Microsecond Pulse Electrical Stimulation Inhibit the Proliferation of Cervical Cancer Cells through the Ras-Raf-MAPK Pathway

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Research Article

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Abstract

Objective: Using microsecond pulsed electrical stimulation (μsPES) applied to treat pelvic floor dysfunctions(PFDs) to stimulate the cervical cancer cells. To explore the effects and mechanisms of different μsPES on the proliferation of cervical cancer cells.

Methods: Scheme #1 (1/4/1 Hz, 270/230/270 μs, 30min), scheme #2 (30 Hz, 500 μs, 20min), and scheme #3 (50 Hz, 250 μs, 20min) were selected from a pelvic floor rehabilitation therapy instrument. Three μsPES-based schemes each at 20, 40, 60, 80, and 100 mA current values were used to stimulate SiHa cells and HeLa cells. Cell counting kit-8 (CCK-8) assay was used to detect cell proliferation. Scheme #2 at 100 mA current was employed to stimulate SiHa cells and HeLa cells and then western blotting was applied to detect the expressions of the Ras-Raf-MAPK pathway.

Results: Current with higer frequency and current intensity tended to have a more proliferation inhibition on cervical cancer cells. μsPES had a stronger proliferation inhibition in SiHa cells than HeLa cells. The expressions of MAPK, MEK1/2 and p-MEK1/2, Raf and p-Raf, Ras protein were significantly decreased in SiHa cells and HeLa cells compared with the control. The p-MAPK was up-regulated in HeLa cells while down-regulated in SiHa cells.

Conclusions: μsPES could inhibit the proliferation of cervical cancer cells by suppressing the Ras-Raf-MAPK pathway. The μsPES applied for functional rehabilitation of pelvic floor nerves and muscles is safe for cervical cancer patients at the cellular level

Introduction

At present, cervical cancer is a fourth-ranked malignant tumor worldwide in women just after breast cancer, colorectal cancer, and lung cancer[1]. Radical hysterectomy plus pelvic lymphadenectomy is the main treatment for early cervical cancer. After this surgery, patients are often associated with lower urinary tract symptoms, such as urinary retention and urinary incontinence.

Lower urinary tract symptoms impose a serious negative influence on the quality of life and the physical and mental health of patients. In 1963, Caldwell et al. proposed functional electrical stimulation (ES) of pelvic floor muscles for treating incontinence and urinary incontinence [2]. Transcutaneous electrical nerve stimulation (TENS) using microsecond pulsed electrical stimulation (μsPES) is helpful to treat patients with various pelvic floor dysfunctions (PFDs), such as urinary incontinence, and pelvic organ prolapse (POP)[3].

At present, the exact mechanism by which μsPES improves urethral symptoms remains elusive, and whether μsPES can change the biological behavior of cervical cancer needs to be further clarified.

Peking University People's Hospital typically uses μsPES to treat PFD patients with postpartum and postoperative pelvic floor dysfunction. The μsPES applied in our clinic is a low-intensity, low-frequency

ES. In previous studies, our research team had previously evaluated the effects of μsPES on the function of pelvic floor nerves and muscles after cervical cancer surgery[4]. What's more, we had assessed the effects of scheme #1 at the current values of 20, 40, and 60 mA on the proliferation and migration of cervical squamous carcinoma cell line SiHa in vitro and in vivo, and our results showed that μsPES did not affect the proliferation and migration of SiHa cells [5–6].

The present study aimed to clarify the effects and mechanisms of μsPES on cervical cancer cells by including more schemes, wider current intensity, and two cervical cancer cell lines using the pelvic floor rehabilitation instrument.

Materials And Methods

ES apparatus and μsPES parameters

A pelvic floor rehabilitation instrument (PHENIX USB4, Electron-IC Concept Lignon Innovation Co., France) (Fig. 1A) was employed for research in vitro. This instrument with dozens of treatment schemes is a kind of TENS device using μsPES, which can treat PFDs (e.g., urinary retention and pelvic organ prolapse (POP)). Three schemes were chosen from this pelvic floor rehabilitation instrument. Scheme #1 with a frequency of 1/4/1 Hz, a pulse width of 270/230/270 μs, and 30 min duration could treat urinary retention by stimulating the pelvic floor muscles. Scheme #2 with a frequency of 30 Hz, a pulse width of 500μs, and 20 min duration could treat POP by stimulating type I muscle fibers. Scheme #3 with a frequency of 50 Hz, a pulse width of 250 μs, and 20 min duration could treat POP by stimulating type II muscle fibers. An oscilloscope (DSO7054A; Agilent Technologies Inc., Santa Clara, CA, USA) (Fig. 1C) was used to detect the waveform of the three schemes (Fig. 2).

