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Effect of metformin on mitochondrial function of glioma cells

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Abstract: *Metformin is a classic treatment for diabetes. In recent years, metformin has been shown to inhibit the growth of tumour cells in vitro and in vivo and to increase the sensitivity of radiotherapy and chemotherapy. This study investigated the effects of metformin on active clonogenic energy and mitochondrial function in human glioma cells U87 and U251 to provide effective data for further understanding the mechanism of metformin in the treatment of glioma. To investigate the effect of metformin on mitochondrial function in U87 and U251 glioma cells. The changes in mitochondrial membrane potential, reactive oxygen species (ROS) and oxygen consumption and respiration in U87 and U251 human glioma cells treated with metformin were determined.*

Keywords: *Metformin; U251 cells; U87 cells; mitochondria; cell proliferation*

1. Introduction

Gliomas are the most common type of malignant brain tumor, accounting for 40% of all intracranial tumors, and the main treatments are surgery, radiotherapy and chemotherapy[1]. The incidence of glioma increases with age, with the highest incidence in the age group 75 years and older[2]. The aging of the central nervous system is also increasing in the general population[3], and the aggressive and diffuse invasiveness of central nervous system tumors often leads to recurrence, tumors are limited by the blood-brain barrier and can only spread to other brain regions[4,5], finding effective anti-glioma drugs is an important research goal of glioma therapy.

Metformin is a classic drug for diabetes and is widely used because of its relative safety, anti-hyperglycaemic activity and weight loss[6]; the inhibitory effects of metformin on a variety of tumors and their potential mechanisms have been observed[7,8,9]. Studies have shown that the use of metformin improves overall survival and progression-free survival in patients with high-grade glioma[10], and metformin in combination with temozolomide has been found to synergistically inhibit glioma stem cell growth in vitro and in vivo and promote apoptosis[11]. The two main effects of metformin are the independent activation of AMPK regulation, which inhibits apoptosis through the AMPK-mTOR signaling pathway[12], and the effects on ATP production and oxygen consumption[13], and through the electron transport chain complexes in mitochondria[14]. This study analyzed the effect of metformin on the mitochondria of human glioma cells and provided important experimental evidence to analyze the mechanism of metformin in the treatment of glioma.

2. Materials and methods

2.1. PageCell viability assay

Cell viability (mitochondrial activity) was detected by the CCK8 reduction assay. Cells were seeded onto 96-well plates at densities of 5× 103 cells/well with three wells in each group (Metformin = 0, 1 mM, 5mM, 15mM, 25mM). Then the cells were incubated with CCK8 detection solution (CCK8:DMEM=1:9) at cell incubator for 2 h. CCK8 metabolism was quantitated spectrophotometrically at 450 nm in a Multi-Mode Microplate Reader (Thermo Fisher Scientific, MA, USA). The results are described as the percentage of CCK8 reduction, with the absorbance of the control group set at 100%.

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2.2. Cell Clone Formation

U87 and U251 cells were digested, counted and then inoculated into 6-well plates at a density of 200 or 250 cells/well. After the cells were attached to the wall, different treatments were performed according to the experimental groups, with 3 replicate wells in each group, and the solution was changed every 3-4 days. Fourteen days after the formation of clonal clusters, cells were fixed with 1 mL of well immunostaining fixative for 15 min. Stain with crystal violet staining solution for 15 min, recover the crystal violet staining solution, wash with ddH2O until the background is clear, then air-dry and take pictures. Clone formation rate = (number of clones in metformin-treated group/number of clones in non-metformin-treated group) × 100%.

2.3. Detection of mitochondrial membrane potential

After 48 hours of metformin treatment, the adherent cells were washed once with PBS and 1 ml of cell culture medium was added, then 1 mL JC-1(5 μL JC-1(200 x)) was added to 1 mL of JC-1 staining buffer (diluted JC-1) staining working fluid and mixed well. The cells were incubated at 37 ℃ for 20 min. After incubation at 37 ℃, the supernatant was removed and washed twice with JC-1 staining buffer. Add 2 mL cell culture medium, the medium can contain serum and phenol red. Laser confocal microscopy (Nikon A1R) was used.

