An Effective Inhibitory Strategy of Low Steady Magnetic Field on Ovarian Cancer

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Abstract

To estimate the effect of a steady-state magnetic field (SMF) with low magnetic intensity gradient on the apoptosis-promoting factors related to cancer cells, we systematically select SMF with 0.2T, 0.4T and 0.6T to study their effect on different ovarian cancer lines. An *in vitro* cell model system about two kinds of ovarian cancer lines is established, whose viability and intracellular factors are detected by CCK-8, confocal microscopy and flow cytometry method. The results demonstrate that the apoptosis rate of ovarian cancer cells is increased with the enhancement of SMF magnetic intensity. Furthermore, we detect an increasing ROS and intracellular Ca^{2+} levels in ovarian cancer cells, which can be caused by SMF. The results suggest that ROS and Ca^{2+} levels are the main reason for the significant apoptosis of ovarian cancer cell lines in SMF. Moreover, an *in vivo* experiment also reveals that SMF has a strong inhibitory effect on ovarian cancer. Therefore, the inhibitory strategy is an effective, which has a great potential in the treatment of drug-resistant ovarian cancer.

Introduction

The use of magnetic therapy has been controversial for thousands of years[1]. Although its physiotherapy principles still lack sufficient theoretical support and are not accepted by mainstream medicine, there are many people voluntarily using magnetic therapy as an alternative and complementary therapy to prevent and treat some chronic diseases[2–4]. Specially, it is well known that steady-state magnetic field (SMF) is widely used in imaging medicine[5–6]. In recent decades, many scholars have studied the effects of SMF on cells including their types and the related parameters in SMF. For example, it has been proved that a negative inhibition on the proliferation of cancer cells and no or a positive effect on normal cell proliferation, and a effect of N-pole and no obvious effect of S-pole[7–12].

Based on previous reports, the magnetic intensity is a key role on cell proliferation. It has been reported that moderate intensity SMF in the tumor treatment is used as an auxiliary chemotherapy method to improve the effect of tumor chemotherapy drugs[8, 13–23]. However, some studies showed that a strong magnetic field of 10-13T did not affect Chinese hamster ovary cells, while a lower magnetic field of 7T significantly inhibited cell proliferation[13–14]. Moreover, many researchers found that SMF affects the cells, including orientation, proliferation, microtubules, division, actin, adhesion, migration, shape and so on, by some factors of cell cycle related to chromosome and DNA, reactive oxygen species (ROS), adenosine triphosphate (ATP), and intracellular Ca^{2+} concentration, etc[14, 24–33]. Among them, ROS and Ca^{2+} plays an indispensable role in cells, because their intracellular levels determine most of the related factors of apoptosis[34–42]. Thus, these reported results may imply that SMF can differentially affect normal ovarian and ovarian cancer cells through a precise adjusting of ROS and Ca^{2+}, which reveals the potential application of SMF in the treatment of ovarian cancer.

At present experiment, to estimate the effect of SMF with low magnetic intensity on the apoptosis-promoting factors related to cancer cells, we systematically select three kinds of SMF with gradient

intensity to conduct *in vitro* cell experiments on two different types of ovarian cancer cell lines. Furthermore, A2780 cell is selected to establish an *in vivo* tumor model in mice. The result mainly explores the effect of gradient SMF on the proliferation and apoptosis of ovarian cancer cell, and on the intracellular ROS and Ca^{2+} concentration, which shows that SMF inhibits the proliferation and activity of ovarian cancer cells and the intracellular ROS and Ca^{2+} are increased significantly in ovarian cancer. Furthermore, an *in vivo* experiment also reveals that SMF has a strong inhibitory effect on ovarian cancer. Therefore, the inhibitory strategy is an effective, which has a great potential in the treatment of drug-resistant ovarian cancer.

Experimental

Materials

The magnets were purchased from Hangzhou Permanent Magnet Group Co., LTD. Ovarian cancer cells of A2780 and Skov-3 were obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). PBS, FBS, and insulin were purchased from Gibco (Carlsbad, CA). Apoptosis kit was purchased from Shanghai Beyotime Institute of Biotechnology Co., LTD. DAPI staining solution was used for the macroscopic analysis of cell number changes in nucleus staining, and cck-8 assay kit was used to determine the survival rate of cells under variable conditions, both of which were purchased from Shanghai Beyotime Institute of Biotechnology Co., LTD. Fluorescent probe was applied to the analysis of intracellular ROS (DCFH-DA) content and calcium ions (Fluo-3-AM) bought level from American AAT Bioquest Inc.

Magnetic intensity measurement

Three magnets with magnetic intensity of 0.2T, 0.4T and 0.6T were selected for the measurement of magnetic intensity along the distance. The magnetic fluxmeter was set to zero, and then a flat table with no other magnetic field interference was selected. The measured magnet was placed in an appropriate position and measured along the center of the measured surface.

Confocal laser observation

Two types of ovarian cancer cells (A2780 and Skov-3) were revived in the same culture environment for a stable cell growth and proliferation. Afterwards, the four-division dish was introduced separately, and the passage of the cells was strictly counted during passage to ensure that the cell concentration and growth viability of the passage were similar. The experiment was divided into four groups, which were the normal training group (0T, control group), 0.2T, 0.4T and 0.6T of SMF training group (experimental group). 200 μL of 4×10^4/mL cell suspension was cultured under appropriate conditions (37°C, 5% CO_2) for 24 hours, which was placed in a fixed magnetic environment at the surface of magnet. The cells were fixed with 4% paraformaldehyde for 30 minutes in both groups and washed with PBS buffer three times. Then they were stained with DAPI for 15 minutes and washed 3 times with PBS buffer before imaging under a confocal laser microscope with a laser range of 360–480 nm.

Cell viability experiment

A2780 and Skov-3 cells were selected to conduct *in vitro* experiments. Both cells (5,000 per well) were seeded in 96-well plates and incubated for 24 hours at 37°C under 5% CO_2 before the treatment. The control group was cultured under normal conditions, and the experimental group was cultured in 0.2T, 0.4T and 0.6T SMF. CCK-8 was added to each well, and then incubated at 37°C for an additional 4 hours. The amount of cell proliferation was measured by a plate reader at 450 nm. The cell viability was calculated using the following formula: viability (%) = (mean of absorbance value of treatment group/mean absorbance value of control) ×100%. The results are shown as an average of five independent measurements.

