Supplementary information

Plasma activated medium induces apoptosis in chemotherapy-resistant ovarian cancer cells: high selectivity and synergy with carboplatin

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Guide through Supplementary Materials:

The Supplementary material and methods provide additional experimental methods (**"Supplementary material and methods"**) that complement the materials shown in the main part of this study.

The Supplementary results provide additional experimental results (**"Supplementary results"**) that complement the results shown in the main part of this study. They are intended to provide additional information about our experimental system.

Contents:

A. Supplementary Material and Methods

A.1 Configuration of CAP devices

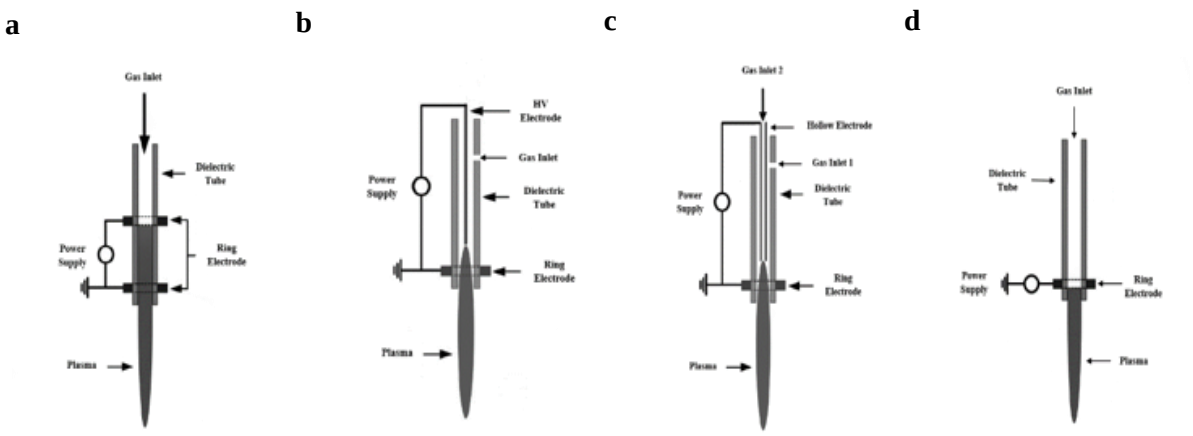

Fig.S1 Configuration of CAP devices that were built in this work. (a) DBD-based plasma jet using two external ring electrodes. (b) DBD-like plasma jet using an axial pin electrode and an external ring electrode. (c) DBD-like plasma jet using a hollow tube electrode, an external ring electrode, and two gas inlets. (d) DBD-based plasma jet using a single ring electrode.

A.2 CAP device diagnostics:

To produce the plasma jet, we used 1-24 kV, and 0-20 kHz sinusoidal high voltage (AC) power supply, with a central electrode in length 8 cm that was covered 10 cm quartz tube and wrapped around with copper electrode. Helim was used as carrier gas with a purity of 99.99%. The operating conditions i.e. applied peak to peak voltage is 2.5 kV, constant frequency is 10 kHz, He gas flow rate was kept at 0.5 l/min which are fixed for entire characterization. The applied voltage

of the CAP is monitored on a Tektronix digital oscilloscope equipped with a high voltage probe (Pintek, HVP-39 PRO, Taiwan) (Fig.S5(a)).

The temperature of the generated plasma jet was measured with a thermocouple thermometer (Jumo iTron 04, Germany) [1,2](Fig.S5(b,c)). The measurable temperature range of this thermocouple is from -200 to +850°C.

A multichannel spectrometer (Avantes, DESKTOP-USB2, Netherlands) was used to acquire the emission profile and active species identification of the plasma discharge. The wavelength range of the spectrometer is 200 –1058 nm with the spectral resolution of 0.19 nm (Fig.S6).

