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Induction of Apoptosis of Cancer Cells Using the Cisplatin Delivery Based Electrospray (CDES) System

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Abstract: Cisplatin, a representative anticancer drug used to treat cancer, has many adverse effects. In particular, it causes significant damage to the kidneys. Thus, many researchers have studied the delivery of drugs, such as cisplatin, to cancer areas using targeted drug-delivery systems. Here, we propose a new way to treat cancer by delivering anticancer drugs directly to the tumor site using the electrospray (ES) technique. We determined the optimal conditions for ES to promote the introduction of cisplatin into cancer cells. In our results, the group with cisplatin delivery-based electrospray (CDES) at 6 kV had an apoptosis of cancer cells approximately two times more than the group treated with cisplatin alone. We also confirmed that ES alone did not affect the survival of cells up to a voltage of 6 kV, but increased the permeability of the cell plasma membrane instantaneously, facilitating the influx of the drug.

Keywords: electrospray; anticancer treatment; cisplatin; drug delivery; cancer

1. Introduction

Drug delivery systems (DDSs) control the transmission and release of pharmacologically active substances to cells, tissues, and organs for optimum effectiveness using various physical and chemical techniques [1]. Examples of chemical delivery systems can use carriers consisting of a wide variety of materials and structures, such as lipid micelles and nanoparticles that can transfer target molecules into cells; physical delivery systems include stimulations by electricity, ultrasonication, heat, and magnetism to penetrate the cell membrane [2–4]. In particular, many studies related to cancer treatment have been conducted. Depending on the target cancer and type of medicine, the delivery system is applied differently. DDS has facilitated the treatment of cancer and pain management associated with cancer progression and chemotherapy [4,5]. Thus, many patients have experienced DDS cancer therapeutics, and many researchers are working to discover superior methods to the existing systems [2,6]. However, anticancer drugs can cause many side effects, such as resistance and various toxicities, including cardiotoxicity, hepatotoxicity, cardiotoxicity, ototoxicity, and neurotoxicity, and secondary malignancy [7]. Thus,

if anticancer drugs can be used to treat locally at a tumor site, the side effects could be minimized, and the treatment effectiveness could be increased [8–14].

Cisplatin, which contains two chlorine atoms and ammonia in platinum atoms, is the most representative anticancer drug used to treat a number of cancers [15]. Cisplatin, which binds DNA, can inhibit the replication of cancer cells. Generally, this drug is administered via an injection. Thus, there are many side effects, such as a large impact on the kidneys [16,17]. Many researchers continue to study cisplatin or other drug delivery systems to improve efficiency and thus reduce these side effects. For example, by decorating biomarkers and magnetic materials to nanocarriers, loaded drugs were delivered to only the cancer site [18–22]. Additionally, the drug was injected directly into the tumor mass [23], hyperthermia was applied in the target area to induce rapid movement of the drug [24,25], or electroporation was used to accelerate the absorption of the drug into the cancer tissue [26–28].

Electrospray (ES) techniques provide micro- or nano-sized and charged droplets. This approach is generally used to manufacture nanoparticles and to encapsulate drugs or cells in polymers [29–33]. However, Ikemoto et al. proposed that an electrospray method can be used to introduce genes into cells [34]. Subsequently, several research teams used this technique to transfer external materials into cells [35,36]. These studies showed that applying an electrical field by electrospraying can facilitate the delivery of exogenous materials, making the ES technique a novel physical drug delivery system without severe damage, similar to traditional electroporation methods, because the delivery is mainly done by the impact of droplets on the cell surface [34]. Our research team also reported previous studies that applied this technique to transfer genes into cells [37]. By applying an appropriate voltage, this technology can promote the influx of external substances while minimizing the impact on cells.

In this study, we endeavored to develop a novel cisplatin delivery-based electrospray (CDES) system for cancer cell lines, proposed a new method for inducing apoptosis of cancer cells by delivering cisplatin using an electrospray method and indirectly proved the mechanism. Figure 1 shows a schematic of the CDES system. All the system facilities were installed on a clean bench. The syringe tip and the ground of the ring shape were connected to a high-voltage generator. Phosphate-buffered saline (PBS) was used as the electrospray solution, as was done in our previous study. Initially, to confirm how much damage was caused to cells by ES, cell viability was measured after treatment with ES at various voltages. Subsequently, the live and dead assay and WST-1 assay were used to confirm the optimal voltage to deliver cisplatin to the cancer cells. Additionally, to compare how apoptosis and necrosis are induced after CDES treatment, an Annexin V analysis was performed. Finally, to analyze the drug delivery mechanism of CDES, changes in plasma membrane permeability were confirmed by propidium iodide (PI) analysis.

