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Effects of focused ultrasound on human cervical cancer HeLa cells *in vitro*.

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Keywords: focused ultrasound; cervical cancer; HeLa cell line; *in vitro* effects.

Abstract. Cervical cancer is the fourth most common malignant tumor in women. Many studies have confirmed that early childbirth, prolificacy, HPV infection, and smoking are some risk factors. This article explored the effects of exposing human cervical cancer HeLa cells to different focused ultrasound intensities *in vitro*. The study employed three groups of cells: 1- a high-intensity treated group, 2- a low-intensity treated group, and 3- a control group. Results showed that after 12 hours of focused ultrasound treatment, the growth inhibition rate of the low-intensity group was 55.6% higher than that of the control group, and the growth inhibition rate of the high-intensity group was 41.2% higher than that of the low-intensity group. Therefore, focused ultrasound had a specific inhibitory effect on the growth of HeLa cells, and the higher the intensity of focused ultrasound, the higher the inhibition rate on cancer cells. In addition, the Cycle Threshold (Ct) values of the three groups of cells before treatment were the same, but the Ct values after treatment had changed. The Ct value of the low-intensity group was 18.1% lower than that of the control group, and the Ct value of the high-intensity group was lower than that of the low-intensity group by 27.8%, showing that focused ultrasound can effectively reduce the activity of HeLa cells *in vitro*.

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Efectos del ultrasonido focalizado sobre células HeLa de cáncer cervical humano *in vitro*.

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Palabras clave: ultrasonido focalizado; cáncer de cuello uterino; línea celular HeLa; efectos *in vitro*.

Resumen. El carcinoma de cuello uterino es el cuarto tumor maligno más común en las mujeres. Muchos estudios han verificado que el parto prematuro, la prolificidad, la infección por VPH y fumar son algunos de los factores de riesgo. El propósito de este artículo fue investigar los efectos del tratamiento con diferentes intensidades de ultrasonido focalizado sobre células HeLa de cáncer de cuello uterino humano *in vitro*. Este estudio utilizó tres grupos de células HeLa: 1- un grupo de tratamiento con alta intensidad, 2- un grupo de tratamiento con baja intensidad y 3- un grupo control. Los resultados mostraron que después de 12 horas de tratamiento con ultrasonido focalizado, la tasa de inhibición del crecimiento del grupo de baja intensidad fue 55,6% más elevada que la del grupo control y la tasa de inhibición del crecimiento del grupo de alta intensidad fue 41,2% más elevada que la del grupo de baja intensidad. Por lo tanto, el ultrasonido focalizado tiene un efecto inhibitorio sobre el crecimiento de células HeLa, y cuanto mayor sea la intensidad del ultrasonido focalizado, más elevada será la tasa de inhibición de las células cancerosas. Además, los valores del Umbral de Ciclos [Cycle Threshold (Ct)] de los tres grupos de células eran los mismos antes del tratamiento, pero estos valores tuvieron cambios evidentes después del tratamiento. El valor del Ct del grupo de baja intensidad fue 18,1% inferior al del grupo de control y el valor del Ct del grupo de alta intensidad fue 27,8% más bajo que el del grupo de baja intensidad; lo que demuestra que el ultrasonido focalizado puede reducir la actividad de las células HeLa *in vitro*.

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INTRODUCTION

Cervical cancer is the most common malignant tumor of the reproductive organs in women. Based on the statistics, more than 500,000 new cervical cancer cases occur yearly; about 5% of all new cancer patients and more than 80% live in developing countries [1]. Many studies have confirmed that sexual disorders, oral contraceptives, bisphenol A, early sexual life, nutritional factors, premature birth, fertility, human papillomavirus (HPV) infection, and smoking contribute to the pathogenesis [2,3]. Zhang *et al*. investigated the relationship between HPV16 E6 and E7 protein expression and telomerase in cervical cancer carcinogenesis. The results showed that these proteins and telomerase increase gradually with the progression of cervical cancer [4]. Sufficient and improved screening programs to detect this kind of cancer increase the knowledge about its relation with HPV, reducing disease cases in developed countries [5].

