supplied by this MF equipment. Li and Chow (2001) used similar values (1.2 mT) in the study of plasmid stability alterations after MF exposure. The voltage waveform characteristics applied to the coils and the electric field waveform associated to the generated MF were published in a previous article (Ruiz-Gómez and Martínez-Morillo, 2005). When the coils are settled as Helmholtz-type (2 coils separated with a common axis, where their distance = coil radius) the value of the MF generated at the peak midpoint was 1.5 mT (homogeneity ± 5 %); factory calibrated and indicated in the technical specifications.

The Faraday's Law of Induction indicates that a time varying MF will induce a nonuniform electric field:

$$\xi = \oint_c \vec{E}.d\vec{l} = -\frac{d}{dt}\iint \vec{B}.d\vec{S} \qquad (1)$$

where ξ = induced electromotive force (V), E = electric field (V/m) and B = magnetic field (T).

If we consider a circular contour (c) where r = radius, coaxial with the coils and located parallel to them, the above equation (1), becomes in equation (2) if we assume that MF is homogeneous in the surface (S) inside the contour:

$$\xi = E.2\pi r = -\pi r^2 \frac{d}{dt} B \qquad (2)$$

At low frequencies, as in our case, the MF is proportional to the intensity that flows through the coils according to Biot-Savart law, and then $\frac{dB}{dt}$ is proportional to $\frac{di}{dt}$.

In this sense, the ξ waveform was measured and visualized in an oscilloscope, for 3 different radii, using a circular pickup coil in air located between coils. Qualitatively it corresponds with the waveform obtained for $\frac{di}{dt}$ in a R-L circuit. The ξ waveform consisted in pulses that decreased exponentially towards zero volts, positive in those intervals, in figure 3, corresponding to 180 μs and negative in those of 20 μs.

After the measurement of the peak values (positive and negative pulses) of ξ, the electric field peak values were calculated from the equation (3):

$$E = \frac{\xi}{2\pi r} \quad (3)$$

The measurements were made using pickup coils of radii 2 cm, 3 cm, and 4 cm. The results obtained were, 0.5 V/m, 0.7 V/m, and 0.8 V/m, for the positive pulses and 2.98 V/m, 3.82 V/m and 3.98 V/m for the negative ones, respectively.

However, these values do not correspond to the electric field experienced by the DNA (López-Díaz et al. 2014). The fact that the DNA samples placed in Eppendorf tubes contained Tris-EDTA buffer makes the medium conductive. This characteristic modifies the electric field peak values. In contrast, the E field waveform is not modified. The effective electric field (Ee) value was measured using pickup coils located inside each well of a 24-well dish. The values obtained for Ee ranged from 65 mV/m to 260 mV/m (for positive pulses in the 180 μs region) and from 650 to 1600 mV/m (for negative pulses in the 20 μs gap), depending on the spatial location of each well (Figure 3).

The spatial distribution of the MF produced by the coils is expected to be due to the vertical component of the MF.

Genomic DNA

Genomic DNA was prepared from yeast cultures by extraction with Phenol-Chloroform and subsequent ethanol precipitation (Hoffman and Winston 1987). This technique, with some modifications, has been previously reported by López-Díaz et al. (2014). Cells were lysed in Tris/Cl Ethylenediaminetetraacetic acid (EDTA) (10 mM, pH 8.0), Triton X-100 (2 %), lauryl sulfate (SDS) (1 %), and NaCl (0.1 M). After Phenol:Chloroform:isoamyl alcohol (25:2:1) and small glass beads addition, samples were vortexed and centrifuged (5 minutes, 15,300 *g*). The DNA, in the aqueous phase, was precipitated with absolute ethanol and RNA digested with RNase

A (50 µg/ml). The isolated DNA was dissolved in Tris-EDTA pH 8.0 buffer. The yield of extracted DNA was 1205.20 ± 284.14 ng.

Experimental protocol

In this study we exposed isolated genomic DNA molecules to DNA damaging agents and pulsed MF. Before exposures, yeast cells were incubated for 48 h in liquid YPD. Then, the culture volume was divided into different test tubes containing 8 ml each ($1x10^8$ cells/ml), for DNA preparation. After DNA extraction, all DNA samples were pooled to be homogeneous. 12 µl from this genomic DNA mix, containing 400 ng of DNA, was used per sample to be incubated with different DNA damaging agents and pulsed MF doses, in Eppendorf tubes. Each sample (25 µl) contained 12 µl of DNA in Tris-EDA plus 13 µl of DNA damaging agent in water. 13 µl of water was added to control samples. After exposures, the entire sample (25 µl) was placed in each well for gel electrophoresis. MF treated samples were exposed to 25 Hz, 1.5 mT peak; for the same length of time as exposure to DNA-damaging agents. The frequency used (25 Hz) was the highest value that could be obtained with the equipment used. The magnetic flux density (1.5 mT) is constant and cannot be varied in this equipment. Both, MF-treated samples and their matched controls in each replicate experiment, were manipulated equally in temporal coincidence of treatment and temperature (23°C). 3 independent experiments were carried out with triplicate samples. A standard fan was used to avoid the excess of heat produced in the coils. The level of temperature was measured during the experiments at control location and in the activated coils. The distance between the coils and the location of control cultures were 5 m. The laboratory room was air conditioned to assure a constant temperature. Therefore, MF-treated and control samples were maintained at the same conditions of temperature during the period of exposure in the experiments. Due to the fact that only one magnetotherapy equipment was available and to ensure that the control DNA and the exposed DNA had been handled exactly the same, were in the same environmental conditions and the possible spontaneous degradation that DNA could suffer since their isolation was the same; we decided not to use sham-exposed samples as control. The temporal mismatch between sham-exposed and MF-exposed samples (especially in the groups treated for 24-72 h) could present differential spontaneous DNA degradation between control and MF-exposed samples.

Control and MF-exposed samples containing the isolated DNA and the adequate concentration of chemical agent were located in different Eppendorf tubes inside test tubes for a better location between coils (Figure 2). After MF exposure time, DNA samples were electrophoresed.