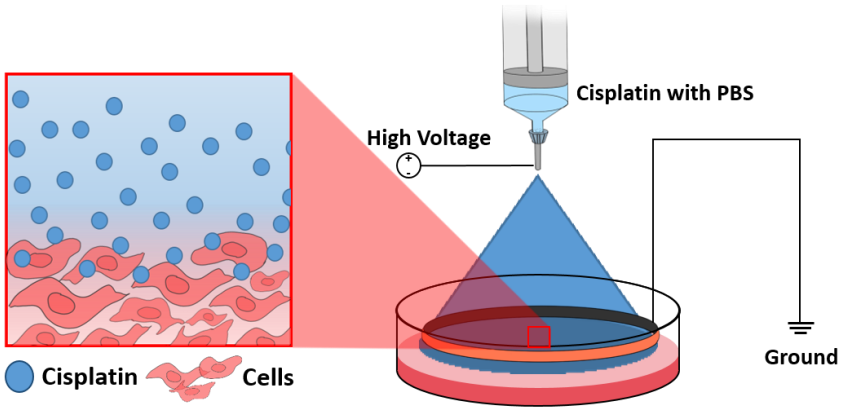

Figure 1. Schematic diagram of the cisplatin delivery-based electrospray (CDES) system. The CDES system facilitated the delivery of cisplatin to cancer cells. This method suggested a way to kill cancer cells by treating drugs locally into the tumor tissue.

2. Materials and Methods

2.1. Materials

Roswell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum (FBS) were purchased from Welgene (Gyeongsan, Korea). A water-soluble tetrazolium salt (WST-1), to measure cytotoxicity, was purchased from Daeil Lab Service Co. (Seoul, Republic of Korea), and a Live/Dead Viability/Cytotoxicity assay kit (Invitrogen (Thermo Fisher Scientific), Waltham, MA, USA) to investigate cytotoxicity, was purchased from MitoSciences (Eugene, OR, USA). For the apoptosis analysis, the FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences (San Jose, CA, USA). A propidium iodide solution (PI, Sigma-Aldrich, St. Louis, MO, USA) was used to observe membrane permeability. A syringe pump (KdScientific, Holliston, MA, USA) and a DC high-voltage generator (TEKKAM, Seoul, Republic of Korea) were also used. Cisplatin (50 mg lyophilized powder) was purchased from Donga ST (Cisplan, Donga ST, Seoul, Korea).

2.2. Cell Preparation

HeLa (ATCC, Manassas, VA, USA) cells were cultured in a 100 mm dish (NUNC) for 2 days in 15 mL complete medium containing RPMI-1640 and antibiotics (Welgene Inc., Gyeongsan, Korea) supplemented with 10% FBS. HeLa cells were seeded at a density of 2×10^6 cells on the plate. After 2 days, the cells were harvested and seeded according to the manufacturer's instructions.

2.3. The Cisplatin Delivery-Based Electrospray (CDES)

The ES system was placed on a clean bench. The system consisted of two main components: the syringe pump and a high-voltage generator (Figure S1). Cisplatin was dissolved in PBS and loaded into a 10 mL syringe, which was then fixed to the syringe pump. A 24-well plate was placed on the support. Subsequently, the syringe tip and the ground of the ring shape with a diameter and thickness of 12 mm and 1 mm, respectively, were connected to the high-voltage generator. The cisplatin solution was sprayed through the needle of the syringe at a constant flow rate of 1.8 mL h^{-1} and applied to a target in each well filled with media. The distance between the syringe tip and the grounded ring was 10 mm, the distance of the ground to the media surface was 1 mm, and the distance of the grounded ring and plate well bottom was 3 mm. The solution was treated at the half maximal inhibitory concentration (IC50, 4 μg/mL) [38] of cisplatin for HeLa cells for one minute. The IC50 value means the drug concentration that inhibits certain biological processes by half. When electrospray was applied to the target, the electrospray voltage was varied (0, 2, 4, 6, and 8 kV).