A.3 Isolation of granulosa cells

NMRI immature female mice aging 21-25 days were purchased from Royan Institute. Mice were sacrificed with cervical dislocation. ovaries were removed and punctured with two needles and then was used collagenase IV as enzymic digestion and after 30 min neutralized with FBS, so granulosa cells were released into media culture (α MEM, 1% antibiotic, 10% FBS). Cells were cultured in α MEM supplemented with 10% FBS and 1% antibiotic at 37°C and 5% CO_2. All the requirements of the Kharazmi University animal care and usage committee under an approved protocol were strictly followed. The approval ID of this protocol is IR.KHU.REC.1399.011. (Fig. S2).

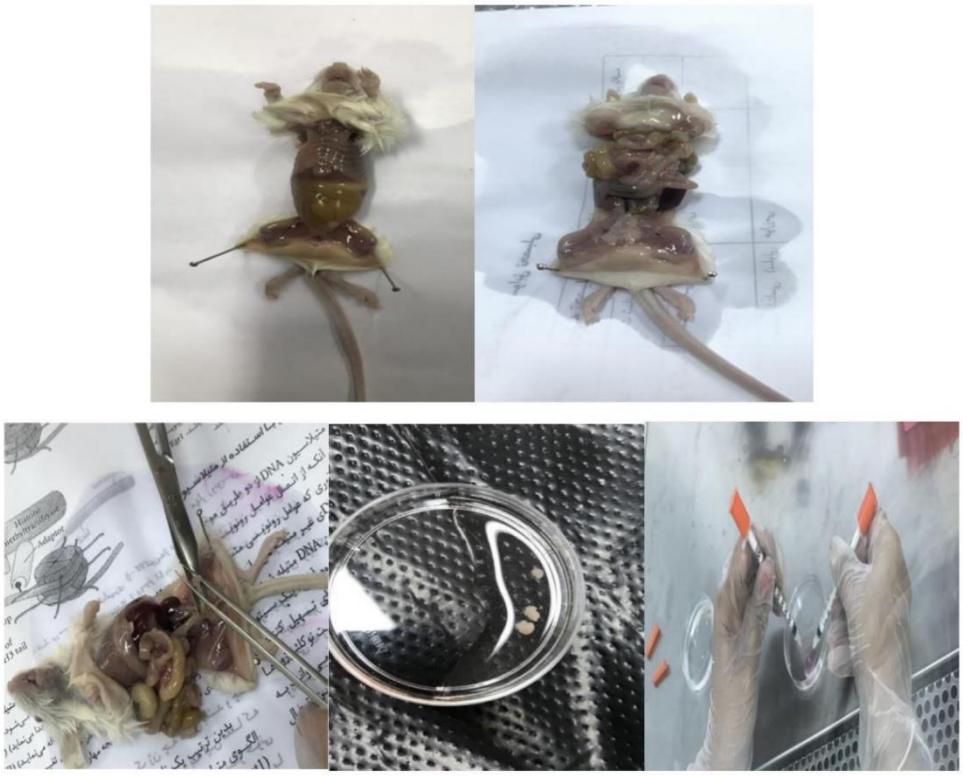

Fig. S2 Granulosa cell extraction steps from the ovaries of NMRI mice.

A.4 Flow cytometry analysis to diagnosis identity of isolated cells:

In brief, the culture medium was removed and respectively trypsinized, pelleted, washed with PBS (Sigma, USA), and cell pellet exposure with triton 0.3% for 30 min cells and then incubated with goat serum 10% for 10 min. the cells were then kept overnight at 2-8 ˚C after primary antibody (α-inhibin) addition. In the next step, after the addition of the secondary antibody (anti-rabbit PE) the cells were incubated at 37 ˚C for 90 min (Fig.S7)