The DC geomagnetic field in Málaga is 42.95 µT (27.225 µT horizontal, 33.20 µT vertical; 3°5' Western (Data from the Institute of National Geographic, Madrid, Spain). The background MF (caused by the lab equipment) in the laboratory was 0.68 µT (Mercado-Sáenz et al. 2021).

Cellular density

The concentration of cells was calculated by OD600 nm measurements (Helios-ε; Unicam; Cambridge-UK). OD600 nm < 1.0 has a linear relationship with the quantity of yeast cells (Amberg et al. 2005).

Agarose gel electrophoresis

Genomic DNA were electrophoresed in agarose gels (1 % in 0.5 X Tris Borate EDTA) at 80 V, 100 min. Gels were stained with ethidium bromide (10 mg/ml). The DNA preparation of all samples was carried out at the same time and under the same conditions, so it is assumed that the yield of DNA from each sample was similar. The same volume of sample (25 µl) was included in each well in the gels. Reagents were purchased from Sigma Aldrich Co, St Louis-MO, USA). 1Kb Blue DNA ladder (Bioron Diagnostics GmbH, Römerberg, Germany) was used as DNA ladder.

Image analysis of agarose gels

The analysis of agarose gels was made from a photography (jpg file) with the software: ImageJ 1.52a (Wayne Rasband, NIH, USA (http://imagej.nih.gov/ij). The level of the signal intensity and the distance migrated by the DNA in each lane permitted to represent in a graph the DNA profile of each sample. From these profiles, the DNA content (measured in arbitrary units) was calculated automatically as the area under the curve. The percentage of untreated control for the undamaged DNA was calculated and plotted on a graph. The DNA profile considered as undamaged was located at the location of the control band in the gel. A DNA ladder was used as a reference for DNA fragments size (López-Díaz et al. 2014; Mercado-Sáenz et al. 2021).

Statistical analyses

The two-way Analysis of Variance (ANOVA) (treatment x dose of chemical agent) were applied to study the differences between treatment curves (MF-treated and Control). In addition, the comparison between each concentration of MMS and bleomycin between treatments (Control and pulsed MF) was analyzed by the Student's *t*-test. The value of $p<0.05$ was consider as statistically significant. 3 independent experiments were carried out with triplicate samples.

Results

The study of pulsed MF effect on the alteration of the damage to DNA induced by the DNA damaging agents MMS and bleomycin was evaluated. The samples were exposed to pulsed MF during the same time as to the drugs.

Exposure of isolated genomic DNA to methyl methanesulfonate and pulsed magnetic field

The damage induced to the genomic DNA by MMS and by co-exposure to MMS and pulsed MF was evaluated at different times of exposure (15, 30, 45 and 60 minutes). After exposure, the genomic DNA was electrophoresed. Figures 4a, 4b, 4c and 4d show the band pattern distribution obtained for genomic DNA exposed to 1.5 mT peak, 25 Hz pulsed MF at different times in co-exposure to MMS, and their respective controls. The area on the gels that corresponds to the width and location of the DNA band in the 0 % MMS control sample was considered as undamaged DNA and hence the area in the gels below was considered as damaged DNA (Figure 4). As it can be seen in the gels, this band did not show degradation. The visual analysis of the gels showed that pulsed MF exposure alone (0 % MMS and MF exposure) did not induce an increase in DNA degradation since the signal obtained in the DNA bands were similar. However, the increment in the concentration of MMS induced the appearance of fuzzy bands with lower intensity signal and even the disappearance of them, in control and pulsed PMF treated samples. Differences were obtained between samples treated with MF and their respective controls. The effect was time and dose dependent in all groups (Figures 4a,b,c,d).

The visual analysis of the gels already indicates that there has been an alteration in the DNA damage pattern due to the exposure to pulsed MF, however it is important to quantify the damage. In this way, for the purpose of quantifying the DNA content, an image analysis was performed to obtain the DNA profiles in each lane. The profiles obtained show in a graph the signal intensity

versus the migration distance (Figures 4e, 4f, 4g, 4h). Figure 4b shows that 15 minutes of pulsed MF exposure induced the disappearance of the DNA peak at 0.8 % MMS. At 30 minutes of exposure the degradation was higher for both, treated and control samples (Figure 4f). The increase in the exposure time to 45 and 60 minutes produced a greater degradation, obtaining the disappearance of the DNA peak at 0.2 % MMS (Figure 4g) and 0.5 % MMS (Figure 4h).

DNA content was calculated from the profiles as the area under the curve by the ImageJ software. The undamaged DNA was located in the profiles as the area under the curve that match with the width of the peaks in the 0 % MMS control samples (from pixel 50 to 150). The area under the curve above this region (from pixel 150 to 819) corresponds to the area of the gels with increasing DNA damage. In this way, the area under the curve for the peak was obtained and the percentage of untreated control for undamaged DNA calculated. The data obtained from the image analysis of bands were normalized for comparison in relation to the DNA content measured in the untreated controls and expressed as percentage. Normalization makes it possible to compare different experiments in which the same amount of DNA can appear with a dimmer or brighter band depending on the different lighting when taking the picture.

