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ORIGINAL ARTICLE

# Pilot in vitro and in vivo study on a mouse model to evaluate the safety of transcutaneous low-frequency electrical nerve stimulation on cervical cancer patients

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## Abstract

**Introduction and hypothesis** To clarify whether the pulse electrical field (PEF) caused by transcutaneous low-frequency nerve electrical stimulation (TENS) enhances the proliferation of cervical cancer cells, leading to recurrence and metastasis, and the effect of such a PEF on a cervical cancer mouse model.

**Methods** 1. In vitro experiment: SiHa cervical cancer cells treated with one session of microsecond PEFs for 30 min were divided into four groups: three experimental groups and the control group. Cell proliferation and migration were determined by CCK-8 proliferation and Transwell chamber Matrigel migration assay. 2. In vivo experiment: A mouse cancer model was established by subcutaneous implantation of SiHa cells that were then were randomly divided into the TENS group and control group. The former group received one session of TENS treatment and the control group received a sham pulse. The growth trend and tumor volume of each group were compared 28 days after PEF treatment. The proliferation and apoptosis of the tumor were determined by an immunohistochemical method.

**Results** (1) The CCK-8 proliferation assay and cell migration ability showed no difference after PEF stimulation treatment ($F = 2.478$, $P = 0.136 > 0.05$ and $F = 0.364$, $P = 0.779$). (2) Tumor growth, size and weight showed no significant difference between the two groups. (3) Expression of VEGF, CD34, caspase-3 and Ki-67 in the tumor tissue showed no significant difference between the two groups.

**Conclusions** In vitro and in vivo experiments (mice) showed that the PEF created by TENS had no effect on the proliferation and migration of SiHa cervical cancer cells and also had no effect on the tumor growth, tumor cell apoptosis and proliferation.

**Keywords** Transcutaneous low-frequency electrical stimulation · Cervical cancer · SiHa cell line · Pulse electrical field

**Abbreviations**

| | |
|---|---|
| PEF | Pulse electrical field |
| TENS | Transcutaneous low-frequency electrical nerve stimulation |
| CC | Cervical cancer |
| LUTS | Lower urinary tract symptoms |
| IRB | Institutional review board |
| CI | Current intensity |
| ANOVA | Analysis of variance |
| OD | Optical density |
| TSHCT | Transplanted SiHa cell tumors |
| IHC | Immunohistochemical |
| DMEM | Dulbecco's modified Eagle's medium |

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## Introduction

There are nearly 131.5 thousand new cases of cervical cancer (CC) per year in China, which accounts for 28.8% of all cases worldwide [1]. Application of cervical cancer screening has increased the proportion of early stage cervical cancer detected in cancer clinics. Class III radical hysterectomy plus pelvic

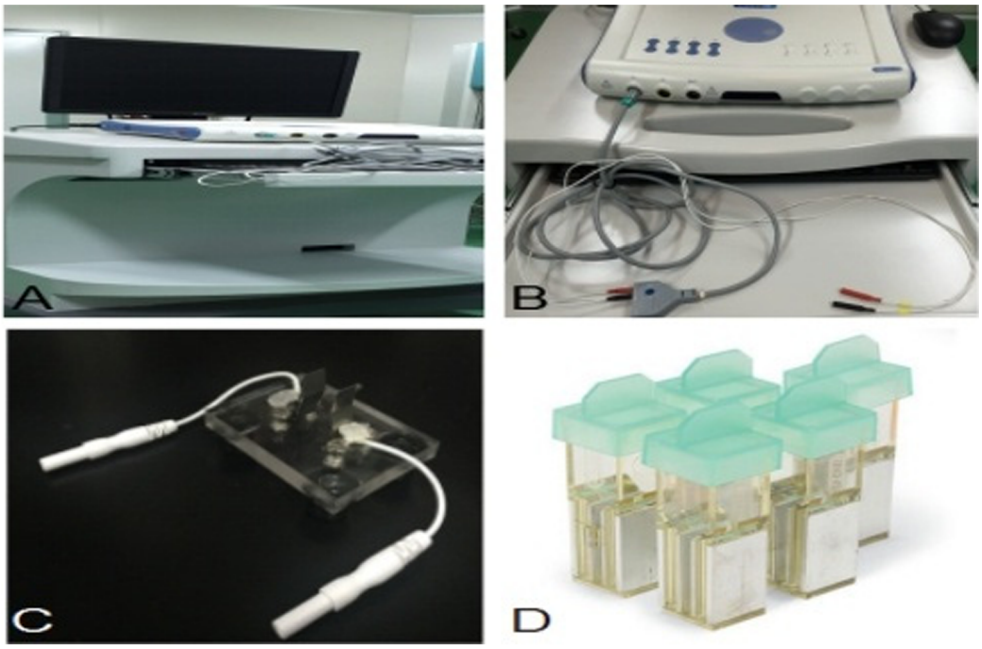


**Fig. 1** Equipment for SiHa cell electric stimulation: **a** Phenix USB4; **b** electrode line; **c** carrier; **d** electrode cup

lymphadenectomy is the standard surgery for early stage cervical cancer patients with the 5-year survival rate being about 90% [2]. However, postoperative pelvic floor disorders, especially bladder dysfunction, which is the common sequela resulting from surgerical damage of the vessels and nerve fibers, increase the patients' suffering [3–5]. Transcutaneous low-frequency electrical nerve stimulation (TENS) is currently used by many clinics for pain relief and functional improvement [6, 7]. TENS has been proved to be effective in relieving lower urinary tract symptoms (LUTS) [8]. Recently, some investigators reported their application of TENS to treatment for CC patients with voiding problems after class III radical hysterectomy and concluded that TENS could shorten the duration of catheter indwelling and improve voiding [9, 10]. However, whether the pulse electrical field (PEF) created by TENS will enhance the proliferation of cancer cells, leading to recurrence and metastasis, what the effect of PEF on cervical cancer is and whether it is safe for patients remain unanswered questions with no current clinical or experimental data available.