2.4. Live/Dead Assay

To investigate cell cytotoxicity after CDES, a live/dead cell assay kit was used 1 day and 3 days after the treatment. For the live/dead assay, calcein and ethidium homodimer (EthD-1) were used as in the kit information sheet. Calcein is retained within live cells and produces green fluorescence at ex/em 495 nm/515 nm, while EthD-1 enters dead or damaged cells and produces red fluorescence at 495 nm/635 nm as stated in the data sheet of the kit. When ES was run on a 24-well plate, cells were seeded at a density of 3×10^4 cells per well, and the electrospray voltage was varied (0, 2, 4, and 6 kV). When dye was used for the assay, the live/dead dye was diluted by 5X in PBS. The cells were stained according to the kit instructions and incubated for 20 min at room temperature. The treated cells were observed by fluorescence microscopy (Nikon N-Storm, Nikon, Tokyo, Japan). The cell density in this section was 3×10^4 because the live/dead assay was part of the optimization process of the assays below. Additionally, considering the existence of the control group and the proliferation rate of HeLa cells, the cell number between day 1 and day 3 was assumed to not make any difference in the results.

2.5. Cell Viability Assay

To investigate cell viability after CDES, a WST-1 assay was used 1 day and 3 days after the treatment. The WST-1 assay is a cell viability assay that uses water soluble tetrazolium salt as an agent, which reacts with mitochondrial dehydrogenase and produces formazan that can be detected quantitatively by spectrometry. For the viability assay, HeLa cells were seeded on 24-well plates at a density of 5×10^4 cells per well. After 1 day and 3 days, CDES was applied to each well. After each period, cell viability was measured using the WST-1 (EZ3000, Daeil Lab Service Co., Seoul, Korea) assay. Each well was washed with PBS, 200 μL of the 1:10 WST-1 agent diluted to the culture media was added and cultured for an hour, and 100 μL of each well was transferred to a 96-well plate to read at a 450 nm wavelength ELISA plate reader (SUNRISE, TECAN, Grödig, Austria).

2.6. Annexin V-FITC Apoptosis Assay

The Annexin V-FITC apoptosis detection assay was followed after the viability assay, and performed according to the supplier's protocol to determine apoptosis after the treatment, followed by fluorescence-activated cell sorting (FACS) for more precise analysis. Annexin V binds to phosphatidylserine, which is located at the inner cell membrane and exposed when cells are in the apoptotic process. After harvesting the cells, the samples were washed twice with cold PBS and resuspended in 1X binding buffer. Subsequently, 5 μL of FITC-Annexin V solution and 5 μL of PI solution were added to the samples. PI exhibits red fluorescence upon binding to DNA inside the cell and is generally used to detect dying or dead cells or opening of the cell plasma membrane. After gentle vortexing, the samples were incubated for 15 min at room temperature in the dark. After incubation, 400 μL of 1X binding buffer was added to the samples and analyzed by FACS within 1 h (FACS Aria II from BD Biosciences, USA).

2.7. Cell Membrane Permeability Analysis Using PI Staining

Simultaneously, after the Annexin V assay, PI staining was performed to determine the permeability of ES, using their nature of exhibiting red fluorescence upon binding to DNA inside the cell. HeLa cells were seeded in 24-well plates at a density of 5×10^4 cells per well. After 1 day, the cells were treated with 6 kV ES. Afterward, at various time periods (after 0, 30, 60, and 120 min), PI solution at a concentration of 3 μM was added, and the cells were incubated for 5 min. After washing the wells with PBS one time, red fluorescence was observed using a fluorescence microscope (Ti-E, Nikon, Tokyo, Japan).

2.8. Statistical Data Analysis

A statistical analysis was performed using R v3.2.1 software (The R Project for Statistical Computing. Available Online: http://www.r-project.org (accessed on 02 October 2020). The least significant difference (LSD) method, Duncan's test, and one-way ANOVA were used to compare the means of the properties of the samples. The level of significance was set at $p < 0.05$. The data are reported as the mean ± standard deviation, with n = 3 or 5.