Figure 5 shows the values of DNA content obtained from the analysis of profiles in figure 4 considering only the undamaged DNA. The data were normalized for comparison in relation to the DNA content measured in the untreated controls and expressed as percentage. In general terms, MF-treated and control curves show statistically significant differences, $p<0.05$ ANOVA (Fig. 5a,b,c,d). As shown in figure 5a, the undamaged DNA was 100 % for MMS doses of 0-0.8 % in the control samples. DNA degradation was clear for 1 % MMS, 15 minutes. However, co-exposure to pulsed MF produced an increase in DNA degradation, causing a decrease in the undamaged DNA until 0.00383 % and 0.000106 % at doses of 0.8 and 1 % MMS, respectively ($p<0.01$ Student's *t*-test). The increase in degradation observed was considerably high due to the action of the MF. For the rest of the exposure times, the observed degradation values were lower. Specifically, exposure to MMS for 30 minutes produced a significant decrease in the levels of undamaged DNA from 0.5 % MMS. Differences were only found after exposure to the pulsed MF in the groups treated with 0.2 % MMS, in which the MF exposure produced an increase in DNA degradation and therefore a decrease in the undamaged DNA to levels of 0.8 % ($p<0.05$ Student's *t*-test) (Figure 5b). Figure 5c shows that exposure to MMS for 45 minutes produced a progressive decrease in the amount of undamaged DNA as the consequence of degradation, for doses between 0.2-1 % MMS, measured undamaged DNA levels of 62.39-33.37 % in controls and 41.22-23.30 % in samples exposed to pulsed MF ($p<0.05$ Student's *t*-test). Finally, the exposure during 60 minutes produced a decrease to 21.10 % the undamaged DNA in the controls and to 9.68 % in the groups treated with MF, for MMS doses of 0.5 %. Higher doses (1 % MMS+MF) decreased the undamaged DNA to 3.30 % ($p<0.05$ Student's *t*-test). MMS alone did

not produce an increase in degradation. Important differences were observed between controls and pulsed MF treated samples (Figure 5d).

These results suggest that exposure to pulsed MF for 15-60 minutes increases the damage induced by the MMS agent. The observed effect was dependent on time and dose.

Exposure of isolated genomic DNA to bleomycin and pulsed magnetic field

The study of another DNA damaging agent such as bleomycin and its modulation by pulsed MF was performed using genomic DNA as a biological model in this case with higher exposure times (24, 48 and 72 hours). Genomic DNA was exposed following the same protocol described and electrophoresed. Figures 6a, 6d and 6e show the band pattern distribution obtained after exposure to 1.5 mT peak, 25 Hz pulsed MF at the indicated times in co-exposure with bleomycin. Also, their respective controls are showed in the same gel. The area on the gels that corresponds to the width and location of the DNA band in the 0 IU/ml bleomycin control sample was considered as undamaged DNA and hence the area in the gels below was considered as damaged DNA (Figure 6). The visual analysis of the gels showed that pulsed MF exposure alone (0 IU/ml bleomycin and MF exposure) did not induce an increase in DNA degradation since the signal obtained in the DNA bands were similar. However, the increment in the concentration of bleomycin induced the appearance of fuzzy bands with a wide area of intensity signal (from 10,000 bp to 300 bp) and even the disappearance of them, in control and pulsed PMF treated samples. Differences were observed between samples treated with MF and their respective controls. The effect was time and dose dependent in all groups (Figure 6a,d,e). An alteration in the DNA damage pattern by the exposure to pulsed MF was evident. In the same way as previously described for MMS, the DNA content was quantified by image analysis and DNA profiles for each lane were obtained. The profiles (intensity signal versus migration distance) (Figure 6b,c,f) shows that 24 hours of exposure to pulsed MF induced the disappearance of the DNA peak at the dose of 0.015 IU/ml bleomycin. At 48 and 72 hours of exposure the disappearance was also evident. Some artifacts were obtained in some electrophoresis that reduced the quality of the image. To avoid the interference of the artifacts found in the analysis, we proceeded to eliminate them with the image analysis software. In this way, the artifacts did not appear in the DNA profiles and therefore did not alter the results.

In order to quantify the DNA content from the profiles, the area under the curve by the ImageJ software was obtained automatically. The undamaged DNA was located in the profiles as the area under the curve that match with the width of the peaks in the 0 IU/ml bleomycin control samples (from pixel 15 to 85). The area under the curve above this region (from pixel 85 to 455)

corresponds to the area of the gels with increasing DNA damage. In this way, the area under the curve for the peak was obtained and the percentage of untreated control for undamaged DNA calculated.

The samples corresponding to figure 6 were exposed for a period of 24-72 hours. The DNA controls suffer spontaneous degradation after this time if they are not at 4ºC (López-Díaz et al. 2014). For this reason, the bands with 0 IU/ml of bleomycin showed a small spontaneous degradation. This spontaneous degradation band makes it possible to check whether the MF alters the spontaneous degradation of DNA during this time. From the analysis it was deduced that there are no significant differences in both control groups (0 IU bleomycin ± pulsed MF), so that the MF did not alter the spontaneous degradation and therefore the comparison is valid as long as the data are normalized, as has been done. In a previous study, altered spontaneous DNA degradation by MF was observed after a period of 16 days (López-Díaz et al. 2014).

The bleomycin concentrations were adequate to obtain a visible result in the time ranges used. Previous analyzes with lower bleomycin doses and shorter exposure times showed no visible DNA degradation effects on the gels.

Figure 7 shows the values of DNA content obtained from the analysis of profiles in figure 6 considering only the undamaged DNA. The data were normalized for comparison in relation to the DNA content measured in the untreated controls and expressed as percentage. In general terms, MF-treated and control curves show statistically significant differences, $p<0.05$ ANOVA (Fig. 7a,b,c). As shown in figure 7a, the undamaged DNA showed a small decrease for low doses of bleomycin in the control samples that remain constant from 0.1 to 0.6 IU/ml. However, samples exposed to pulsed MF during 24 hours showed a higher gradual increase in DNA degradation, showed in figure 7a as an important and gradual decrease in the levels of undamaged DNA, reaching values of 0.015 % ($p<0.01$ Student's *t*-test). The increment in the time of exposure to pulsed MF showed a similar pattern with small decrease in undamaged DNA at low bleomycin doses and a constant level of DNA from 0.1 to 0.6 IU/ml bleomycin in control samples exposed during 48 and 72 hours. DNA degradation was clear by exposure to bleomycin during 24-72 hours. However, co-exposure with pulsed MF produced a gradual increase in DNA degradation in relation to controls, causing a gradual decrease in the undamaged DNA at doses from 0.015 to 0.6 IU/ml of bleomycin. Differences between MF-treated and control samples were statistically significant ($p<0.05$ Student's *t*-test). The DNA levels obtained decreased until 18.84 % (48 hours) (Figure 7b) and to 21.98 % (72 hours) (Figure 7c). The increase in DNA degradation obtained was considerably very high in relation to their matched controls due to the action of the applied pulsed MF, in the conditions of the assays.