Cell Lines and Cell Culture

Cervical squamous cell carcinoma SiHa cells and cervical adenocarcinoma HeLa cells were supplied by the Gynecology Laboratory of Peking University People's Hospital (Beijing, China). Two groups of cells were both grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA) and 1% antibiotics (100 U/ml penicillin/streptomycin; Gibco, New York, NY, USA). Cells were cultured at 37 °C with 5% CO2 and passaged after 3 days. SiHa cells and HeLa cells were harvested and resuspended in saline solution with a concentration of 106 cells/ml. Besides, 1000 μl cell suspension was placed in 2-mm gap cuvettes (Bio-Rad Laboratories Inc., Hercules, CA, USA) (Fig. 1B) and exposed to μsPES. Three μsPES-based schemes at current values of 20, 40, 60, 80, and 100 mA were applied to stimulate cervical squamous cell carcinoma SiHa cells and cervical adenocarcinoma HeLa cells. The temperature of the suspension was measured during the experiment.

Cell proliferation assay

A total of 2 × 103 cells/well were seeded into 96-well plates (Corning Inc., Corning, NY, USA) after treatment with three μsPES-based schemes at current values of 20, 40, 60, 80, and 100 mA each. 10 μl of the cell counting kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added to each well and incubated at 37 °C for 4, 24, 48, and 72 h and then every 24 h if necessary. The absorbance was measured at 450 nm on a spectrophotometric plate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA) at each time-point. All experiments were performed in triplicate.

Western blot assay

SiHa cells and HeLa cells (treated and untreated cells) were harvested and lysed using cell lysis reagent containing NP40 and protease inhibitor cocktail for protein isolation after the treatment with μsPES scheme #2 at 100 mA current. Protein concentration was detected using the Bradford assay. Next, 30 ug of total protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto nitrocellulose membranes (Millipore, Burlington, MA, USA). After blocking with 5% non-fat dry milk, the membranes were incubated with the following primary antibodies: mouse anti-β-actin (Proteintech, Rosemont, IL, USA), rabbit anti-MAPK (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-Phospho- MAPK (Cell Signaling Technology), rabbit anti-MEK1/2 (Cell Signaling Technology, Inc.), rabbit anti-Phospho-MEK1/2 (ImmunoWay Biotechnology Co., Plano, TX, USA), rabbit anti-Raf (Abcam, Cambridge, UK), Phospho-Raf (Cell Signaling Technology, Inc.), The blots were washed thrice, and antigen-antibody complexes were detected by a fluorescence method using fluorescent secondary anti-mouse (Cell Signaling Technology, Inc.) IgG and anti-rabbit IgG (Cell Signaling Technology, Inc.). Fluorescent signals were detected by a bicolor infrared laser imaging system (LI-COR Biosciences, Lincoln, NB, USA) and quantified using the ImageJ software.

Statistical analysis

All trials were repeated 3 times. Data were analyzed using a one-way analysis of variance (ANOVA) for making comparisons among multiple groups and a t-test for making comparisons between the two groups. Statistical analyses were performed using SPSS 26.0 software (IBM, Armonk, NY, USA). The results were expressed as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

Results

1. The effects of μsPES on the proliferation of cervical cancer cells

Compared with the control group, the μsPES scheme #1 at 100 mA current could inhibit the proliferation of SiHa cells (P = 0.033) and at 40 mA current may inhibit proliferation of HeLa cells (P = 0.001). No significant effect of μsPES scheme #1 at other current values on the proliferation of SiHa cells and HeLa cells was found (P > 0.05). (Fig. 3A, Fig. 3B)

Compared with the control group, the μsPES scheme #2 at current values of 80 and 100 mA could inhibit proliferation of SiHa cells (P = 0.017 and 0.008, respectively), and at current values of 40, 60, 80, and 100 mA could inhibit proliferation of HeLa cells (P = 0.006, 0.010, 0.005, and 0.025, respectively). No significant effect of the μsPES scheme #2 at other current values on the proliferation of SiHa cells and HeLa cells was detected (P > 0.05). (Fig. 3A, Fig. 3B)

