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Carnosine as a Glycolysis Modulator: Implications for Glioma Cell Growth Inhibition

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Abstract: *Carnosine is a dipeptide, which plays a variety of biological activities in different tissues, given its easy brain barrier permeability, and minimal side effects, indicating potential in glioma therapy. In this paper, the effects of carnosine on the proliferation and glycolysis of U87 and U251 cells were discussed. U87 and U251 cells were treated with 50 mmol/L carnosine for 72 h under normoxia (21% O_2) and hypoxia (1% O_2), cell viability, clonogenicity, and proliferation were assessed via MTT, clonogenic, and Edu assays. Glucose, lactate, ATP, G6P kits were used to detect changes in glucose, lactate, ATP and G6P content. Western Blotting assessed AMPK, p-AMPK, GLUT1, and PFK1 protein expressions. Intracellular glycolytic intermediates were analyzed via ^{13}C-labeled metabolic flux analysis. Carnosine can inhibit the glycolytic metabolism of glioma U87 and U251 cells, thereby inhibiting the proliferation of tumor, and its mechanism may be related to the reduction of the expression of glycolysis-related enzymes GLUT1 and PFK1, and the expression of p-AMPK.*

Keywords: *Carnosine; Glioma cells; Glycolysis; Metabolic flux analysis*

1. Introduction

Glioblastoma (GBM) is the most common malignant solid brain Glioblastoma multiforme in adults, accounting for about 80% of all brain tumors [1]. Although the standard dose of chemoradiation during the treatment procedure has brought strong toxic side effects to patients [2], the recurrence rate of the disease still exceeds 90%, and the 5-year survival rate is currently only 5.8%[3]. GBM is located in the blood-brain barrier (BBB), and the immune microenvironment is complex, unique and heterogeneous. Therefore, carnosine has shown unlimited potential as a natural small molecule drug that can cross the BBB with minor side effects. Carnosine is a kind of dipeptide, which exists in the state of amphoteric ions in physiological state. It exerts various biological activities in different tissues, these include anti-oxidation, anti-carbonylation, anti-glycosylation and as pH buffers[4].However, the effect of carnosine on glycolysis in glioma is unknown. This study discussed the effects of carnosine on the viability, proliferation and glycolytic metabolism of human glioma cells, and explored its possible mechanism, it provides new evidence for further understanding the systemic changes of carnosine in glycolytic energy metabolism.

2. Manuscript Preparation

2.1. Materials and methods

2.1.1. Materials

Dulbecco's modified Eagle medium (DMEM) was from GIBCO-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Clark Bioscience (Shanghai, China). Metformin, trypsin-EDTA Solution, BCA protein concentration detection kit, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) powder, 4% Paraformaldehyde Fix Solution, Immunostaining Permeabilization Solution with Triton X-100 and Trizol Reagent were purchased from Beyotime Institute of Biotechnology (Nanjing, China). Cell-Light EdU Apollo567 In Vitro Kit was from RiboBio (Guangzhou, China), Glucose test kit and Glucose-6-Phosphate(G6P) test kit were from Biyun Tian Biotechnology Institute(Shanghai,China), Lactic acid test kit was purchased from Kaiji Biotechnology(Jiangsu,China). Human glioma U-251MG (#TCHu 58, national collection of authenticated cell cultures, Shanghai, China)

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and U-87MG (#TCHu 138, national collection of authenticated cell cultures, Shanghai, China).

2.1.2. Cell culture and drug preparation

U87 and U251 cells were cultivated in a high-glucose medium supplemented with 10% FBS, and were subjected to treatments under normoxic (5% CO2) and hypoxic (1% O2) conditions respectively. Carnosine was dissolved in the dark and sterilized by filtration, then added to the cell culture solution at the designated concentration. The control group received an equal volume of drug-free medium. Both groups were cultured under their respective conditions for 72 hours.

2.1.3. Cell viability assay

Cell viability was assessed using an MTT assay. Cells (3×10^3/well) were plated in triplicate in 96-well plates. Post-treatment, cells were incubated with 0.5 mg/mL MTT for 4 h. After removing the supernatant, 100 μL DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a Multi-Mode Microplate Reader (Thermo Fisher Scientific). Results were expressed as %MTT reduction relative to controls set at 100%.

2.1.4. Cloning experiment

U87 and U251 cells were inoculated with 300 cells per well in 6-well plates. After the cells adhered to the wall, each group was divided into 3 multiple wells. The supernatant was washed with PBS, fixed with 4% paraformaldehyde solution at room temperature for 15 min, and stained with crystal violet for 30 min.