Apoptosis experiment

A2780 and Skov-3 cells respectively represented two groups, the same cells to extend equal density. The control group was cultured under normal conditions, and the experimental group was cultured in 0.2T, 0.4T and 0.6T magnetic field. After 24 hours of culture, the cells were harvested for several minutes with trypsin digestion without EDTA for 5 minutes. The cells were harvested and washed three times with PBS, and the cells were re-suspended in buffer to adjust the concentration of the control cell suspension at 10^6-10^7/mL. Afterwards, 10 μL of the apoptosis reagent propidium iodide staining solution and 5 μL of Annexin V staining solution were added and stained for 10 minutes at 4°C in the dark before the flow cytometry test.

ROS and Ca^{2+} measurements

After loading DCFH-DA fluorescent probes for 1 hour, the laser confocal microscopy tested the neutrophils ROS generation in the cell for excitation wavelength of 488 nm. The passage cells were collected separately, and then the probes were loaded. The cells were washed and re-suspended with PBS, whose concentration was adjusted to be consistent, and then the fluorescence intensity was measured by flow cytometry to relatively quantify intracellular ROS.

The Fluo-3 AM fluorescent probe was loaded during 40 minutes for the laser confocal microscopy detection of intracellular Ca^{2+} distribution of excitation wavelength of 488 nm. The passage cells were collected separately, and then the probes were loaded. The cells were washed and re-suspended with HEPES buffer, whose concentration was adjusted to be consistent. The fluorescence intensity was determined by flow cytometry to relatively quantify the intracellular Ca^{2+} level.

Growth inhibition of SMF on tumor in vivo

Animal experiments were approved by local governmental authorities and carried out in compliance with the ARRIVE guidelines. To investigate therapeutic efficacy of SMF *in vivo*, comparative studies of inhibiting ovarian tumor have been performed. 16 female nude mice, supplied by the Department of Experimental Animals, Shanghai Jiaotong University, were divided into four groups (a control group and three experimental groups). A2780 tumor bearing mice were established by subcutaneous injection of

about 1×10^7 (0.2 mL×5×10^7/mL). Four groups including control group and experimental groups were controlled at 0, 0.2T, 0.4T and 0.6T SMF intensity. The tumor volumes were tracked every 2 days using vernier calipers from 1 day, 3 day ... until to 31 day. On the 31th day, all animals were euthanized and the tumors were dissected and weighed. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Shanghai Jiaotong University.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Statistical analysis was conducted using Student's t-test. Differences were considered significant at $P < 0.05$.

Results And Discussion

As shown in Fig. 1a, we design a strategy to investigate the effective inhibitory in SMF with low magnetic intensity, on which the cells and mice are placed on the N-pole. Specially, the cells including A2780 and Skov-3 cells are incubated in a cell culture flask with 1 mm thickness to detect their factors. After the cells are injected into mice subcutaneously, the experimental tumor bearing mice are established. To ensure that the distance between the tumor and magnet surface is less than 1 mm, these mice are fixed on the surface of magnet constantly. Furthermore, a precise surface magnetic intensity is measured. As shown in Fig. 1b, three magnets with surface magnetic intensities of 0.2T, 0.4T and 0.6T are used to evaluate the relationship of their surface magnetic intensity and the distance. Figure 1c shows that the curve shape is approximate inverse function, whose intensity is decreased gradually with the increase of distance away from the magnet surface and to near zero at the distance of 10 cm no matter their initial magnetic field strength. Closer observation finds the surface magnetic intensity falls to 0.185T, 0.392T and 0.587T at the distance of 1 mm for 0.2T, 0.4T and 0.6T magnet, respectively. Based on the result, the controlled distance precisely for *in intro* and *in vivo* model is important, leading to the same actual experimental conditions.

A2780 cells and Skov-3 cells are used to establish an *in vitro* model of ovarian cancer cells in gradient SMF. Figure 2 is the confocal microscope images of the both ovarian cancer lines under gradient SMF about 0.2T, 0.4T and 0.6T. As shown in Fig. 2, there is a big different nucleus number in the same size within the vision field in different magnetic fields. Specially, as seen from these images, the number of two kinds of ovarian cancer cell nucleus is decreased with the increasing of SMF magnetic intensity, which indicates that both ovarian cancer cells are inhibited under SMF magnetic field. Additionally, there is an abrupt inhibition under 0.6T SMF compared to that of 0.2T and 0.4T. The reason could contribute to the appearance of compensatory hyperplasia and the increase of nucleus, which leads to the aging of apoptotic cells. The results demonstrate that SMF has a very good inhibitory effect on ovarian cancer cells, revealing that SMF is a great potential treatment of ovarian cancer.

Furthermore, CCK-8 is performed to evaluate the survival rate of two kinds of ovarian cancer cells under different SMF. As shown in Fig. 3, the magnetic intensity with 0.2T, 0.4T and 0.6T can reduce the survival rate of two kinds of ovarian cancer cells. Among them, the survival rate of A2780 cells Skov-3 cells for 24

hours is decreased by 2.3%, 4.7% and 13.5%, and 2.1%, 4.68% and 10.1%, respectively, for 0.2T, 0.4T and 0.6T SMF. In other words, the inhibition degree of 0.6T SMF is about 2–4 times that of 0.2T and 0.4T SMF, which shows the largest inhibition on the survival rate of A2780 about 13.5%. Compared with Fig. 2, the results mean that high magnetic field strength is sensitive to the inhibitory effect on ovarian cancer cell lines.

To better quantify the inhibitory effect on both ovarian cancer cell lines, we perform the apoptotic rate detection experiments on two ovarian cancer lines under the same conditions. Flow cytometry analysis about two kinds of ovarian cancer cells under different SMF reveal the effect of ovarian cancer cells apoptosis on 0, 0.2T, 0.4T and 0.6T magnetic intensity for 24 hours. As shown in Fig. 4, Q1 is mechanical damaged cell, Q2 is late apoptotic or necrotic cell, Q3 is living cell, and Q4 is early apoptotic cell. Additionally, A2780 cell early apoptosis rates are 7.1%, 10.4%, 10.3% and 9.1%, late apoptosis rates are 5.1%, 11.8%, 16.5% and 25.3%, and the total apoptosis rates are 12.2%, 22.2%, 26.8% and 34.4% under control, 0.2T 0.4T and 0.6T SMF, respectively. Moreover, the early apoptosis rates of Skov-3 cell are 5.8%, 14.2%, 16.5% and 18.6%, the late apoptosis rates are 6.0%, 12.4%, 21.0% and 24.8%, and the total apoptosis rates are 11.8%, 26.6%, 37.5% and 46.1% under control, 0.2T 0.4T and 0.6T SMF, respectively. Therefore, the results prove that the effect of gradient SMF on the apoptosis of ovarian cancer cells in different lines is different, and the apoptosis rate gradually increases with the increasing SMF intensity, showing a magnetic intensity-dependent relationship of ovarian cancer.

