Combination of metformin and cold atmospheric plasma induces glioma cell death to associate with c-Fos

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Glioma is the most common type of brain cancer. Chemotherapy combination with surgery and radiotherapy is a standard clinical treatment for patients. Although many advances in glioma therapy, the prognosis of glioma patients has not significantly been improved over the past decades. Hence, there is still an urgent need to develop a new therapy to treat glioma. Cell viability was assessed by CellTiter Blue assay; flow cytometry (FCM) was used for detecting cell apoptosis; ROS detection was detected by ROS Assay; H2O2 detection was performed by hydrogen peroxide detection kits; real-time PCR and WB were used to determine gene expression. Using the glioma cell line U251 and U87, we investigated a possible combination inhibitory effect includes metformin and cold atmospheric plasma (CAP). The combination treatment showed a synergistic inhibitory effect on cell viability, significantly inducing cell apoptosis. Furthermore, we also found H2O2 produced by CAP has an important role in the synergistic inhibitory effect, eliminating H2O2 with catalase reversed the synergistic inhibitory effect. In addition, the transcript and protein levels of c-FOS were robustly increased after co-treated with metformin and CAP. Taken together, we propose that pre-treatment of glioma cells with metformin sensitize tumor cells to CAP, which may serve as a potential therapeutic strategy for glioma.

Key words: metformin, cold atmospheric plasma, glioma, synergistic inhibitory effect

Glioma is the most common primary brain tumor and glioblastoma (GBM) is the most malignant form of glioma with 5-year survival rates of 5% [1]. The incidence of the tumor in China is 1–4/100,000 [2]. The 2016 World Health Organization classification of central nervous system tumors separates glioma into circumscribed gliomas (WHO grade I) and diffusely infiltrating gliomas (WHO grades II–IV) based on their pattern of histological feature and genetic mutant [3]. Chemotherapy combined with surgery and radiotherapy is a standard clinical treatment for patients [4]. Temozolomide (TMZ), is the first-line treatment for glioblastoma multiforme and second-line treatment for astrocetoma. TMZ is an alkylating agent that attenuates glioma through alkylating guanine at the O6 position along with additional contributions at the N3 and N7 positions of adenine [5]. However, accumulating evidence points toward resistance of GBM patients or no response to TMZ-based chemotherapy. Hence, there is still an urgent need to develop a new therapy to treat glioma.

Plasma is the fourth state of matter after solid, liquid, and gas, formed under high-temperature. The plasma with a room temperature called cold atmospheric plasma (CAP). CAP is composed of multiple reactive free groups including reactive oxygen species (ROS, such as hydroxyl radical (OH•), hydrogen peroxide (H2O2), ozone (O3), and superoxide (O2–)) and reactive nitrogen species (RNS, such as nitric oxide (NO•) and anionic (OONO•) and protonated (ONOOH) forms of peroxynitrite) [6]. In the past decades, CAP or CAM has been used in combination with some agents to attenuate tumors. Adachi et al. confirmed that histone deacetylase (HDAC) inhibitors have the potential to enhance the susceptibility of A549 to CAM [12]. Pre-sensitization of breast cancer cells with HSP90 inhibitor (PU-H71) followed by the treatment with CAP, synergistically induce cell death [11]. TMZ-resistant GBM cells restore sensibility after treat-
ment of CAP [13]. This year, FDA approved a clinical trial of cold plasma "scalpel" for cancer treatment. Thus, CAP has the potential to become an auxiliary physical therapy for chemotherapy.

Metformin (Met) is the most important drug for patients with type 2 diabetes mellitus (T2DM) [14]. metformin inhibits electron transport chain (ETC) and elevates AMP/ATP ratio, resulting in AMP-activated protein kinase (AMPK) activation and the mechanistic target of rapamycin complex 1 (mTORC1) inhibition, finally change cell progress [15]. In recent years, metformin has been used to inhibit the tumor cells proliferation directly or to increase the sensitivity of multiple tumors to chemotherapeutic agents [16–22]. In this study, we found that Met had a weak toxic effect on U251, U87 cell lines even in a high concentration of Met (16 mM), while CAP had an obviously dose-dependent toxic effect. In addition, pre-treated glioma cells with Met significantly enhanced the sensitivity of the cells to CAP. The combination therapy remarkably induced cell death. In a further study, upregulation of c-fos was observed in combination therapy, however, the role of c-fos in synergistic effect needs to be further explored. Our findings suggest that CAP combined with Met may serve as a potential therapy for glioma patients.