Matthew et al. [11] chose electrical stimulation as the therapy for lung cancer patients with weak constitutions postoperatively to help their rehabilitation. Robert [12] reported a case treated with TENS that resulted in apparent relief of bone pain. Some investigators used TENS and high-frequency electrical stimulation to treat the pain of patients with malignant tumors and achieved satisfying results [7, 13]. It was reported that electrical stimulation could improve the life quality of postoperative cancer patients. Mariotti et al. [6] randomized 60 patients with prostate cancers into two groups; the study group received electrical stimulation on their pelvic floors and biofeedback therapies for 7 days after the operations and

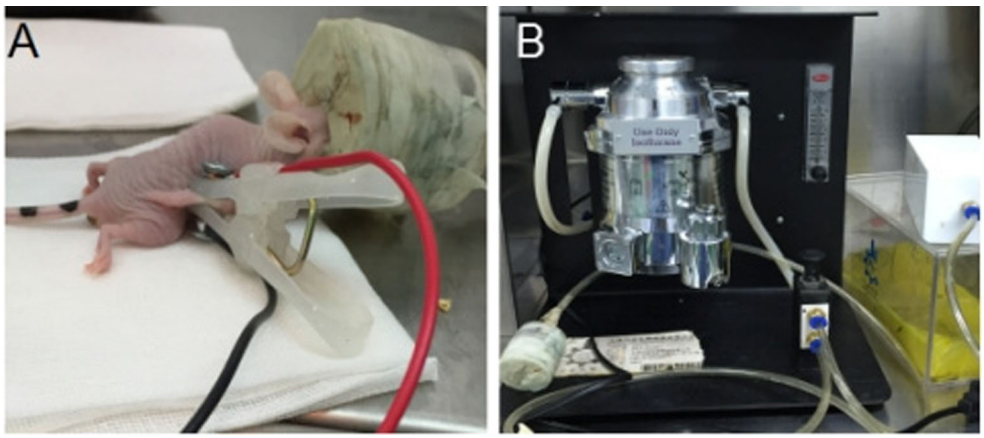


**Fig. 2** Equipment for nude mouse electric stimulation: **a** mouse and clamp electrode; **b** anesthesia machine

**Table 1** OD values of the four groups (mean ± SD)

| | 4 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|
| Control group | 0.81 ± 0.017 | 0.88 ± 0.082 | 1.26 ± 0.067 | 2.57 ± 0.083 |
| 20 mA group | 0.84 ± 0.017 | 1.05 ± 0.049 | 1.35 ± 0.250 | 2.63 ± 0.070 |
| 40 mA group | 0.86 ± 0.013 | 0.91 ± 0.166 | 1.20 ± 0.069 | 2.62 ± 0.110 |
| 60 mA group | 0.89 ± 0.055 | 0.91 ± 0.144 | 1.30 ± 0.049 | 2.64 ± 0.136 |

the control group only received routine nursing treatments. Their 1–6 months of follow-up showed that the voiding function of patients in the study group was significantly better than those in the control group.

Some studies suggest that electrical stimulation promotes vessel formation in vivo by increasing the expression of vascular endothelial growth factor [14]. Electrical characteristics of the malignant tumor tissues may change, and the tumor can metastasize its galvanotaxis, which affects the metastasis of carcinoma cells in circulation [15]. Electrical stimulation can promote healing of wounded skin and bones, repair of nerve muscles, spinal repair and neuronal migration, all of which make us think about the potential migration and proliferation of cancer cells when being electrically stimulated.

Our study aims to identify whether PEFs caused by electrical stimulation enhance the proliferation of cervical cancer cells, leading to recurrence and metastasis, and the effect of such PEFs on cervical cancer in a mouse model.

## Materials and methods

The research protocol was approved by the Institutional Review Board (IRB) of the Peking University People's Hospital.

Our study assesses the safety of TENS on cervical cancer in vivo and in vitro in three steps: (1) using TENS to stimulate cervical cancer SiHa cells; (2) inducing human cervical cancer tumors in Balb/c(nu−/nu-) mice and (3) treating tumors with TENS.

A Phenix USB4 PEF generator from Electron-IC Concept Lignon Innovation Co., France, was adopted in our study. The carrier and clamp electrode was designed by Frontier Discipline Institute of Peking University (Figs. 1c and 2a). MsPEFs (microsecond pulsed electric field stimulation) applied to treat the urinary retention after type III hysterectomy were used to stimulate the SiHa cells and tumor in the mice. We performed only one session of electrical stimulation in the in vivo and in vitro study.

The SiHa human cervical cancer cell line was supplied by the Gynecology Laboratory of Peking University People's Hospital. SiHa cells were cultured in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% fetal calf serum. No antibiotics were used. The cells were cultured in a monolayer and maintained in a humidified air atmosphere with 5% $CO_2$ at 37 °C for exponential growth. SiHa cells were collected and treated with 0.25% trypsin at 80–90% of their maturity to prepare for the following in vitro and in vivo experiments.