It is generally believed that the free radical content of ROS in cells determines the oxidative senescence and death of cells, which is the key factor to promote the process of apoptosis[43–44]. So, we explored the effect of SMF on the total active oxygen content of two ovarian cancer cells within 24 hours. Figure 5a is the result of ROS laser confocal microscopy images with the same inoculation concentration for two ovarian cancer lines. The DCFH-DA fluorescence intensity in a single cell is increased although the number of cells in the same area is decreased because of the external SMF according to Fig. 2. However, the quantitative analysis of the total ROS in the same concentration cells by flow cytometry is conducted, as shown in Fig. 5b. Compared with the control groups, the total ROS in A2780 cells is increased by 24.5%, 69.7% and 135.0%, respectively, and the total ROS in Skov-3 cells is increased by 17.8%, 62.5% and 112.7%, respectively. The result suggests that SMF can stimulate the production of a large number of ROS in ovarian cancer cells to promote apoptosis. The reason may be that ovarian cancer cells causes its own oxidative damage to oxidative stress of SMF, or ROS oxidizes intracellular macromolecules, destroys the normal function of macromolecules, and thus causes damage to cells and even death[45–46].

Ca^{2+} plays an indispensable role in the signaling cascade reaction between cells, and it has been proved that increased intracellular Ca^{2+} level can accelerate the apoptosis process of tumor cells[41–42]. Therefore, the influence of intracellular Ca^{2+} level on two kinds of ovarian cancer lines under SMF of 0, 0.2T, 0.4T, and 0.6T for 24 hours. As shown in Fig. 6a, the laser confocal microscope shows that the number of cells in the same area is decreased and the Fluo-3 fluorescence intensity in a single cell gradually is strengthened, with the increase of SMF magnetic intensity, in both two kinds of ovarian cancer cells,

Furthermore, the total intracellular Ca^{2+} is quantitatively analyzed by flow cytometry in Fig. 6b. Compared with the control group, the intracellular Ca^{2+} level of A2780 under 0.2T, 0.4T, and 0.6T SMF is increased by 8.7%, 27.5% and 68.8%, respectively, and that of Skov-3 increases by 12.6%, 37.5% and 82.7%, respectively. The result suggests that gradient homeostasis magnetic field can stimulate Ca^{2+} influx in ovarian cancer cells or change signaling factors between ovarian cancer cells to promote apoptosis of ovarian cancer cells[47]. Thus, the intracellular Ca^{2+} level of ovarian cancer is a magnetic intensity-dependent.

As we well known, several references have reported that cancer cells may evade apoptosis through decreasing calcium influx into the cytoplasm[47]. The reasons can be mainly shown as following: on the hand, it can be achieved either by the downregulation of plasma membrane Ca^{2+}-permeable ion channels or by the reduction of the signaling pathways to activate Ca^{2+}-permeable ion channels[48,49]. On the other hand, the defensive mechanism against apoptosis would involve the cancer cell adaptation for the reducing basal [Ca^{2+}]-endoplasmic reticulum (ER) without the induction of pro-apoptotic ER stress response that usually accompanies ER luminal calcium imbalance[50]. Therefore, in the present experiment, based on the inhibition of SMF on two kinds of ovarian cancer cells and the detection of apoptosis-related factors (ROS and Ca^{2+}) in cancer cells, we suggests that the intracellular Ca^{2+} concentration is increased stimulated by SMF, which activates the Ca^{2+}-dependent nuclear factors of activated T cells (NFAT) protein through ER luminal calcium imbalance. The results could activate Ca^{2+}-permeable ion channels. Furthermore, the activated Ca^{2+}-dependent NFAT protein can stimulate the mitochondria production of a large number of ROS in ovarian cancer cells that oxidize the key cysteine residues of transient receptor potential A1 (TRPA1) to activate proteins to promote apoptosis. Therefore, as shown in Fig. 7, ROS can synergize with Ca^{2+} to promote apoptosis in cancer cell[51].

Furthermore, the results of *in vivo* nude mice experiments on A2780 ovarian cancer further confirm the above results. As shown in Fig. 8, different results in different treatment groups indicate that the low SMF magnetic intensity can effectively inhibit the growth and proliferation of ovarian cancer cells. It is obvious that the size of the tumor in the control group is increased significantly during the experiment. Compared with the control group, three SMF treatment groups show significant inhibitory effect on ovarian cancer of A2780 line. Among them, 0.6T SMF treatment group has the most obvious effect. As shown in Fig. 8b, the mean tumor volume of 0.6T SMF group increases most slowly among the four groups, which shows that 0.6T SMF have considerable growth inhibitory effect on tumors. Furthermore, on the 31 day, mice are sacrificed and tumors are excised for weight. The tumor photograph and their volume and weight in each group after the treatment are shown in Figs. 8a, 8b and 8c. 0.6T SMF group also shows a higher inhibition activity than that of control, 0.2T and 0.4T SMF groups. The mean tumor weight in 0.6T SMF group (0.186 ± 0.073g) is smaller than that of control group (1.170 ± 0.109g, $P < 0.05$), 0.2T SMF group (0.689 ± 0.098g, $P < 0.05$) and 0.4T SMF (0.278 ± 0.018g, $P < 0.05$). The results indicate that the treatment with SMF displays a significant antitumor performance. Thus, the above results reveal that SMF are a powerful candidate for combining therapy of cancer *in vivo*.

Conclusions

In our study, an effective inhibitory strategy of SMF on ovarian cancer is reported. A low magnetic intensity of 0.2T, 0.4T and 0.6T are selected to evaluate the cell viability and tumor treatment. *In vitro* cell experiment proves that the apoptosis rate of ovarian cancer cells is increased with the enhancement of SMF magnetic intensity. The reason comes from an increasing ROS and intracellular Ca^{2+} levels in ovarian cancer cells, which means that SMF can induce ovarian cancer cells to produce large amounts of ROS free radicals to ultimately promote cell apoptosis, and also induce Ca^{2+} influx between ovarian cancer cells to interfere with signal transduction to reduce the proliferation of ovarian cancer cells. Furthermore, *in vivo* experiment also reveals that SMF has a strong inhibitory effect on ovarian cancer. And the stronger the SMF is, the better the inhibitory effect on ovarian cancer tumor is. Therefore, the inhibitory strategy is an effective, which has a great potential in the treatment of drug-resistant ovarian cancer.