Materials and methods

Cell culture and plasma treatment. U251 and U87 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in high glucose Dulbecco’s minimum essential media (DMEM, HyClone) supplemented with 10% fetal calf serum (FCS) and 100 units/ml penicillin, 100 mg/ml streptomycin (Gibco). Cells were cultured at 37°C in an incubator (Thermo Fisher Scientific) containing 5% CO2. In total, U251 cells and U87 cells were seeded for 12 h in 96-well plates and then used in experiments. Cells were treated with the indicated concentrations of metformin. ddH2O was used as vehicle control. The atmospheric pressure argon plasma jet kINPen 11 served as a reactive species-generating source and was operated at a frequency of 1 MHz, a voltage of 3 kV with a feed gas flux of 4 standard liters/min. The distance between the CAP source and the bottom of the plates was fixed at 3 cm in height. Cells were exposed to CAP for the indicated times.

Cell viability. For the Met or CAP treatment only, cells were treated with indicated concentrations or exposure times and further incubated for 24 h or 48 h. For the combination group, cells were incubated with Met for 12 h, and further incubated for 12 h after exposure to CAP. Then fresh complete DMEM 100 µl with 20 µl Cell Titer-Blue Cell Viability Assay reagents (Promega) was added. The plate was incubated for 4 h at 37°C, and fluorescence was measured in a multimode plate reader (Thermo Fisher) at λem 560 nm and λex 590 nm. Cell viability was shown as the percentage of untreated control.

Flow cytometry. Cell apoptosis analysis was carried out by flow cytometry (BD). Cells were seeded in 12-well plates for 12 h and pre-treated with metformin (16 mM) for another 12 h. Then cells were exposed to CAP (30 s) and incubated for 12 h. After treatment, the apoptosis ratio was analyzed using an eBioscience Annexin V Apoptosis Detection Kit APC (Thermo Fisher) according to the manufacturer’s protocol.

ROS detection. 30 s after CAP treatment, the intracellular ROS and the H2O2 concentration in the medium were detected according to the manufacturer’s protocol. Briefly, for the ROS detection, DCFH-DA (ROS Assay Kit, Beyotime) was diluted with a serum-free medium at 1:1000 to a final concentration of 10 µM. Afterward, the culture medium was removed and the appropriate volume of diluted DCFH-DA was added. Incubation was made in a 37°C cell incubator for 20 minutes. The cells were washed three times with a serum-free cell culture medium to sufficiently remove DCFH-DA that did not enter the cells. The fluorescence was observed through the Olympus fluorescence microscopy. For the H2O2 detection, hydrogen peroxide detection reagent (Beyotime) was thawed on ice or an ice-water bath. 50 µl of treated culture medium was added to the detection well, and 100 µl of the hydrogen peroxide detection reagent to each well. Gently shake or beat to mix. Left at room temperature (15–30°C) for 30 minutes, and then measured A560 immediately on a multimode plate reader (LEICA).

qRT-PCR. After treatment (described in Flow cytometry), cells were washed once with ice-cold PBS and total RNA was extracted from cells with 0.2 ml of RNAiso Plus reagent (TaKaRa). The preparation of cDNA and reverse transcription-polymerase chain reaction (RT-PCR) were performed according to the manufacturer’s protocol. Changes in mRNA expression were measured by using the SYBR Green Realtime PCR Master Mix gene expression assay (TOYOBO). Real-time PCR was performed on Applied Biosystems 7500 Real-time System (Applied Biosystems) using primers shown in Table 1.

Western blotting. Cells were washed twice using ice-cold PBS and lysed in RIPA lysis buffer (WB3100, NCM Biotech)

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Table 1. Primers for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>GADD45β</td>
<td>F: CGCCCTGGCGCAGCTCCTG</td>
<td>R: AGGGCAGGGTAATGGGTT</td>
</tr>
<tr>
<td>c-Fos</td>
<td>F: TGCCCTCTCCTCAATGACCCTGA</td>
<td>R: ATAGGTCCATGTCTGGCACGGA</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: AGTGTGACGTGGACATCCGCA</td>
<td>R: ATCCACATCTGCGGAAGGTCGAC</td>
</tr>
<tr>
<td>c-jun</td>
<td>F: AGGGGCAGGGTAATGGGTT</td>
<td>R: ATCCACATCTGCGGAAGGTCGAC</td>
</tr>
<tr>
<td>HSPA6</td>
<td>F: CTTCCATGAAGTGGTTCACGA</td>
<td>R: CTCCGTCATCTGCGACAGGGA</td>
</tr>
<tr>
<td>PPM1A</td>
<td>F: AGGCCCAGGGTAATGGGTT</td>
<td>R: CTTCCATGAAGTGGTTCACGA</td>
</tr>
</tbody>
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supplemented with protease inhibitors and phosphatase inhibitors for 30 mins on ice followed by centrifugation at 12,000 g for 15 min and supernatants were collected. The protein concentration was quantified by the BCA Protein Assay Kit (23227, Thermo Scientific). Proteins (10 µg) per lane were resolved by SDS-PAGE and transferred to NC membrane (GE Healthcare Life Sciences). Non-specific binding sites were blocked using 1× TBS containing 0.05% (v/v) Tween 20 and 5% (w/v) skimmed milk for 1 h at room temperature (RT). After washing, the membranes were incubated with the indicated primary antibody HSPA6 (A7688, 1:1000) and c-FOS (A16641, 1:1000) from ABclonal, PARP (#9532, 1:1000 Cell Signaling Technology), β-actin (HC201, 1:1000, TransGen Biotech) overnight at 4 °C, washed and further incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (1:5000, Sangon Biotech) at RT for 1 h. Visualization of reactive protein bands was performed using ChemiScope 6000 Touch (Clinx).