Patients may have no apparent symptoms in the early stage of this disease. With

the disease progression, vaginal bleeding and vaginal drainage, and even systemic failure signs like anemia and cachexia in the later stages may appear [6], which seriously threaten the lives and health of most women.

The common symptoms of cervical cancer are vaginal bleeding, vaginal discharge, frequent urination, urgency, constipation, swelling of the lower limbs, and abdominal pain caused by the involvement of adjacent tissues, organs, and nerves. In recent decades, due to the wide application of cervical cytology screening technology, early diagnosis and treatment of cervical cancer and precancerous lesions have become the main reason for reducing morbidity and mortality, thereby improving patient survival. A study investigated the positive effect of using WhatsApp (the internet messaging app) on the health promotion of older women's behavior for early detection of cervical cancer through the visual acetic acid examination [7].

Focused ultrasound technology is a high-tech that has gradually matured and developed in recent years. Its basic principle is to use ultrasound to have good permeability inside the tissue and focus on identification. Through the computer control system, the thermal effect, cavitation effect, and mechanical effect eventually make the target tumor cell degeneration and necrosis to achieve the purpose of treatment[3]tissue disintegration is also possible because of the interaction between the distorted HIFU bursts and either bubble cloud or boiling bubble. Hydrodynamic cavitation is another type of cavitation and has been employed widely in industry, but its role in mechanical erosion to tissue is not clearly known. In this study, the bubble dynamics immediately after the termination of HIFU exposure in the transparent gel phantom was captured by high-speed photography, from which the bubble displacement towards the transducer and the changes of bubble size was quantitatively determined. The characteristics of hydrodynamic cavitation due to the release of the acoustic radiation force and relaxation of compressed surrounding medium were found to associate with the number of pulses delivered and HIFU parameters (i.e. pulse duration and pulse repetition frequency. In recent decades, because focused ultrasound has the advantages of non-invasiveness, quick recovery after surgery, and less pain for patients, it has been increasingly used to treat various solid tumors, such as cervical cancer, uterine fibroids, and pancreatic cancer [8]. Many clinical studies have confirmed that focused ultrasound can play a better role in treating cervical cancer [9,10].

Imankulov *et al*. evaluated the feasibility of using high-intensity focused ultrasound (HIFU) to treat tumors and proved that high-intensity focused ultrasound can effectively inhibit the growth of various tumor cells [11]. Hong *et al*. discussed the effect of HIFU irradiation on the apoptosis-related genes of human pancreatic cancer xenograft tumors. By establishing a nude mouse-human pancreatic cancer YY-1 cell xenograft model and HIFU irradiation, the original TUNEL labeling method was used to detect the apoptosis rate of tumor cells, and it was found that the tumor cell apoptosis rate in the irradiated group was higher [12]. Yuan *et al.* reported that the mechanism by which HIFU enhances anti-tumor immunity has not been well elucidated, and there is emerging evidence that miRNA plays an essential role in the immune response [13].

Focused ultrasound for the treatment of cervical cancer uses the directionality of ultrasound, tissue penetration, and focusing and uses special focusing equipment to focus ultrasound from the outside of the body to the selected treatment area in the body[14]. In some studies, focused ultrasound treatment was recorded, and the average number of treatments was eight times. Most patients completed the follow-up. Among patients who completed follow-up, survival rate statistics were conducted every six months for the 24 months after treatment. The survival rate in the 6th month was 100.00%; the survival rate at the 12th month was 75.93%; the survival rate at the 18th month was 66.67%; the

survival rate at the 24th month was 55.56%. These results show that focused ultrasound can kill cancer tissues in a targeted manner while preserving most normal tissues [15].

This article aimed to analyze the effect of focused ultrasound on human cervical cancer HeLa cells *in vitro*.