Declarations

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AUTHOR CONTRIBUTIONS

Data curation, validation and writing - Xiaodi Li and Yanwen Fang.

Conceptualization and project administration - Zhicai Fang

Funding acquisition, resources and supervision - Ping Wang and Jun Zhu.

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COMPETING INTERESTS

The authors declare no competing interest.

STATEMENT

The study is carried out in compliance with the ARRIVE guidelines.

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Figures

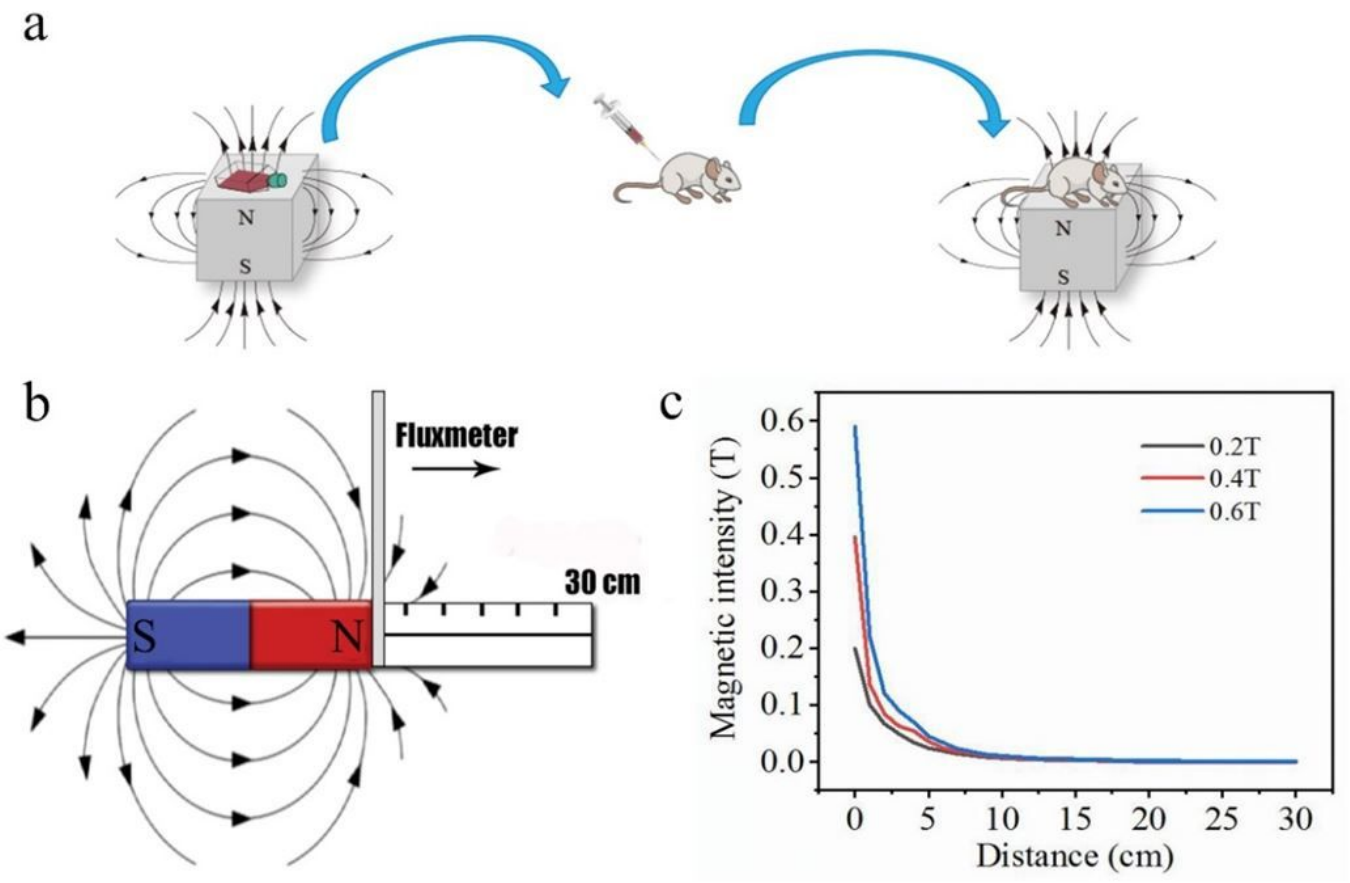

Figure 1

Schematic diagram of (a) experimental method and (b) magnetic intensity measurement, and (c) the relationship of the surface magnetic intensity and the distance.