2.1.5. EdU incorporation assay

The EdU incorporation assay was performed with the Cell-Light EdU Apollo567 Kit on U87 and U251 cells seeded at 5000 cells/well in 96-well plates. After 24h incubation with 10 μM EdU, cells were fixed, permeabilized, and stained with Apollo® dye. Hoechst 33342 was used for nuclear counterstaining. Positive nuclei were identified under fluorescence microscopy, and the percentage of positive cells was calculated from at least ten random fields per well.

2.1.6. ATP content assay

The level of ATP was detected using the ATP Content Assay Kit. Protein concentrations in the samples were quantified using the BCA assay, and the average ATP content per cell was calculated based on the protein concentrations.

2.1.7. Glucose consumption assay

The glucose content was measured with Glucose Content Assay Kit (Beyotime) by following the manufacturer's protocol. Calculated the glucose content based on the standard curve, protein concentrations in the samples were quantified using the BCA assay, and the average glucose consumption per cell was calculated based on the protein concentrations.

2.1.8. Lactate assay

The level of lactate was detected using the Lactate Content Assay Kit (KeyGEN BioTECH). As directed by the manufacturer, both the supernatants and lysates of cultured cells were harvested to detect the extracellular and intracellular lactate content, respectively. Protein concentrations in the samples were quantified using the BCA assay. The average lactate content per cell was calculated based on the protein concentrations.

2.1.9. G6P assay

The level of G6P was measured with G6P Content Assay Kit (Beyotime) by following the manufacturer's protocol. Protein concentrations in the samples were quantified using the BCA assay. The average G6P content per cell was calculated based on the protein concentrations.

2.1.10. Stable-isotope tracing analysis

Take the experimental group and control group glioma cells U251 that have been treated with drugs, discard the supernatant, wash with PBS, and replace with 10% FBS culture medium containing U-[13C] d-glucose. Continue to culture for 24 hours. Discard the supernatant, wash it with 37℃ physiological saline, add mass spectrometry grade methanol, collect it with a cell scraper into an EP tube, and label it properly. Send to Metabo-Profile Biotechnology (Shanghai) Co., Ltd. for testing and analysis.

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2.1.11. Western blot analysis

Cells at 80%-90% confluence were treated with carnosine. Post-treatment, cells were washed twice with PBS, lysed in RIPA buffer with PMSF on ice for 10 min, then centrifuged at 14,000 g for 30 min at 4°C. Supernatants were collected and protein concentration determined using a BCA kit. Standard western blot protocols were followed. Antibodies included: β-actin (#db14040), AMPKα2 (#db11167), P-AMPK172 (#db2535), GLUT1 (#db384), PFK1 (#db58470), GAPDH (#db12905) from DiagBio, and HRP-conjugated goat anti-rabbit IgG (#dbA0208) from Beyotime Institute of Biotechnology.

2.1.12. Statistical analysis

All data were represented three or more independent experiments as mean ± SD. Statistical analyses were conducted by SPSS 20.0. Comparisons between two groups were performed using Student's t-test. $P < 0.05$ was considered as statistically significant.

2.2. Results

2.2.1. Effect of carnosine on U87 and U251 cell viability

U87 and U251 cells were exposed to carnosine (25, 50, 75 mmol/L) for 24, 48, and 72 h under normoxic and hypoxic conditions. MTT assay results (Fig. 1) showed increased cytotoxicity with higher carnosine concentrations and longer exposure times. Given physiological constraints and efficacy, a 50 mmol/L, 72 h treatment model was chosen. U87 cell viability fell to 75.5% ± 4.5% normoxia, 71.1±5.8% hypoxia; U251 viability dropped to 68.7% ± 4.1% normoxia, 82.2±4.7% hypoxia.