A.5 Diagram of treatment methods

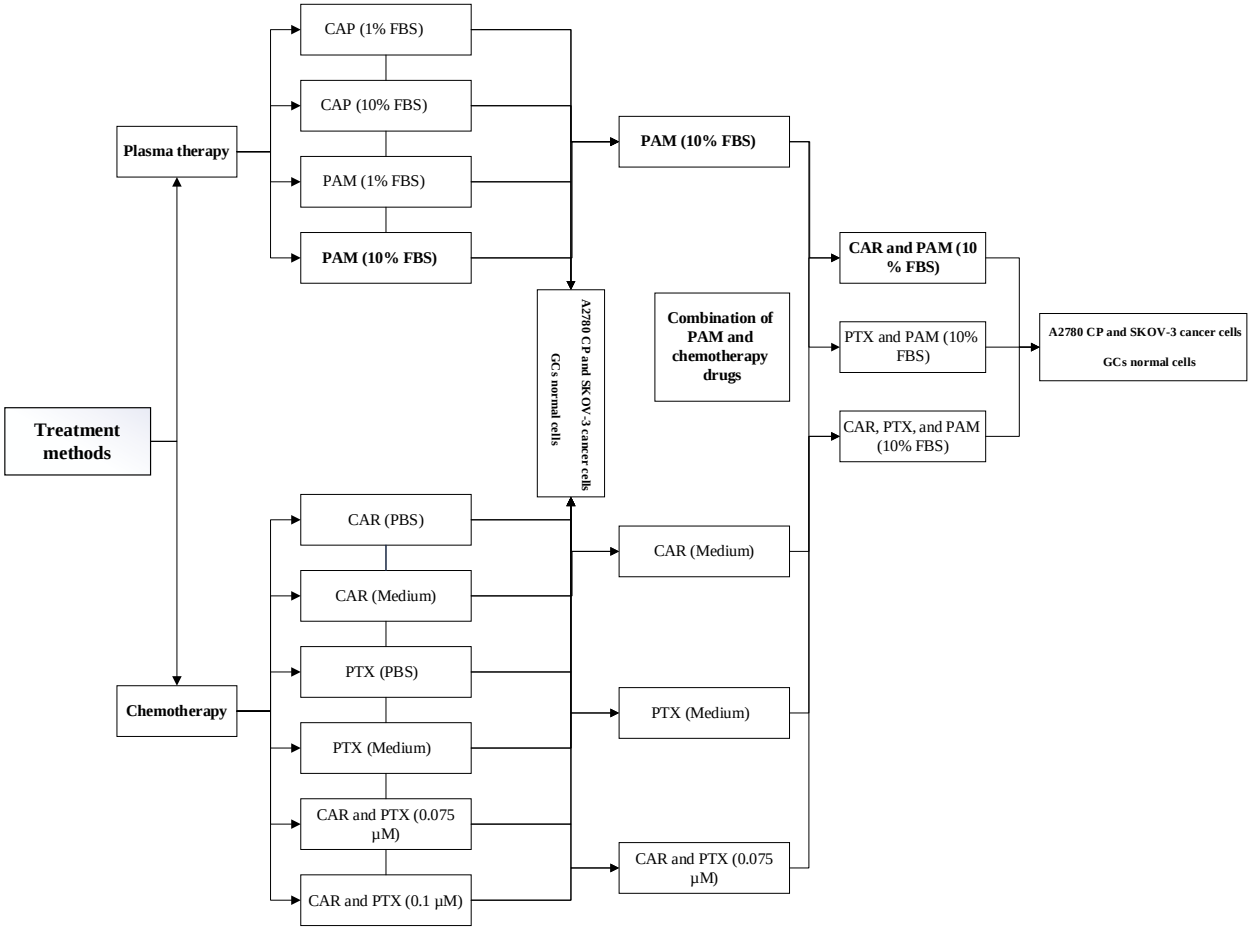

Fig.S3 Diagram of treatment methods in this study. CAP (cold atmospheric plasma), PAM (plasma activated medium), PTX (paclitaxel), CAR (carboplatin). All treatment methods performed on three A2780 CP, SKOV-3, and GCs cells.