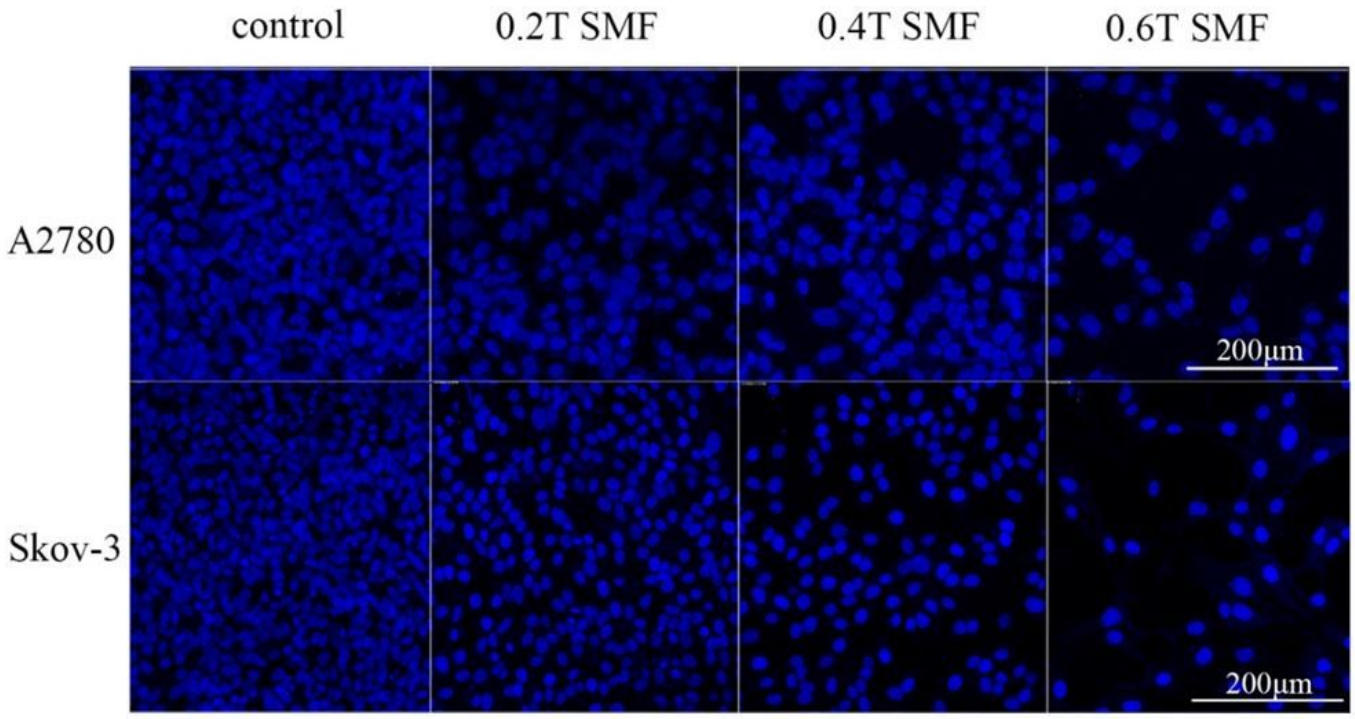

Figure 2

The confocal microscope images of different ovarian cancer cells under different SMF with 0.2T, 0.4T and 0.6T magnetic intensity (blue is the cell nuclear).

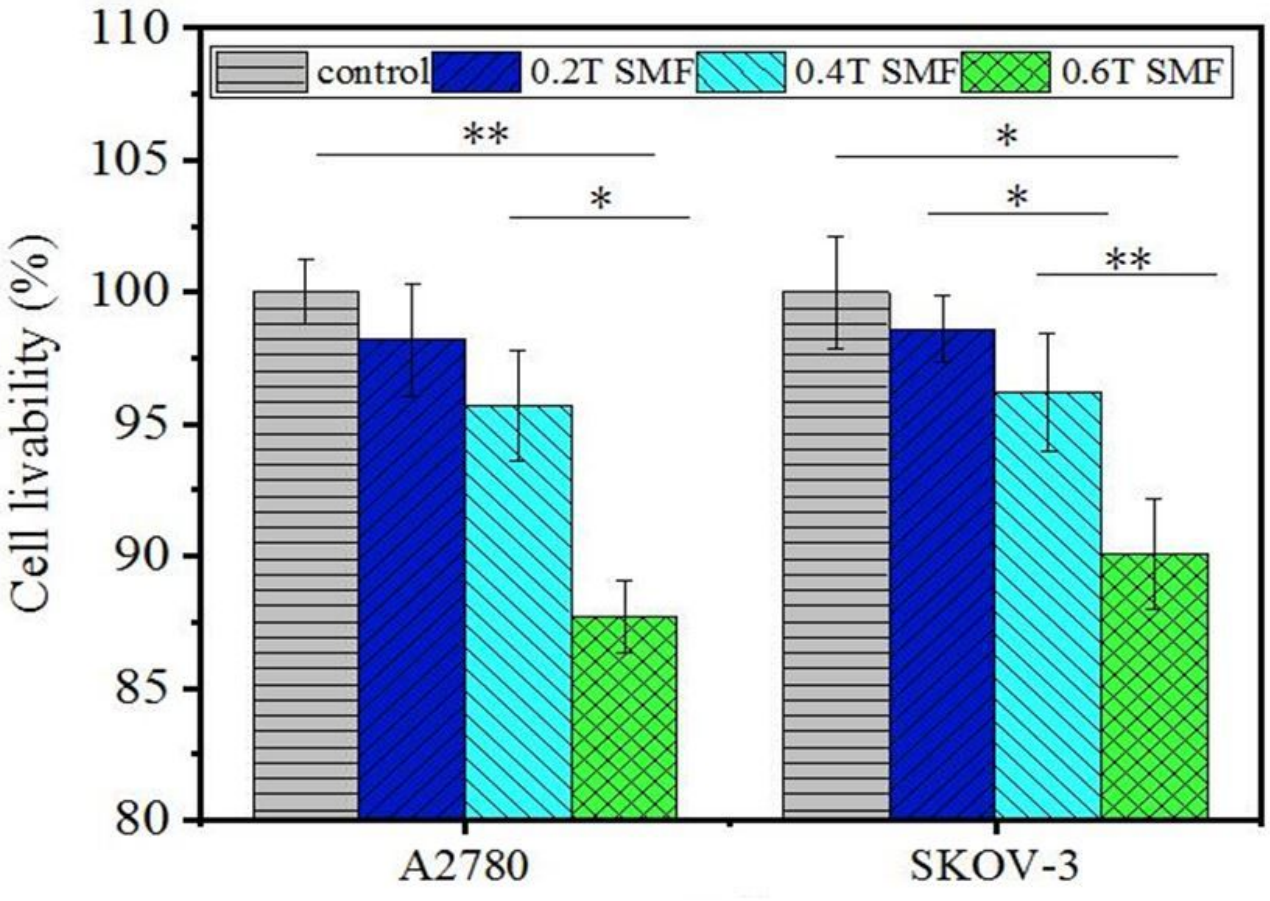

Figure 3

The cell livability of different ovarian cancer cells under different SMF with 0, 0.2T, 0.4T and 0.6T magnetic intensity.