3. Results and Discussion

3.1. Cell Viability Analysis after ES at Each Voltage

To use the electrospray system as a tool for drug delivery to cells, it is necessary to check how much cell damage is done by the high voltage of the process. Thus, we measured cell viability after treating ES at various voltages. Figure S2 shows the results of the WST-1 assay after ES at various concentrations (2, 4, 6, 8 and 10 kV). In the ES treatment group below 6 kV, no significant cytotoxicity was measured. However, the ES treatment group with 8 kV or more showed more red fluorescence in the live/dead assay and significantly lower viability in the WST-1 assay than the control. These results showed that ES above a specific high voltage caused significant damage to the cells, and up to 6 kV allowed the delivery of a drug using ES. Thus, we decided to use a voltage of 6 kV or less for the ES drug delivery system.

3.2. The Induction of Apoptosis by CDES

We hypothesized that treatment with a higher voltage ES would promote the delivery of cisplatin. However, CDES was performed with a voltage of 6 kV or less since ES alone at more than 6 kV impacted cells. To determine the optimal voltage, CDES was performed at various voltages. One day after treatment, cell proliferation was investigated by live/dead analysis (Figure S3A,B). Living cells (green fluorescence) were classified according to intracellular esterase activity, which involved the release of red dye (ethidium homodimer-1). In contrast, the dead cells (red fluorescence) degraded esterase activity and did not release red dye. As a result, there was no significant difference between the cisplatin-only, 2 kV CDES, and 4 kV CDES treatment groups. The fewest live cells were found in the 6 kV CDES treatment group. The analysis showed that the ratio of dead to live cells was almost twice that of the group treated with cisplatin alone. In the WST-1 analysis, it was found that the cell viability was the lowest in the 6 kV CDES treatment group, similar to the live and dead results (Figure S3C). Based on these results, we confirmed that the optimal voltage of CDES for inducing apoptosis in cancer cells is 6 kV. Figure 2 shows the cell viability of the 6 kV CDES and cisplatin treatment groups 3 days after treatment. Interestingly, in the group treated with only 6 kV ES, most of the cells were alive, similar to the control group. However, it was confirmed that the 6 kV CDES treatment group had significantly lower proliferation than the group treated with cisplatin alone. After the fluorescence image analysis for the live and dead assays, it could be seen that the ratio of dead/live cells in the 6 kV CDES treatment group was approximately 7 times higher than that of the cisplatin-treated group (Figure 2B). Cell viability using the WST-1 assay also showed similar results to the live and dead assays (Figure 2C). Based on these results, we expected that cisplatin would be delivered to the cells with higher efficiency through the CDES method, and accordingly, apoptosis of cells was better induced. To determine accurately whether apoptosis was caused by CDES, FACS analysis was performed using the Annexin V assay (Figure 3). Apoptotic cells were detected by staining with phosphatidylserine with green fluorescence (FITC), and necrotic cells were detected by PI through red fluorescence. Early apoptotic cells showed only green fluorescence, while late apoptotic cells showed both green and red fluorescence. Surprisingly, it was confirmed that more than 85% of the cells were in the process of apoptosis in the 6 kV CDES treatment group. In the group treated with cisplatin alone, approximately 40% of the cells were undergoing apoptosis. Few cells were apoptotic in the 6 kV ES treatment group. This means that the ES method promoted the delivery of cisplatin, dramatically increasing the induction of apoptosis, even though it did not cause much damage to the cells by itself.