MATERIALS AND METHODS

Preparation of Experimental Materials

A medical university's ultrasound engineering institute provided human cervical cancer HeLa cells. The cultured HeLa cells were randomly divided into control, low-intensity, and high-intensity irradiation groups. The control group was treated with conventional interferon in vitro, the low-intensity group was treated with focused ultrasound, and the high-intensity group was treated with an intensity-focused ultrasound instrument (Table 1).

The conventional cell culture method

The cryopreserved human cervical cancer HeLa cells were recovered in a culture bottle containing 10% fetal bovine serum RPM H-1640 nutrient solution, and a single-cell suspension was prepared. Cell growth inhibition was determined by MTT colorimetry (tetrazolium dye colorimetric assay).

The density of human cervical cancer HeLa cells was adjusted to $3x10^3$ cells/mL. The MTT method was used to determine the concentration, and the inhibition rate (R) was calculated as follows:

Inhibition rate (R) = (1-experimental group A value, control group A value) × 100%

The experiment was repeated three times with the same method.

Focused ultrasonic field treatment

Logarithmically grown cells were taken and digested with 0.25% trypsin. They were centrifuged and washed with PBS solution 2~3 times. The cell density was adjusted to 2 x 10^4 cells/mL. The experimental group was divided into low-dose and high-dose groups according to the different intensities of focused ultrasound (0, 1000 and 3000 Watts/cm^2). A volume of 500 μL was taken out of cell suspension from each group and placed in the smallest electric chamber. The fixed electric field intensity was 250 kV/cm, the repetition frequency was 3 Hz, and the pulse width was 800 ps. The control group did not receive focused ultrasound treatment.

Observing the cell morphology under an optical microscope

The cell density was adjusted to $3x10^4$ cells/mL and inoculated into a 24-well culture plate with a preset cover glass. Each well was placed in an incubator for 1ml culture, and the medium changed in the logarithmic growth phase. After 72 hours, the coverslip was removed and stained, and the cell morphology was observed under an optical microscope.

Observing the cell morphology by transmission electron microscopy (TEM)

The cells were inoculated in a culture flask according to the above density. They were collected, fixed before washing through 4% glutaraldehyde, fixed after washing with 1% acid, periodically dehydrated, and then soaked with epoxy resin. They were then embedded to make a fat mass and cut into ultra-thin sections. The cell morphology was observed with TEM.

Table 1
The condition and treatment of Hela cells in different groups.

Serial number	Number of cells	Passages	Treatment
Control group	1.83×10^4	5	Alpha interferon
Low-intensity group	1.75×10^4	5	Low-intensity Focused ultrasound
High-intensity group	1.82×10^4	5	High-intensity focused ultrasound

Detecting cell apoptosis by the TUNEL method

Cell slides were obtained according to the method of observing cell morphology under an optical microscope and cultured in an incubator. After the logarithmic growth phase, the medium was changed, and drugs were added, according to the TUNEL method, which was utilized for detecting apoptotic DNA fragmentation and identifying and quantifying apoptotic cells.

Western blot analysis of BAX and Bcl-2 protein expression

After cell treatment, each group of cells was incubated for 12 hours, suspended in PBS, and counted. The $1x10^6$ cells were pelleted by centrifugation, lysed with 200 mL PMSF cell lysis buffer for one hour, and then centrifuged at 12000 g at 4 °C for 10 minutes. Bcl-2 and BAX primary antibodies were added overnight at 4 °C. The samples were washed three times at room temperature in TBST for 10 minutes each time. Quantity One ® 1-D image analysis software (Bio-Rad) was used to analyze the results. The absorbance of the blot was equal to the average absorbance × area. The ratio of BAX / Bcl-2 was equal to the absorbance of the BAX band/Bcl-2 band.

Immunohistochemical staining (SP method) was performed, and the steps of the SP kit instructions were followed. PBS was used instead of the primary antibody as a negative control, and Fuzhou Maxim Biological Company provided photos of the positive pair.

