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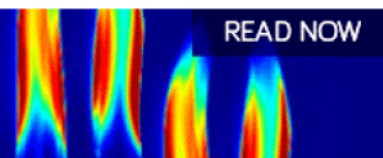
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ABSTRACT

Glioblastoma is one of the most aggressive brain cancers. Chemotherapy is a standard modality for its therapy. Here, we demonstrated a novel strategy using a helium gas discharge tube as a tunable electromagnetic emission source to sensitize glioblastoma cells to the cytotoxicity of temozolomide (TMZ), a widely used drug for glioblastoma. After a single 7-min of treatment, the efficacy of TMZ was enhanced in two typical glioblastoma cell lines U87MG and A172 without affecting a normal human astrocyte cell line hTERT/E6/E7. The discharge tube is a non-invasive approach, which provides a safe, controllable, stable, and low-cost modality to improve the conventional chemotherapy.

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Cold atmospheric plasma (CAP) is a near room temperature ionized gas,¹ which can interact with organic materials without causing thermal damage to the cells.² Traditional CAP sources produce a highly reactive environment, composed of reactive oxygen species (ROS) and reactive nitrogen species (RNS).³ Physical factors such as electromagnetic (EM) emission, ultraviolet (UV) irradiation, and thermal irradiation can also be produced.⁴ The biological response to these physical factors particularly EM emission is largely unknown.

So far, nearly all studies only focused on the description of the cytotoxicity of CAP treatment via several ROS-dependent pathways.¹ Recently, we found that cancer cells would enter the activation state after a direct CAP treatment.^{5,6} In the activation state, the CAP treated cancer cells will become sensitive to ROS. The short-lived reactive species or the physical factors from CAP may cause activation phenomenon.⁷ To date, it is still unknown whether CAP can sensitize cancer cells to chemotherapy drugs.

Chemotherapy is a common glioblastoma treatment. The use of temozolomide (TMZ) is limited due to its severe side effects.⁸ Besides, the nature of glioblastoma in deep and critical areas of the brain makes a surgical method very difficult. It will be important to develop a non-invasive way to increase the efficacy of TMZ. Here, we used the helium gas discharge tube to sensitize glioblastoma cells to TMZ. The discharge tube overcomes disadvantages of traditional CAP sources and provides a non-invasive strategy to improve glioblastoma therapy.

The discharge tube was 100 mm long. An electrode in one end was connected to DC output [Fig. 1(a)]. Helium gas with a 99.995% purity (Roberts Oxygen, grade 4.5) was sealed in the tube. One copper electrode was grounded. The discharge voltage (18 kHz) was 5.18 kV (peak-to-peak). The 96-well plate was above the discharge tube with a gap [Fig. 1(b)]. The gap was 1 or 5 mm. The temperature of the treated 96-well plate was shown in Fig. 2(a). The maximum temperature after 7 min of treatment was 32.4 °C, which was lower than the culture temperature (37 °C). Therefore, the thermal effect is ignorable. UV irradiation was measured using a UV radiometer. The bottom of the 96-well plate blocked 80% UV irradiation [Fig. 2(b)]. The background UV irradiation at the lab was even much higher than the UV intensity of the discharge tube. Such an extreme weak UV irradiation is not likely to affect the cells' normal function.

The EM emission was measured by using a heterodyne setup for radio frequency (RF) range [Fig. 3(a)], which has been used in our recent study.⁹ There are five peaks in the microwave power spectrum [Fig. 3(b)]. Briefly, the hardware includes a WR75 horn antenna, waveguide, coaxial cables, frequency multiplier, and frequency mixer. All these components work in 10–40 GHz. The high voltage (HV) circuit of the plasma generator works at 18 kHz, which emits the RF radiation with the same frequency. The GHz radiations should be due to the plasma oscillations. 18 kHz is the discharge frequency. During every discharge period, there is a streamer propagation in the