These results suggest that exposure to pulsed MF for 24, 48 and 72 hours increases the DNA damage induced by bleomycin. The observed effect was dose dependent.

Discussion

The carcinogenic processes induced by genotoxic agents are strongly related to the formation of DNA breaks, which lead to the appearance of mutations as a consequence of the abnormal repair of these lesions. In this study we found that co-exposure of isolated DNA molecules to a pulsed MF (1.5 mT, 25 Hz) and MMS or bleomycin, increases the degradation of DNA induced by these drugs.

Due to the low energy that low-frequency MF deposit in the tissues they pass through, the occurrence of direct DNA damage (DNA single or double strand break) (genotoxic effect) is unlikely. DNA breaks are very efficiently repaired in cells, so they are hidden and hardly noticeable. The co-exposure to MF and genotoxic agents (additive effect) allows to observe with greater probability the alterations in the DNA damage that could occur (Ruiz-Gómez and Martínez-Morillo, 2009). Even so, there are many articles that describe direct damage to the DNA molecule by exposure to MF alone. In this sense, we reported an increment in the degradation of genomic DNA in *S. cerevisiae* cells that were exposed to the same type of pulsed MF but with different exposure time (8 h/day) during chronological aging (40 days) (Mercado-Sáenz et al. 2021). Considering isolated genomic DNA molecules as an experimental model, we found previously a 20.7-fold increase in genomic DNA degradation, in relation to controls, after exposure of DNA to pulsed MF during 16 days (López-Díaz et al. 2014).

In order to use a biological model that allows a better observation of the induced damage, genomic DNA molecules prepared from *S. cerevisiae* cultures were used in co-exposure with MF and DNA damaging agents. In this way, cellular repair mechanisms are avoided so that any minimal alteration in the damage produced can be observed.

The results show a greater effect of the MF for higher doses of DNA damaging agents. However, a clear relationship with the longest exposure time is not observed. For both MMS and bleomycin, the greatest differences are obtained with respect to the control for lower exposure times. The applied MF enhances the effect of MMS and bleomycin in a time dependent manner, but not in all cases. This result is in principle contradictory to what was expected. However, in this sense we hypothesize that there could be some kind of competition effect for causing DNA damage. On the one hand, the damage caused by the DNA damaging agent by itself and, on the

other hand, the damage induced by the combination of the chemical agent and the MF. Both types of damage could occur at the same time competitively (Ruiz-Gómez and Martínez-Morillo, 2009).

It has been published that isolated genomic DNA is unstable since it is subjected to continuous oxidation reactions that cause its degradation (Billen 1990; Muratori et al. 2003). In the conditions of the assays, the DNA undergoes spontaneous degradation produced by oxidation reactions to which the effects of genotoxic agents are added, which also act by increasing ROS levels. According to the experimental conditions of this work and the results found, we hypothesize that the increase in DNA degradation by pulsed MF could be induced by an increment in ROS generation. This hypothesis is supported by the previous studies performed by Solek et al. (2017) and Sherrard et al. (2018), which show an increase in DNA breaks by pulsed MF (0-10 mT, 2-120 Hz, 15 min-96 h), also suggesting the increase in ROS levels as a mechanism of action. In relation to this theory, the interaction produced between MF and living matter may involve the mechanism known as Radical Pair. In this way, MF drive pairs of free radicals from singlet to triplet spin state. This process produces a longer lifetime of the generated radical pairs when the levels of MF are low. Thus, MF modify the probability of interaction of free radicals (Eveson et al. 2000; Liu et al. 2005; Till et al. 1998).

The results reported in this article and the proposed hypothesis is also supported by other authors who have used other types of in vitro DNA molecules. In this way, Li and Chow (2001) observed DNA degradation after exposure of plasmid pUC18 to 50 Hz MF, 1.2 mT; and Potenza et al. (2004) found alterations in DNA stability of plasmid pGEM exposed to static MF, 200 mT and 250 mT. These authors conclude that MF increase the activity of free radicals, enhancing the oxidative damage to DNA.

The published literature shows results of DNA damage from both short and long-term exposures to MF. In the same way, an increase in DNA damage is observed using different types of DNA damaging agents, at different doses and protocols of MF exposure. It is clear that MF is capable of causing damage to DNA despite the low energy that it can supply to the cell or tissue, so the existence of an intermediate molecular mechanism responsible for the damage is more plausible, such as oxidation reactions by ROS. However, as dose-response relationships have not yet been established, this mechanism proposed by many authors remains a hypothesis to date awaiting definitive confirmation. In addition, it happens that the results are the sum of a multifactorial process that can be modified by numerous parameters such as the type of MF, frequency, magnetic flux density, exposure time, cell state, repair capacity, etc.., making difficult the replication of studies.

In an attempt to study whether oxidation reactions by ROS are involved in the mechanism of DNA damage by MF, bleomycin and MMS were used as DNA damaging agents. Bleomycin

generates ROS which react with DNA, but MMS is an alkylating agent. Thus, exposure to MMS does not produce ROS and does not induce oxidative DNA damage.

It is clear that MF induced an increment in DNA damage in co-exposure to both agents, which suggests *a priori* the existence of another mechanism of action of MF other than the generation of ROS. However, this is not correct, as it has been demonstrated by Salmon et al. (2004) that the presence of DNA damage alone is sufficient to produce an increment in the levels of intracellular ROS. Therefore, MMS is capable of induce an increment in intracellular ROS, independently of its mechanism of action. This fact supports our hypothesis that MF interacts with living matter increasing the action of free radicals. In addition, the image analysis performed contributes to a better evaluation of the damage observed in the electrophoresis gels that allows the detection of DNA quantities as low as 26 ng/band.

These results, which link co-exposure to MF and DNA damaging agents to intracellular ROS generation, may provide insight into the role of MF on carcinogenesis.