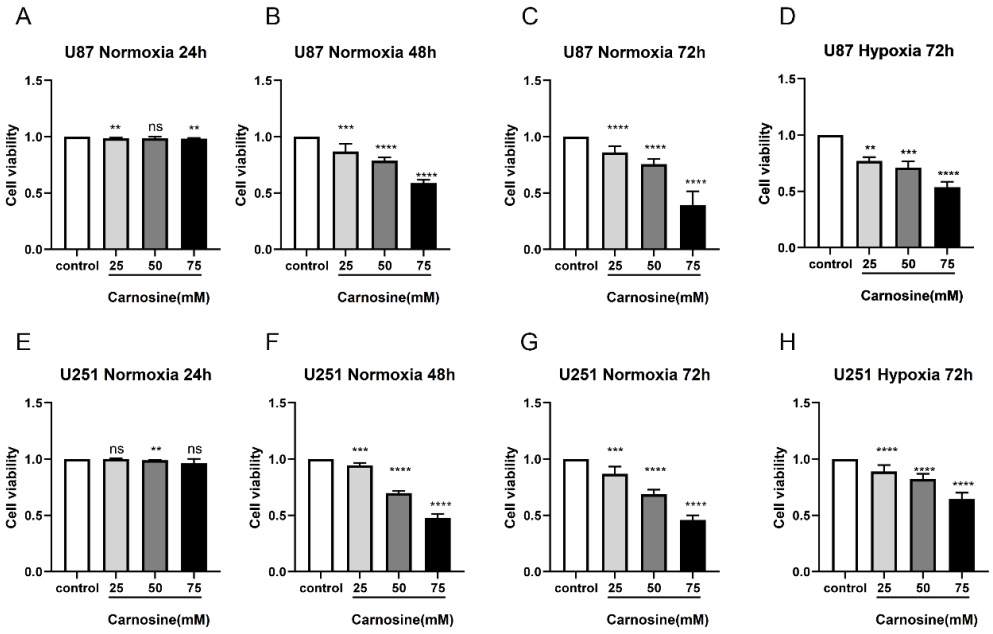

Figure 1: Effect of carnosine on U87 and U251 cell viability

2.2.2. Effect of carnosine on the clonal formation ability of glioma U87 cells and U251 cells

As shown in Figure 2, the cloning ability of U87 cells decreased to 52.4±%, 1.4 %, 39.1% ± 1.0% of the control group under normal oxygen and hypoxic conditions, respectively, and the cloning ability of U251 cells decreased to 70.0%± 1.5%, 83.7% ± 1.8%, respectively.

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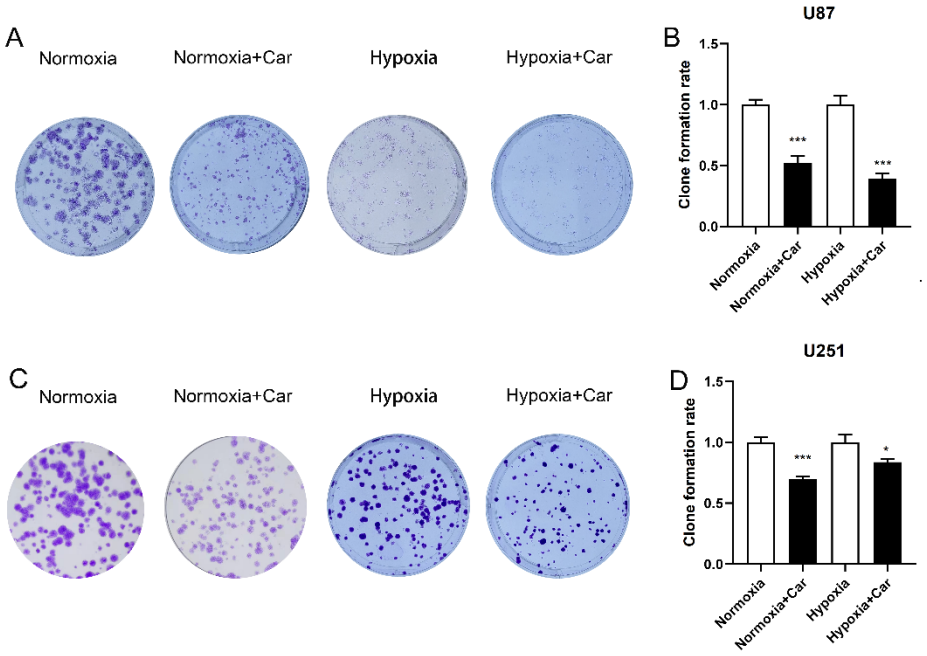
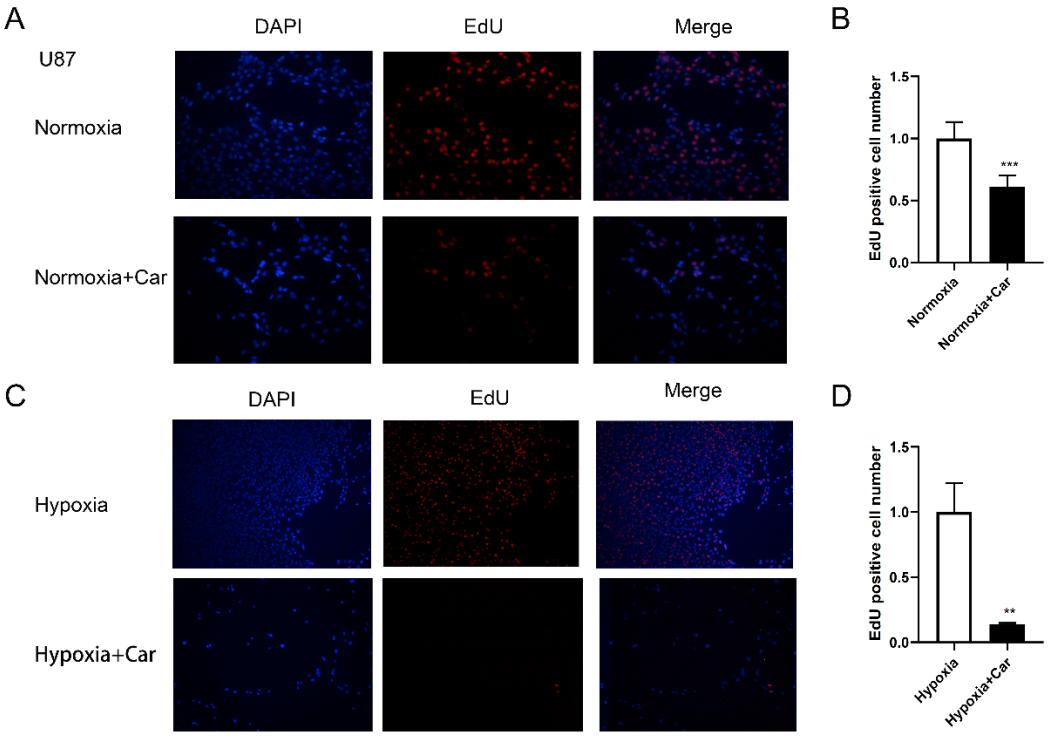


