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Nanosecond electric pulses induce DNA breaks in cisplatin-sensitive and -resistant human ovarian cancer cells

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ABSTRACT

Human ovarian cancer cells COC1 and COC1/DDP (cisplatin-resistant subline) were exposed to 6 kV/cm nanosecond electric pulses (nsEP) with a pulse length of 8, 16 or 24 ns. The potential in a subcellular unit was calculated using a multilayer dielectric spherical model, and area under the voltage–time curves (AUC) integrated with a lower limit of 0.2 V. Cell viability was determined, and double-stand and total DNA breaks detected with the neutral and alkaline comet assays. nsEP evoked a higher voltage and AUC in nucleoplasm, and the levels in COC1 cells was just above those in COC1/DDP cells. Comets only appeared in the alkaline assay demonstrating single-stand DNA break. Fewer DNA break (16.51% *vs.* 35.13% at 24 ns, $p = 0.0150$) and more survival (22.42% *vs.* 13.19% at 24 ns, $p = 0.0015$) occurred in COC1/DDP cells despite an equal electric energy and almost equal cell sizes. 24-ns EP led to higher rates of cell-death and comet. The comet rate correlated with cell-death fraction in either cell line ($r = 0.5701$, $p = 0.0135$; $r = 0.5110$, $p = 0.0302$). There was no a correlation between the tail length, tail moment or Olive tail moment and cell-death rate. The data showed that response of chemosensitive cells differed from that of chemoresistant cells and DNA damage contributed to percent of cell death.

1. Introduction

The property of electric pulses (EP) such as the intensity and pulse length is the determinant of biological effects induced. EP provokes high potentials on a cell thereby triggering biophysical responses, when a potential above the threshold sustains long enough. EP with a pulse length of microseconds evokes a high potential in cell membrane leading to pore formation (*i.e.*, electroporation), which is a technique for transmembrane drug delivery. Nanosecond EP (nsEP) can penetrate through a cell membrane inducing a high potential in the cytoplasm, nuclear envelop or nucleoplasm, which results in alterations in a subcellular structure without affecting cell membrane (*i.e.*, intracellular electric manipulation). nsEP can induce apoptosis, influx of molecules into an organelle and non-thermal tissue ablation, thereby being a promising anticancer means [1,2].

An equivalent circuit is widely applied to simulate the distribution of potentials within a cell. A subcellular structure can be considered as a capacitor and/or a resistor. The provoked voltage in a specific unit is dependent upon the electric properties (conductivity, permittivity and resistivity) of this and adjacent structures [3,4]. The threshold potential of damage induction varies between subcellular units [1]. A cell consists of membranous structures. The critical potential for membrane poration therefore can serve as a reference for other subcellular structures. A transmembrane potential of $\geqslant 1$ V results in irreversible electroporation, which can be used to estimate the biophysical response of a subcellular structure under nsEP [5].

Cisplatin is the first-line regimen for many types of cancer. The gradual development of chemoresistance during treatment decreases the therapeutic efficiency, and ultimately leads to the failure of treatment. Cisplatin kills cells *via* the formation of DNA crosslinks and an improved DNA repair capacity plays an important role in chemoresistance [6]. A chemical sensitizer is usually limited by the toxicities to normal tissues and the acquired resistance to the modulator. A non-drug physical means such as ultrasound may provide an alternative [7]. Physical energies can be efficiently delivered into the lesion without affecting adjacent structures, thereby being a targeted therapy.

The aim of this study was to investigate nsEP-induced DNA damages and cell-death in cisplatin-sensitive and -resistant human ovarian cancer cells. Potential in the nucleoplasm was theoretically calculated using an equivalent circuit with a multilayer dielectric model, and DNA breaks were detected with the alkaline and neutral comet assays. Preliminary data suggested that nsEP led to weaker damages in chemoresistant cells.

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2. Materials and methods

2.1. Calculating potentials in subcellular units

A cell was divided into cell membrane, cytoplasm, nuclear envelop and nucleoplasm (each was homogenous). Each unit was considered as a capacitor and a resistor in parallel. The potentials in subcellular units were calculated using a multilayer dielectric spherical model with following electric and geometric parameters (Table 1) [3,4,8,9].

The duration of a high voltage affected the response. Area under the voltage–time curve (AUC) was therefore integrated with a lower limit of 0.2 V, as which was considered as the lowest critical potential for tissue damages [5].