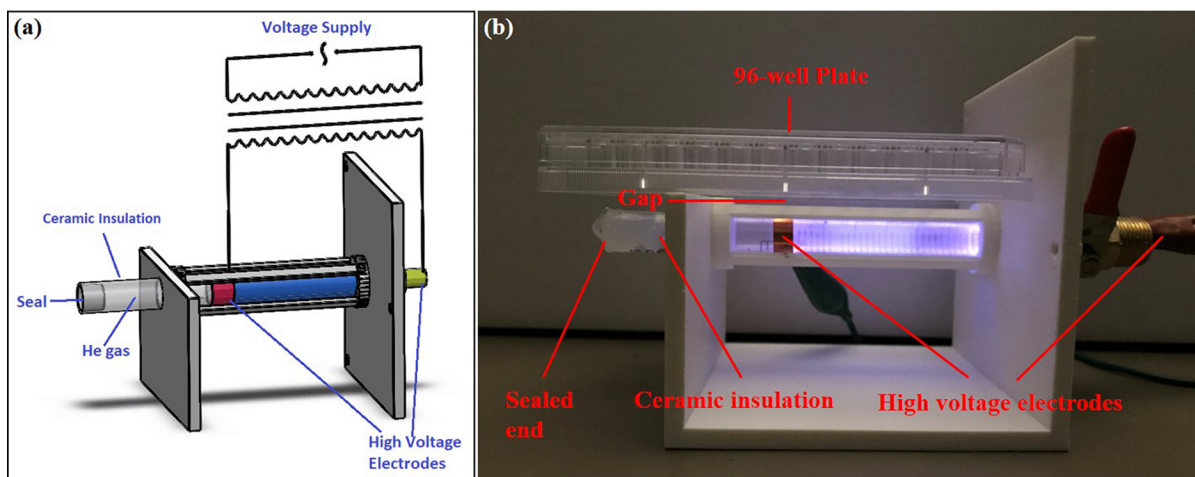


FIG. 1. The helium gas discharge tube. (a) The schematic illustration of setup; (b) the photo of discharge tube in operation.

discharge tube, which is also known as the ionization wave. The streamer propagates along the plasma in the discharge tube.¹⁰ However, the cells were exposed to the side of the discharge tube in this study. There may not be an electric field on side of the plasma discharge tube. Here, we just focused on the relatively short-range effect of the radial emission rather than the axial emission. Considering the significant electric field decay of streamer, we can neglect such an effect when we place our cells at a short radial distance from the tube. The electric field due to the ionization waves propagation may not trigger the observed cellular response.

Human glioblastoma cancer cell line U87MG, A172 and normal astrocyte cell line hTERT/E6/E7 were cultured in Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with 10% fetal bovine serum (GE Healthcare, SH30396) and 1% penicillin-streptomycin solution (Life Technologies) under standard cell culture conditions (humidified environment with 5% CO₂ at 37 °C). After one passage, 10 000 cells in 100 μ l/well medium were seeded in 96-well plate and cultured for 24 h. After the treatment, cells were further

cultured for 72 h before the thiazolyl-blue tetrazolium bromide (MTT) assay (Sigma-Aldrich, M2128). The absorbance at 570 nm was measured using an H1 microplate reader (Hybrid Technology). The cell viability was normalized by the division between all the experimental groups and the control group. In each experiment, the number of samples was 15. Each experiment was repeated for six times. Results were expressed as the mean value $\pm$ standard deviation (s.d.) of independent experiments.

In all cases, the overnight media were renewed by 10 μ l fresh media before the test. The first experimental case was just the control group. For each well of plates, 90 μ l/well fresh cell culture media for each well was added to the cells after the test. The second experimental case was the group just treating cells by the discharge tube-only, in which the cells would be sensitized. In this case, cells were treated by placing the plate above the discharge tube with a gap of 1 or 5 mm for 1, 4, or 7 min with 10 μ l/well of fresh cell culture media. After that, 90 μ l/well of fresh cell culture media was added to keep culturing cells. The third experimental case was the group just using the drug TMZ.