In relation to the relationship between yeast genome and human genome, it is important to highlight that *S. cerevisiae* genome has 12 Mb while the human genome has 3200 Mb. This enormous difference in size means that the effects described in this article depend not only on the concentration of the chemical agent and the time of exposure, but also on the amount of DNA exposed. In this case, the use of human genomic DNA will likely require longer exposure times and/or higher doses of agents.

Conclusions

The results found in this work indicate that exposure to a pulsed MF (1.5 mT peak, 25 Hz) could induce the modulation of the action of DNA damaging agents. Specifically, this work concludes that co-exposures to pulsed MF and chemical agents, both at short times (15, 30, 45 and 60 minutes, in the case of MMS) and at long times (24, 48 and 72 hours, for the case of bleomycin), produce an increase in the activity of the toxic agents, which is manifested with a greater degradation of the genomic DNA molecules used as a model. This could be the result of a multifactorial process influenced by the type of agent that damages DNA, the dose, and the duration of the exposure to the pulsed MF.

Even today the intimate mechanisms of interaction between MF and tissues, cells or both organic and inorganic molecules are not known with certainty. The investigation carried out in

comparison with the literature cited seems to indicate a relationship with the generation of ROS and therefore with reactions with free radicals in oxidative reactions.

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FIGURE LEGENDS

Figure 1. DNA damaging agents. **a)** Chemical structure of bleomycin. **b)** Chemical structure of methyl methanesulfonate (MMS).

Figure 2. Equipment used to generate the pulsed magnetic field. **a)** Photography of the Pulsatrón CEM–84/J magnetotherapy equipment. **b)** Diagram of the equipment and location of samples inside eppendorf tubes.

Figure 3. Tension waveform produced by the equipment used in the generation of pulsed magnetic field (Pulsatron–CEM-84/J). The equipment was used at its maximum frequency (25 Hz). The number of rectangular pulses (180 µs) was 15. The magnetic field generated has the same waveform.

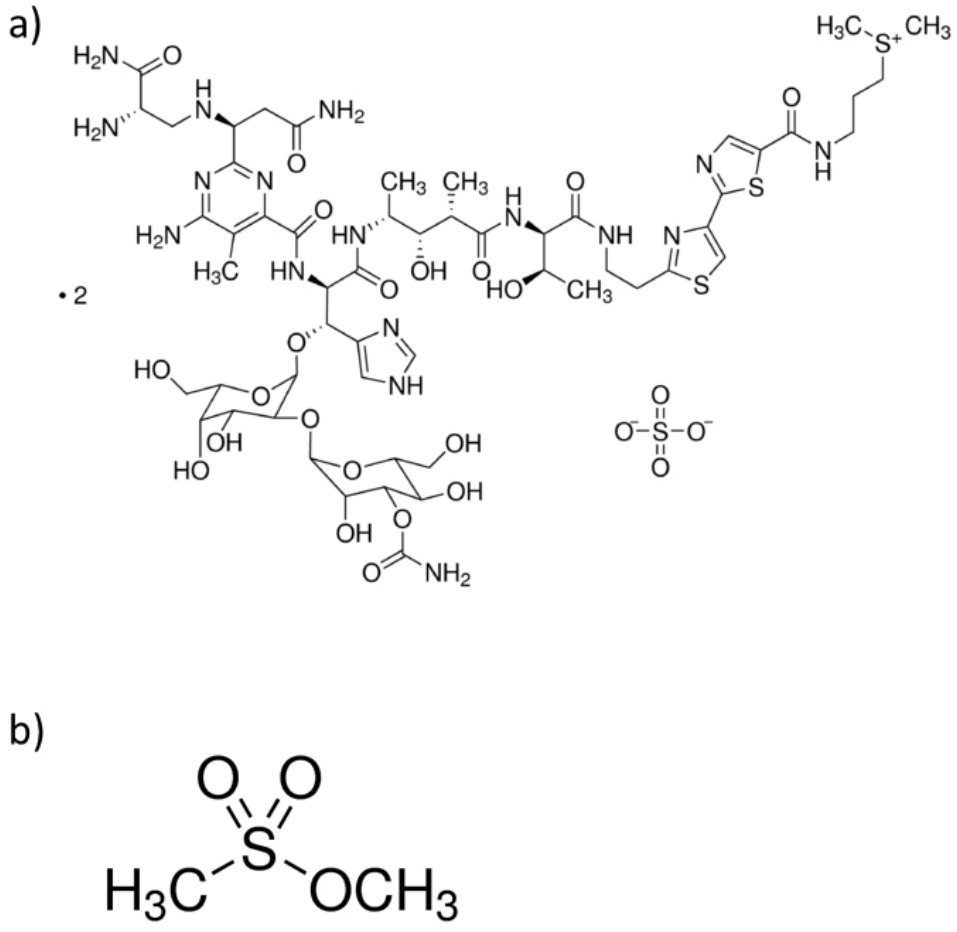
Figure 4. Analysis of DNA damage after exposure of yeast genomic DNA to different doses of methyl methanesulfonate (MMS) and pulsed magnetic field (PMF) (1.5 mT peak, 25 Hz). **(a)(b)(c)(d)** DNA patterns obtained after co-exposure during 15, 30, 45 and 60 minutes and their respective controls. All samples (MF exposed and controls) contained 400 ng of genomic DNA. After each exposure period, samples were electrophoresed in a 1 % agarose gel. DNA for control sample with 0 % MMS was considered undamaged. **(e)(f)(g)(h)** DNA profiles obtained for each electrophoresis lane in (a,b,c,d) and analyzed after exposure of genomic DNA to MMS and PMF and their respective controls. M: Molecular weight marker 1Kb Blue DNA ladder.