Real-time Quantitative Polymerase Chain Reaction (rtPCR) and Cycle Threshold (Ct) Determination

Cycle Threshold (Ct) values were determined using the real-time quantitative polymerase chain reaction (rtPCR) method. Total RNA was extracted from the control, low-intensity, and high-intensity HeLa cell samples before and after treatment using the TRIzol reagent. The quality and concentration of the extracted RNA were measured using a NanoDrop spectrophotometer.

The extracted RNA was then reverse-transcribed into cDNA using a reverse transcription kit. The cDNA was used as the template for real-time PCR amplification. The reaction mixture included SYBR Green PCR Master Mix, forward and reverse primers specific to the gene of interest, and the cDNA template.

The cDNA was amplified by real-time PCR using SYBR Green PCR Master Mix on an Applied Biosystems 7500 Fast Real-Time PCR system. The amplification conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 1 min, and extension at 72 °C for 30 sec.

The Ct value indicates the number of cycles required for the fluorescence signal to surpass the threshold level, which is inversely proportional to the amount of target nucleic acid in the sample. Lower Ct values correspond to higher levels of target nucleic acid.

The Primer Premier software designed PCR primers specific for the reference gene GAPDH, and the target gene was used. Relative expression levels of the gene of interest were calculated using the 2-ΔΔCT method, with GAPDH as the internal control. Fold changes in gene expression were analyzed using the 2-ΔΔCt method, and qRT-PCR was performed in triplicate for each sample. The Ct values of the control, low-intensity, and high-intensity groups before and after treatment were compared to evaluate the effect of various treatments on HeLa cell activity. Lower Ct values were indicative of higher gene expression and cell activity.

Statistical processing

The IBM SPSS 18.0® statistical analysis software was used for statistical processing. All experiments were performed at least three times. MTT results were analyzed for the variance of repeated measurement data, and laser scanning confocal and Western blot detection was performed using the ANOVA test. The difference was statistically significant with $p<0.05$.

RESULTS

Comparison of the mortality of each group of cells after focused ultrasound treatment

The mortality of each group of cells was measured using focused ultrasound treatment of human cervical cancer HeLa cells at three h, six h, 12 h, 24 h, and 48 h. The mortality of cells in each group at 48 h showed an upward trend with time, but the rising speed of each group was different. It can be seen that there is a difference between the death rate of human cervical cancer HeLa cells and the intensity of focused ultrasound have a proportional relationship. In addition, the cell death rate of HeLa cells in the same treatment group reached a peak 12 hours after treatment, which was significantly higher than other time points ($P<0.01$) (Table 2).

Comparison of the growth inhibition rate of focused ultrasound

The growth inhibition of human cervical cancer HeLa cells by focused ultrasound after different times was statistically analyzed. It can be seen from the data in Fig. 1 that focused ultrasound has a specific inhibitory effect on the growth of HeLa cells, and the higher the intensity of focused ultrasound, the higher the inhibition rate on cancer cells. After 12 hours of focused ultrasound treatment, the growth inhibition rate of the low-intensity group was 55.6% higher than that of the control group, the growth inhibition rate of the high-intensity group was 41.2% higher than that of the low-intensity group, and 72 hours after the focused ultrasound treatment, the growth inhibition of the low-intensity group was 55.6% higher than that of the control group. The growth inhibition rate of the high-intensity group was 11.1% higher than that of the low-intensity group.

Western blot detection of BAX and Bcl-2 expression

It can be seen from the data in Fig. 2 that the expression of BAX protein increased significantly after focused ultrasound treatment. The expression of BAX in the low-intensity group was 13.6% higher than that in the control group, and the expression of BAX in the high-intensity group was 2.1 higher than that in the low-intensity group. The expression of Bcl-2 protein decreased after focused ultrasound treatment. The expression of Bcl-2 in the low-intensity group was 15.9% lower than that in the control group, and the expression of Bcl-2 in the high-intensity group was 51.7% lower than that of the low-intensity group.

