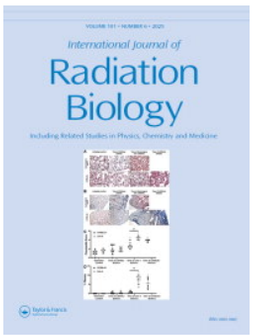

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ORIGINAL ARTICLE

Sensitive organelles of U251 MG glioblastomas to boron neutron capture therapy

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ABSTRACT

Purpose: The micro-distribution of boron compounds within cells influences the effectiveness of boron neutron capture therapy (BNCT) in killing tumor cells. Boron neutron capture therapy-sensitive organelles within the range of α particles and ^{7}Li recoil nuclei can enhance killing effects on tumor cells. However, comprehensive studies on the sensitive organelles to BNCT are currently lacking. In the present study, we explored the sensitivity of organelles in U251 MG glioblastomas to BNCT.

Materials and methods: Trypan blue exclusion assay, the level of autophagic proteins, cell counting kit-8, and clonogenic formation assay were used to evaluate the sensitivity of the cellular membrane, the endoplasmic reticulum, the mitochondria, and the cell nuclei of U251 MG glioblastomas to BNCT (^{10}B atoms of L-4-boronophenylalanin capture neutrons).

Results: Boron neutron capture therapy induced a trypan blue exclusion rate of 93.33%, no changes in the levels of LC3-II and Beclin-1, a survival rate of 77.78%, and reproductive death of 90.25% in U251 MG glioblastomas. These results indicate a hierarchical sensitivity of U251 MG glioblastoma organelles to BNCT ranked as the cell membrane (the endoplasmic reticulum) < the mitochondria < and the cell nucleus. Reproductive death, the main type of U251 MG glioblastoma death induced by BNCT, is attributed to the shortening of the S phase duration.

Conclusions: The comprehensive understanding of sensitive organelles within tumor cells to BNCT lays the foundation for significantly improving the efficacy of BNCT in killing tumor cells.

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BNCT; sensitive organelle; reproductive death; cell nuclear; cell cycle

Introduction

Glioblastoma is the most aggressive brain tumor in adults (Sheehan and Lee 2014). Despite the availability of various treatment options, including surgical resection, radiotherapy, and chemotherapy, the survival period for patients remains short (Cam et al. 2020; Mangel 2024). Boron neutron capture therapy (BNCT) primarily induces tumor cell death through a reaction where boron atoms capture neutrons. In this treatment, a patient is injected with a boron compound that selectively accumulates in tumor cells (Malouff et al. 2021). During subsequent neutron irradiation, ^{10}B atoms capturing thermal neutrons produce charged particles, i.e. alpha particles and ^{7}Li recoil nuclei. These two charged particles exhibit a higher linear energy transfer (LET) at the cellular level than traditional radiotherapy. In addition, the short range (5–10 μm) of the particles allows BNCT to target tumor cells while minimizing damage to normal brain tissue. Therefore, BNCT is considered an effective method for glioblastoma, particularly for cases of refractory recurrence (Miyatake et al. 2020).

Some clinical studies have demonstrated that BNCT can extend the survival of patients with malignant glioblastoma. For example, Kageji et al. (2011) reported a median survival of 19.5 months for 23 glioblastoma patients treated with BNCT. Among these patients, five survived for more than 3 years. The boron drugs used in this trial were sodium borocaptate (BSH) and L-4-boronophenylalanin (BPA). BSH offers significant advantages for BNCT: it contains a high concentration of boron (with 12 ^{10}B atoms per BSH molecule) and is highly soluble in water. However, its use has been limited due to poor cellular uptake. In contrast, BPA has a high uptake by cells but only contains a ^{10}B atom per molecule. Increasing the accumulation of ^{10}B in tumor cells is crucial for enhancing the effectiveness of BNCT. One strategy is to modify BSH to improve its cellular uptake. Specifically, FITC-BSH-encapsulated extracellular vesicles modified with hexadeca oligoarginine, a cell-penetrating

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peptide, induce macro-pinocytosis, leading to efficient cellular uptake and remarkable BNCT killing of C6 gliomas (Hirase et al. 2022). BSH encapsulation within cell membrane-derived vesicles has also been developed (Balboni et al. 2024).

In BNCT, the alpha particles and ^{7}Li recoil nuclei are the primary sources of energy deposition that kill tumor cells. The different intracellular distributions of the boron compound result in various killing effects on tumor cells. In other words, BNCT-sensitive organelles within the range of α particles and ^{7}Li recoil nuclei can enhance killing effects on tumor cells. By gaining a comprehensive understanding of these sensitive organelles within tumor cells, we have the potential to significantly improve the efficacy of BNCT by directing boron atoms to localize near these sensitive organelles. This presents another strategy for improving BNCT effectiveness. Consequently, it is crucial to assess the sensitivity of organelles to BNCT.