Figure 2: Effect of carnosine on the clonal formation of U87 and U251 cells.

2.2.3. Effect of carnosine on the proliferation of glioma U87 cells and U251 cells

The results of EdU showed that the number of EdU-positive cells in U87 cells decreased to 61.1% ±9.1% and 13.8% ± 1.1% in the control group under normal oxygen and hypoxia conditions, respectively, and the number of EdU-positive cells in U251 cells decreased to 55.7%±1.05% and 82.5% ± 1.5% under normoxia and hypoxia conditions, respectively, and the difference was statistically significant, as shown in Figure3.



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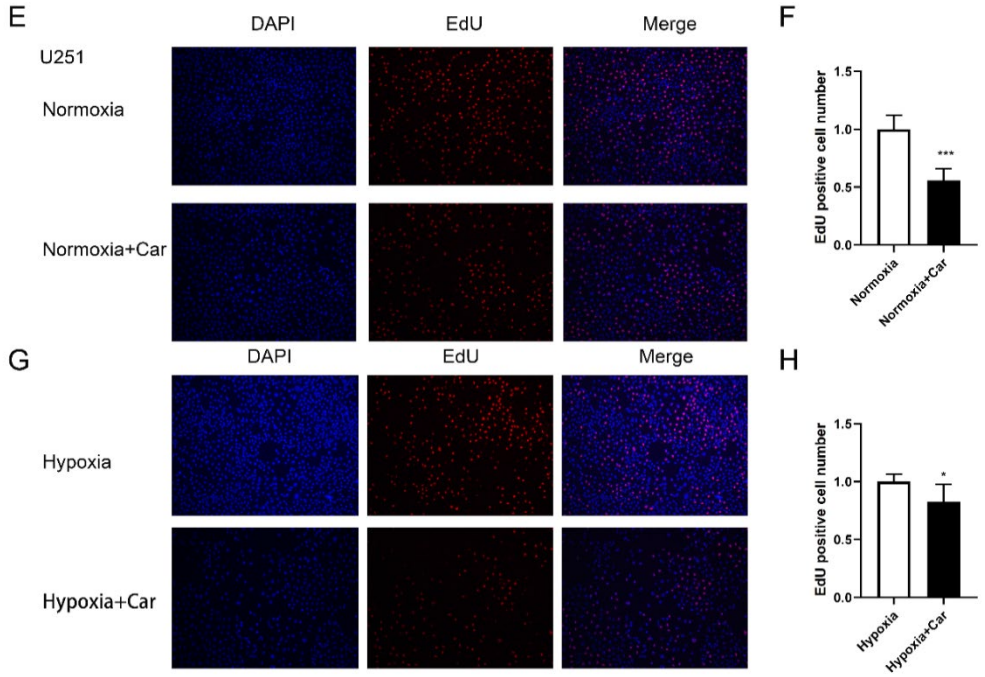