A.6 Schematic representation of the treatment methods

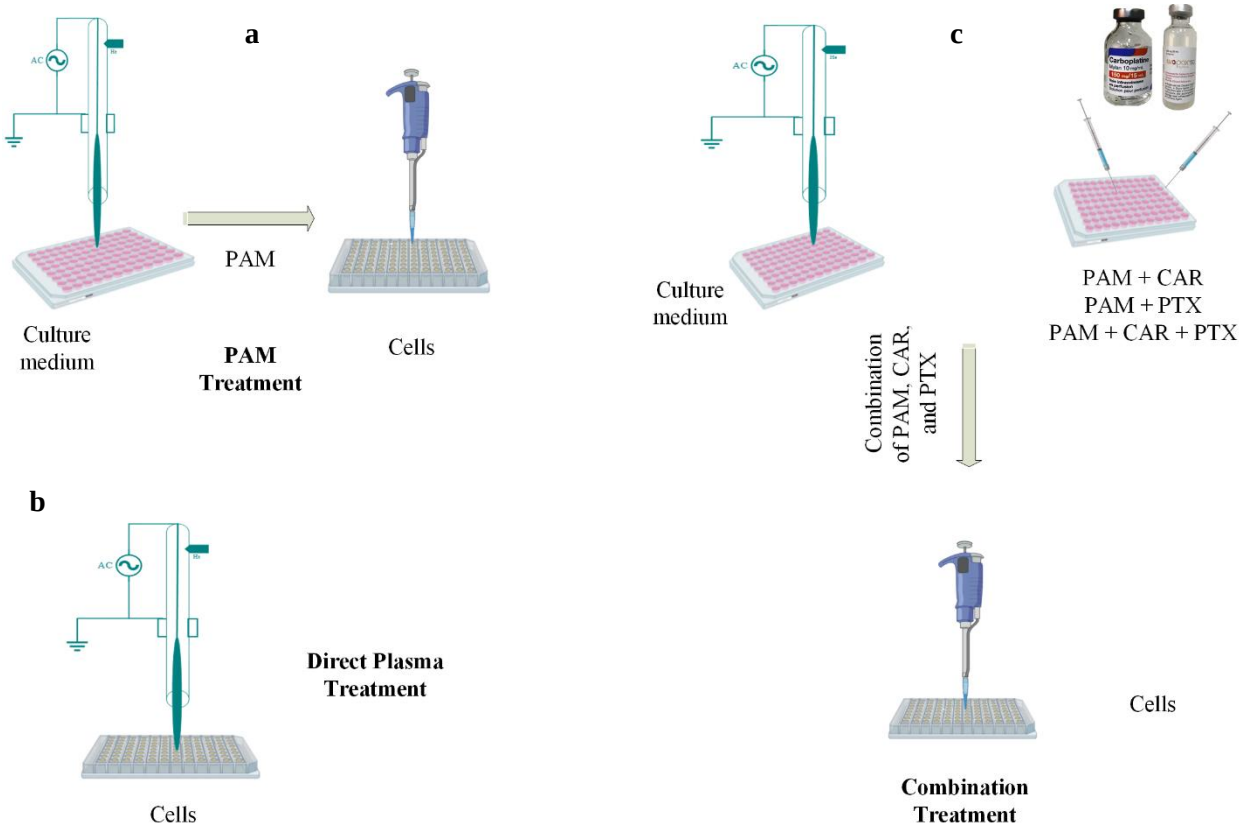

Fig.S4 Schematic representation of the treatment methods used in this work. (a) Plasma activated medium (PAM) treatment. (b) Direct plasma treatment (CAP). (c) Combination treatment of PAM and common chemotherapy drugs.

A.7 Primers used for the real-time quantitative polymerase chain reaction

gene	Primer sequencing (5´-3´)
β-actin	F: CAGCAGATGTGGATCAGCAAG R: GCATTTGCGGTGGACGAT
Bax	F: AACATGGAGCTGCAGAGGAT R: CAGTTGAAGTTGCCGTCAGA
Caspase3	F: CATGGAAGCGAATCAATGGACT R: CTGTACCAGACCGAGATGTCA
p53	F: GCCAAAGAAGAAACCACTGGATG R: TGAGTTCCAAGGCCTCATTCAG
SOD1	F: GGTGGGCCAAAGGATGAAGAG R: CCACAAGCCAAACGACTTCC

Table 1. Primers used for the real-time quantitative polymerase chain reaction

A.8 Combination effect of CAR and PTX on ovarian cells

A2780 CP, SKOV-3, and GCs cells were seeded into a 96-well cell-culture plate with a density of 1×10^4 cells in the 200 µM. After 24 h, all three cell types were treated with a combination of Cis-diammine-1, 1-cyclobutane dicarboxylate platinum II (carboplatin, Mylan®, USA) and paclitaxel (Nano Alvand, Iran) alone, then with these drugs. According to IC_{50} values obtained from the chemosensitivity section, we utilized a lower dosage than the recommended concentration values for clinical application in the present study. We evaluated the low concentration of carboplatin with IC_{50} concentration of paclitaxel which is the conventional therapy for ovarian cancer

treatment. The results are shown in Fig. S8 expressed that this combination treatment has higher cytotoxicity for GCs cells compared with OC cells.