2.4. Ros Detection

After metformin treatment, centrifuge with trypsin digestion, wash PBS twice, add DCFH-DA diluted in serum-free medium at 1:1 000 to a final concentration of 10 μmol/L. Cells were incubated in the incubator for 20 minutes in 37 ℃. Mix it upside down every 3-5 minutes to bring the probe into full contact with the cells. The cells were washed three times with serum-free cell culture medium to remove DCFH-DA completely. Run it through Flow cytometry.

2.5. OCR detection

After the cells were digested with trypsin, the cells were seeded on seahorse cell plate at a density of 2 × 104/ml, leaving more than 4 background wells and adding PBS only. After the cells were attached to the wall, the treatment group was treated with metformin at the final concentration of 25 mmol/L for 48 hours. One day before the machine, 200 μl sterile water was added into each hole of Seahorse probe plate and placed in 37 ℃, CO2-free incubator overnight. The next day, at ultra-clean Taichung, the cells were rinsed with a prepared Seahorse test solution, then 175 μL of Seahorse test solution was added to each well and placed at 37℃ CO2-free incubator for more than 30 min. 25 μL of final concentration of 1.5 μmol/L of drug A (oligomycin), 2 μmol/L of drug B (FCCP), and 1 μmol/L of drug C (rotenone/antimycin A) were added to the drug-adding holes of the probe plate cover, respectively. Set up the program on the computer, monitoring on the machine. Correction of protein concentration: after the end of boarding, cell plates were removed, hippocampal test fluid and drugs were aspirated, and cells were washed once with PBS, then 30 μl 1% Trixon-X 100 was added per well, and lysed at room temperature for 20 min, after mixing, 10 μL was sucked out from each hole to a new 96-well plate. BCA method was used to detect the concentration of protein in the hippocampus. 10 μL protein samples were added into the pore to record the serial number of the samples. The BCA working fluid is prepared according to the number of samples (test holes and sample holes) and mixed thoroughly. 100 μL BCA solution was added into the holes of standard holes and sample holes and incubated in 37 ℃ oven for 30 min. At the wavelength of 562 nm, the absorbance of each pore was detected, the protein concentration was recorded, and the protein concentration was input into the hippocampal calibration program, and the curve was calibrated.

2.6. Statistical analysis

All data were represented three or more independent experiments as mean ± SD. Statistical analyses were conducted by SPSS 20.0. One-way ANOVA (analysis of variance) was applied for multiple comparisons, whereas comparisons between two groups were performed using Student's t-test. $P < 0.05$ was considered as statistically significant.