Figure 3: Effect of carnosine on the proliferation of U87 and U251 cells

2.2.4. Effect of carnosine on the ATP content of U87 and U251 cells under normoxic and hypoxic conditions

Following carnosine treatment, ATP levels in U87 cells declined significantly to 75.25% ± 7.6% under normoxia and 71.0% ± 14.3% under hypoxia, as depicted in Figure 4. In U251 cells, ATP levels dropped to 88.5% ± 3.2% under normoxia and sharply to 51.1% ± 11.1% under hypoxic conditions, as shown in Figure4.

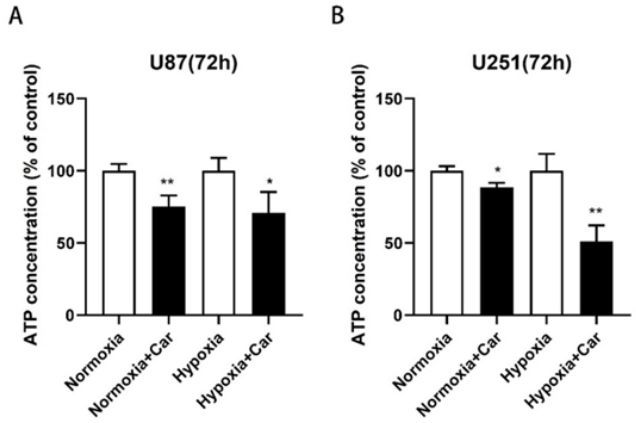

Figure 4: Effect of carnosine on the ATP content of U87 and U251 cells

2.2.5. Effects of carnosine on glucose consumption, glucose-6-phosphate and lactate production in U87 and U251 cells

Under normoxic and hypoxic conditions, U87 cell glucose consumption rose to 107.7%±9.7% and 199.0%±48.7% of controls, respectively. After 72h with 50 mmol/L carnosine, U251 cell glucose consumption escalated to 230.3±65.5% (normoxia) and 225.3%±35.7% (hypoxia), with significant differences, see Figure 5A and 5B.

Carnosine notably reduced lactate production in glioma cells under normoxia , as shown in Figure 5C and 5D, decreasing U87 and U251 cells to 43.8%±%, 23.7%, and 42.5%±14.5% of controls, respectively. Additionally, carnosine curbed U251 extracellular lactate content under normoxia (Fig. 5E, 5F), dropping to 68.4%±28.1% with significant changes, whereas U87 alterations were insignificant.

Glucose-6-phosphate content in U87 cells under normoxia and hypoxia dropped to 52.2%±12.5% and 74.8%±20.7% of controls, respectively. Post-carnosine treatment, U251 cells' glucose-6-phosphate

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content decreased to 14.9%±5.9% (normoxia) and 46.2%±10.8% (hypoxia) (Fig. 5G, 5H).