Changes in Ct values before and after treatment of HeLa cells

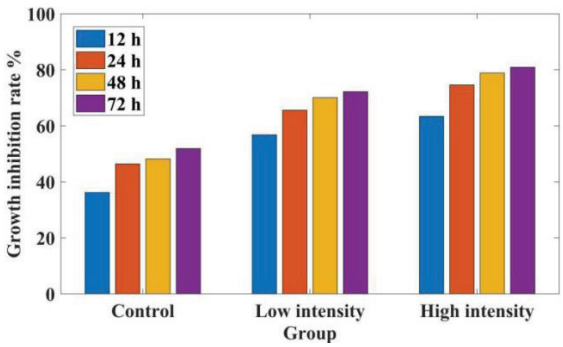

Fig. 1. Comparison of the growth inhibition rate of focused ultrasound on human cervical cancer HeLa cells after different times.

Table 2
The percentage of mortality of different groups of cells at different time points.

Group	3 h	6 h	12 h	24 h	48 h
Control group	1.05±1.25	2.57±0.98	4.67±1.28	5.60±1.22	7.05±3.44
Low-intensity group	35.94±1.68	71.23±0.99	74.22±0.87	57.56±0.99	74.88±1.96
High-intensity group	56.94±3.68	83.24±0.25	85.23±0.45	85.68±2.97	85.88±1.01

Values are mean ± standard deviation.

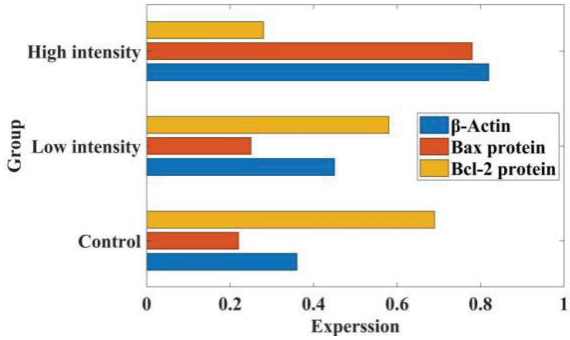

Fig. 2. Comparison of the results of Western blot detection of BAX and Bcl-2 expression.

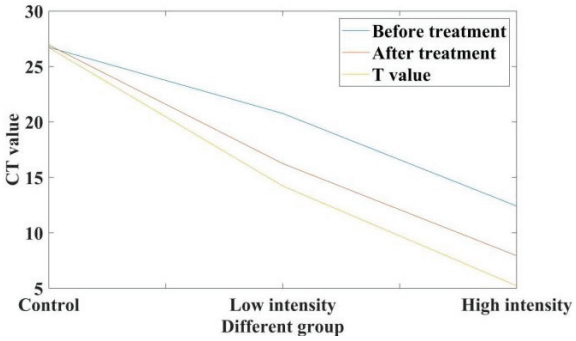

Fig. 3. Changes of Ct values before and after treatment of HeLa cells.

This study recorded the Ct value changes of three groups of human HeLa cells before and after treatment. It can be seen from the data in Fig. 3 that the Ct values of the three groups of HeLa cells before treatment are the same, but the Ct values after treatment have apparent changes. After treatment, the Ct value of the low-intensity group is 18.1% lower than that of the control group. The Ct value of the intensity group is 27.8% lower than that of the low-intensity group, which shows that focused ultrasound can effectively reduce the activity of HeLa cells.

DISCUSSION

This study analyzed the effect of focused ultrasound on human cervical cancer HeLa cells *in vitro,* and it showed that. This study showed that HIFU could be used to treat cervical cancer. Specifically, the following three points: first, this article uses a controlled experiment to compare the *in vitro* effects of low-intensity and high-intensity focused ultrasound. It accurately reflects the inhibitory effect of focused ultrasound on HeLa cells. Secondly, this article uses Western blot to detect the expression of BAX and Bcl-2 proteins, which can accurately reflect the changes in protein expression in HeLa cells and the tumor suppressor effect of focused ultrasound at the protein level. Thirdly, MTT results for repeated measurement data analysis of variance, laser scanning confocal, and Western blot detection using ANOVA test to ensure the experimental results are rigorous and credible.