2.2. Cells

Human ovarian cancer cells COC1 and COC1/DDP were cultured in RPMI 1640 medium (GIBCO) enriched with 10% fetal calf serum (HyClone), at 37 °C and 5% CO_2. COC1/DDP was a chemoresistant subline, and can grow at 0.5 μg/ml cisplatin [10]. Cell and nuclear sizes were calibrated under a microscope, with values of 23.01 μm and 17.46 μm in COC1, and 23.19 μm and 16.28 μm in COC1/DDP. Single cell suspensions were prepared for nsEP exposure.

2.3. nsEP exposure

2.0 ml suspensions (1.0×10^6 cells) were added into a cultural dish, and two cylindrical electrodes were inserted into the cell suspension to treat cells. Electric strength was 6 kV/cm with a pulse length of 8, 16 or 24 ns, and exposure duration was set 60 s. The electric energy applied was dependent upon the conductivity, intensity, pulse length and the number of pulses (*i.e.*, total exposure duration) [11]. Thus, eclectic doses for three types of nsEP were equal. Control cells were sham-exposed.

2.4. Cell viability

Cell viability was determined with the WST-8 assay 1 and 2 h after nsEP exposure, and then the cell-death rate was calculated [12].

Table 1
List of parameters for calculating potentials in subcellular units.

Parameter	Value
Conductivity (σ, *S/m*)	
Cell membrane	5.3×10^{-6}[a]
Cytoplasm	0.13[a]
Nuclear envelop	4.3×10^{-3}[a]
Nucleoplam	0.18[a]
Relative permittivity (ε)	
Cell membrane	7.0[a]
Cytoplasm	60.0[a]
Nuclear envelop	22.8[a]
Nucleoplasm	120.0[a]
Geometry (m)	
Diameter of cell	23.01×10^{-6} (COC1)[c] 23.19×10^{-6} (COC1/DDP)[c]
Diameter of nucleus	17.46×10^{-6} (COC1)[c] 16.28×10^{-6} (COC1/DDP)[c]
Thickness of cellular membrane	5.0×10^{-9}[b]
Thickness of nuclear envelop	40.0×10^{-9}[a]

[a] From Ref. [8].
[b] From Ref. [9].
[c] Calibrated in our laboratory.

2.5. Detecting DNA damages with comet assay

DNA damages were determined 1 and 2 h after nsEP. Cells were centrifuged and washed with phosphate buffer saline. Double-stand and total DNA breaks were detected with the neutral and alkaline comet assays, respectively [13]. Slides were stained by 4,6-diamidino-2-phenylinclode (DAPI; Invitrogen), and then scanned under a fluorescent microscope (Ti-E; Nikon). Images were captured and analyzed with the software CASP (http://casp-lab.com). Comet-formed cells were counted, and the rate of comet-formation was calculated. The tail length (TL, μm), tail moment (TM; tail DNA% × tail length) and Olive tail moment (OTM; tail DNA% × tail moment length) in those damaged cells were calibrated. At least 50 cells were analyzed in each experiment and data from 3 trials were used for statistic process.

2.6. Statistics

All data were processed with the statistics software SAS (SAS Inst.). Rates of comet formation and cell-death were compared with the analysis of variance (ANOVA), and the relationship between comet and cell-death with the analysis of correlation (CORR). The critical value was set $p < 0.05$.

3. Results

3.1. Potentials in subcellular units

nsEP provoked high potentials in nucleoplasm (>1.98 V) and cytoplasm (>0.95 V), with the highest value in nucleoplasm. There was a slight increase in the voltage in cell membrane (<0.26 V) or nuclear envelop (<0.32 V). The potentials in cell membrane and nuclear envelop were reliant upon the pulse length; the level attributable to 24-ns EP was 2.28–2.83 times as high as that due to 8-ns EP. However, there was only slight difference in cytoplasm or nucleoplasm under three types of nsEP (Fig. 1).

There were no differences in potentials in cell membrane and nuclear envelop between COC1 and COC1/DDP cells. The level in nucleoplasm in COC1 cells was just above that in COC1/DDP cells (2.16 V *vs.* 1.98 V at 24 ns), and the voltage in cytoplasm in COC1 cells was just below that in COC1/DDP cells (0.95 V *vs.* 1.17 V at 24 ns).

AUC in each subcellular unit increased with increasing pulse length, and higher values occurred in the nucleoplasm and cytoplasm. Interestingly, AUC in cell membrane at 24 ns was $>12 \times 10^{-8}$ V s despite a much lower level of peak potential. The difference of AUC in subcellular units between COC1 and COC1/DDP cells was similar to that of potential (Table 2).

3.2. Cell death

A higher cell-death rate occurred at the 2nd hour, in COC1 cells ($p < 0.0001$) and in COC1/DDP cells under 24-ns EP ($p = 0.0451$). Compared with 8- or 16-ns EP, 24-ns EP led to a higher death fraction in either COC1 ($p < 0.0001$) or COC1/DDP cells ($p < 0.0001$).