A.9 RONS and pH measurements of PAM

Washed the cells and preincubation for 5 min in prewarmed incubation buffer, added to each well of 200 ml reaction mixture ferric-xylenol orange complex (Sulfuric acid, Sorbitol, Xylenol orange, Ammonium iron sulfate, Merck, USA), after a further 45-min incubation at room temperature absorbance of the solution determined at 560 nm with Perkin Elmer UV/VIS spectrophotometer. Additionally, to determined Nitrite and Nitrate concentration in PAM, was used colorimetric Nitrite/Nitrate Assay kit (Sigma–Aldrich Co., Ltd) according to instructions provided by the manufacturer.

The pH measurements of RPMI-1640 and α-MEM culture mediums were carried out immediately after plasma exposure with a pH meter (Bel, PHS3-BW, Italy).

B. Supplementary Results

B.1 Cold atmospheric plasma device characterization:

In this set of experiments, we investigated the CAP jet (CAPJ) under different conditions, including voltage and frequency, to generate the discharges for different configurations of cold plasmas (data not shown). CAPJ was operated at a frequency of 10 kHz of a high-voltage AC power supply which resulted in the most stable electrical characteristics. The temperature of He-based CAPJ was measured at a different distance from the nozzle, and different applied voltage as

shown in Fig.S5. the temperatures increased from 27.98°C to 30.69°C when the applied voltage increased from 2.5 kV to 5 kV, respectively. We utilized a single jet cold plasma system with 10 kHz and 2.5 kV of applied AC power to study the effects of plasma treatment on ovarian cells. The voltage waveform is shown in Fig.S5 for the CAPJ device.