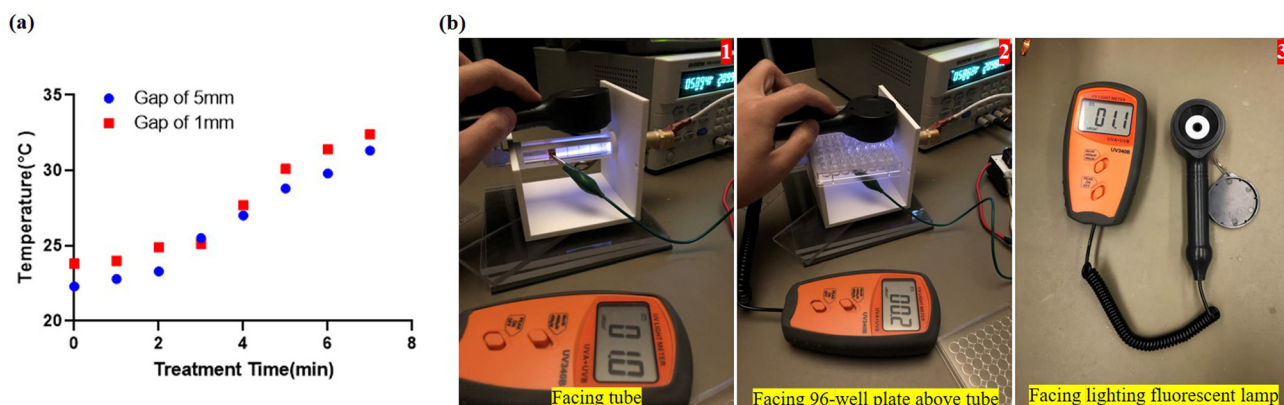


FIG. 2. Heating effect and UV irradiation of discharge tube. (a) The temperature of the treated plate. An HT-02D Handheld Digital IR Infrared Thermal Imaging Camera Thermometer was used for the thermal imaging. (b) The UV intensity measured by a UV radiometer (UV340B, KONGZIR). From left to right, the UV intensity from the discharge tube, the UV intensity after the blockage of 96-well plate, and the UV intensity of lighting at lab was 1.0 μ W/cm², 0.2 μ W/cm², and 1.1 μ W/cm², respectively.