Figure 5. DNA content obtained from the analysis of profiles in figure 4. The undamaged DNA was calculated as the area under the curve of profiles in figure 4e, 4f, 4g and 4h in the location of the DNA band obtained for the control not treated (0 % MMS). The values obtained were normalized in relation to the DNA content of the untreated control. **(a)** Exposure to MMS ± pulsed MF, during 15 minutes. **(b)** Exposure to MMS ± pulsed MF, during 30 minutes. **(c)** Exposure to MMS ± pulsed MF, during 45 minutes. **(d)** Exposure to MMS ± pulsed MF, during 60 minutes. PMF: Pulsed magnetic field. MMS: Methyl methanesulfonate. Data points are Mean±SD of 3

independent experiments. The general tendency of MF-treated and control curves showed statistically significant differences in figures 5a, 5b, 5c and 5d; $p<0.05$ (ANOVA). Data points statistically different were those marked as: *$p<0.05$, **$p<0.01$ (Student's *t*-test).

Figure 6. Analysis of DNA damage after exposure of yeast genomic DNA to different doses of bleomycin (BL) and pulsed magnetic field (PMF) (1.5 mT peak, 25 Hz). **(a)(d)(e)** DNA patterns obtained after co-exposure during 24, 48 and 72 hours and their respective controls. MF-exposed and control samples contained 400 ng of genomic DNA. After each exposure period, samples were electrophoresed in a 1 % agarose gel. DNA for control sample with 0 % bleomycin was considered undamaged. **(b)(c)(f)** DNA profiles obtained for each electrophoresis lane in (a)(d)(e) and analyzed after exposure of genomic DNA to bleomycin and PMF and their respective controls. M: Molecular weight marker 1Kb Blue DNA ladder.

Figure 7. DNA content obtained from the analysis of profiles in figure 6. The undamaged DNA was calculated as the area under the curve of profiles in figure 6b, 6c and 6f in the location of the DNA band obtained for the control not treated (0 IU/ml bleomycin). The values obtained were normalized in relation to the DNA content of the untreated control. **(a)** Exposure to bleomycin ± pulsed MF, during 24 hours. **(b)** Exposure to bleomycin ± pulsed MF, during 48 hours. **(c)** Exposure to bleomycin ± pulsed MF, during 72 hours. PMF: Pulsed magnetic field. Data points are Mean±SD of 3 independent experiments. The general tendency of MF-treated and control curves showed statistically significant differences in figures 7a, 7b and 7c; $p<0.05$ (ANOVA). Data points statistically different were those marked as: *$p<0.05$, **$p<0.01$ (Student's *t*-test).



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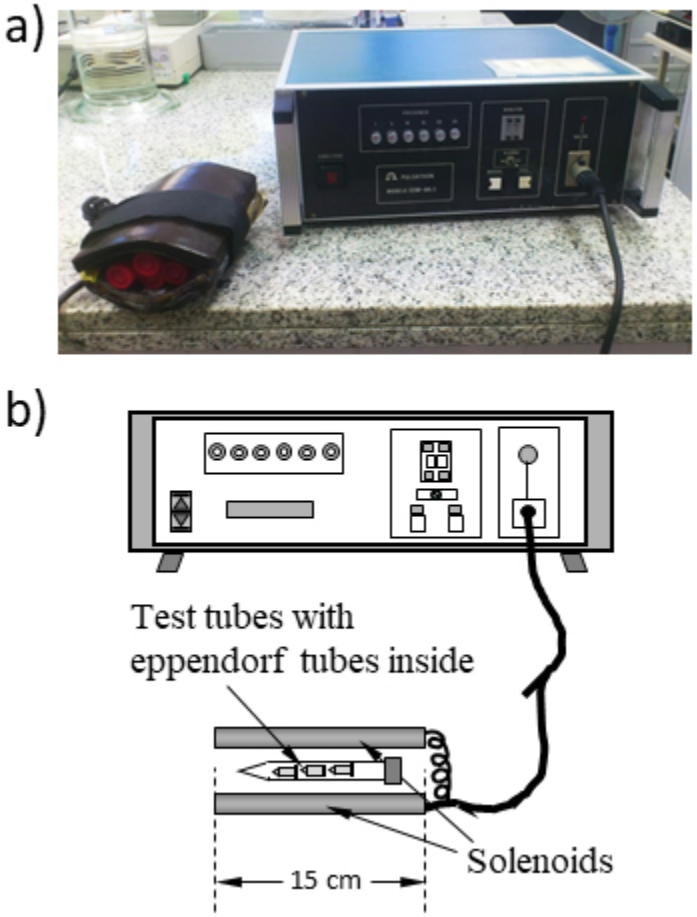

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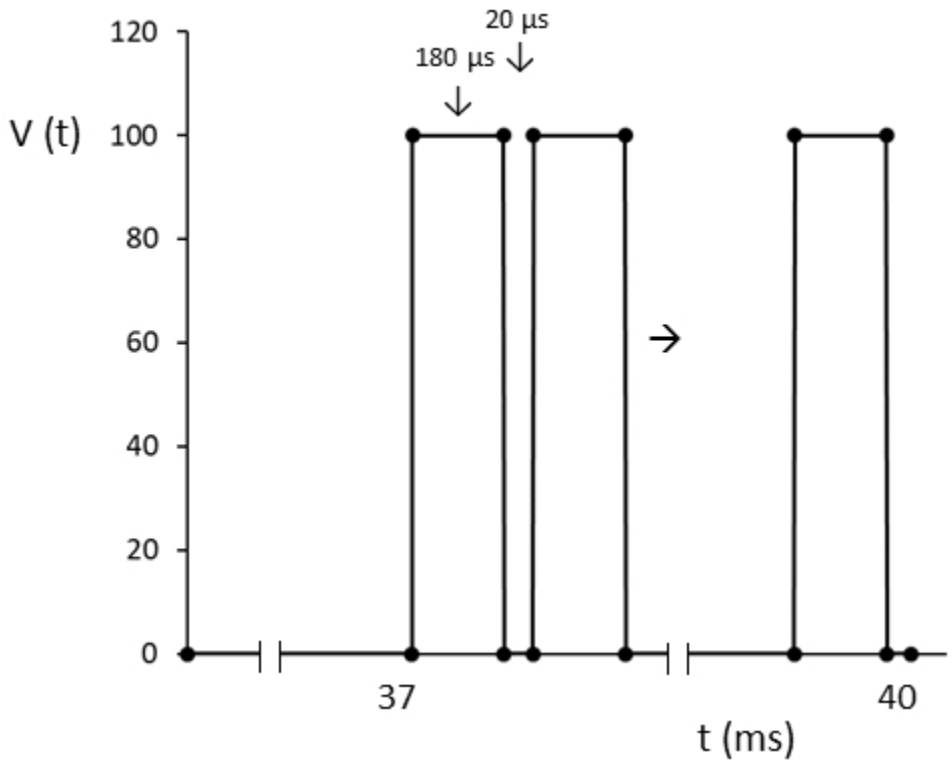