In the in vitro study (Figs. 1 and 8), an average of 2000 ul cell suspension was put into four 2-mm electric rotors (Fig. 1d), one in the control group and three in the experimental groups, followed by msPEF stimulation with the specific frequency of 1/4/1 HZ, pulse width of 230/270/230 us, pulse number of 1710, duration of 30 min and current intensity (CI) of 0 mA, 20 mA, 40 mA and 60 mA, respectively. The control group had no electrical stimulation treatment.

In the in vitro study, 2000 cells from the four groups were seeded into each well of the 96-well flat-bottom (Costar) plates, which were placed in a 5% $CO_2$ cell culture medium and kept at 37 °C and saturated humidity; 10 ul CCK-8 was added to each well 4, 24, 48 and 72 h after incubation followed by a further incubation at 37 °C for 2 h. The medium was then removed, and the dye intensity was then read on a microplate reader (Bio-Rad) at 450 nm.

Cell invasion was assayed using Transwell chambers (Costar, Cambridge, MA) with 8-mm-pore polycarbonate

**Table 2** Repeated measures ANOVA of the four goups at four time poins

| Source | III Sum of squares | df | Mean square | *F* value | *P value* |
|---|---|---|---|---|---|
| Intercept | 97.043 | 1 | 97.043 | 12,287.160 | 0.000 |
| Time | 24.033 | 3 | 8.011 | 650.266 | 0.000 |
| CI | 0.059 | 3 | 0.020 | 2.478 | 0.136 |
| CI*time | 0.055 | 9 | 0.006 | 0.492 | 0.866 |
| Deviation | 0.296 | 24 | 0.012 | | |

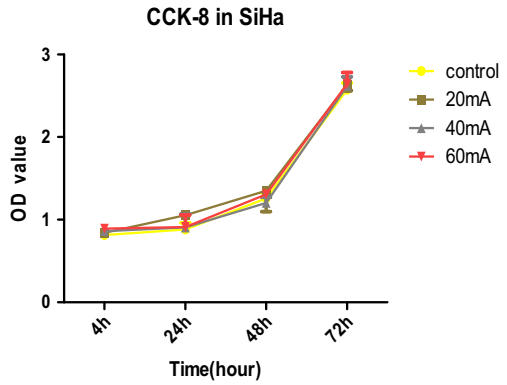


**Fig. 3** Proliferation curve of cervical cancer SiHa cells after stimulation for the four groups

filters coated with 50 mg/ml of Matrigel™ (BD, Biosciences, Bedford, MA) diluted in serum-free medium. SiHa cells were treated with msPEFs (20 mA, 40 mA and 60 mA). Cells (10,000 cells/chamber) were seeded in the top of the cylindrical cell culture inserted in the DMEM without FBS. Ten percent FBS was placed in the wells to allow the cells to migrate through the filter for 36 h at 37 °C in 5% $CO_2$. Cells that did not migrate were removed from the upper surfaces of the chambers by scrubbing with a cotton swab. Cells that migrated to the lower membrane were fixed in 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (Invitrogen) for 30 min. Invasion cells were counted under a microscope.

For the in vivo study, 30 6-to-8-week-old female Balb/c (nu/nu) mice were housed in the Peking University People's Hospital Laboratory Animal Center for 1 week before inoculation. Thirty tumor species were prepared by making a 100-ul suspension with $5 \times 10^7$/ml SiHa cells mixed into 100 ul Matrigel on ice in each of the 11 1.5-ml EP vials. The tumor species were then transferred to the animal center at 4 °C. The tumor cells were centrifuged at 1000 turns per 3 min. After dumping the supernatant, all the cells in each of the vials were injected subcutaneously into the back of each mouse, ensuring only one tumor on the back of each mouse. Treatments began when the tumors on the backs of the mice grew to 4–5 mm in diameter. Eleven nude mice were successfully subcutaneously implanted with SiHa cervical cancer cells and were randomly divided into two groups. Six mice were randomized as the control group and treated with a sham pulse (with the electrode clipped on the tumor but no pulse applied), while five mice comprised the study group and were treated with PEF. All mice were anesthetized with isoflurane inhalation during the treatments.

The TENS parameters used in the in vivo study (Figs. 2 and 9) were as follows: 1/4/1 Hz frequency; 230/270/230 μs pulse width; 20 mA current intensity. TENS was placed directly on the tumor for 20 min at 1100 pulses. Each mouse only received TENS once. No electrical stimulation treatment was applied to the control group.

All the tumors were measured every day one by one on their major and minor axes as each tumor was nearly an ellipse. The measurements were done with a caliper after the electric stimulation. The mice were killed by dislocation of the cervical spine, and the tumors were removed and weighed. The tumors were fixed in 4% paraformaldehyde.