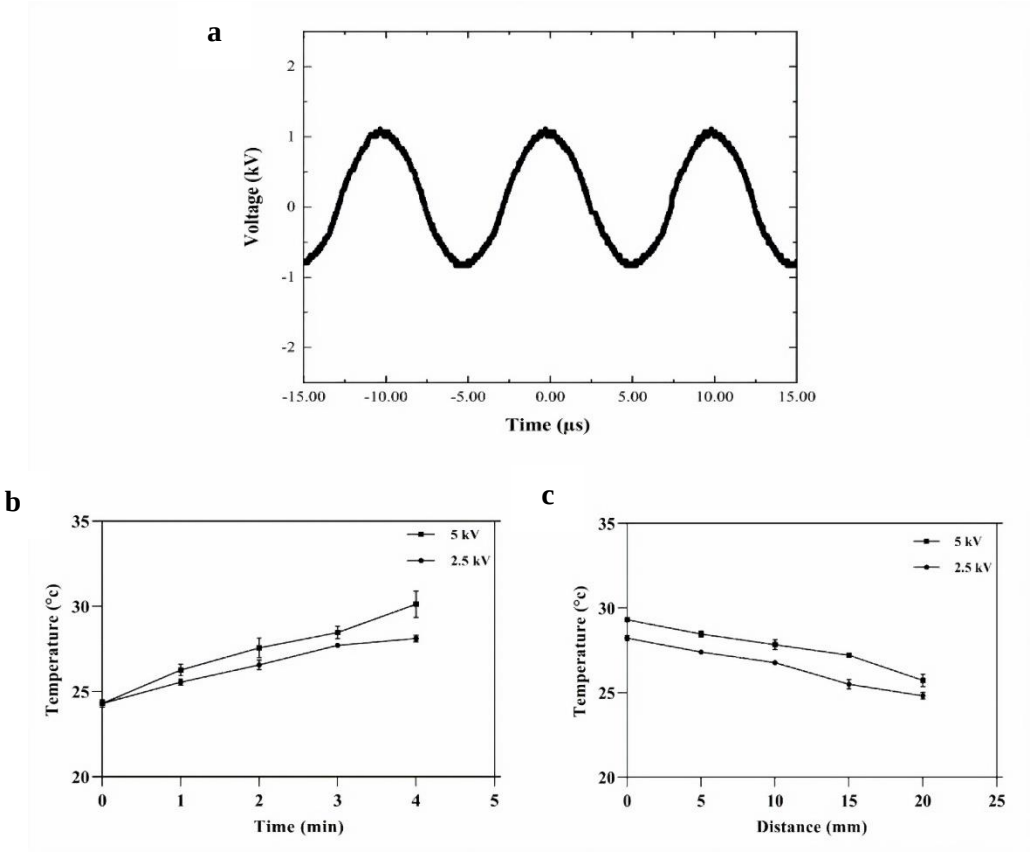

Fig. S5 Voltage waveforms and temperature of the CAPJ. (a) Voltage waveforms μs for CAP, we work with a 2.5 kV peak to peak voltage, and a flow rate of 0.5 lpm. (b) The temperature of the plasma plume according to treatment time. (c) The temperature of the plasma plume according to a distance of the nozzle. The temperature was detected at the position of 1 cm below the downstream of CAPJ.

Fig.S6 presents the optical emission spectra between 200 and 1100 nm from the CAPJ. We recorded the intensities of the most prominent He lines at 587, 667, 706, and 728 nm, neutral oxygen (OI) at 777, 788, 844 nm. The hydroxyl (OH) radicals produce optical emission at 309 nm.

The N_2^+ first negative system produced the emission line at 391 nm. Also, the detected reactive species associated with nitrogen are excited nitrogen molecules producing emission between 300 and 400 nm.

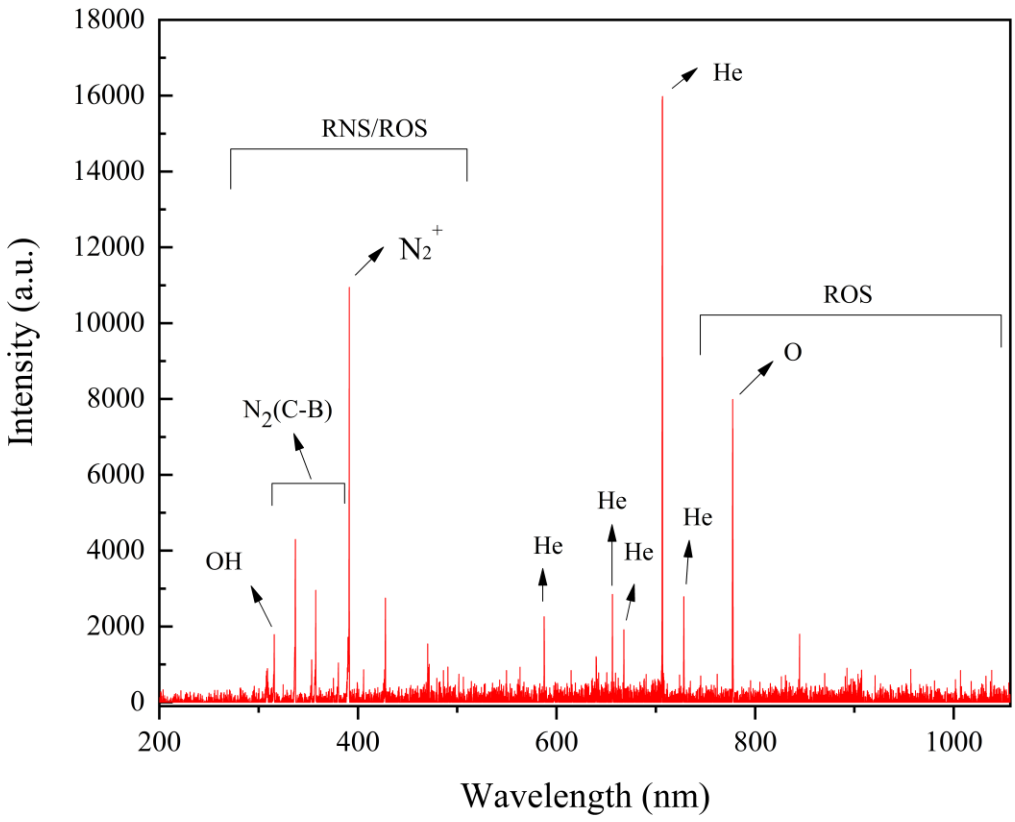

Fig.S6 The OES spectra of the CAP operated at a fixed 2.5 kV peak to peak voltage and fixed He gas flow rate of 0.5 lpm.