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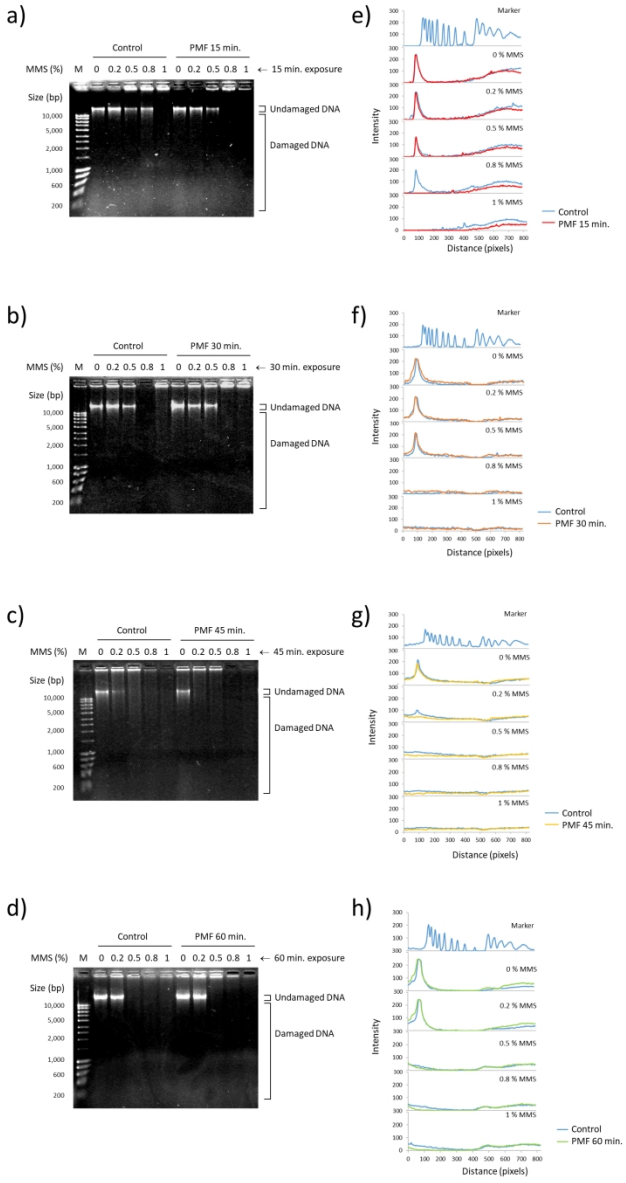

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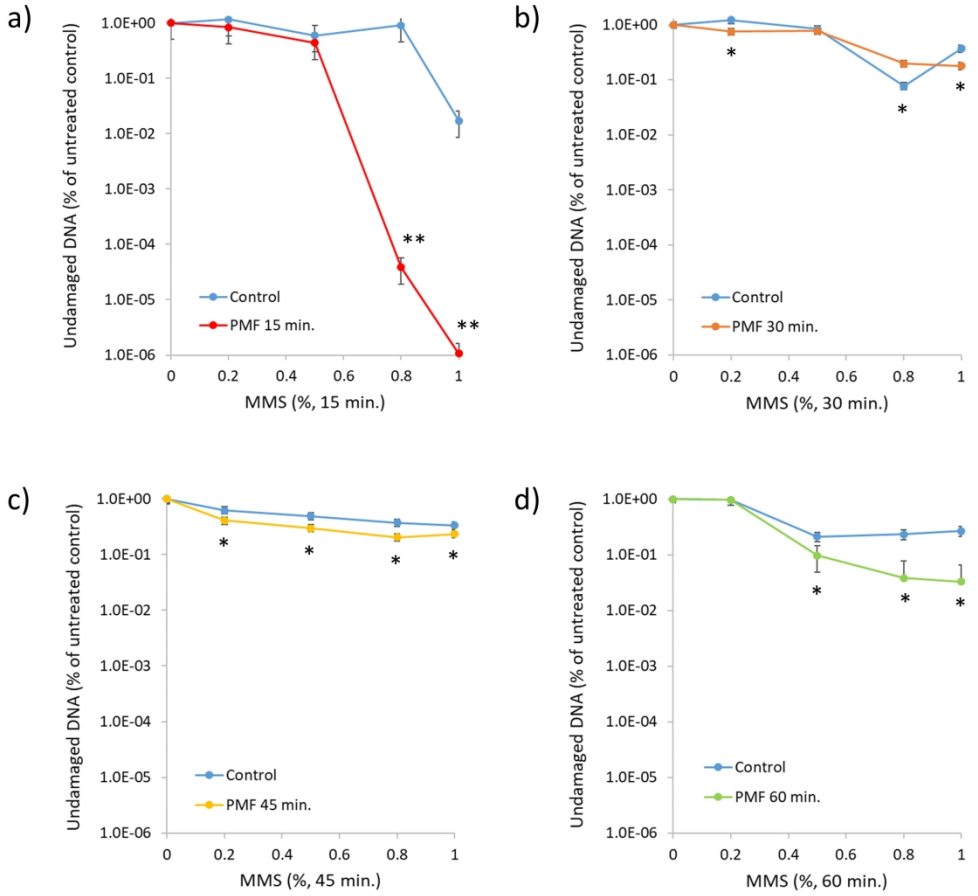