**Statistical analysis.** Prism 8.0 software was used for statistical analysis. The values were presented as the mean ± SD. Statistical analyses were performed using Student’s t-test. The analysis of multiple groups was performed with one or two-way ANOVA with an appropriate post-hoc test.

**Results**

**Metformin pre-treatment enhances the inhibitory effects of CAP to glioma cells.** To explore the inhibitory effects of CAP or Met on tumor cells, glioma cell lines U251, U87 were exposed to CAP for variable time (10, 20, 40, 60 s) or concentrations (2, 4, 8, and 16 mM) of Met for 24 h or 48 h, respectively. U251/U87 cells were plated in 96 well, the cell density of U251 was 1×10^4, U87 was 0.5×10^4. The value CI<1 means synergy. The data represented as the mean±SD from 3 independent experiments. *p≤0.05; **p≤0.001; CAP – cold atmospheric plasma; Met – metformin

Figure 1. Inhibition of cell viability by metformin, CAP only, or combination. A, B) CAP inhibited Cell viability in a dose-dependent way. C, D) Cell viability of two glioma cell lines upon 24 h or 48 h treatment with metformin (0, 2, 4, 8, and 16 mM) or CAP exposure (0, 10, 20, 40, and 60 s). E, F) The viability of cells treated with metformin combine with CAP. Pretreated glioma cells with metformin (0, 2, 4, 8, and 16 mM) for 12 h followed by exposure to CAP (0, 10, 20, and 30 s), Cell viability was measured at 12 hours after CAP treatment. G, H) Synergy was analyzed by CompuSyn software. U251/U87 cells were plated in 96 well, the cell density of U251 was 1×10^4, U87 was 0.5×10^4. The value CI<1 means synergy. The data represented as the mean±SD from 3 independent experiments. *p≤0.05; **p≤0.001; CAP – cold atmospheric plasma; Met – metformin
density of U251 was 1×10⁴/well, U87 was 0.5×10⁴/well. Using the Cell Titer-Blue Cell viability assay, the effect of different CAP exposures and Met dose on glioma cell viability was investigated. For the CAP treatment, an exposure time-dependent but not the incubation time-dependent reduction in viability was observed in both cell lines. Moreover, CAP has a more obvious inhibitory effect on U87 compared to U251 (Figures 1A, 1B). For the Met treatment, Met has a weak toxic effect on both cell lines in a dose-dependent manner and irrespective of exposure time (Figures 1C, 1D). Based on current observations and previous reports that Met could sensitize tumor cells to multiple chemotherapeutic drugs [16, 18, 19], we tried to ask whether Met could sensitize glioma cells to CAP. Therefore, glioma cells were pre-treated with Met for 12 h and then exposed to CAP for specific timelines (10, 20, and 30 s). Figure 2 shows the effect of the combination on normal cells and the relationship between the cell density and the effect of the combination. Mouse primary astrocyte cells at a density of 1×10⁴ were selected as the nonmalignant control group (Figure 2A). Although a combination of Met and CAP suppressed the astrocyte cells viability, the extent of inhibition was lower than the effect on U251 and U87 (Figures 2B, 2C) especially in the low concentration of Met (2 and 4 mM) and 30 s CAP.