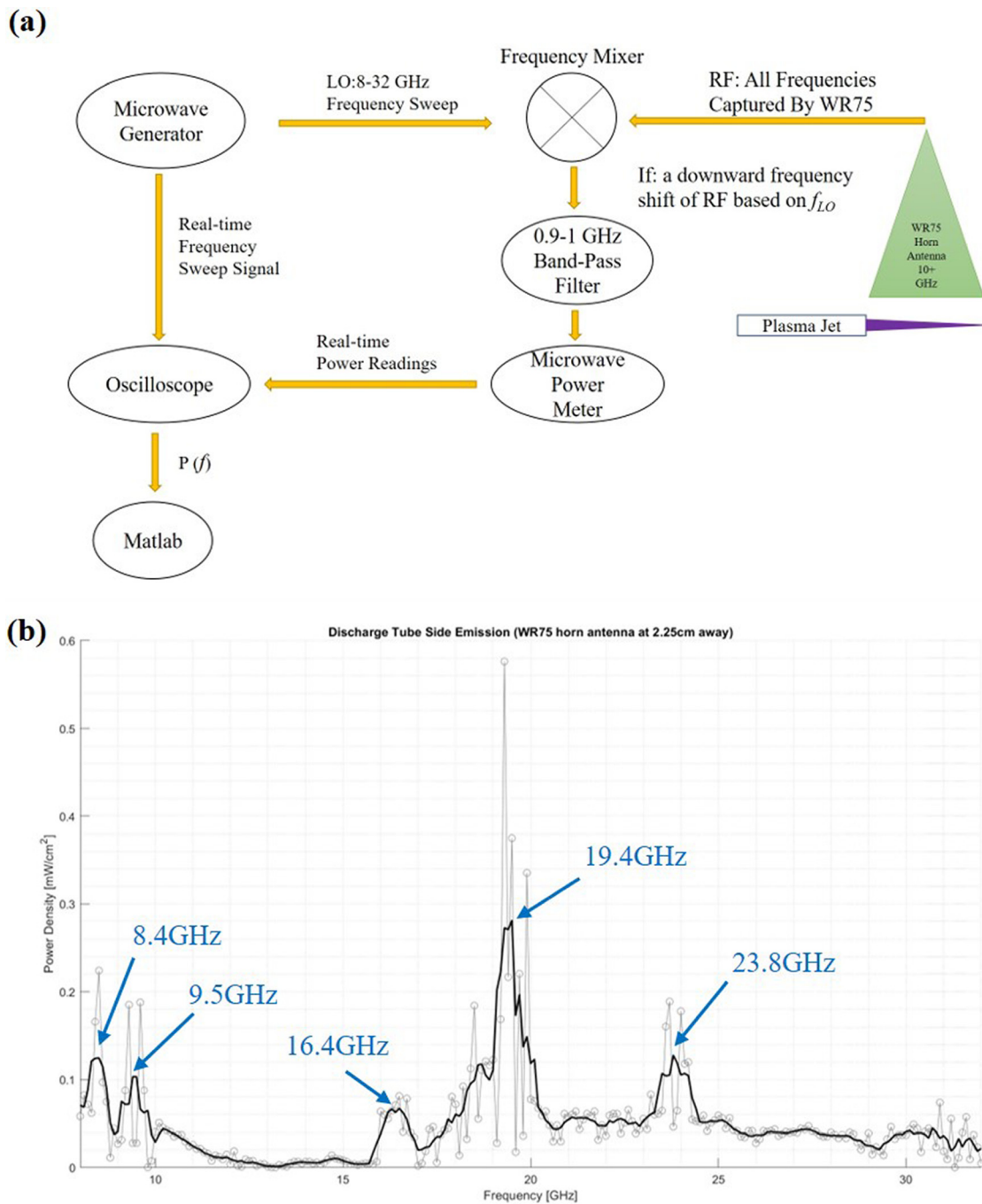


FIG. 3. The EM spectrum of discharge tube. (a) The schematic illustration of the heterodyne setup for RF power spectrum measurement.⁹ (b) An electromagnetic spectrum measurement of the ionized helium in the discharge tube. There are five main peaks (8.4 GHz, 9.5 GHz, 16.4 GHz, 19.4 GHz, and 23.8 GHz) in the microwave power spectrum.

In this case, 100 μl /well of fresh media with 180, 250, 320 μM of TMZ was added to culture cells. The last case was designed to test the sensitization state of cells to the cytotoxicity of TMZ. Cells were treated by the discharge tube for 1, 4, and 7 min with a gap of 1 or 5 mm with 10 μl /well of fresh cell culture media with 180, 250, 320 μM of TMZ.

After that, 90 μl /well fresh cell culture media with 180, 250, 320 μM of TMZ was added to culture the treated cells.

The discharge tube alone did not cause noticeable growth inhibition on glioblastoma cells. As shown in Fig. 4(a), even 7 min of treatment just caused a 10% growth inhibition. The TMZ treatment alone