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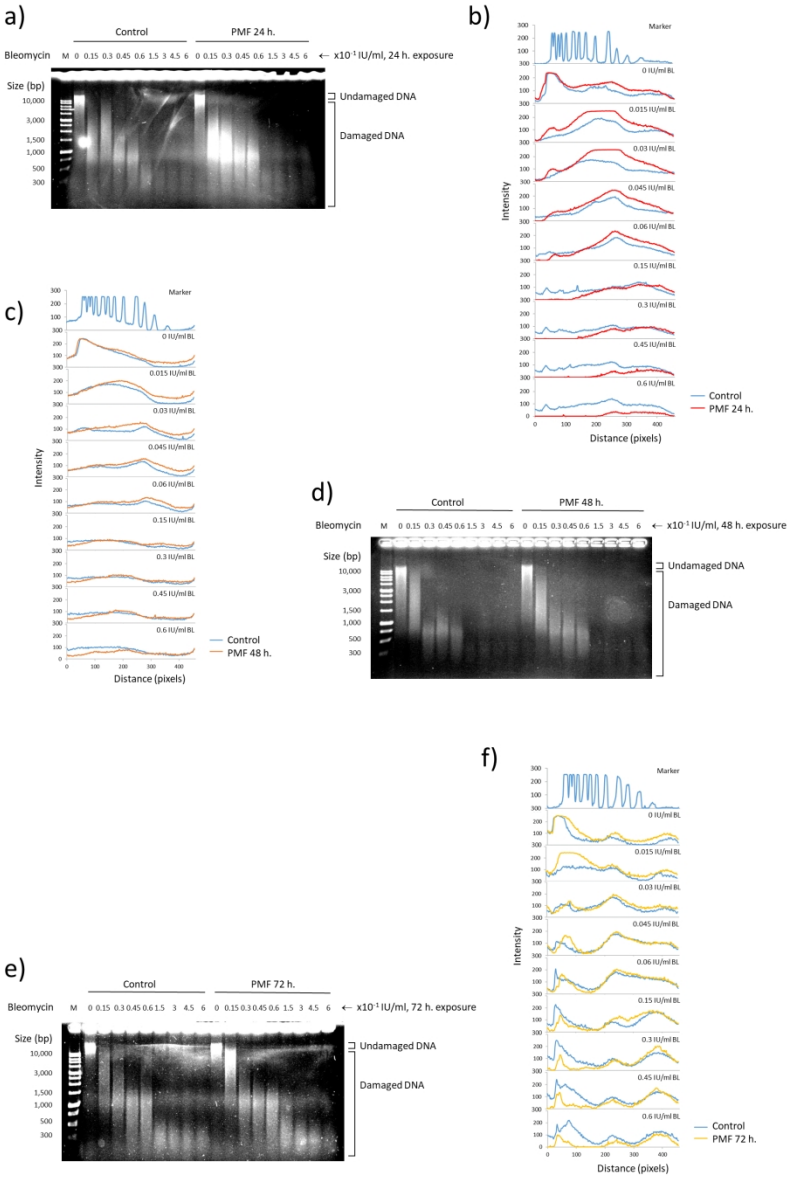

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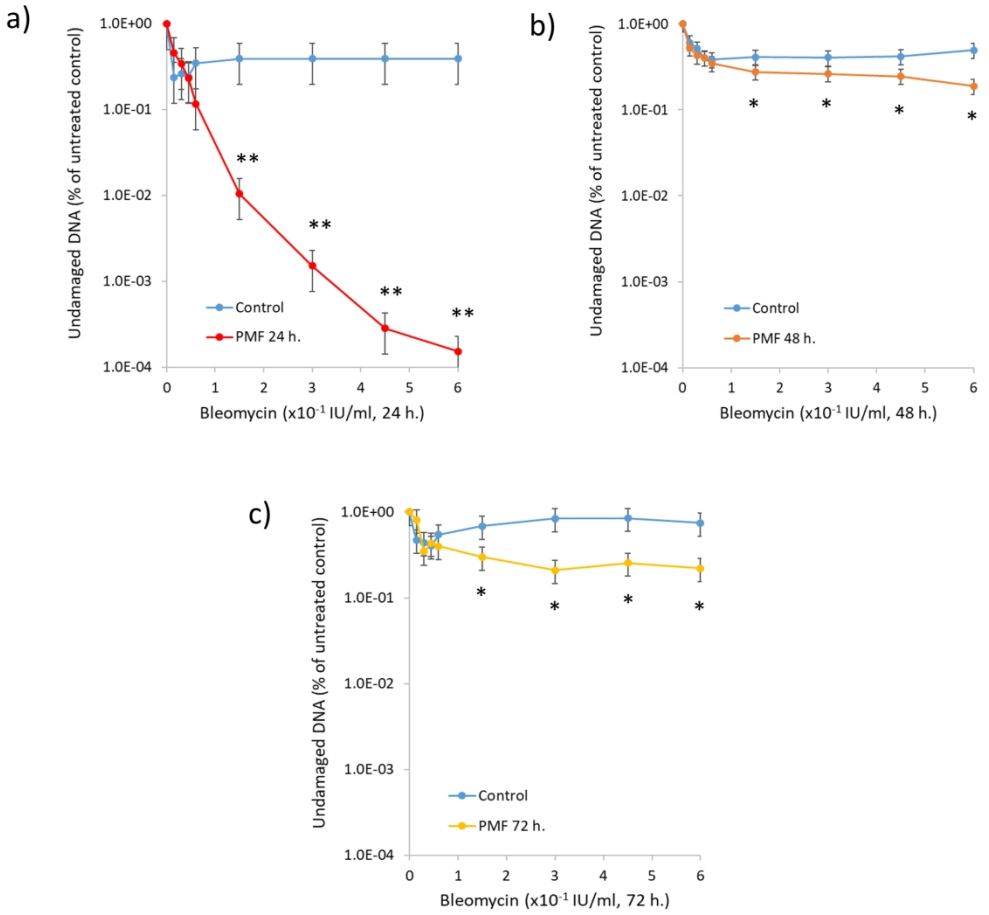

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