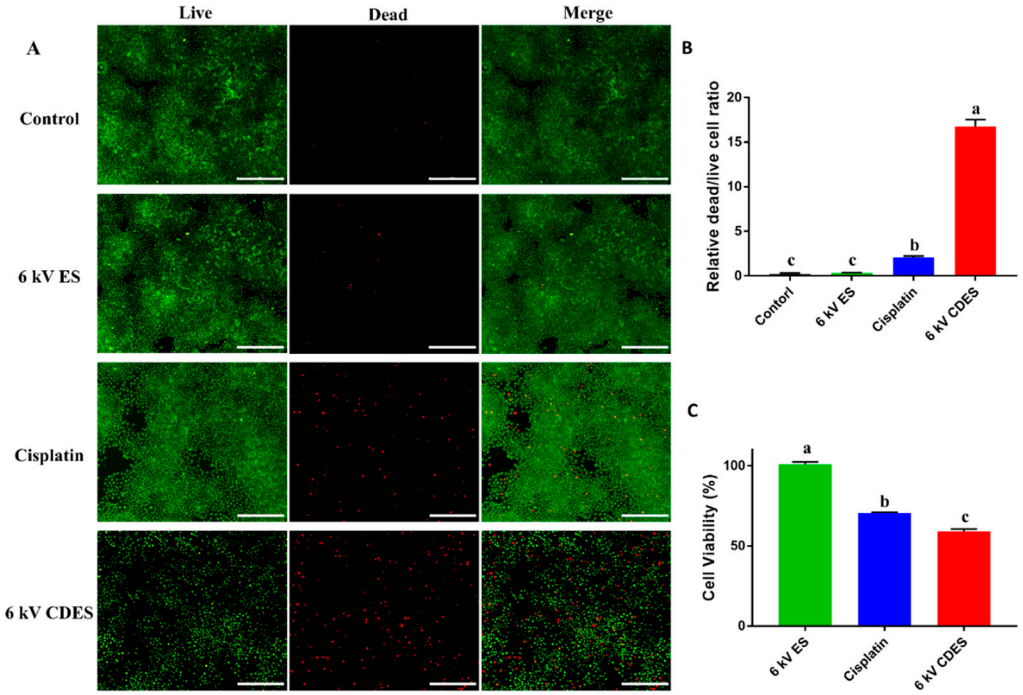

Figure 2. Comparison of cell viability of 6 kV CDES and cisplatin, (**A**) live and dead assay of 6 kV electrospray with cisplatin (Control, 6 kV ES, Cisplatin and 6 kV CDES), (**B**) the bar graph of the dead/live ratio, (**C**) water-soluble tetrazolium salt (WST) assay of 6 kV CDES (scale bar: 1 mm, n = 3, $p < 0.05$. Columns with different letters are significantly different according to the Duncan test). The ratio of dead/live cells in the CDES-treated group was approximately 7 times larger than that in the cisplatin-treated group 3 days after treatment.