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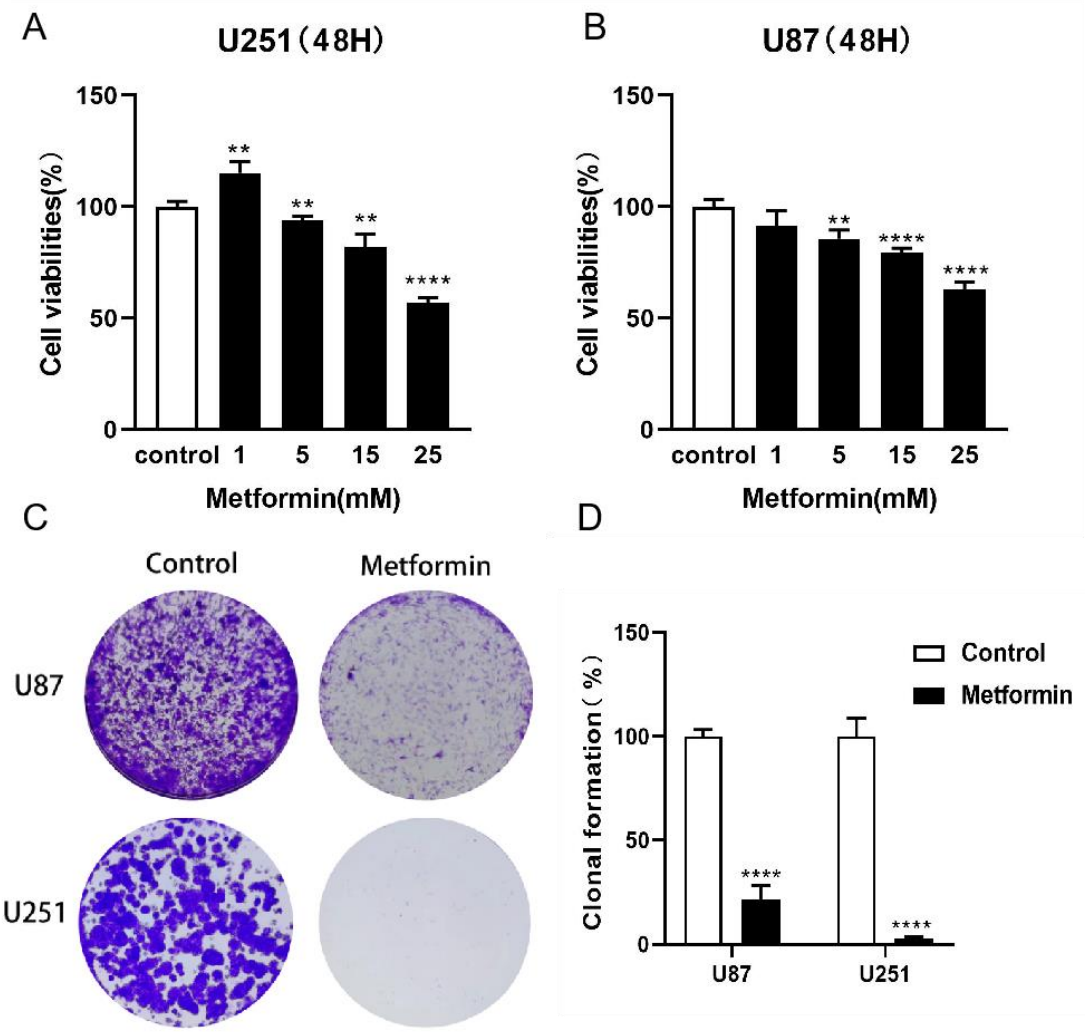
3. Results

3.1. Effects of metformin on the proliferation of U87 and U251 cells

CCK8 results showed that the activity of U87 and U251 cells treated with metformin for 48 h decreased with increasing metformin concentration, the difference was statistically significant ($p<0.05$). At 1 mmol/L metformin, U251 increased its activity, possibly because low doses of metformin increase cell activity (Figure 1 A-B).

Cloning experiments showed that metformin inhibited colony formation in U87 and U251 cells (Fig 1 C-D). Metformin may inhibit glioma cells.



*A-B:U87 and U251 cells were treated with different concentrations of metformin for 48 h;C-D:Effects of metformin on the clonogenic ability of U87 and U251 cells. Mean ± SD (n=3); **P<0.01, ****P<0.0001, vs. Control group.*

Figure 1: Effects of metformin on U87 and U251 cells

3.2. Effects of metformin on mitochondrial function in U87 and U251 cells

Metformin may inhibit glioma proliferation by partially affecting methionine metabolism, but it has been reported that most of the action of metformin is through the mitochondria. Under normal conditions, the inner mitochondrial membrane potential is high and remains at a negative potential, while the outer membrane potential is low and remains at a positive potential. In healthy cells, a small amount of green fluorescence can be seen, mostly red fluorescence; cells with depolarised mitochondria mostly show only green fluorescence. After metformin treatment, JC-1 was seen to shift from polymer to monomer, with a decrease in red fluorescence, an increase in green fluorescence in the cytoplasm and a decrease in membrane potential (Fig 2 A). The ROS content of the cells was measured by flow assay and it was found that ROS increased in 87 and decreased in 251 after metformin treatment (Fig 2 B).