B.2 Proving of granulosa cells isolation

Flow cytometry analysis was performed to prove the granulosa cells and the results that are displayed in Fig. S7 shows that 89.3% of the cultured cells are GCs cells.

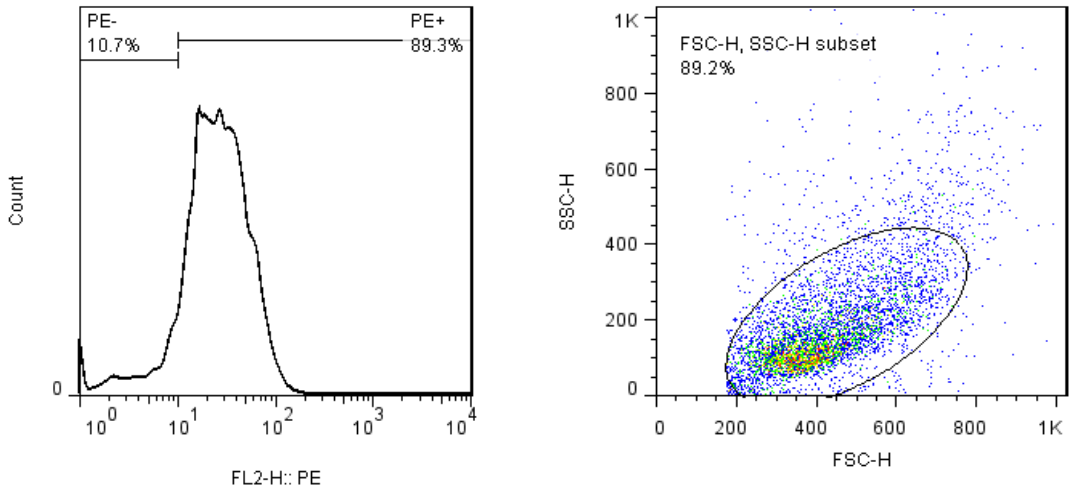

Fig. S7 Flow cytometry analysis for proving the existence of cultured granulosa cells with α-inhibin as a marker of granulosa cells.

B.3 Evaluation of ovarian cells viability treated with combination CAR and PTX by MTT assay

According to IC_{50} values obtained from the chemosensitivity section, we utilized a lower dosage than the recommended concentration values for clinical application in the present study. We evaluated the low concentration of carboplatin with IC_{50} concentration of paclitaxel which was the conventional therapy for ovarian cancer treatment. The results are shown in Fig. S8 expressed that this combination treatment has higher cytotoxicity for GCs cells compared with OC cells.