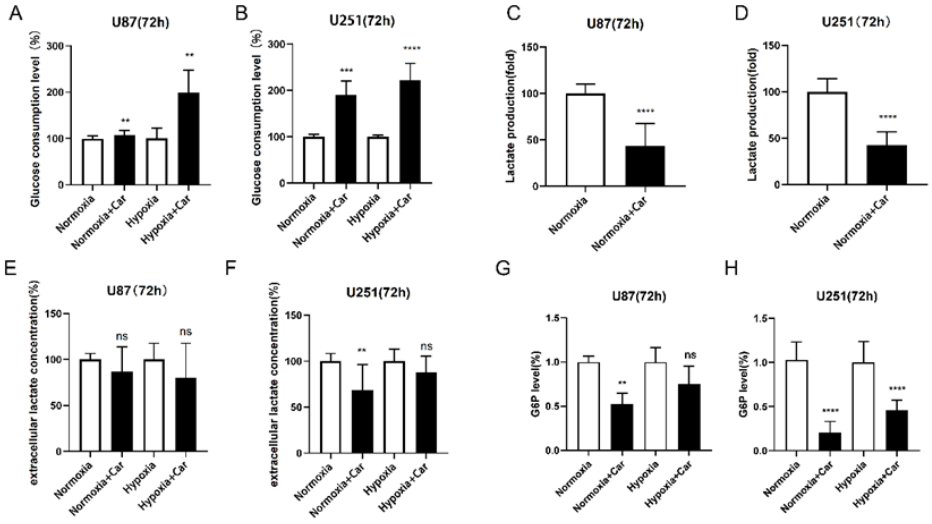

Figure 5: Effects of carnosine on glucose consumption, glucose-6-phosphate content, intracellular lactate content and extracellular lactate content in glioma cells.

2.2.6. Effect of carnosine on glycolytic metabolites in U251 cells

^{13}C isotope tracing metabolic flux analysis post 72 h 50 mmol/L carnosine treatment revealed a marked decline in glycolysis-derived M+3 pyruvate and lactate production in U251 cells. We noted a decrease in M+0 F1P and an increase in M+6 F1P, resulting in an overall reduction in total F1P levels under carnosine influence. Citrate, α-ketoglutarate, succinate, malate, and oxaloacetate synthesis in carnosine-treated U251 cells was significantly inhibited compared to the normoxic control, with isocitrate levels showing a particularly pronounced decrease. Glycolytic intermediate-originated aspartate and serine, along with glutamate and glutamine levels, were significantly reduced (P<0.05) (Fig. 6).

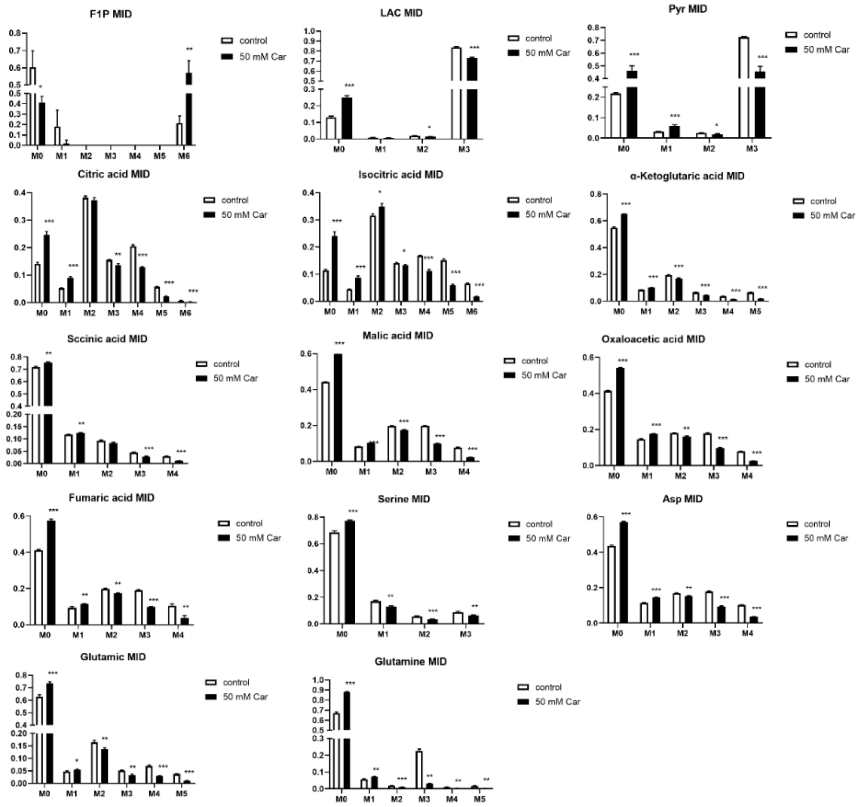

Figure 6: Effect of ^{13}C isotope tracer on metabolites of U251 cells

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2.2.7. Effect of carnosine on protein expression of GLUT1, PFK1 in U87 and U251 cells

The protein expressions of GLUT1 and PFK1 in glioma cells U87 and U251 were down-regulated after carnosine treatment under normoxia and hypoxia conditions, and the difference was statistically significant (Fig.7).