The death rate in COC1 cells was higher than that in COC1/DDP cells. At the 2nd hour, 8-, 16- and 24-ns EP led to values of 17.45% *vs.* 4.28% ($p = 0.0015$), 15.31% *vs.* 10.38% ($p = 0.0319$) and 22.42% *vs.* 13.19% ($p = 0.0015$), respectively (Fig. 2A–C).

3.3. DNA breaks

No comet was observed in the neutral assay (Fig. 2D–G). The quantitative analysis here was based on the data in alkaline comet

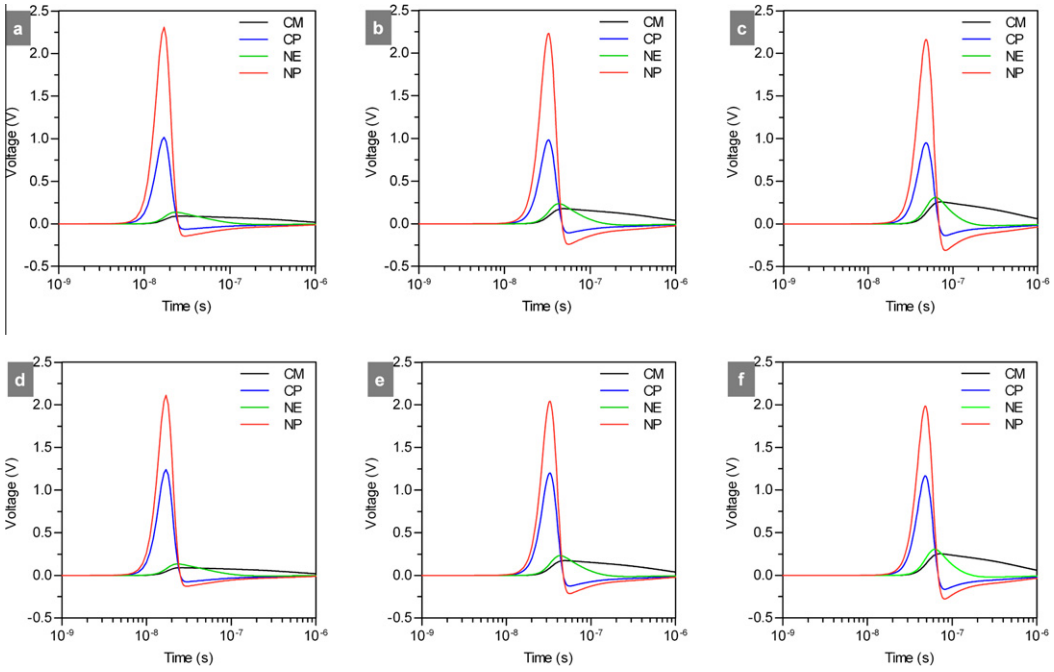

Fig. 1. Potentials in subcellular units provoked by 6 kV/cm electric pulses. Electric pulses with a pulse length of 8 (a, d), 16 (b, e) or 24 ns (c, f) evoked high potentials in nucleoplasm and cytoplasm in either COC1 (a–c) or COC1/DDP (d–f) cells. There was a slight increase in potential in cell membrane or nuclear envelop. Voltages in COC1 cells did not significantly differ from those in COC1/DDP cells. CM, cell membrane; CP, cytoplasm; NE, nuclear envelop; NP, nucleoplasm.

Table 2
List of the peak potentials and areas under the voltage–time curves (AUC).

	8 ns				16 ns				24 ns			
	CM	CP	NE	NP	CM	CP	NE	NP	CM	CP	NE	NP
COC1												
Peak potential (V)	0.0912	1.0167	0.1366	2.3100	0.1766	0.9819	0.2354	2.2310	0.2579	0.9526	0.3108	2.1645
AUC ($\times 10^{-8}$ V s)		0.8306		1.8872		1.5665	1.1433	3.5591	12.6858	2.2344	1.7133	5.0768
COC1/DDP												
Peak potential (V)	0.0895	1.2423	0.1340	2.1138	0.1734	1.2009	0.2311	2.0435	0.2534	1.1660	0.3052	1.9840
AUC ($\times 10^{-8}$ V s)		1.0158		1.7284		1.9184	1.1278	3.2643	12.6364	2.7397	1.6901	4.6617

CM, cell membrane; CP, cytoplasm; NE, nuclear envelop; NP, nucleoplasm.

assay. Comet rates were 1.32% and 1.89% in control COC1 and COC1/DDP cells, respectively.