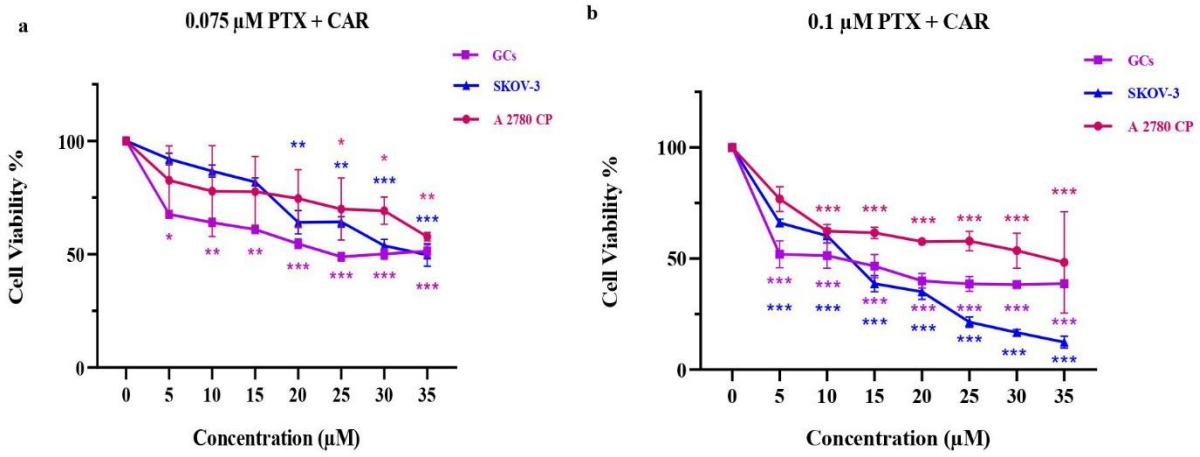

Fig. S8 Combination effect of CAR with PTX on ovarian cells. (a) 0.075 µM concentration of PTX with different concentrations of CAR. (b) 0.1 µM concentration of PTX with different concentrations of CAR. Data are presented as means ± S.D. and statistical analysis was carried out using two-way ANOVA with Dunnett's multiple comparison test (*p < 0.05, **p < 0.01, ***p < 0.001 versus control).

B.4 pH measurements

The pH of two culture mediums in this work measured immediately after the plasma exposure. Fresh medium, medium supplemented with 1% FBS, medium supplemented 1% FBS with 4 min plasma exposure time, medium supplemented 10% FBS, medium supplemented 10 % FBS with 4 min plasma exposure time, were the five sets for the pH measurements. We found that the pH of RPMI1640 and αMEM decreased to 6.24 and 6.72 at 1% FBS, and also reached 6.41 and 6.99 in 10% FBS, respectively (Fig. S9).

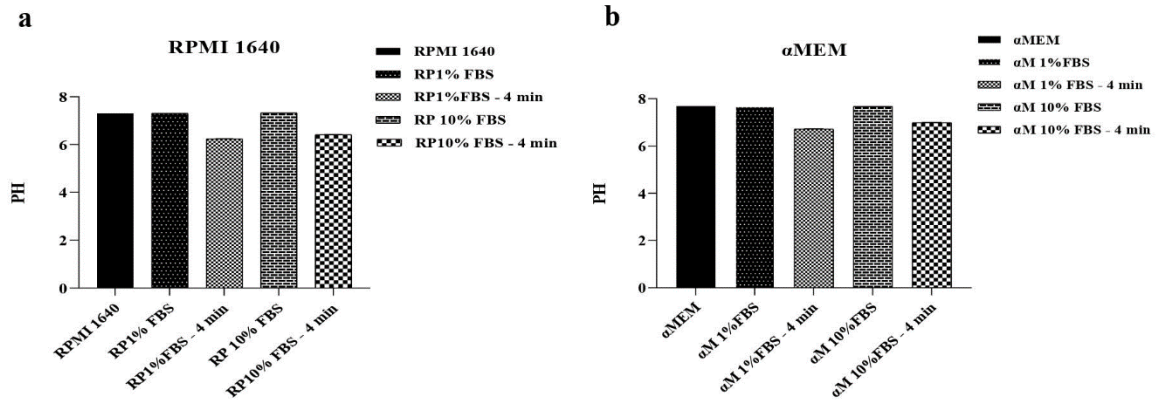

Fig.S9 (a) pH of RPMI 1640 culture medium. (b) pH of αMEM culture medium. Results from three independent experiments are shown as means ± SD.

References:

1. DENG, G. *et al.* Experimental study on bacteria disinfection using a pulsed cold plasma jet with helium/oxygen mixed gas. *Plasma Sci. Technol.* **20**, 115503 (2018).

2. Mehrabifard, R., Mehdian, H., Hajisharifi, K. & Amini, E. Improving Cold Atmospheric Pressure Plasma Efficacy on Breast Cancer Cells Control-Ability and Mortality Using Vitamin C and Static Magnetic Field. *Plasma Chem. Plasma Process.* **40**, 511–526 (2020).