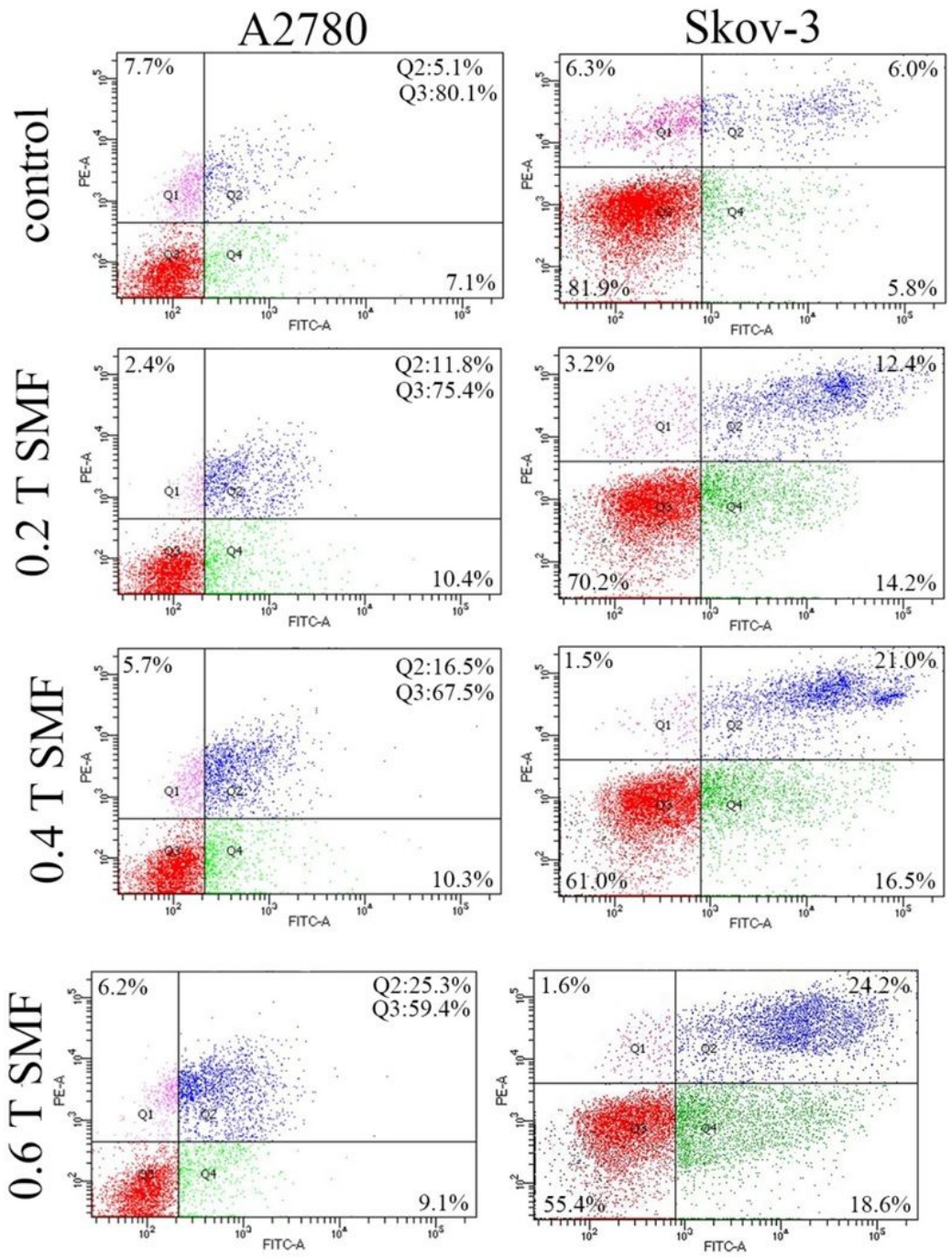

Figure 4

Flow cytometry of different ovarian cancer cells under different SMF with 0, 0.2T, 0.4T and 0.6T magnetic intensity.

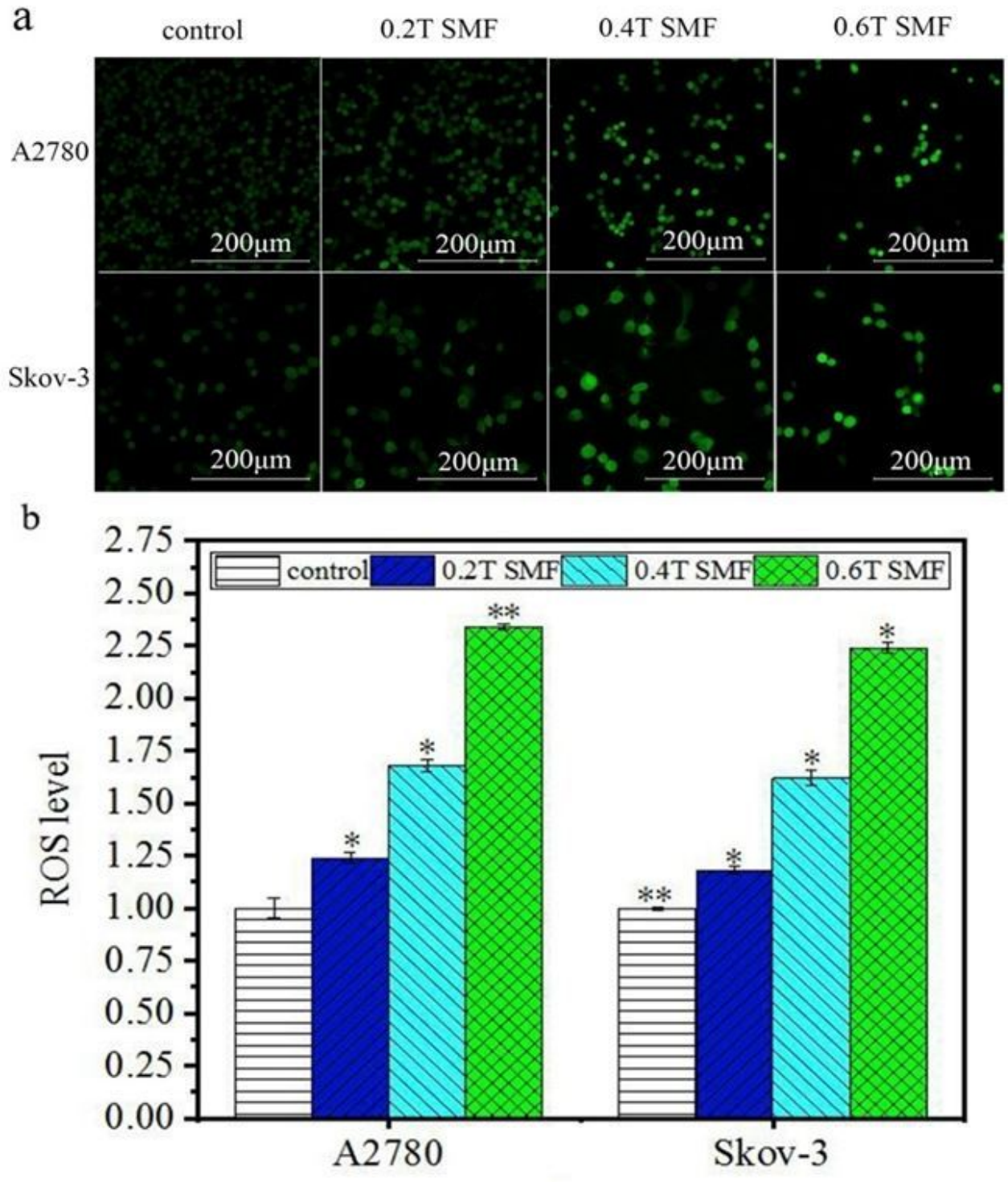

Figure 5

ROS content of different ovarian cancer cells under different SMF with 0, 0.2T, 0.4T and 0.6T magnetic intensity. (a) the confocal microscopy images of ROS and (b) the ROS quantitative results detected by flow cytometry.