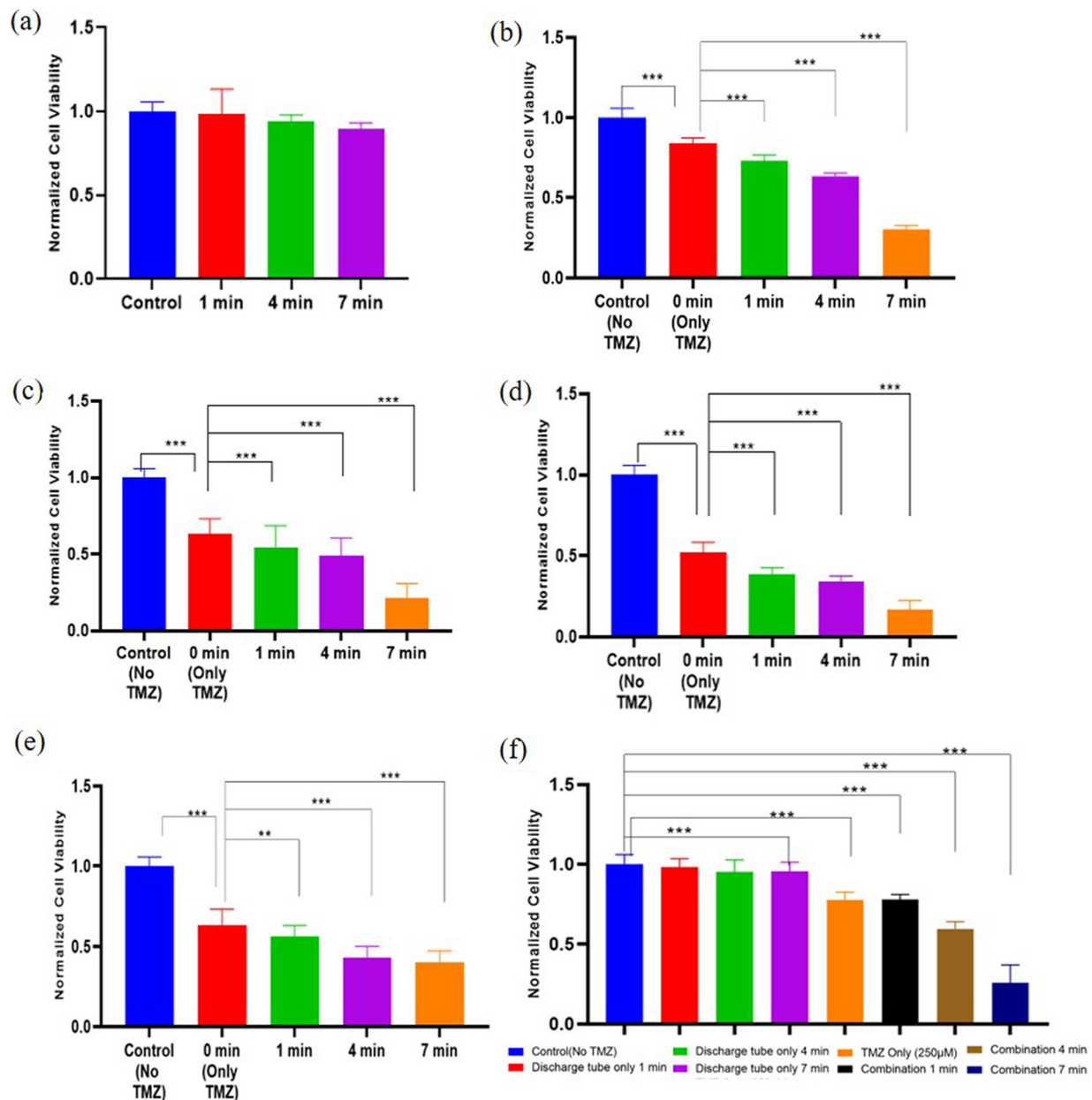


FIG. 4. The sensitization of the glioblastoma cells to the cytotoxicity of TMZ by the discharge tube. (a) The impact of discharge tube on U87MG cells. (b) The cytotoxicity of TMZ (180 μM) on the sensitized U87MG cells. (c) The cytotoxicity of TMZ (250 μM) on the sensitized U87MG cells. (d) The cytotoxicity of TMZ (320 μM) on the sensitized U87MG cells. (e) The case with a larger gap (5 mm) with TMZ (250 μM). (f) The cytotoxicity of TMZ (250 μM) on the sensitized A172 cells. The gap was 1 mm for all figures except (e). (Student's t-test, $p < 0.05$, ***)

caused noticeable inhibition on U87MG cells. The growth of U87MG cells was inhibited by 21%, 37%, 50% by the TMZ treatment with a concentration of 180 μM , 250 μM , and 320 μM TMZ, respectively [Figs. 4(b)–4(d)]. The cytotoxicity of TMZ on U87MG cells could be considerably enhanced by the discharge tube treatment [Figs. 4(b)–4(d)]. For example, 180 μM TMZ alone caused a 21% growth inhibition rate on U87MG cells [Fig. 4(b)]. The pre-treatment by the discharge tube of

1 min, 4 min, and 7 min caused 24%, 38%, and 72% of growth inhibition rate, respectively. A similar pattern was also observed with the TMZ concentrations was 250 μM and 320 μM [Figs. 4(c) and 4(d)]. Furthermore, when the gap increased from 1 mm to 5 mm, the sensitization strength decreased [Fig. 4(e)]. The same sensitization was also observed in the discharge tube treated A172 cells [Fig. 4(f)]. The discharge tube treatment alone also did not cause a noticeable impact on A172 cells.