The removed tumors were fixed in 4% paraformaldehyde and then stained with hematoxylin and eosin. After deparaffinization, the antigen was retrieved via high temperature and pressure for 2.5–3 min. Then, the primary antibody,

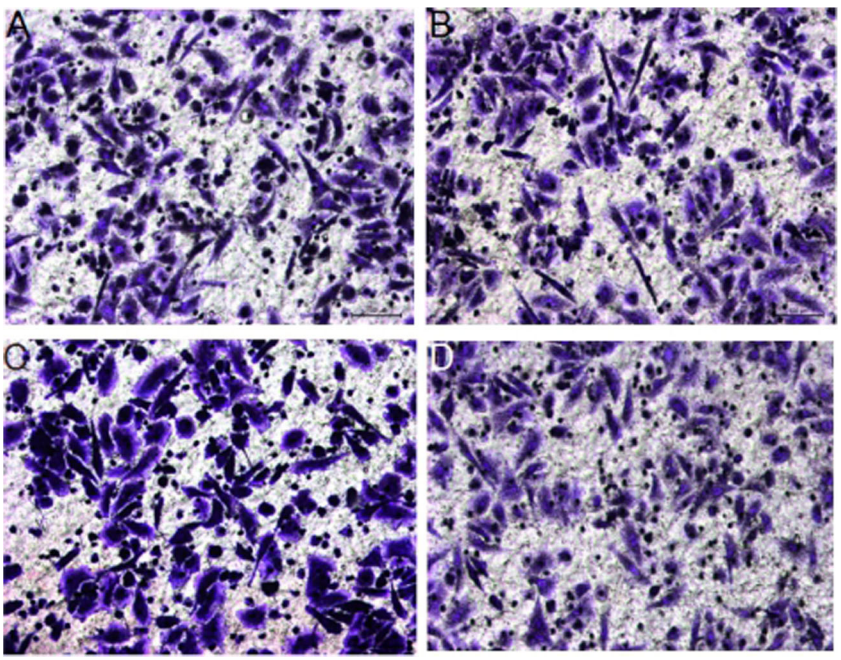


**Fig. 4** Number of cells penetrating the Transwell (100×): **a** 20 mA group; **b** 40 mA group; **c** 60 mA group; **d** control group

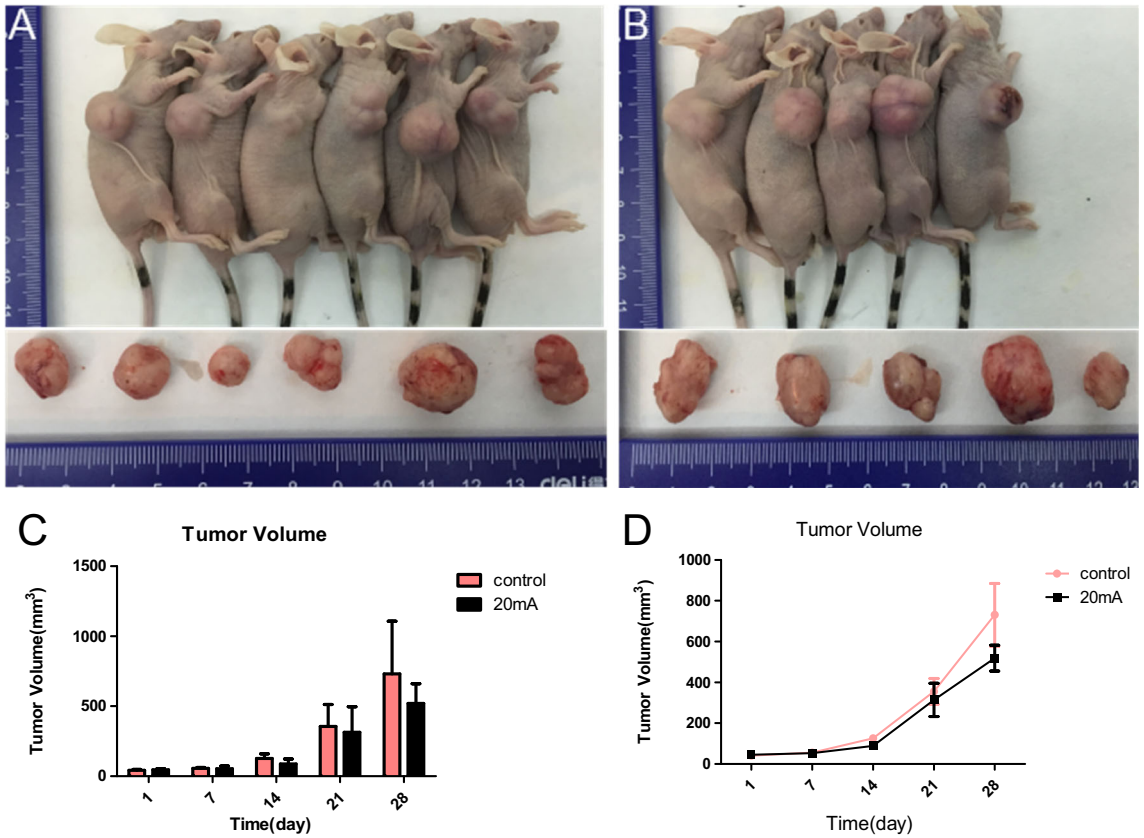


**Fig. 5** Effect of PEF on mouse tumors: **a** Control group **b**; 20 mA group. **c** Comparison of tumor sizes of the two groups. **d** Tumor growth speed of the two groups

Ki-67 (mouse anti-human Ki-67 monoclonal antibody, BioZSGB), VEGF (rabbit anti-human VEGF polyclonal antibody, BioZSGB) and CD34 (mouse anti-human ki-67 monoclonal antibody, BioDako) were added to the sample followed by incubation at 25°C for 10–20 min. The antigen was retrieved once again by microwaving for 15 min in citrate buffer, followed by adding the primary antibody, caspase-3 (mouse anti-human caspase-3 monoclonal antibody, Santa Cruz Biotechnology, Inc.), to the sample. The samples were incubated at 25 °C for 2 h before the secondary antibody was added and then incubated for 30 min at room temperature. 3,3-Diaminobenzidine (DAB) was added as chromogen. Slides were observed under an Olympus microscope after being sealed with balsam. Ten fields of each slide without overlap were selected randomly at 40× magnification under the microscope.