U251/U87 glioma cell lines were chosen for study at different densities. At the cell density of 1.5×10⁴, the inhibitory effect of the combination was weakly decreased compared with CAP or Met treatment alone on both cell lines. At the U251 cell density of 0.5×10⁴ (Figure 2B, left panel), the inhibitory effect of the combination was weakly decreased compared with CAP alone. Conversely, at the U251 cell density of 1×10⁴ (Figure 1E), U87 cell density of 0.5×10⁴ (Figure 1F), a combination showed a more obvious inhibitory effect compared to CAP or Met alone, respectively. These results illustrated that the combination effect depended on the density of the cells. Further analysis demonstrated a synergistic effect of Met and CAP in the combination treatment (Figures 1G, 1H). These findings demonstrate that CAP leads to synergistic or additive toxicity in glioma cells sensitized with Met.

**Combined treatment significantly induces glioma cell apoptosis.** Met treatment alone had no effect on cell morphology, while cell antennae were recovered after CAP treatment in U251, U87 cells (Figures 3A, 3B, third panel, arrows). This also confirms previous studies that CAP can inhibit cell migration and promote cell detachment [7, 23]. However, combination Met and CAP can significantly change cell morphology, producing a death-like phenotype (Figures 3A, 3B, fourth panel). Apoptosis was tested by flow

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**Figure 2.** The effect of a combination on normal cells and the relationship between the cell density and the effect of a combination. A) The combination effect on mouse primary astrocyte cells in the cell density of 1×10⁴, cell viability was tested by CellTiter Blue kits. The combination effect on different cell density of U251 (B) and U87 (C). *p<0.05; **p<0.01; ***p<0.001
cytometry analysis. A combination significantly induced cell apoptosis (FITC stain, Figures 3C, 3D). Apoptosis kinetics showed that after CAP treatment, the apoptosis rate of the combination group increased with the incubation time (Figures 3E, 3F). It seems that Met enhanced the vulnerability of glioma cells to CAP, and CAP induced cell death through the apoptosis pathway. Pretreated U251 (at the cell density of $1 \times 10^4$) and U87 cells (at the cell density of $0.5 \times 10^4$) with Met for 12 h, the cell-permeable pan caspase inhibitor (Z-VAD-FMK, 40 μM) was added to the culture medium 1 hour before CAP treatment and cell viability was not reversed after caspase inhibitor was added. This result demonstrates that combination-induced cell apoptosis may not through the caspase pathway.

$H_2O_2$ plays an important role in the synergistic effect. Previous studies have shown that CAP can increase the intracellular ROS and the concentration of hydrogen peroxide in the medium [24, 25]. To test whether it also has the same effect in this study, we measured intracellular ROS through the fluorescent probe DCFH-DA. Indeed, CAP significantly...
increased the intracellular ROS level in both cell lines (Figure 5A). Next, we measured the hydrogen peroxide concentration in the medium. The concentration of hydrogen peroxide was also found to be remarkably increased (Figure 5B). Based on changes in intracellular ROS level and hydrogen peroxide concentration in the medium, we proposed that hydrogen peroxide may have a role in the synergistic effect of Met and CAP. Therefore, we added catalase directly to the medium in the presence or absence of Met before CAP treatment. No wonder, after the addition of catalase the toxic effect of the combination was completely reversed and no change in cell morphology was observed (Figures 5C–5F). In addition, a similar synergistic killing effect was observed when the hydrogen peroxide was directly added after tumor cells were pretreated by Met (Figures 5C–5F). However, this phenomenon was not observed with hydrogen peroxide treatment alone. These findings indicate that CAP synergistically kills glioma cells through the production of hydrogen peroxide.

The combination of CAP and Met increases the expression of c-fos. Previous studies reported that the upregulation of c-fos, c-jun, GADD45A, HSPA6, and PPM1A had been constantly found in Met treated colorectal cancer [20]. Therefore, we also examined the expression of these genes in this study. A dramatic upregulation of HSPA6 was found in combination-treated U251 and U87 cells and a slight upregulation of GADD45A in U87 cells. Interestingly, combination therapy significantly increased the expression of c-fos in U251, U87 cells (Figures 6A, 6B). Next, we performed western blot to identify the changes in the expression of respective genes at the protein level. Although CAP only and combination therapy markedly increased HSPA6 mRNA, it had a little effect at the protein level in both cell lines. Consistently, the combination can increase the expression of c-fos at both mRNA and the protein level (Figure 6C). Moreover, after catalase was added to the combined group, the expression of c-fos was downregulated compared with the combined group. The combination of Met and hydrogen peroxide had a similar c-fos protein expression as the combination of Met and CAP (Figures 6D–6E). These findings further proved that hydrogen peroxide produced by CAP and Met has a synergistic killing effect.