Compared with the control group, the μsPES scheme #3 at 60 mA, 80 mA, 100 mA current could inhibit SiHa cells proliferation (P values were 0.030, 0.004, 0.001 respectively), the proliferation effect of μsPES scheme #3 at 40 mA current on SiHa cells need further study. The μsPES scheme #3 at 100 mA current had stronger inhibition than 60 mA current intensity in SiHa cells (P = 0.026);at the current intensity of 40 mA, 60 mA, 80 mA, 100 mA could inhibit the proliferation of HeLa cells (P values were 0.002, 0.035, 0.002, 0.003 respectively), and 100 mA current had stronger inhibition than 80 mA current (P = 0.004) and 60 mA current (P = 0.036) in HeLa cells, and 80 mA current had stronger inhibition than 60 mA current in HeLa cell (P = 0.033). (Fig. 3A, Fig. 3B)

The effects of three μsPES schemes on SiHa cells and HeLa cells were not the same. Compared with the control, μsPES scheme #1 at 100 mA current intensity could inhibit the proliferation of SiHa cells, but had no effect on the proliferation of HeLa cells. Compared with the control, μsPES scheme #2 at 40 mA and 60 mA current intensity could inhibit HeLa cell proliferation, but had no effect on SiHa cells proliferation. At the current of 60 mA, μsPES scheme #3 had a stronger proliferation inhibition than μsPES scheme #2 in cervical cancer HeLa cell(P = 0.013).

μsPES Scheme #2, at the current of 80 mA, μsPES had a stronger proliferation inhibition in SiHa cells than HeLa cells(P = 0.028); at the current of 100 mA, μsPES had a stronger proliferation inhibition in SiHa cells than HeLa cells (P = 0.031). μsPES Scheme #3, at the current intensity of 60 mA, μsPES had a stronger proliferation inhibition in SiHa cells than HeLa cells (P = 0.017). This showed that the sensitivity to different μsPES between cervical squamous cell carcinoma and adenocarcinoma was not the same. Overall, the above-mentioned results showed that the effects of μsPES on inhibiting the proliferation of cervical cancer cells increased with the elevation of current intensity.

2. The effects of μsPES on the Ras-Raf-MAPK pathway of cervical cancer cells

The expressions of MAPK (P = 0.046), p-MAPK (P = 0.048), MEK1/2 (P = 0.002), p-MEK1/2 (P = 0.021), Raf (P = 0.033), p-Raf (P = 0.009), and Ras (P = 0.009) were remarkably decreased in SiHa cells after treatment with μsPES scheme #2 at 100 mA current compared with the control group. The expression of p-MAPK

was downregulated in SiHa cells (P = 0.048), whereas that was upregulated in HeLa cells (P = 0.008) after treatment with scheme #2 at 100 mA current. The expressions of MEK1/2 (P = 0.017) and p-MEK1/2 (P = 0.033), Raf (P = 0.038), p-Raf (P = 0.020), and Ras (P = 0.048) were significantly decreased in HeLa cells after treatment with μsPES scheme #2 at 100 mA current compared with the control group (Fig. 4A, Fig. 4B)

There were no significant differences in protein expressions between SiHa cells and HeLa cells in the Ras-Raf-MAPK pathway except for p-MAPK downregulated in SiHa cells, while that was upregulated in HeLa cells. The subtle different mechanism between SiHa cells and HeLa cells was unclear, which may be related to the different regulation in different tumor subtypes.

Discussion

The μsPES studied in this experiment is used in the clinical, however, there are some subtle differences between the experiment and the clinical. In the clinical applications, the current intensity is adjusted according to the patient's feelings which is normally below 0–60 mA while in the cellular experiment, the current intensity is in a range of 0-100 mA. What's more, the clinical application of the Pelvic Floor Rehabilitation Therapy Instrument placed electrode patches on the abdomen while tumor cells were secured in gap cuvettes during experiments. Although there are differences between the experiment and the clinic, to some extent, the results of the experiment still can explain the clinical question.