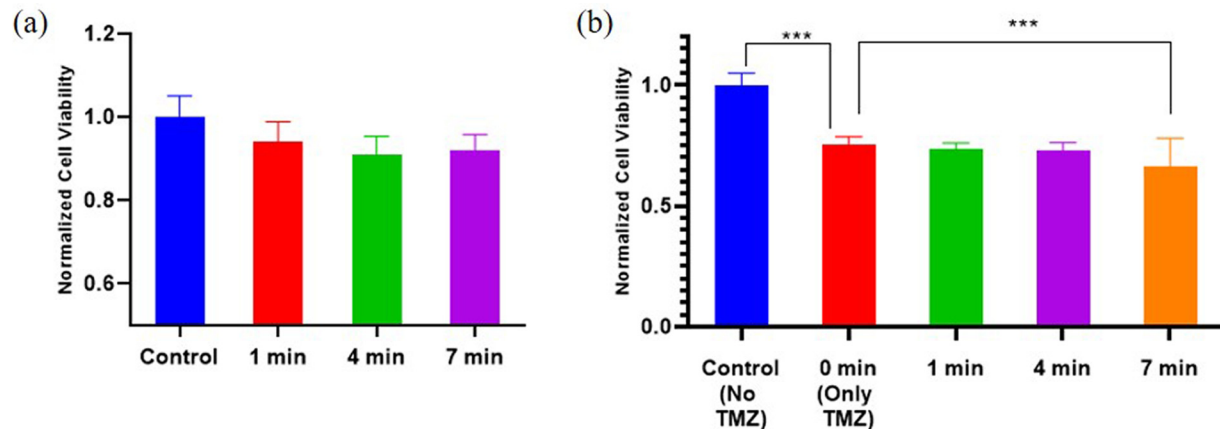


FIG. 5. The limited side effect on normal astrocyte cell line hTERT/E6/E7. (a) The discharge tube treatment alone. (b) The cytotoxicity of TMZ (250 μ M) on the tube-treated cells. The gap was 1 mm. (Student's t-test, $p < 0.05$, ***)

More importantly, the discharge tube is the primary driver of the selective sensitization toward the glioblastoma cells. The same tests were performed on the normal astrocyte cell line hTERT/E6/E7. Like two cancer cell lines, the discharge tube treatment alone did not cause a noticeable impact on hTERT/E6/E7 [Fig. 5(a)]. The pre-treatment of the plasma discharge tube has not increased the cytotoxicity of TMZ on hTERT/E6/E7 cells [Fig. 5(b)]. This trend suggests that the discharge tube is very promising to decrease the side effect of TMZ on normal brain tissue without sacrificing its anti-glioblastoma efficacy.

In this study, we investigated the sensitization effect of the electromagnetic emission generated by the plasma discharge tube on glioblastoma cells to a widely used drug TMZ. UV will not be able to penetrate the bottom of 96-well plate. In addition, the thermal effect was also deemed to be inconsequential in this study. The EM emission of the discharge tube also shows a 19 GHz peak and a 24 GHz peak, which suggests a microwave effect on the cancer cells (19 GHz and 24 GHz are in microwave range) (Fig. 3). However, the underlying mechanism is still unknown. The EM field has been demonstrated to lower the number of metastatic tumor sites and slow tumor growth rate when compared to the control group, without showing harmful side effects.^{11,12} EM emission may weaken the glioblastoma cells' DNA repairing function because TMZ mainly affects brain cancer by causing serious DNA damage.¹³

Compared with the traditional CAP sources, the discharge tube shows a much better stability. The chemical and physical stability of CAP will be affected by the change of local environments, particularly the relative humidity.¹⁴ Due to the sealing of helium in the discharge tube, the local experimental environments do not affect the discharge process. The stabilization of plasma discharge tube has been drastically improved compared with the normal CAP jet sources. This provides an approach to realize the application in precise medicine. The discharge tube does not need the continuous supply of noble gases, which will drastically decrease the research cost. Its small size and its portable nature also make it as a potential wearable device.

In summary, through the demonstration of the sensitization of glioblastoma by the helium gas discharge tube, we provided a new modality to enhance the selective anti-glioblastoma effect of TMZ. The discharge tube alone did not cause significant cancer cell growth inhibition. It activated two glioblastoma cell lines to be very sensitive

to the cytotoxicity of a widely used anti-glioblastoma drug TMZ, which would decrease the side effect of such drugs without sacrificing its efficacy. The discharge tube used its physical factors to achieve these promising effects, which is a non-invasive approach and might have wide translational applications in the treatment of glioblastoma as well as other solid tumors.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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