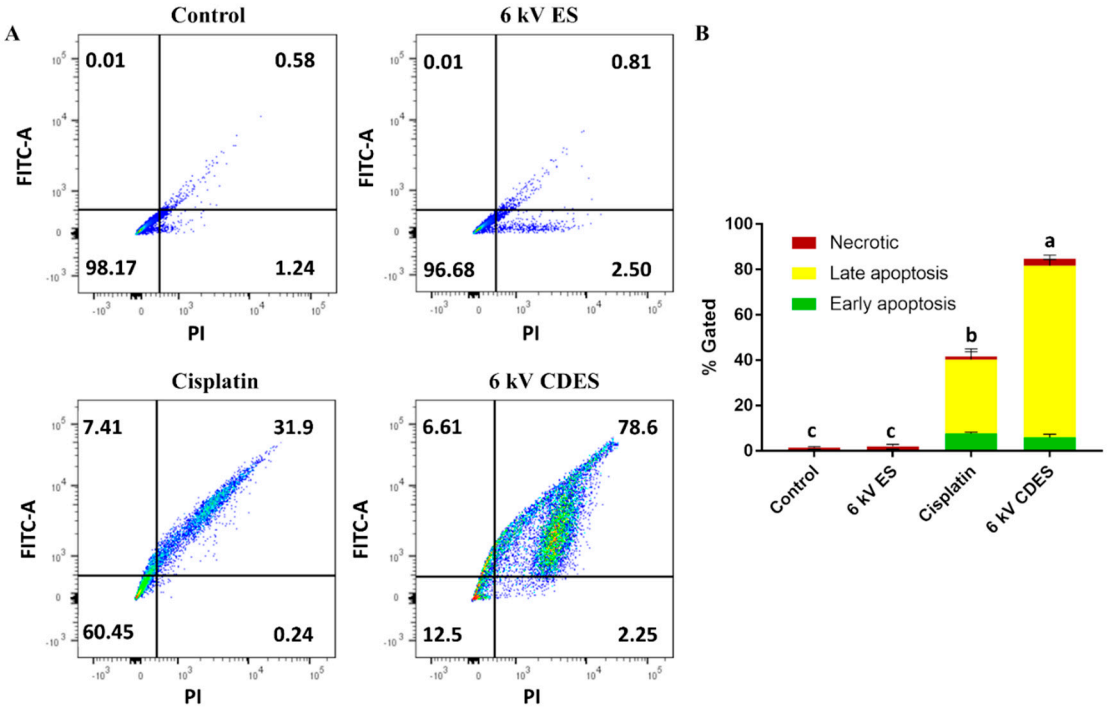

Figure 3. (**A**) The results of fluorescence-activated cell sorting (FACS) for Annexin V staining after 6 kV CDES (Control, 6 kV ES, Cisplatin, and 6 kV CDES). (**B**) FACS analysis showed more than 80% cell death in the 6 kV CDES group. Approximately 80% of apoptotic cells were shown in the 6 kV CDES treatment group (n = 3, $p < 0.05$. Columns with different letters are significantly different according to one-way ANOVA and Duncan's test).

3.3. The Proposed Mechanism

PI staining analysis was performed to confirm indirectly the mechanism of how drugs are forcibly injected into the cells by ES. PI is a substance that exhibits red fluorescence upon binding to DNA inside the cell. PI is generally used to detect dying or dead cells because the cell membranes of healthy cells do not allow PI to pass. On the other hand, it is also used as a tool to detect the opening of the plasma membrane when a specific stimulus is applied to a cell [39,40]. We hypothesized that the ES method temporarily increased the permeability of the cell plasma membrane, thereby increasing the influx of drugs into the cells, so PI was administered at increasing time intervals (0, 30, 60, and 120 min) after ES treatment. In our results, numerous red spots resulting from PI, which indicates dying or dead cells, appeared in the group treated with 6 kV ES at 0 min. However, as time passed, the expression of red fluorescence rapidly decreased, and there were almost no red spots at 60 min (Figure 4A). Compared with the control, the amount of red fluorescence in the 6 kV ES treatment group was approximately 20 times higher at 0 min (Figure 4B). After ES treatment, the red fluorescence derived from DNA-bound PI was different at each time interval, indicating that the opening of the plasma membrane was changed. Thus, we indirectly indicated that when a specific substance is delivered to cells using ES, it can facilitate substance transfer by increasing the permeability of the cell plasma membranes. Figure 4C shows the proposed CDES mechanism. The charged droplets generated by ES increase the plasma membrane permeability of the cells and greatly increase the influx of cisplatin.

