Intracellular DNA Damage Induced by Intense Burst Sinusoidal Electric Fields

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Non-thermal, intense electric fields with frequencies exceeding 1 MHz are known to cause intracellular effects to mammalian cells. In this paper, intracellular DNA break induced by an intense burst sinusoidal electric field (IBSEF) are investigated by means of the alkaline comet assay. The 200 μs long, 100 MHz IBSEF with the amplitude up to 200 kV/m was applied to Chinese hamster ovary (CHO) cells. The application of the IBSEF gives non-thermal effects to CHO cells since the temperature rise owing to Joule heating in this study is at most 1.2 ºC, which is considered to be sufficiently small to influence the biological activity. Degree of the DNA break was evaluated by the tail extent of the comet pattern. It has been experimentally shown that the application of the IBSEF with the amplitude more than 100 kV/m induces a significant damage to the intracellular DNA. The comet pattern of the cells subjected to the IBSEF are different from that of the cells exposed to an ultraviolet radiation (312 nm), whereas their comet extents are the same level. The difference in the comet pattern implies that the DNA damage process by the IBSEF application might be different from that of ultraviolet irradiation.

Keywords: intense burst sinusoidal electric field (IBSEF), non-thermal effect, alkaline comet assay, Chinese hamster ovary (CHO) cell, comet extent

1. Introduction

Recently, biological effects of intense pulsed electric fields (PEFs) have been investigated since the intense PEFs gives unique non-thermal stresses to biological targets in the cellular level. Intense electric fields with frequencies exceeding 1 MHz are capable of giving intracellular stimulation because they pass through the plasma membrane that is regarded as a dielectric thin film. Nanosecond pulsed electric fields (nsPEFs) with amplitudes on the order of 10 MV/m were reported to cause various kinds of intracellular effects in mammalian cells [1-5]. Since the frequency spectrum of the nsPEF is scattered from 0 to hundreds MHz, the pulse energy is distributed to all components of the cell, which is regarded as a complex of dielectric materials, and only a small portion of the energy penetrates into the cell. We proposed the use of the intense burst sinusoidal electric field (IBSEF), which has narrow band frequency spectrum, to apply well-defined fields to biological targets. We have demonstrated in the previous work [6] that the MHz range IBSEF with amplitude level around 100 kV/m causes the rapid degradation of intracellular deoxyribonucleic acid (DNA) in mammalian cells. There are several in vitro studies on the non-thermal biological effect of radio frequency (RF) exposure. Most of these studies used continuous waves (CW) and their power densities were on the order of W/kg. They conclude RF exposures do not induce DNA strand breaks, sister chromatid exchanges, DNA repair synthesis, phenotypic mutation, or transformation (cancer-like changes) [7]. The power density in our study is on the order of MW/kg and the electric field strength is on the order of 100 kV/m, which is sufficiently large to give a momentum or a stress to intracellular bio-molecules [8].

We have investigated the IBSEF induced DNA degradation by means of the comet assay, single cell electrophoresis. The comet assay is more sensitive to the DNA strand break and needs only a small sample in comparison with agarose gel electrophoresis. 200 μs long, 100 MHz IBSEFs with various field strength in the range between 1 and 200 kV/m were applied to Chinese hamster ovary (CHO) cells. The frequency of 100 MHz is sufficiently high for the electric field to penetrate into the cell. The minimum field strength to induce the intracellular DNA damage is discussed in this paper.

2. Materials and Methods

2.1 IBSEF Application System

The IBSEF generator consists of a signal generator to provide a sinusoidal wave, a pulse generator to determine the burst duration, and a radio frequency amplifier. Sinusoidal signals are generated by a signal generator (Agilent E4408 250 kHz - 1.0 GHz) and the duration of the signals is determined by the pulse length from a pulse generator (Berkeley Nucleonics, BNC555). The amplified signals from an amplifier (EM Power model 2088, 1 - 100