The research results show that focused ultrasound has a particular inhibitory effect on the growth of human HeLa cells, and the higher the intensity of focused ultrasound, the higher the inhibition rate on cancer cells. After 12 hours of focused ultrasound treatment, the growth inhibition rate of the low-intensity group was 55.6% higher than that of the control group, and the growth inhibition rate of the high-intensity group was 41.2% higher than that of the low-intensity group. A study found that HIFU combined with cisplatin facilitates tumor volume reduction and could be beneficial in treating patients with cervical cancer [16]. In addition, Abel *et al*. reported that HIFU is a potentially safe way to treat cervical cancer [17].

Bcl-2 is a member of the apoptotic gene family. It is known that Bcl-2 is expressed in solid tumors and is mainly located in the cytoplasm and nucleus. Scientists speculate that this may be related to the formation of certain malignant tumors [18]. In the related studies of cervical cancer, although the expression of Bcl-2 has no apparent relationship with tumor histological type, tumor stage, or lymph node metastasis, the five-year survival rate of Bcl-2-positive patients is significantly higher than in BAX-positive patients [19].

In addition, BAX protein expression was significantly increased after focused ultrasound treatment. The expression of BAX in the low-intensity group was 13.6% higher

than that in the control group, and the expression of BAX in the high-intensity group was 2.1 times higher than that in the low-intensity group, while the Bcl-2 protein after focused ultrasound treatment, the expression level decreased. The expression level of BAX in the low-intensity group was 15.9% lower than that of the control group, and the expression level of BAX in the high-intensity group was 51.7% lower than that of the low-intensity group.

According to the study results, the Ct values of the three groups of HeLa cells before treatment were the same, but the Ct values after treatment had changed. After treatment, the Ct values of the low-intensity group were 18.1% lower than those of the control group, and the Ct value of the high-intensity group was 27.8% lower than the low-intensity group, showing that focused ultrasound can effectively reduce the activity of HeLa cells. In addition, focused ultrasound has the highest proportion in treating pancreatic cancer, reaching 51.4%, followed by bone tumors, accounting for 21.1%, while cervical cancer only accounts for 3% (Fig. 4) [20,21]. It can be seen that focused ultrasound has a more favorable prospect for treating cervical cancer.

In summary, this article proposed focused ultrasound as a new treatment method for cervical cancer. This study briefly introduced focused ultrasound as a treatment method, studied the in vitro effects of focused ultrasound on HeLa cells, and analyzed the inhibitory effect of focused ultrasound on cancer cells. The research results show that focused ultrasound has a specific inhibitory effect on the growth of HeLa cells, and the higher the intensity of focused ultrasound, the higher the inhibition rate on cancer cells.

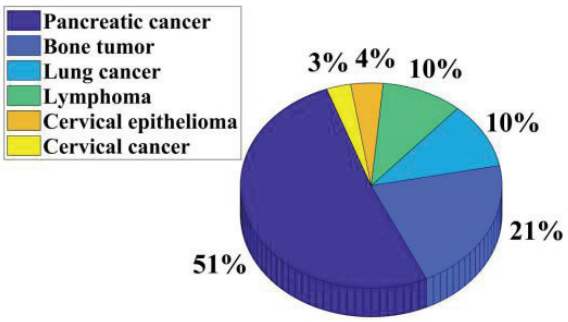

Fig. 4. Application of focused ultrasound in the treatment of solid tumors and its proportion.

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Conflict of interests

All of the authors had no personal, financial, commercial, or academic conflicts of interest separately.

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Authors' Contribution

YLiu: Manuscript editing, revising and final approval of manuscript. QZ: Manuscript editing, revising and final approval of manu-

script. PL: Administrative support. YLi: Conception and design, Collection and assembly of data. LY: Provision of study materials or patients. HY: Data analysis and interpretation.

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