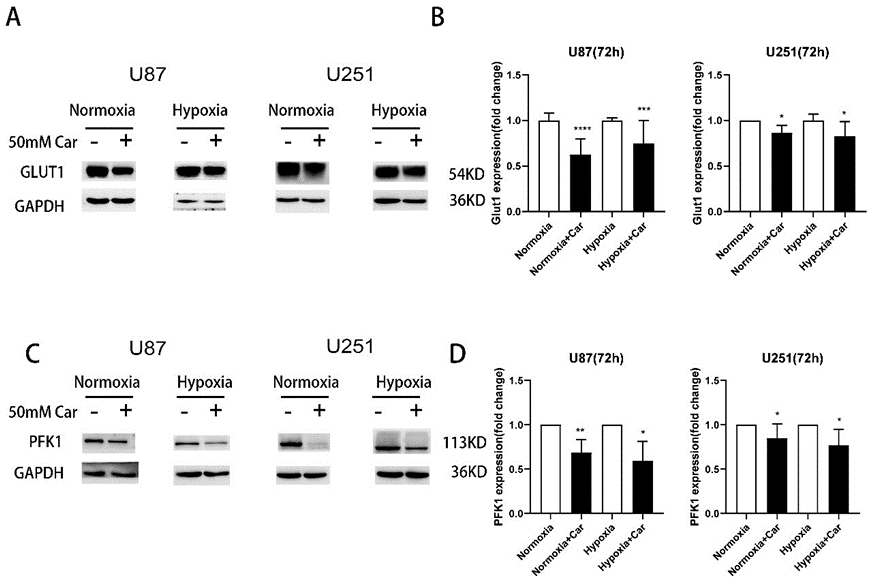

Figure 7: Effects of carnosine on protein expression of GLUT1 and PFK1 in U87 and U251 cells.

2.2.8. Effect of carnosine on AMPK and p-AMPK expression in U87 and U251 cells

The protein expressions of p-AMPK in U87 and U251 cells were down-regulated after 50 mmol/L carnosine treatment under normoxia and hypoxia conditions, and the difference was statistically significant (Fig.8).

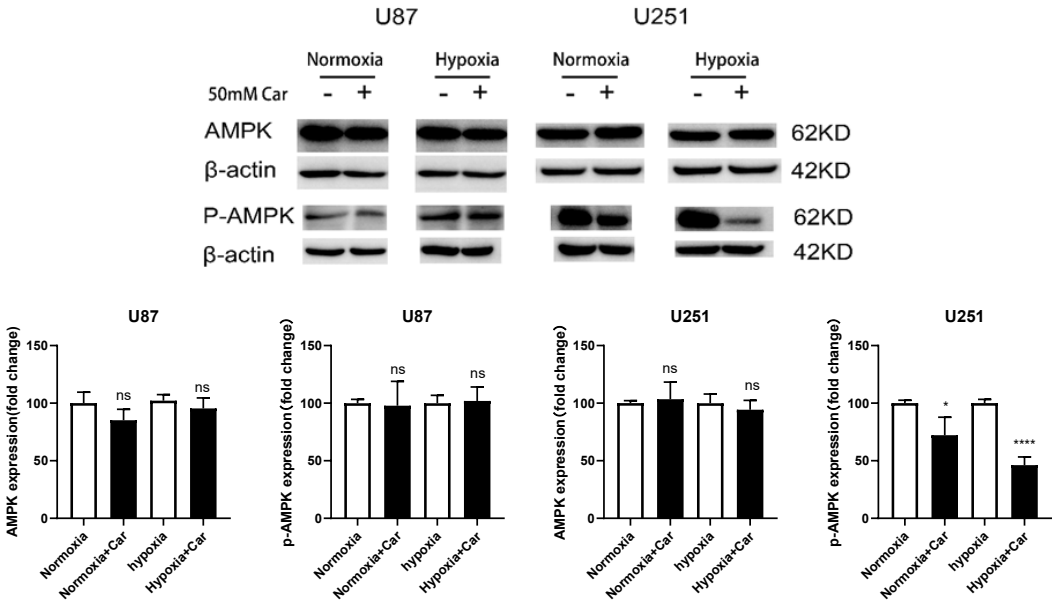

Figure 8: Effect of carnosine on AMPK and p-AMPK protein expression in in U87 and U251 cells.