## Statistical analysis

Data were analyzed using repeated measures ANOVA (analysis of variance) for comparisons of one sample at different time points, T test for analyses of two-group comparisons and ANOVA for analyses of multiple group comparisons. Statistical analyses and graphics were performed using SPSS version 19.0. Results were expressed as SD. In all cases, $P < 0.05$ was taken as the level of significance.

## Results

### CCK-8 and Transwell assay showed TENS had no influence on the proliferation and migration of SiHa cervical cancer cells

After stimulation with different currency intensities, the optical density (OD) value of the control group was compared with those of the test groups. Table 1 shows

**Table 3** Weekly tumor volumes of the control and 20 mA groups ($mm^3$)

| | 20 mA group (mean ± SD) | Control group (mean ± SD) |
|---|---|---|
| Day 1 | 45.5 ± 8.2 | 41.6 ± 6.0 |
| Day 7 | 52.9 ± 20.5 | 55.2 ± 8.4 |
| Day 14 | 88.6 ± 34.8 | 126.3 ± 32.9 |
| Day 21 | 313.9 ± 181.3 | 355 ± 156.1 |
| Day 28 | 518.9 ± 142.2 | 731.1 ± 376.9 |

**Table 4** Repeated measures ANOVA of tumor volume between the control and 20 mA groups

| Source | III Sum of squares | df | Mean square | *F* value | *P value* |
|---|---|---|---|---|---|
| Intercept | 2,959,926.353 | 1 | 2,959,926.353 | 68.875 | 0.000 |
| Time | 2,701,593.604 | 4 | 675,398.401 | 36.280 | 0.000 |
| CI | 45,788.995 | 1 | 45,788.995 | 1.065 | 0.329 |
| CI*time | 85,623.357 | 4 | 21,405.839 | 1.150 | 0.349 |
| Deviation | 386,776.823 | 9 | 42,975.203 | | |

**Table 5** Comparision of the IHC expression rate between stimulated and control group

| Group | VEGF | CD34 | Caspase-3 | Ki-67 |
|---|---|---|---|---|
| Stimulated | 43.3% | 15% | 66% | 82% |
| Control | 40% | 11.7% | 70% | 75% |
| *P* value | 0.635 | 0.581 | 0.677 | 0.284 |

the absorbance values of the four groups. As shown in Table 2, the repeated measures ANOVA OD values of the four groups increased across time, and there were significant differences among those values from the four time points ($F = 650.226$, $P = 0.000 < 0.05$). There was no interaction between the absorbance and time ($F = 0.492$, $P = 0.866 > 0.05$) in the four groups and no significant difference in OD values among the four groups ($F = 2.478$, $P = 0.136 > 0.05$). Figure 3 shows the proliferation curve of the cervical cancer SiHa cells of the four groups after stimulations, with no significant differences among them.

The 24-h SiHa cell migrations of both the control and experimental groups under different electrical stimulations were compared with the values of the control group, 20 mA group, 40 mA group and 60 mA group being $101.07 \pm 10.79$, $103.6 \pm 12.79$, $103.33 \pm 13.95$ and $99.6 \pm 11.23$, respectively, showing no significant differences ($F = 0.364$, $P = 0.779$) using ANOVA. Figure 4 shows the numbers of migrating cells in the four groups.

## TENS did not induce tumor growth or metastasis in a CC mouse model

The experimental results in transplanted SiHa cell tumors (TSHCT) in nude mice are shown in Fig. 5, demonstrating that the tumor growth states of the 20 mA group and control group were the same. ANOVAs of the weekly repeated measurements were used to analyze the tumor size of the two groups. Table 3 shows the tumor volumes in nude mice after