24-ns EP resulted in the highest comet-formation rate at the 1st hour in either COC1 or COC1/DDP cells ($p = 0.0009$, $p = 0.0303$), compared with 8- and 16-ns EP. The comet rate in COC1 cells was higher than that in COC1/DDP cells under 8- (12.74% *vs.* 9.08%, $p = 0.0296$) or 24-ns EP (35.13% *vs.* 16.51%, $p = 0.0150$) at the 1st hour, and under 16-ns EP (28.77% *vs.* 15.25%, $p = 0.0456$) at the 2nd hour (Fig. 2A–C). The comet rate correlated with the cell-death rate in either COC1 ($r = 0.5701$, $p = 0.0135$) or COC1/DDP cells ($r = 0.5110$, $p = 0.0302$).

TL, TM and OTM varied between cells, and did not correlate with the cell-death rate in both COC1 ($p = 0.8643$–0.9478) and COC1/DDP cells ($p = 0.5379$–0.8630) (Fig. 2H–J).

4. Discussion

Simulations demonstrated the highest potential in nucleoplasm in both cell types. This accorded with the occurrence of DNA damages. These manifested that nsEP can penetrate through cell membrane and nuclear envelop provoking a high voltage in the nucleoplasm. This was due to the formation of cutoff on cell and nuclear membranes when applying ultrashort pulses [14]. An expected nucleoplasm potential can be realized by adjusting the intensity and pulse length, thereby manipulating the nucleus.

Comets were only detected in the alkaline assay, which showed that nsEP merely led to single-stand DNA breaks. We assumed that this was related to electric forces. There were positively- and negatively-charged molecules in a DNA chain, thereby producing a torque under nsEP exposure. Twisting off single stand needed weaker torsion, so it was easier to induce single-stand DNA breaks.

Rates of cell-death and comet in COC1/DDP cells were lower than those in COC1 cells despite an equal electric energy applied. The findings showed that nsEP induced slighter damages in chemoresistant cells. Cisplatin-resistant cells had a higher capacity of DNA repair, thereby protecting cells against attack by a toxic factor [6]. Some cells can survive DNA breaks *via* repairing damages. The repair was evoked rapidly and most single-stand breaks can be rejoined; a fraction of single-stand breaks evolved into double-stand breaks resulting in cell death [15]. This accorded with the present data that the rate of comet was higher than that of cell death. DNA repair in nsEP treatment required further explorations.

Cell and subcellular sizes were the determinant for the evoked potential in a subcellular unit [3,4]. The cell size of COC1 was almost equal to that of COC1/DDP and the nuclear diameter of COC1/DDP was 1 μm smaller than that of COC1. Calculation demonstrated a slight difference (<8.49%) in the potential in nucleoplasm between