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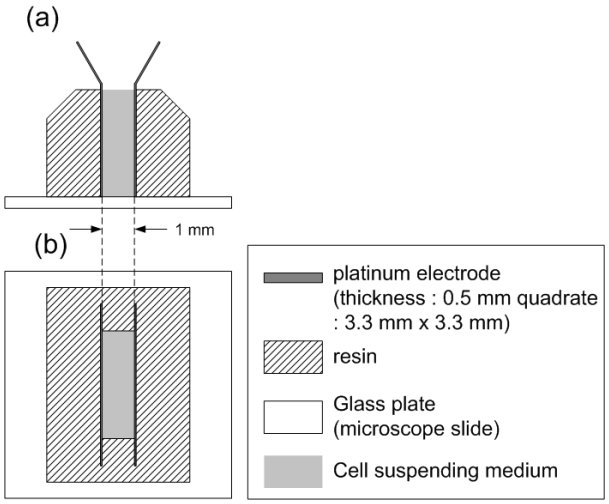

Fig.1 Schematic of the cross sectional (a) side and (b) top views of the application vessel..

MHz, 500 W) are delivered to the application vessel through a 50 Ω coaxial cable. Duration of IBSEF is fixed at 200 μs in order to minimize the temperature increase of the cell suspending medium [6]. The application vessel consists of two quadratic parallel platinum electrodes fixed on a glass plate using a heat resistant resin, which enables to sterilize the vessel with hot air (200°C, 2 hour). The vessel, of which the electrode separation and the cross section are 1 mm and 10 mm^2, respectively, is regarded as a 51 Ω load including 7 pF capacitance. The simple diagram of the application vessel is shown in Figure 1, The energy dissipation by a single 100 kV/m, 200 μs IBSEF in the 10 μl suspending medium is only 15 mJ, which increases the temperature of approximately 0.3°C. The specific absorption rate (SAR) value during the burst time is estimated to be approximately 8 MW/kg by dimensional calculation evaluated by the vessels volume, and induced electric field energy, in which the voltage between the electrodes was monitored with a voltage probe (Tektronics, P6139A, 500 MHz) for all shots. The application vessel was placed in the Faraday cage to protect us from electro magnetic radiation.

2.2 Preparation of Biological Cells

All experiments were conducted using CHO cells cultured in an incubator (5% CO_2, 95% air, 37°C) with α minimum essential medium (αMEM) including 10% fetal bovine serum (FBS). Cells were moderately passage through every 2 or 3 days, before getting 80% confluent. Cells for experimental use were therefore assumed to be in logarithmic growth phase. Cultured CHO cells were washed by PBS(-) (phosphate buffered saline pH 7.4) twice, detached by PBS base 5% trypsin-EDTA (ethylene diamine tetra acid), suspend by 10% FBS-αMEM, centrifuged at 1000 rpm for 5 minutes at room temperature, and re-suspended to 5×10^5 cells/ml with αMEM before experimental use and settled in a 1.5 ml micro tube, and finally stored in the incubator.

There are three kinds of treatments. One is IBSEF exposed sample, and was prepared by injecting the cell suspension of 10 μl gently into the application vessel which would be exposed to IBSEF. The second is sham control, which is prepared by the same procedure as the first one, except for the IBSEF exposure. The last one is a positive control, which was subjected to ultraviolet (UV) irradiation for 10 seconds using transilluminator (λ = 312 nm, ATTO TP-15MP). This is considered to cause oxidative stress leading to a DNA lesion, and/or to damage the DNA by forming the thymine dimmer [9,10]. The positive control was prepared for verifying whether the comet assay was performed successfully or not. The energy level of the UV irradiation is comparable to the 200 μs long, 200 kV/m IBSEF applications.

2.3 Alkaline Comet Assay Protocol

Comet assay is used for detecting DNA damage, which is defined as a DNA strand breakage. Comet assay method was operated in a dim room with the constant temperature of 25°C. Experimental materials which are in contact with the biological target were pre-sterilized for avoiding nuclease contamination.