3. Conclusions

In recent years, Carnosine has been found to exhibit anti-tumor effects, distinguished by its selective killing of transformed cells without harming normal cells [5], and its remarkable ability to cross the blood-brain barrier, being producible by glial cells [6]. These characteristics have propelled Carnosine forward

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as a highly promising natural small molecule anti-cancer agent, particularly in the context of gliomas. Recent studies have illuminated its inhibitory actions against gastric cancer [7], bladder cancer [8], and colorectal cancer [9], exerting its effects through mitochondria suppression, induction of autophagy, and apoptosis. This study ventures into uncharted territory by examining Carnosine's impact on glycolysis in U87 and U251 glioma cells, and how this influences cell proliferation. Our findings reveal that, under both normoxic and hypoxic conditions, Carnosine inhibits the viability, colony formation capacity, and proliferative ability of U87 and U251 cells in a dose- and time-dependent manner.

Tumor cells display a propensity for relying on glycolysis to fuel their metabolic needs, a phenomenon known as the "Warburg effect" [10]. Moreover, numerous studies emphasize that the majority of anabolic processes required for accelerated tumor growth are accomplished via heightened glycolytic activity [11][12]. Inhibiting glycolysis, therefore, holds significant implications for suppressing tumor proliferation. Evidence from literature suggests that Carnosine can impede the generation of ATP derived from glycolysis [13]. This experiment further corroborates that following Carnosine treatment in glioma cells, there is a reduction in intracellular ATP levels, alongside decreased concentrations of G6P (glucose-6-phosphate) and lactate, indicating a diminished supply of energy and substrates for cellular proliferation. These findings imply that Carnosine effectively inhibits the process of glycolysis in these cells.

Building upon the aforementioned experimental outcomes, we delve further into investigating whether carnosine influences cell proliferation by affecting enzymes related to glycolysis. Glucose transporter 1 (GLUT1), a pivotal protein in glucose uptake [14], is frequently overexpressed in malignant tumors [15] and has been correlated with cancer prognosis[16]. Research has revealed that elevated GLUT1 expression fosters proliferation in pancreatic cancer [17] and colorectal cancer cells [18], whereas downregulating GLUT1 suppresses proliferation in hepatocellular carcinoma and melanoma cells [19]. In this study, carnosine was found to significantly reduce the protein expression of GLUT1, which is consistent with the results of our G6P decrease. Phosphofructokinase-1 is a second rate-limiting enzyme involved in glycolysis and uses ATP to catalyze the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate[20]. Adenosine monophosphate-activated protein kinase (AMPK), a crucial cellular energy sensor and regulator of metabolic homeostasis, activates to phosphorylate downstream substrates, upregulates GLUT1, and modulates other rate-limiting enzymes of glycolysis upon activation [21]. Carnosine might inhibit glycolysis by suppressing the expression of GLUT1, PFK1, and phosphorylated AMPK (p-AMPK), thereby decreasing ATP production and restricting the energy supply to glioma cells, leading to impaired proliferation. Consequently, carnosine may inhibit glycolytic activity in U87 and U251 cells, in turn suppressing their cellular proliferation.

The changes in glucose metabolism in tumor cells are affected by a variety of factors, and focusing only on the changes in one aspect is not enough to accurately reflect the dynamic changes of the entire pathway. In this study, isotope-stable tracer technology was used for the first time to observe the changes of glucose metabolites in glioma cells after carnosine action. The results showed that carnosine inhibited the production of lactate and pyruvate from glycolysis of M+3 in glioma cells, but increased glucose consumption, which led to lactate production in tumor cells. The ratio of glucose consumption decreased, which was not conducive to the vital activity of the tumor, while we observed an increase in the production of fructose-1 phosphate. Studies have shown that the relative mRNA expression of glucokinase (GCK) in glioma cells increases after carnosine treatment[22], which may explain this phenomenon to a certain extent, but the exact mechanism needs to be further explored. However, this experiment did not detect a decrease in M+6 G6P, nor was a reduction in glycerol-3-phosphate levels observed, as reported in the literature[22]. This may be related to the fact that glycolytic metabolism reaches homeostasis in a shorter time than 24 hours[23].Carnosine not only inhibits glycolytic flux, but also affects the tricarboxylic acid cycle, which is consistent with the results of carnosine inhibition of mitochondrial function in gastric cancer cells reported by our research group in the previous stage. We also found that carnosine can inhibit the metabolism of aspartic acid, serine, glutamic acid and glutamine derived from glycolysis, which is consistent with the previous findings of the research group that carnosine can reduce the expression of LAT1 and GS and inhibit the metabolism of amino acids.

The results of this study suggest that carnosine is a potential antitumor drug that can inhibit glucose metabolism in human glioma cells under normoxic and hypoxic conditions. Targeting GLUT1 and PFK1 proteins, and reducing glycolytic flux.

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