Discussion

In the past decades, CAP has shown a broad-spectrum anti-tumor effect on different cancer types [6]. Although a low level of CAP can selectively kill tumor cells, high doses of CAP can also kill normal cells [26, 27]. Recent publica-

![Figure 5](image-url)
tions have determined the window of selectivity of CAP action, this window is defined by the concentration of H$_2$O$_2$ in CAP [28, 29]. There is accumulating evidence that CAP combined with chemical agents has a more deleterious toxic effect compared with CAP or chemical agents alone [8, 12, 13, 30]. In recent years, Met, the first-line drug for type 2 diabetes, has also exhibited a broad-spectrum anti-tumor effect [15]. Many studies showed that Met sensitizes tumor cells to chemotherapy drugs [16, 18, 19]. Based on these studies, we wonder whether the killing-effect of combined CAP with Met will be better than the CAP or Met only on tumor cells.

CAP changes cell membrane permeability or expression of certain transporters to facilitate the entry of drugs into cells [8]. Initial experiments were performed to determine the sequence of combination therapy in tumor cells. Preliminary results indicated that pre-treated glioma cells with CAP followed Met incubation for 24 h or co-treated with CAP and Met did not significantly decrease cell viability (data not shown). Inversely, pretreatment of the Met for 12 h robustly enhanced the sensitivity of tumor cells to CAP. A combination could significantly induce cell apoptosis. At least, those preliminary experiments showed that CAP did not increase the sensitivity of tumor cells to chemical drugs. Instead, pretreatment with Met changes the physiological state of the glioma cells, sensitizing cells to CAP.

In vitro, CAP does not directly contact with the cells due to the presence of a layer of medium between the cells and the CAP. CAP interacts with the culture medium to produce H$_2$O$_2$, NO, NO$_2^-$. Among diverse plasma-originated reactive species, H$_2$O$_2$ has been proved as the main anti-cancer reactive species participate in CAP-induced cancer cells death in vitro [7, 9, 24, 30]. But CAP-associated apoptosis was not directly induced by H$_2$O$_2$, it has been shown to be involved in at least three steps during CAP and PAM action. See references for more details [28, 29, 31, 32]. Consistent with these studies, the concentration of H$_2$O$_2$ in the culture medium significantly increased after CAP exposure in this study. At the same time, the intracellular ROS levels also raised after treatment of CAP (Figure 3), DCFH-DA-based fluorescence was not always reflecting the generation of ROS but may be related to heme or cytochrome c content [33–35]. The real ROS levels may be detected by DCFA-based ROS test kits using cell-permeable inhibitors/scavengers of DCFH-DA competitor. Next, we examined the role of hydrogen peroxide in a synergistic killing-effect. After catalase was added, the combination effect was totally reversed. Meanwhile, pre-treatment with Met and then supply with H$_2$O$_2$ had a similar synergistic killing effect with a combination of Met and CAP. Bauer et al. suggested that H$_2$O$_2$ and nitrite, two long-lived molecular species from CAP, triggers tumor cells to induce their own cell death [28]. We speculated that the synergistic killing-effect in our study was due to Met providing ROS or RNS to CAP, enhancing the CAP-associated cell death. Although Met has been reported as an antioxidant regent in diabetic patients [36], there was research suggested Met increased intracellular ROS [37]. The radical (OH or O$_2^-$)-induced oxidation...
byproducts of metformin did not contain ROS and RNS [38], this means that the interaction between CAP and Met may not contribute to the synergistically inhibitory effect. In freshly isolated mouse hepatocytes, Met treatment increased ONOO− production [39]. The Met-pretreated U251/U87 cells may produce ONOO−, this species involved in CAP-associated cell death.

In the previous study, Met suppressed the proliferation of colon cancer cells and induced a time-dependent metabolic and transcriptional alteration [20]. We looked for the changes in the expression of genes reported previously. However, those genes (JUN, PPM1A, HSPA6, GADD45A, and c-FOS) were not significantly increased at the transcriptional level after the treatment of Met in this study. Interestingly, the c-FOS transcript and the protein levels were significantly upregulated after a combination of Met and CAP. c-FOS has been reported to regulate retinal neuronal cell death andBrazilin induced T24 cell death [40, 41]. Therefore, the role of c-FOS in this study needs to be explored further. Meanwhile, experiments also need to be performed to determine the effect of the combination of metformin and CAP in vivo. Because of the limitation of penetration depth, CAP was difficult to apply to solid tumors in vivo. Keidar et al. has developed a micro-CAP device that allows CAP to treat glioma in the mouse brain [10]. Miniaturization of CAP devices and implantable CAP devices may be a development direction for the treatment of brain tumors in the future.

In general, this study may provide a new method for gliomas therapy, but more substantial mechanisms need to be explored.

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