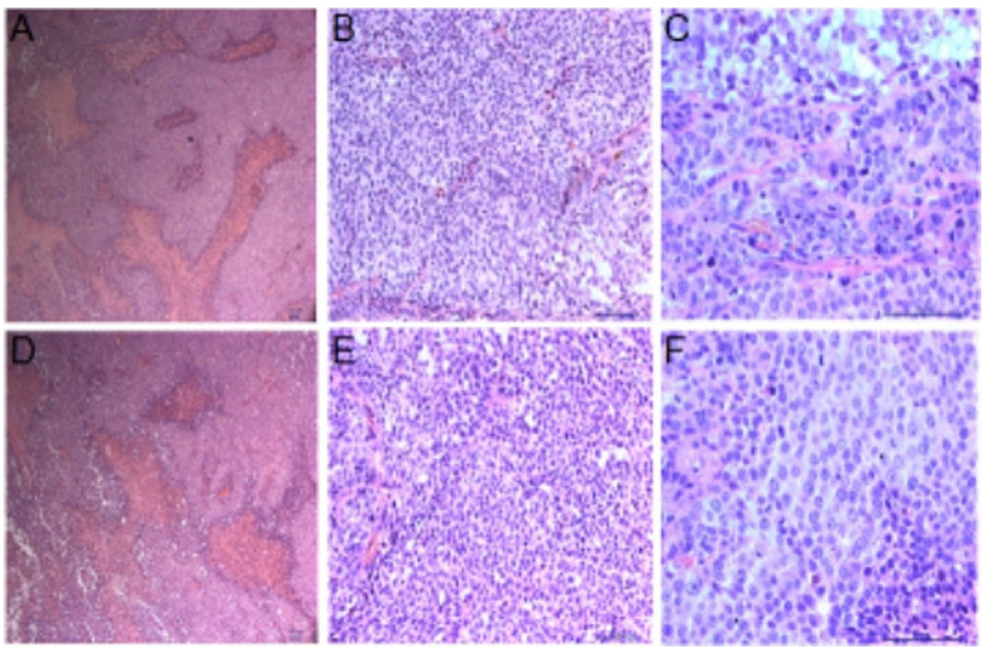


**Fig. 6** H&E staining of transplanted SiHa cells on nude mice. **a** Control group (40×). **b** Control group (100×). **c** Control group (200×). **d** Stimulated group (40×). **e** Stimulated group (100×). **f** Stimulated group (200×)

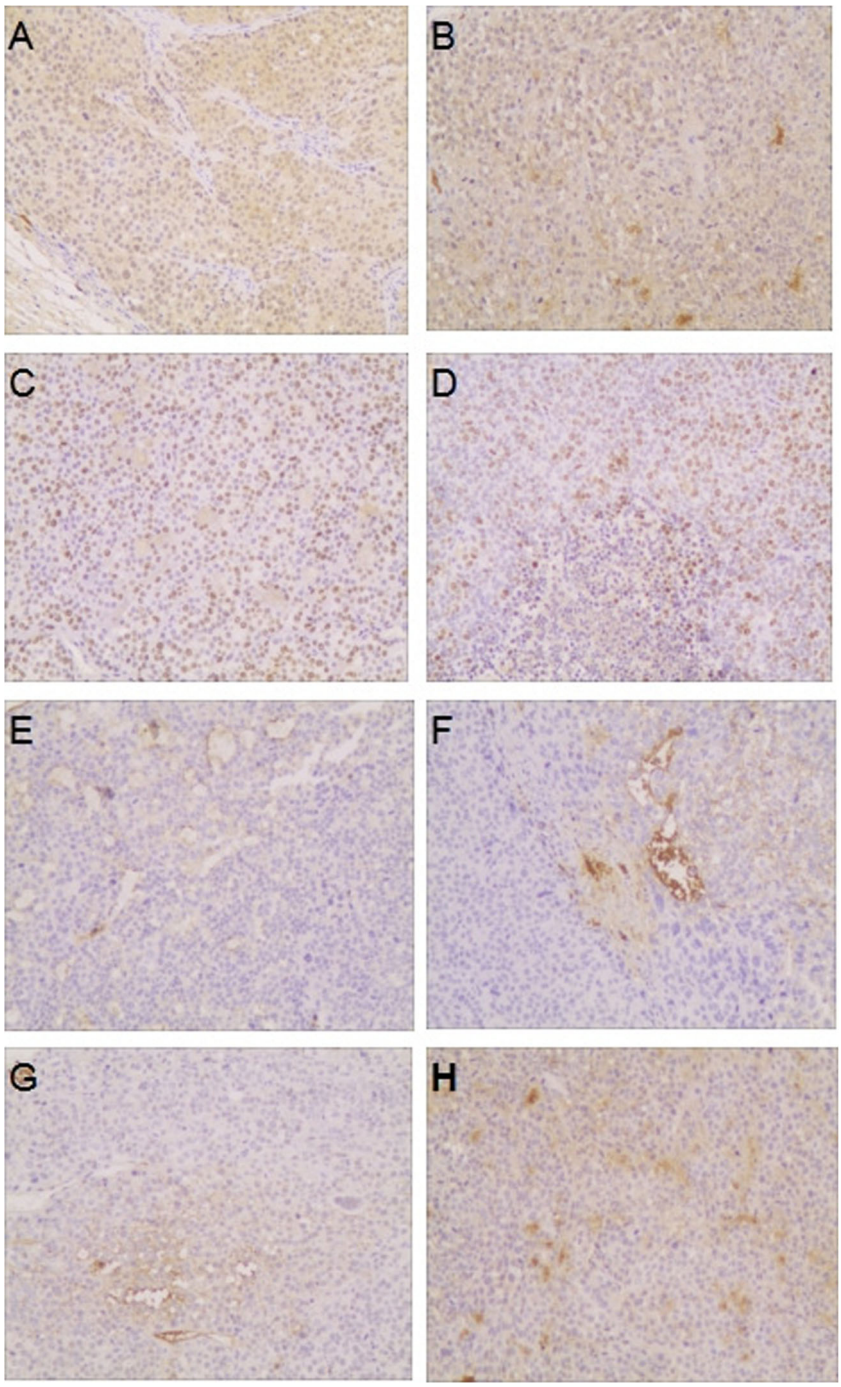


**Fig. 7** IHC staining of transplanted SiHa cells on nude mice (100×). **a** Control group caspase-3 staining. **b** Stimulated group caspase-3 staining. **c** Control group Ki-67 staining. **d** Stimulated group Ki-67 staining. **e** Control group CD34 staining. **f** Stimulated group CD34 staining. **g** Control group VEGF staining. **h** Stimulated group VEGF staining

stimulation for the five episodes (mean ± standard deviation) in which the tumor volumes of the two groups increased ($F = 68.875$, $P = 0.000 < 0.05$), but there was no relation between the tumor sizes and time ($F = 1.150$, $P = 0.349 > 0.05$) as well as the tumor sizes between the two groups ($F = 1.065$, $P = 0.329 > 0.05$) (Table 4). Figure 5d shows the tumor growth conditions of the two groups. When comparing the weight of tumors in nude mice, we found that the tumors of the 20 mA group were weighted at 1.26 ± 0.59 g on the 28th day after stimulation, while those of the control groups weighed 0.98 ± 0.5 g, with a t value of −0.875 and $P$ value of 0.41 > 0.05, showing no significant difference between the two groups.