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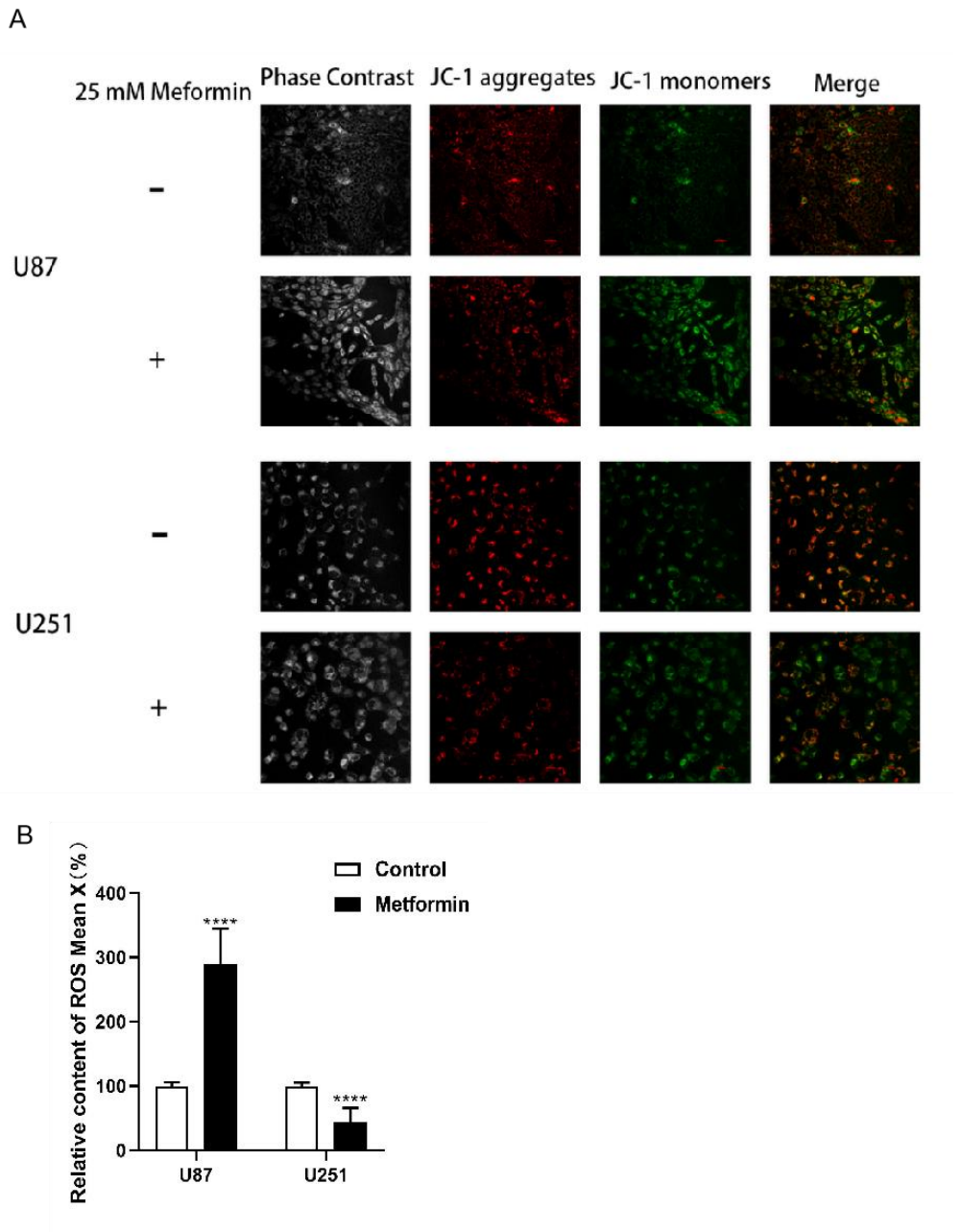

*A:The mitochondrial membrane potential of U87 and U251 cells treated with 25mM metformin for 48 h was photographed by laser confocal; B:ROS levels in U87 and U251 cells treated with 25mM metformin for 48 h were detected by flow-through; Mean ± SD (n=4); ****P<0.0001, vs. Control group.*

Figure 2: Metformin's effect on glioma Cellular mitochondrial effects.