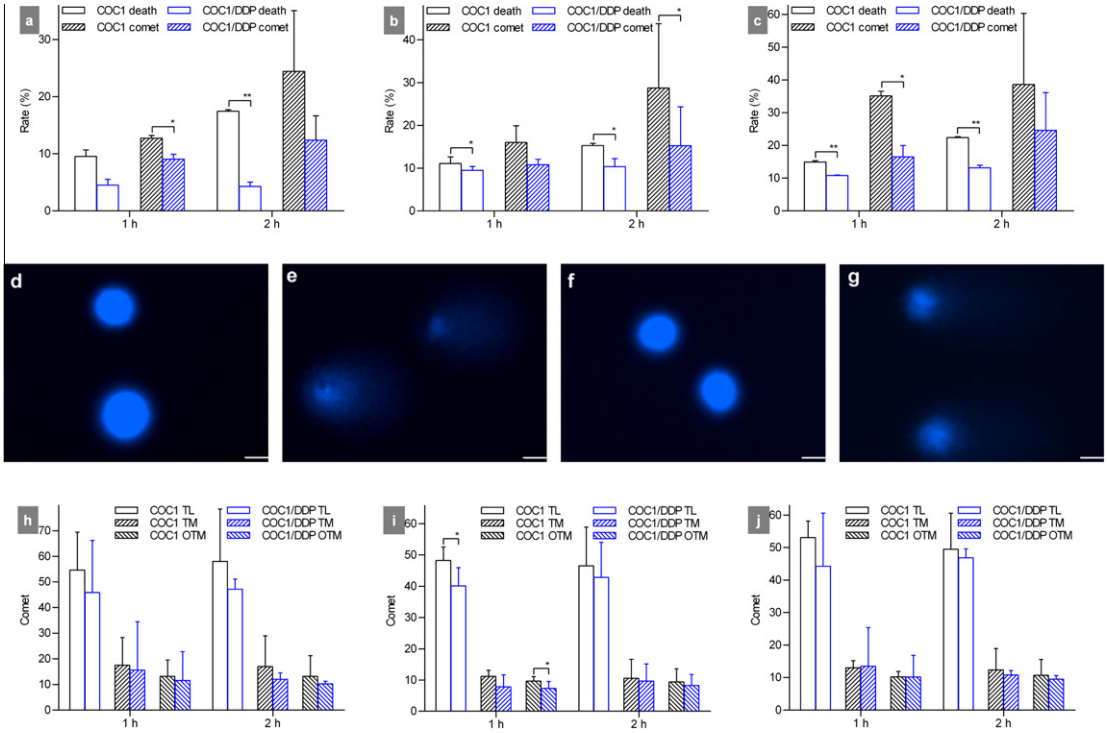

Fig. 2. Cell death and DNA damage in COC1 and COC1/DDP cells after exposure to electric pulse. Rates of comet formation and of cell death under a pulse length of 8 (a), 16 (b) or 24 ns (c); lower rates occurred in COC1/DDP cells. DNA breaks in COC1 (d, e) and COC1/DDP (f, g) cells detected with the neutral and alkaline comet assays; comets were not observed in the neutral assay (d, f) and appeared in the alkaline assay (e, g); the scale was 20 μm. Tail length (TL), tail moment (TM) and Olive tail moment (OTM) under 8- (h), 16- (i) or 24-ns (j) electric pulses; there parameters varied between cells. $^{*}p < 0.05$; $^{**}p < 0.01$.

two cell types. Fewer DNA break in COC1/DDP cells therefore was not mediated by electric mechanisms. The responsive difference between cisplatin-sensitive and -resistant cells should be considered when applying nsEP to treat cancers. The present data indicated that the invoked potential in nucleoplasm increased with increasing nuclear size. This favored the killing of cancer cells because malignant cells usually had nuclear enlargement [16].

The variations of AUC between subcellular units and between cell types were similar to those of potential. A higher value in nucleoplasm was consistent with the occurrence of DNA breaks. Previous investigation indicated that a transmembrane potential of 1 V with duration of 2 μs (*i.e.*, 2×10^{-6} V s) was necessary for poration [17]. In the present study, AUC in the nucleoplasm was lower than the alleged critical value but DNA damages did occur. This suggested that the evoked potential was the leading determinant of biological effect; the lasting time played a part only when the invoked voltage reached the threshold. A higher provoked potential indicated that a shorter duration was needed to induce tissue damages. Consequently, a much higher AUC in cell membrane at 24 ns failed to result in tissue insults because of a lower peak potential. Indeed, the higher AUC value was due to a long polarization time. This should be considered in cancer treatment with nsEP.

Comets appeared and the comet rate correlated with the cell-death rate in this study, which was consistent with the finding of Stacey et al. [18]. However, 60-ns EP (1.0–2.5 MV/m) reduced the comet-assayed DNA migration in Jurkat cells in the investigation of Romeo et al. [19]. It was difficult to understand the data, because such a high strength concurrently invoked high potentials in the nucleoplasm, nuclear envelop, cytoplasm and cell membrane. Thus, DNA pattern observed may not be the direct outcome of nsEP but a subsequent response resulted from damages to other subcellular structures. Cell type may be involved in, *i.e.*, the biologic property of a cell affected the response to nsEP. A higher comet rate but a lower death fraction manifested that percent of cell-death was mediated by DNA breaks. Other types of cell death should be explored. There was a high voltage in cytoplasm, which may impair organelles. Electric insults to mitochondria can initiate apoptosis, which may be involved in cell death [1].

The present findings implied that TL, TM and OTM can not reflect the degree of DNA damages attributable to nsEP. Variance of these parameters between cells demonstrated the heterogeneity of DNA damages, where the inhomogenous distribution of electric field played a role. Comets were uniform when treating COC1/DDP cells with ultrasound, cisplatin and cyclosporin, and the combination led to the longest comets [7]. This was a clue for nsEP-the addition of specific chemicals can counteract the heterogeneity and enhance the chemoresistance reversal due to a physical factor. The interaction between a drug and nsEP (practically the potency and toxicity of a drug) therefore should be explored [20,21]. Lower values of TL, TM and OTM in COC1/DDP cells demonstrated fewer DNA breaks.

In summary, nsEP induced fewer DNA break and more survival in chemoresistant cells. The correlation between the comet rate and cell-death rate indicated that DNA damages contributed to percent of cell death. The responsive difference between cisplatin-sensitive and -resistant cells should be considered when applying nsEP to treat cancers.

Acknowledgments

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