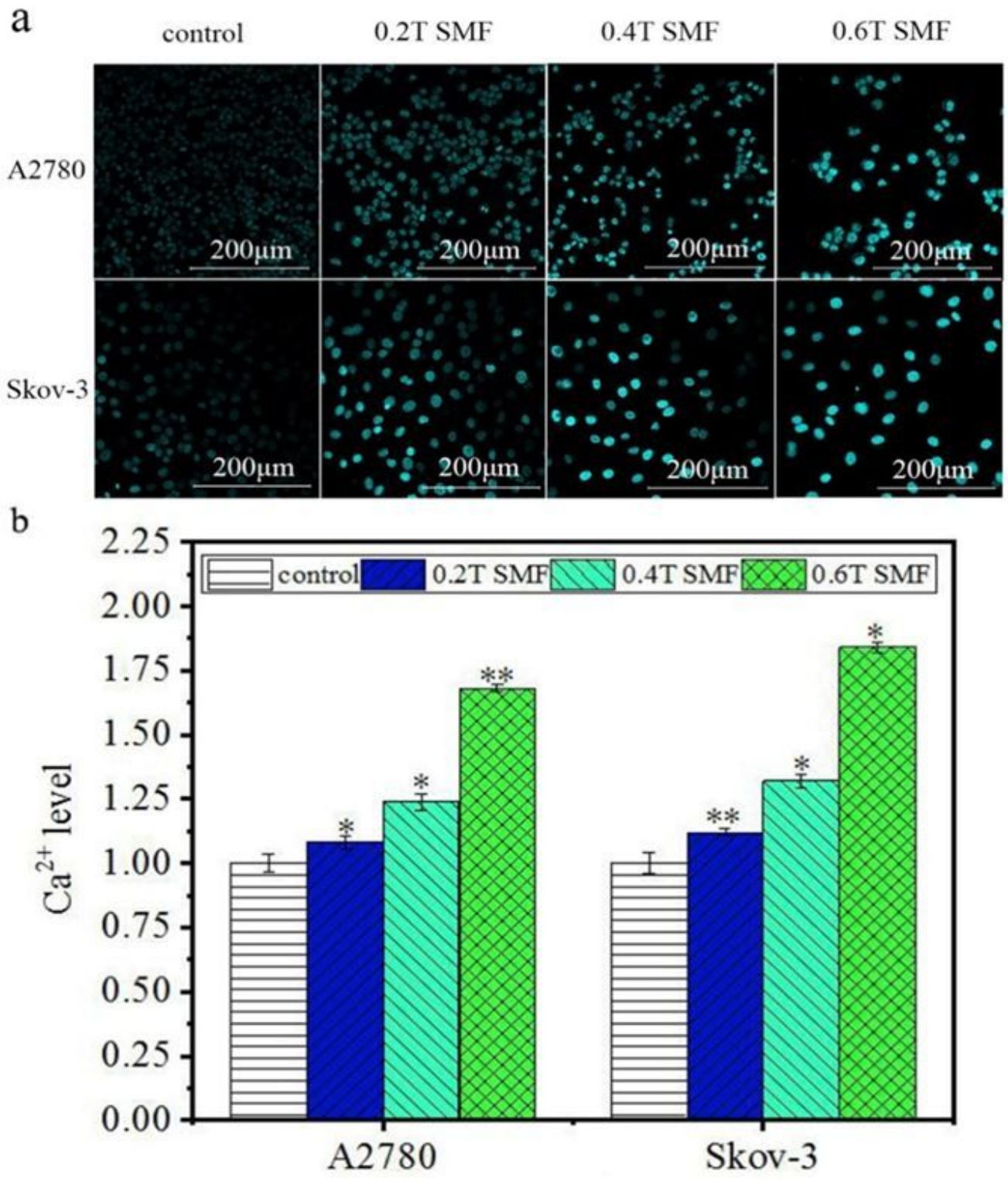

Figure 6

The intracellular Ca2+ levels of different ovarian cancer cells under different SMF with 0, 0.2T, 0.4T and 0.6T magnetic intensity. (a) the confocal microscopy images of intracellular Ca2+ and (b) the quantitative results of intracellular Ca2+ detected by flow cytometry.