3.3. Effect of metformin on mitochondrial oxygen consumption and respiration in glioma cells

The main metabolic pathways contributing to energy balance are glycolysis and mitochondrial respiration. Cellular aerobic respiration can be measured by the rate at which the cell consumes the extracellular oxygen concentration, known as the oxygen consumption rate (OCR). To confirm mitochondrial damage in the detection of cellular oxygen-consuming respiration, after U87 and U251 administration, basal OCR, maximal respiratory capacity and respiratory capacity were significantly decreased, while ATP production and ATP coupled oxygen consumption, mitochondrial respiration and non-mitochondrial respiration were significantly decreased, Compared with U87 and U251, U251 switched to enhanced glycolysis after the addition of mitochondrial inhibitors, whereas there was no significant change in the U87 group, but basal ECRA of U87 and U251 increased after the addition of metformin, cells may switch to glycolytic process to increase ATP production (P< 0. 0001) (Fig 3).

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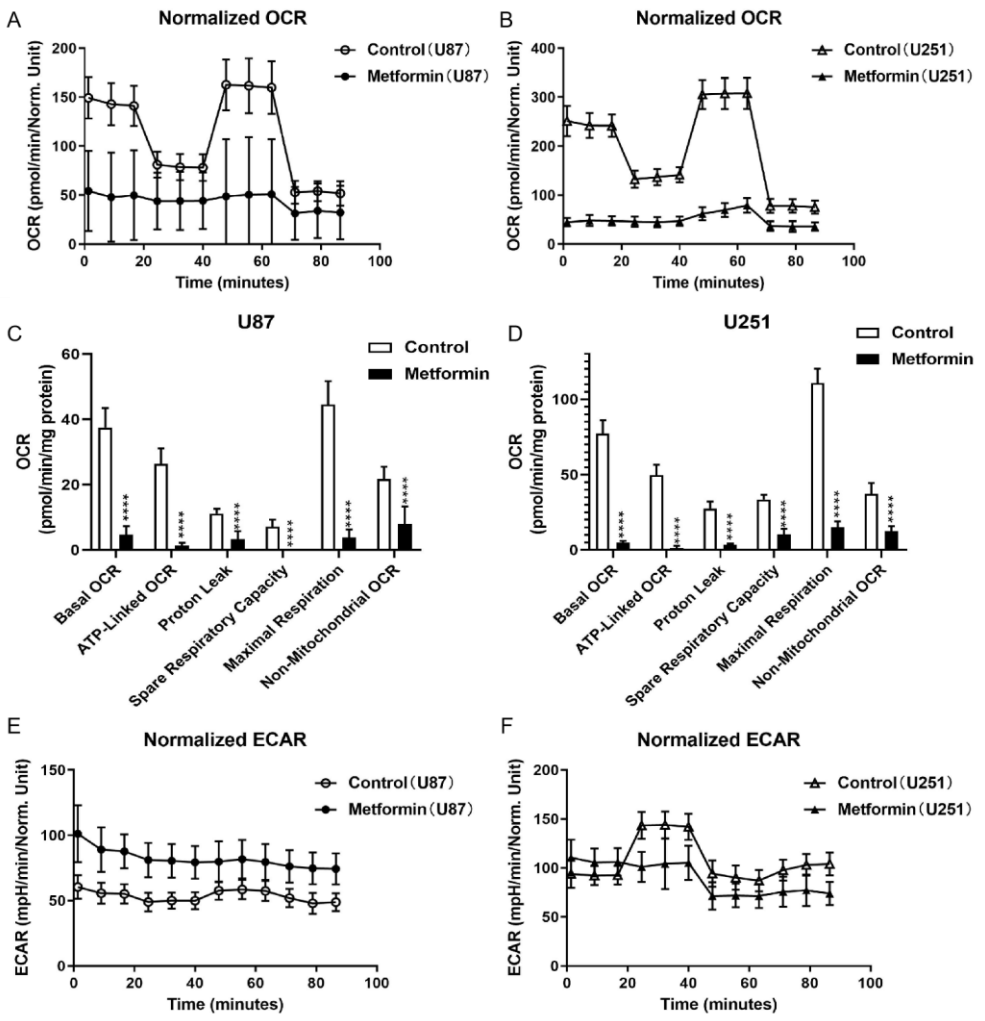