## PEF had no effect on angiogenesis and tumor growth as indicated by VEGF, CD34 and Ki-67, nor did it affect tumor apoptosis

IHC (immunohistochemical) staining on the specimens showed that the stimulated and control groups were both

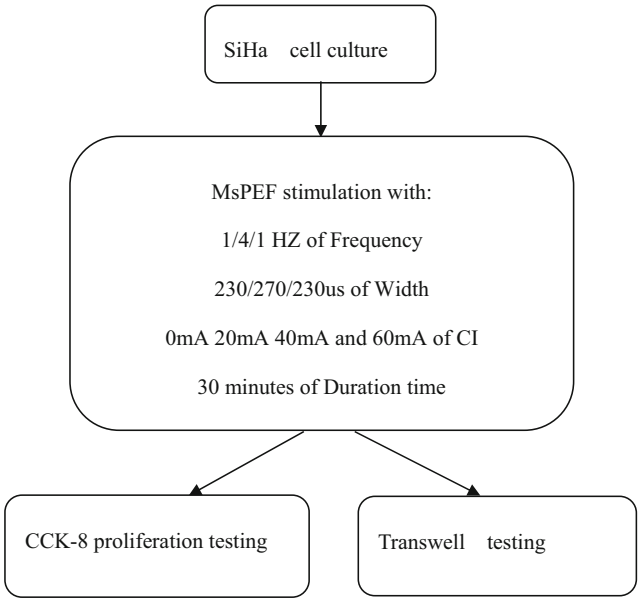


**Fig. 8** Flowchart for the in vitro study

positive for vascular antibody VEGF cytoplasm in the transplanted SiHa cell tumors on mice; the two groups were also positive for CD34, apoptosis of cytoplasmic caspase-3 antibody and proliferation antibody Ki-67. The positive rates of these indicators in the stimulated and control groups were 43.3% vs. 40%, 15% vs. 11.7%, 66% vs. 70% and 82% vs. 75%, respectively. However, there was no significant difference between the two groups for any of those indicators ($P = 0.635$, $P = 0.581$, $P = 0.677$ and $P = 0.284$, respectively) (Table 5 and Fig. 6). H&E stains confirmed the diagnosis of cervical squamous carcinoma cells (Figs. 7, 8, and 9).

## Discussion

The decreased life quality of cervical cancer patients after standard surgery has drawn extensive attention. Among the several treatment methods, TENS seems to be the most effective. But is it safe for cervical cancer patients? Would it induce recurrence or metastasis of the cancer? It has been proved that TENS can trigger neuromuscular electrophysiologic changes, stimulate the neuromuscular junction, improve nerve conduction and enliven nerve fibers, but no study of its effect on cervical cancer cells could be found. To investigate the safety of TENS for cervical cancer patients, we conducted this in vivo and in vitro study.

In the in vitro experiment, we treated SiHa cervical cancer cells with low-frequency electrical stimulation, which simulated the pulse width, frequency and intensity that are used in clinics to treat LUTS patients. The device creating low-frequency electrical stimulation was designed and kindly provided by Jue Zhang, a professor at the College of Engineering, Academy for Advanced Interdisciplinary Studies, Peking University. Our CCK-8 proliferation test showed the same trend of proliferation in the control and three experimental groups. The fact that there was no significant difference in cellular absorbance at different time points indicated that the low-frequency electrical stimulation had no impact on SiHa cervical cancer cell proliferation. In brief, low-frequency electrical stimulation neither had an effect on cell proliferation nor changed the migration of cervical cancer SiHa cells.

According to the literature, different frequencies of electrical stimulation may have different effects on tumors. Weaver [16] studied the mechanism and found that when receiving an electrical pulse with an intensity of KV/cm and a duration of seconds to milliseconds, the cell membranes generated reversible perforations, which largely increased the permeability and improved the absorption of drugs, proteins and other macromolecules. When strengthening the electrical pulse, the cell membrane sustains irreversible perforation, which can lead to cell death [17]. If the pulse electrical field (PEF) created by electrical stimulation was large enough, there would be rupture and cell death, which is irreversible electroporation [16, 18–23]. Both reversible and irreversible electroporations have already been applied to biomedicine and oncologic treatments [21, 24, 25]. Wu et al. [26] conducted in vivo research on breast cancer and found that nsPEF could induce tumor apoptosis and inhibit tumor growth. Chen et al. [27] demonstrated that microsecond electrical stimulation could lead to liver cancer cell apoptosis and necrosis. The electrical stimulation we studied was a low-frequency microsecond pulse with the parameters being as follows: 1/4/1 Hz frequency, 230/270/230 μs pulse width, 20 mA currency and 1100 pulses. The PEFs created by such parameters belong to msPEF. They are not strong enough to induce cell membrane rupture and cell death like nsPEF and do not enhance cell proliferation and migration, indicating that they may not be related to the recurrence or metastasis of cervical cancer.