10 μl of each sample was instantly mixed with 100 μl PBS agarose gel (1% agarose in PBS, stored in 37°C in advance), and were spread onto a microscope slide coated with super pure water agarose gel (1% agarose in pure water). Then the cells were placed in a 4°C incubator to freeze the gel and to stop all biological processes. The above mentioned procedure was completed within approximately 1 minute after each treatment. The cells on the slide glass were soaked for 3 hours in a lysing solution (5 % sodium N-lauroyl sarcocinate, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Triton, containing 0.02 % protenase K, pH 10 by NaOH) to dissolve the membrane and proteins. The electrophoresis was controlled with a voltage of 25 V for 20 minutes on ice after 20 minutes equilibration in the electrophoresis buffer (1 mM EDTA, 0.3 M NaOH) for unwinding the DNA. Then, the samples were equilibrated with 0.4 M Tris-HCl buffer (pH 7.5) for 15 minutes. Finally, the samples were instantly stained with 1/10000 0.4 M Tris-HCl (pH 7.5) diluted SYBR® Green I by dropping 20 μl of the diluted solution. SYBR Green I is a fluorescent dye which is permeable to living cells and intercalates with double strand DNA (dsDNA), and fluoresces in green (519 nm). It is noted that SYBR Green I also attracts single strand DNA (ssDNA), but its fluorescent intensity is approximately 11-fold lower than that of dsDNA [11]. Therefore, the fluoresced dots are indicated to be dsDNA fraction. Each sample was examined at 10× magnification on fluorescent microscope (Nikon ECLIPSE E600) in the dark.

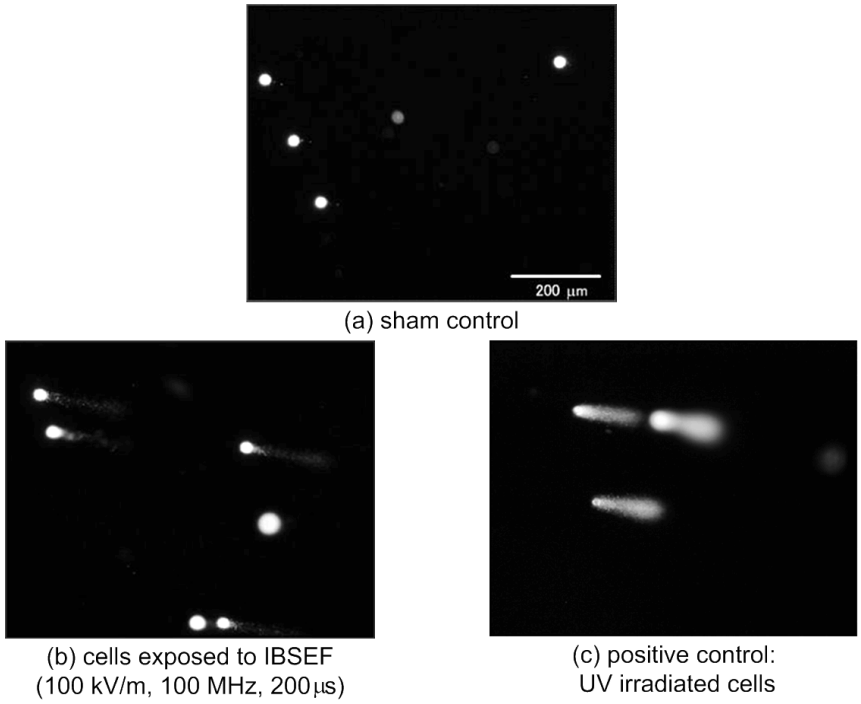

Fig.2 Typical images of the comet assay result from the CHO cells treated with (a) sham, (b) cells exposed to IBSEF (100 kV/m, 100 MHz, 200 μs), and (c) UV irradiation for 10 seconds. All images are in the same scale.

3. Results and Discussions

Figure 2 shows the typical inverted fluorescence images from the DNA for three cases: (a) the sham control, (b) the positive control, and (c) cells exposed to the 100 MHz, 100 kV/m, 200 μs IBSEF. The sham control shows a short and thin tail, indicating a slight DNA damage. This damage is assumed to be produced by the various stresses during the procedure, such as temperature, pressure, or illumination. These stresses might directly or indirectly influence DNA. The positive control shows a long, thick and bright comet tail, indicating that there is a serious DNA damage. Cells exposed to the IBSEF shows longer tail than the sham control, which clearly indicates that DNA was damaged. However, the comet pattern of the cells exposed to the IBSEF is different from that of the positive control. The ratio of the DNA migrating out of the comet head (nucleus) is apparently different.