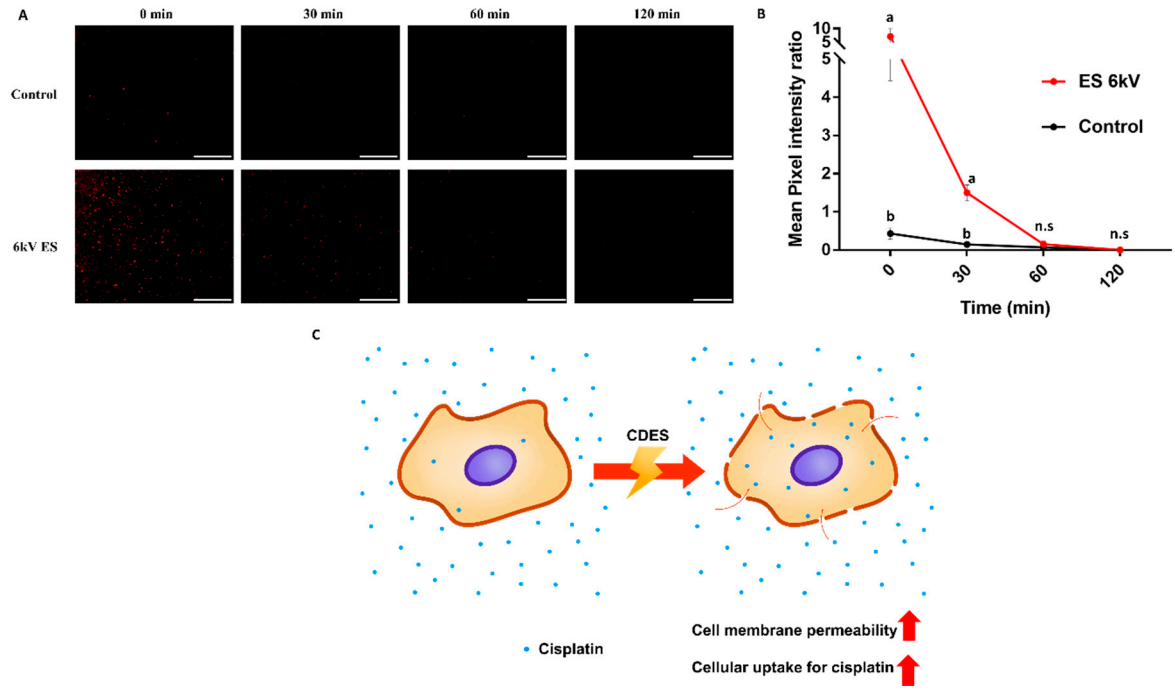

Figure 4. Proposed CDES mechanism. (**A**) Comparison of plasma membrane permeability after electrospray (ES) using propidium iodide (PI) staining (scale bar: 1 mm). (**B**) PI staining analysis over time. Compared to the control, when treated with 6 kV ES, red fluorescence derived from DNA-bound PI was significantly higher, but over time, the expression of PI decreased to a similar level (n = 3, $p < 0.05$. Columns with different letters are significantly different according to one-way ANOVA and Duncan's test). (**C**) Proposed CDES mechanism. The charged droplets generated by ES increase the permeability of the plasma cell membrane. Then, it increases the influx of cisplatin.

In this study, we proposed a new method for inducing apoptosis of cancer cells by delivering cisplatin using an ES method and indirectly proved its mechanism. Several studies have reported the transfer of genes and proteins to cells using ES, but our study is the first to induce apoptosis by delivering anticancer drugs to cancer cells using ES.

Recently, a study on the delivery of genes using a miniaturized ES device was reported [36]. If an anticancer drug can be delivered using such a miniaturized ES device, it is expected that a new anticancer treatment method can effectively deliver the drug to a limited target area. In the future, we plan to use technology to reduce the size of skin cancers and in vivo tumors.

4. Conclusions

In summary, we have confirmed that electrospray technology facilitates the introduction of cisplatin, an anticancer drug, into cancer cells and discovered that electrospray at 6 kV is the proper voltage for delivery of the drug to the target. Compared with the group treated only with cisplatin, the 6 kV CDES-treated group effectively inhibited cell proliferation and induced apoptosis of cancer cells. Additionally, after ES treatment, the plasma membrane permeability was measured at regular intervals. This result indirectly showed that CDES increased the influx of the drug by increasing the permeability of the cell membrane. If this technology is further improved with additional in vivo studies, we expect it could be advanced to local anticancer treatment to apply the drugs selectively to tumor sites only. With our research, another anticancer delivery system fundamental for local treatment has been established.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11073203/s1, Figure S1: Components of CDES system, Figure S2: Cell viability after electrospray (ES) at various voltage, Figure S3: Comparison of cell viability at various voltage CDES.

Author Contributions: Conceptualization, M.C.L., S.P. (Shambhavi Pandey), H.S. (Hoon Seonwoo) and J.H.C.; methodology, M.C.L. and J.W.L.; validation, J.W.L., M.C.L., S.P. (Sangbae Park), H.S. (Hyunmok Son) and S.P. (Shambhavi Pandey); formal analysis, M.C.L., J.W.L. and S.P. (Shambhavi Pandey); investigation, M.C.L., S.P. (Shambhavi Pandey), J.W.L., H.S. (Hyunmok Son), S.P. (Sangbae Park) and J.E.K.; resources, S.P. (Shambhavi Pandey); data curation, S.P. (Shambhavi Pandey) and J.W.L.; writing—original draft preparation, M.C.L., S.P. (Shambhavi Pandey) and J.W.L.; writing—review and editing, H.S. (Hoon Seonwoo), M.C.L., P.G. and J.H.C.; visualization, J.W.L. and J.H.; supervision, H.S. (Hoon Seonwoo), P.G. and J.H.C.; funding acquisition, P.G. and J.H.C. All authors have read and agreed to the published version of the manuscript.

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