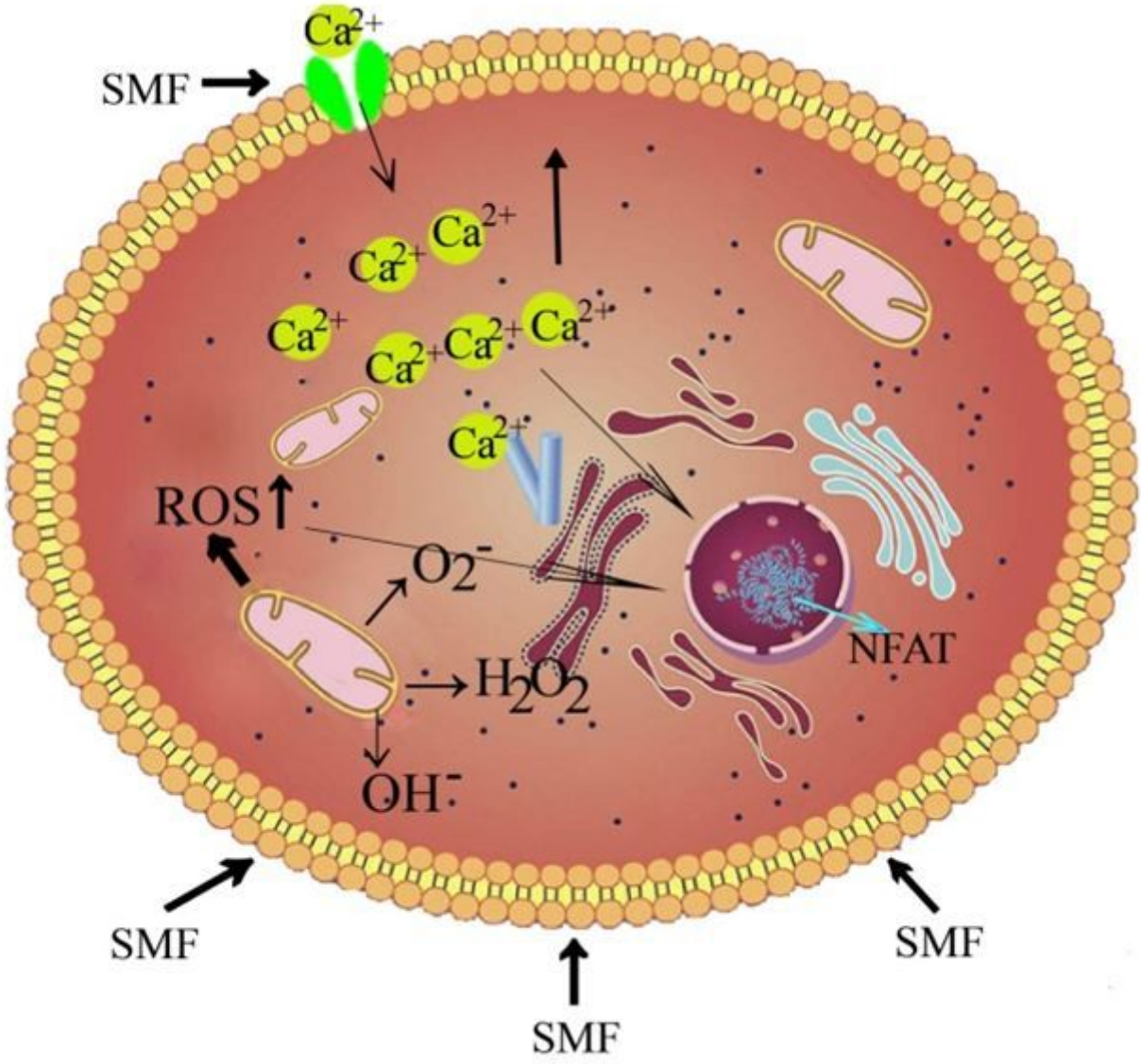

Figure 7

Schematic diagram of SMF inhibiting ovarian cancer cell.

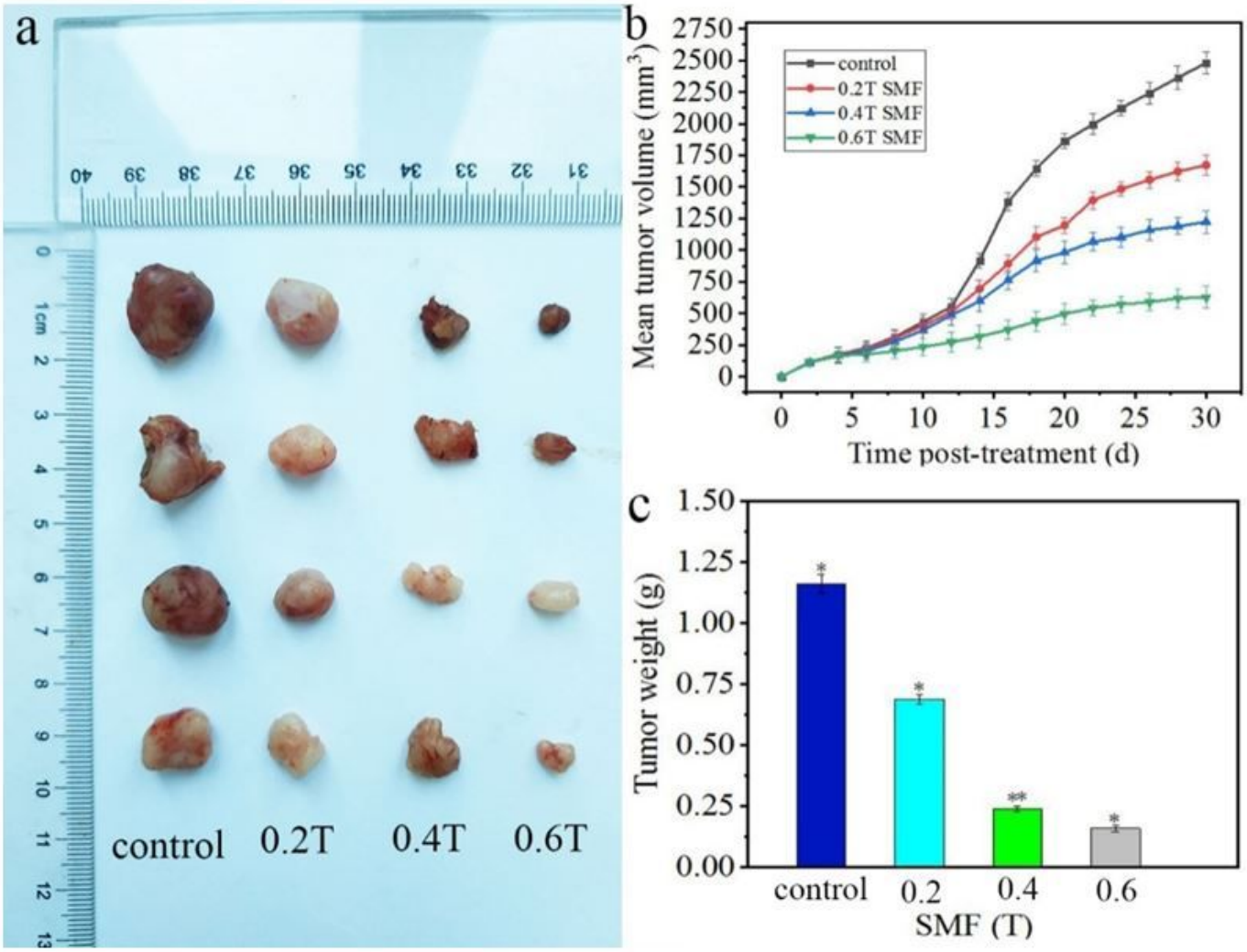

Figure 8

(a) Photograph of the tumors of A2780 ovarian cancer, (b) their mean tumor volume and (c) mean tumor weight from different groups of different SMF with 0, 0.2T, 0.4T and 0.6T magnetic intensity. Data represent the mean ± standard deviation of sixteen mice. $P < 0.05$ was considered to be statistically significant difference and shown by asterisks.