We further performed an in vivo experiment to verify the hypothesis. First, we established a mouse model by injecting SiHa cells subcutaneously and then gave TENS to mice in the study group by using the device we used in the clinic with exactly the same parameters as for treating LUTS patients. The growth of subcutaneous grafts, vascular expression, proliferation and apoptosis antigen expression were compared between the two groups after 28 days of stimulations. The results showed that the tumor growth of the two groups was

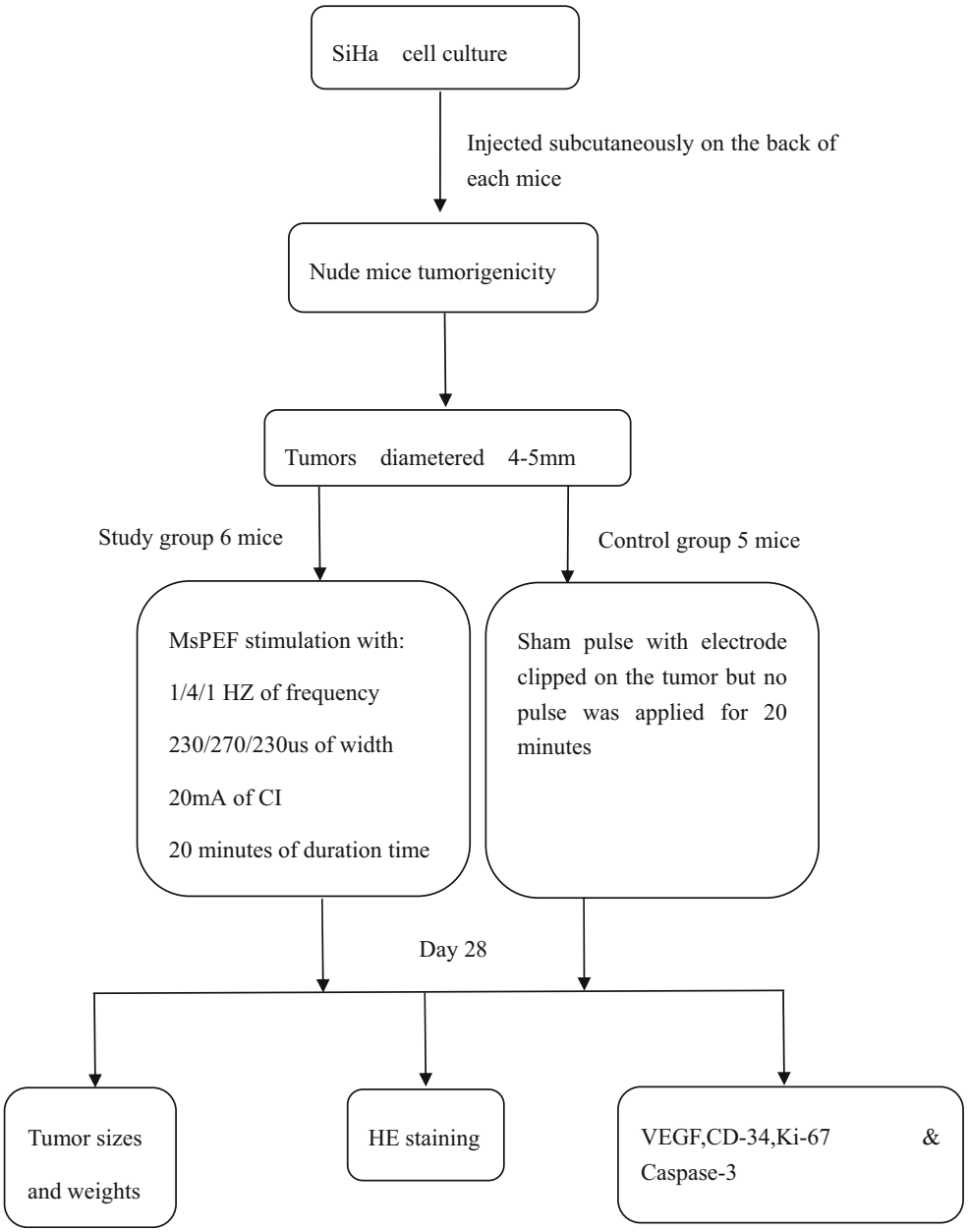


**Fig. 9** Flowchart for the in vivo study

similar, and the VEGF and CD34 vascular antibody immunohistochemistries were all moderately expressed with no statistically significant difference. Also no significant difference was shown in the Ki-67 immunohistochemical staining between the groups. In conclusion, low-frequency electric stimulation had no effect on cervical cancer tumors; it did not promote tumor growth or lead to cancer metastasis.

To our knowledge, this is the first study to investigate the effect of TENS on cervical cancer. The result has a certain significance for guiding clinical practice, but more accurate research on the safety is still in needed.

A limitation of this study might be the limitation of our study's subject was limited to cells and animals with no focus on human being. Due to the relatively small number of mice, the analyses of the in vivo study results had limitations, but we had the support of pathological data, so the results still could be of certain value.

In summary, low-frequency msPEF used for rehabilitation does not generate changes in the proliferation and invasion of SiHa cells, suggesting that the current clinical application of TENS may not cause recurrence of cervical cancer. However, before being applied to humans, fully informed consent, prognosis and survival follow-up are required.

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## Compliance with ethical standards

**Conflicts of interest** None.

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