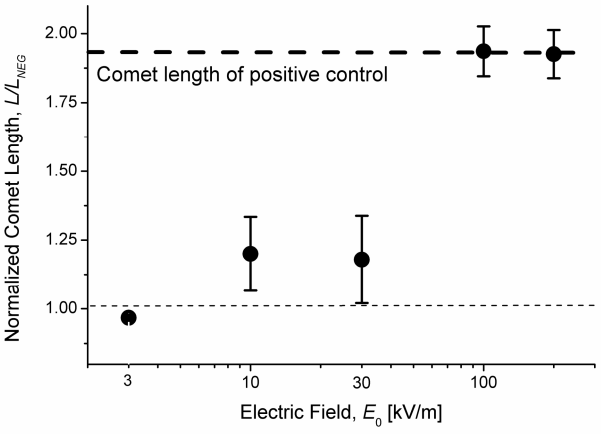

Fig.3 Level of DNA fragmentation expressed by comet extent L/L_S, as a function of electric field amplitude E_0 for a 100 MHz, 200 μs long IBSEF. Error bars are standard error of the mean on approximately 60 cells per condition on 3 or 4 independent trials.

The DNA damage was evaluated by the comet extent, which is defined as the distance between the leading edge and the trailing edge along the direction of electrophoresis. The comet extent describes how small the DNA was broken into. The edges were determined by the position to be the intensity level of 10% of the maximum of the comet. This procedure was done by using an imaging software (Transform, Fortner). Figure 3 shows the comet extent as a function of the electric field amplitude E_0. The comet extent was normalized by the average of the sham control for each trial. Each point contains information of approximately 60 cells in 3 or 4 independent trials, and the error bar shows the standard error of the mean (SEM). There is the significant difference between the sham control and the cells subjected to IBSEF with amplitude more than 100 kV/m. The comet extents for the amplitude lower than 30 kV/m do not pass the student t test, a statistical analysis to determine if the average difference of two pair of data is significant or not. In order to investigate the direct effect to the isolated DNA, the 200 kV/m IBSEF was applied to the 1 kbp Genomic Human DNA (COSMO

BIO). No DNA break was detected by an agarose gel electrophoresis [12].

The comet patterns of the cells subjected to the IBSEF are different from those of the cells exposed to the UV irradiation, while their comet extents are the same level. This indicates that the minimum size of the DNA fragments is the same level, but the amount of the fragments caused by the IBSEF application is less than that by the UV exposure even though the energy deposition is approximately the same. The difference in comet pattern implies their biological processes leading to the DNA break might be different from each other. It was reported that the UV irradiation to the cell causes both direct and indirect effects to the DNA break Covalent bonds (1 eV) such as phosphodiester bond composing the DNA strand are directly broken by the photons of the UV irradiation. As indirect effects, the UV radiation oxidize intracellular molecules and forms pyrimidine dimmer in DNA, resulting in faults in the DNA duplication and recovery [9,10]. As for the effect of the IBSEF, the electric field strength to break the covalent bond in DNA strand owing to the electrostatic force is estimated to be more than 100 MV/m [13], which is larger by three orders of magnitude than that used in this study. It is supported by the experimental fact that the application of 200 kV/m IBSEF does not break the isolated DNA. Therefore, we reach the conclusion that the DNA damage caused by the IBSEF is indirect effects, for instance, and electrostatic stress to intracellular biomolecules, which activates the biological processes leading to the DNA break.

Apoptosis, which is a programmed cell death and is accompanied by DNA fragmentation, can be one of the biological processes leading to the DNA break [14]. Generally apoptosis is initiated at the membrane mitochondria or endoplasmic reticulum, and it takes more than couple of hours to reach the DNA fragmentation. However, the DNA breakage detected in this study does not seem to be the general apoptotic DNA fragmentation because there is only 1 minute given for the biological activity after the IBSEF application.

4. Conclusion

We have experimentally demonstrated that the application of 200 μs long 100 MHz IBSEF with the amplitude more than 100 kV/m induces the significant damage to intracellular DNA in CHO cells. The DNA damage occurs as a result of the indirect effect of the IBSEF, which activates biological processes leading to the DNA break. The comet assay indicates that the DNA damage process induced by the IBSEF application might be different from that due to an ultraviolet irradiation.

5. Acknowledgment

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6. References

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