The μsPES of three schemes were all asymmetry bipolar sharp electrical pulses, and the shape of waves was similar to an action potential, which was biomimetic. Bipolar electrical pulses did not have the same effects as the monopole electrical pulse. A previous research concentrated on Chinese hamster ovary cells demonstrated that the energy produced by bipolar electrical pulses was twice a unidirectional waveform in the same parameters, and monopole electrical pulse was more lethal than bipolar electrical pulses [7]. Determining the waveform enables us to better understand the possible effects and mechanisms of μsPES on cervical cancer.

In our results, proliferation inhibition was found after the stimulation of μsPES in SiHa cells and HeLa cells. The expressions of the Ras-Raf-MAPK pathway was decreased in both SiHa cells and HeLa cells compared with those in the control group except p-MAPK was downregulated in SiHa cells while upregulated in HeLa cells. In conclusion, μsPES could inhibit the proliferation of cervical cancer cells by inhibiting the Ras-Raf-MAPK pathway. However, the molecular pathways did not exist in isolation, they are intertwined and connected. At present, it is not clear that the inhibition of μsPES on cervical cancer proliferation is the direct or indirect effect of the Ras-Raf-MAPK pathway. And, it is also not clear whether other signaling pathways could influence the proliferation inhibition of cervical cancer cells. The upregulated p-MAPK in HeLa cells may be influenced by other molecular pathways, which indicated that other molecular mechanisms existed in the proliferation inhibition in HeLa cells. Anyway, the Ras-Raf-MAPK pathway was suppressed by μsPES both in SiHa cells and HeLa cells.

Current with higer frequency and current intensity tended to have a more proliferation inhibition on cervical cancer cells. And the pulse width of μsPES may had a effect on proliferation inhibition of cervical cancer cells which needed further study to confirm this hypothesis. Meanwhile, Silve et al. pointed out that ES with the frequency of 0.1 Hz is more likely to cause enhanced cell permeability and 1 and 10 Hz have a more remarkable effect on permeability than 100 Hz and 1 kHz in plant cells and mouse liver cells[8]. Simultaneously, another study demonstrated that the pulse width did not affect the electroporation, while the intensity of the electric field could[9]. There are also some studies shown that proliferation was increased used a lot higher frequency(50 Hz) than a lower frequency(2 Hz)[10].

Controversial results exist as several studies have shown that ES can increase the rate of cell proliferation in tumors and other studies show that ES can decrease or have no effect on cell proliferation in tumors. Hernandez-Bule et al. found that CRET sine wave could decrease cell proliferation in HepG2 hepatocarcinoma[11]. Matsuki et al. found that LVEP square wave could increased apoptosis but have no effect on cell proliferation in B16 malignant melanoma[12]. Linkov et al. found that NMES(neuromuscular electrical stimulation) and BPC(biphasic pulsed current) have no effect on cell proliferation and apoptosis in Cutaneous squamous cell carcinoma[13].

To date, little is known on how ES can control important cell-biological events such as proliferation[14]. Several scholars reported that a physiological ES can reduce the proliferation and inhibit G1-S phase transition by downregulating cyclin-E and upregulating p27kip1 in rat lens epithelial cells and bovine endothelial cells[15]. Conversely, an endogenous ES controls the corneal epithelium wound healing rate in vivo [16]. Besides, ES also can regulate the expression of type-I collagen in keloids[17].

In summary, we, in the present study, found that μsPES could inhibit the proliferation of cervical cancer cells by inhibiting the Ras-Raf-MAPK pathway. Thus, it can be concluded that μsPES applied for functional rehabilitation of pelvic floor nerves and muscles is safe for cervical cancer patients at the cellular level.

Abbreviations

Electrical stimulation (ES)

Low-voltage electric field pulses (LVEPs)

Microsecond pulse electrical stimulation (μsPES)

Pelvic floor dysfunctions (PFDs)

Pelvic organ prolapse (POP)

Transcutaneous electrical nerve stimulation (TENS)

neuromuscular electrical stimulation (NMES)

biphasic pulsed current (BPC)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Xiaoyue Xu: Protocol, Data collection, Data analysis, Manuscript writing/editing; Shiyan Wang: Protocol, Data collection; Tingting Cao: Protocol, Data collection; Bing Xie: Protocol, Data collection; Xiuli Sun: Protocol, Data collection, Data analysis; Jianliu Wang: Protocol, Data collection, Data analysis, Writing review&editing, Funding acquisition.

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