*A-B: 25 mM after metformin treatment of U87 and U251 cells for 48 h; C-D:The data of OCR of U87 and U251 cells treated with 25 mM metformin for 48 h were statistically analyzed; E-F:The changes of ECAR of U87 and U251 cells treated with 25 mM metformin for 48 h were compared. Mean ± SD (n=8); ****P<0.0001 vs. Control group.*

Figure 3: Effect of metformin on OCR and ECAR changes in U87 and U251 cells

4. Discussion

Glioma is difficult and complex to treat, so it is important to find a new treatment. New Use of Metformin as a hypoglycaemic drug, metformin was first discovered to have anti-tumour properties in 2005[15], a report suggested that metformin has dual anti-glioma effects in vitro on C6 rat and human U251 glioma cell lines[16]. The inhibitory effect of metformin on tumor cell growth has been demonstrated in a variety of tumors, but metformin has been reported to be suboptimal in early breast cancer clinical trials[17]; clinical trials of metformin face enormous challenges. The main pathways of metformin antitumour activity are inhibition of complex I in the mitochondrial respiratory chain or activation of adenosine monophosphate activated protein kinase (AMPK) mammalian target of rapamycin (mToR) targets[18], Metformin has also been found to inhibit fibulin-3 expression at the transcriptional level[19], reduce temozolomide resistance[20], induce cell cycle arrest and mitochondrial-dependent apoptosis in glioma cells[16,21], inhibit glioma proliferation through the AMPK-FOXO3 axis[22,23], and so on. Metformin can also be given in combination with simvastatin[24], diclofenac[25], statins[26], cisplatin[27], sorafenib[28], 5-fluorouracil[29], gefitinib[30]and gemcitabine[31].

To investigate whether metformin can inhibit glioma via mitochondria, the present study focused on mitochondrial membrane potential, ROS generation and OCR. In this study, CCK8 and cloning experiments showed that U87 and U251 exhibited a significant decrease in proliferation after metformin treatment, while mitochondria are important regulators of cellular energy and metabolism, it plays a vital

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role in maintaining cell growth and survival. The core function of mitochondria is to synthesise ATP through oxidative phosphorylation. This is known as mitochondrial bioenergetics. ATP production is linked to the mitochondrial membrane potential. ROS, mainly produced by mitochondria, and signaling dysregulation have been found to be associated with increased levels of ROS, including in glioma[32], and the literature has demonstrated the ability of glioma cells to survive in a highly stressful microenvironment[33]; making them more susceptible to damage caused by anti-tumor drugs, through oxidative therapy, induced ROS production, thus triggering their apoptosis[34]. Theoretically, metformin can inhibit mitochondrial superoxide dismutase activity and excessive ROS formation[27]. This experiment verified that metformin decreased mitochondrial membrane potential and promoted ROS formation in U87, but decreased ROS formation and significantly inhibited mitochondrial respiration in U251. While ROS production induced by metformin treatment was not consistent across different glioblastoma cell lines, other studies have shown similar results[35], with significant inhibition of cellular OCR and a significant reduction in basal oxygen consumption, the effect of inhibitor accelerator was attenuated, and ATP production from mitochondrial respiration, mitochondrial respiration and non-mitochondrial respiration were all reduced. Compared with the control group, the drug-treated group had a low and straight line. Combined with ECAR analysis, it was found that U251 switched to glycolysis after oligomycin inhibition and U87 did not switch to glycolysis after oligomycin inhibiting, and that after the addition of metformin, the cells could increase ATP synthesis by switching to glycolysis; This suggests that metformin may not have a significant inhibitory effect on cell glycolysis and that the main effect may be at the mitochondrial respiratory level.

5. Conclusions

The study of the mechanism of metformin not only plays an important role in glioma cells, but also in other tumors. Taken together, these results suggest that metformin, as an anti-tumor drug, may act mostly through mitochondria, but the specific mechanism remains to be further studied.

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