Currently, the research on the biological effects of BNCT remains limited, primarily due to the general unavailability of BNCT apparatus. Among these limited studies, most focus on reactor-BNCT-induced DNA damage, repair processes, and apoptosis in various tumor cell lines. Specifically, Chen et al. (2019) demonstrated that boric acid (BA)-mediated BNCT induces DNA double-strand breaks (DSBs) in Huh7 hepatocellular carcinoma cells, thereby activating the homologous recombination repair (HRR) pathway primarily for DNA repair. Additionally, BA-mediated BNCT induces apoptosis in Huh7 cells via caspase-3 activation. BNCT also causes DNA damage in the human thyroid follicular cancer cell line, leading to the increased expression of HRR repair enzymes Rad51 and Rad54 (Rodriguez et al. 2018). In human squamous carcinoma SAS cells, BNCT induces apoptosis 24 h after irradiation, with the cellular response involving the modulation of DNA repair proteins at the early post-irradiation stage (Sato et al. 2015). To date, comprehensive studies on the sensitivity of cellular structures to BNCT are still lacking.

In this investigation, we explored the sensitivity of the cellular membrane, the endoplasmic reticulum, the mitochondria, and the cell nuclear of U251 MG glioblastomas to BNCT. This therapy utilizes ^{10}B atoms of a BPA molecule, the clinically approved boron drug, to capture neutrons irradiated from an accelerator. And then, the mechanism of BNCT-sensitive organelles was elucidated. The results suggest that the organelles have different sensitivities to BNCT, which can be categorized into three levels: the cell membrane (the endoplasmic reticulum) < the mitochondria < and the cell nucleus. The reduction in duration of the S phase resulting in inadequate DNA synthesis contributes to the reproductive death of U251 MG glioblastomas, which is one of the mechanisms for the sensitivity of the cell nucleus to BNCT.

Materials and methods

Cell culture and experimental design

U251 MG glioblastomas were purchased from Wuhan Pricella Biotechnology Co., Ltd. (Wuhan, China). The cells were seeded in a 10-cm culture dish and cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Austin, TX). The medium was supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Austin, TX) and 1% penicillin–streptomycin, and the cultures were maintained at 37 °C in a 5% CO_2 atmosphere.

The exponentially growing U251 MG glioblastomas were divided into the following experimental groups: (1) *Blank group*: Cells without any treatment; (2) *BPA group*: Cells incubated with 54 ppm BPA for 3 h; (3) *Neutron group*: Cells irradiated with neutrons for eight minutes; (4) *BNCT group*: Cells irradiated with neutrons for eight minutes after being incubated with 54 ppm BPA for 3 h. Each experimental group included three replicates.

BPA administration and boron concentration detection

A stock solution of BPA (NBB-001; Neuboron Bio-SciTech Co., Ltd., Xiamen, China) with a concentration of 2280 ppm was prepared by dissolving 1 g of BPA in 20 ml of ultrapure water. This solution was then stored at −80 °C. Before neutron irradiation, the stock solution was diluted to 54 ppm using a complete culture medium and incubated with the U251 cells for 3 h.

The boron concentration in the U251 glioblastomas was measured using inductively coupled plasma mass spectrometry (ICP-MS; Agilent, Santa Clara, CA). The procedures were as follows: the BPA-incubated U251 cells were washed with PBS and then digested using a trypsin solution. After performing two rounds of centrifugation at 1200 rpm/min for 5 min, the resulting cell suspension was analyzed with a cell counter (Countess 3; Thermo Fisher Scientific, Austin, TX), adjusting the cell concentration to 5×10^5 to 1×10^6 cells per milliliter. Following another centrifugation, the cell pellet was collected. To lyse the cell pellet, 5 ml of an acid digestion diluent (1% HNO_3 and 1% Triton X-100 diluted in ultrapure water) was added and incubated for five minutes. The boron concentration in the lysate was then detected using the ICP-MS under the following conditions: radio frequency power at 1550 W, in no gas mode, with a sample extraction rate of 0.1 rps, a nebulizer gas flow rate of 1.15 l/min, and peak hopping acquisition mode. This analysis yielded a boron measurement of 146 ng per10^6 cells.

BNCT treatment of U251 MG glioblastomas

The U251 MG glioblastomas were incubated with 54 ppm BPA for 3 h before being irradiated with neutrons from the NeuPexTM Block-l accelerator-based BNCT system (Neuboron Therapy System Ltd., Xiamen, China). The thermal neutron flux was 2.07×10^9 neutrons per cm^2 per second. The physical dose rates for thermal neutrons, epithermal neutrons, fast neutrons, and gamma rays were 0.00031, 0.00015, 0.00011, and 0.0018 Gy/s. The physical doses for BNCT and neutrons were calculated using the NeuMANTA system (Neuboron Therapy System Ltd., Xiamen, China) and recorded as 2.09 and 0.8 Gy, respectively. The dosage of 2.09 Gy for BNCT was chosen based on pre-experimental results indicating it could induce a reproductive death rate

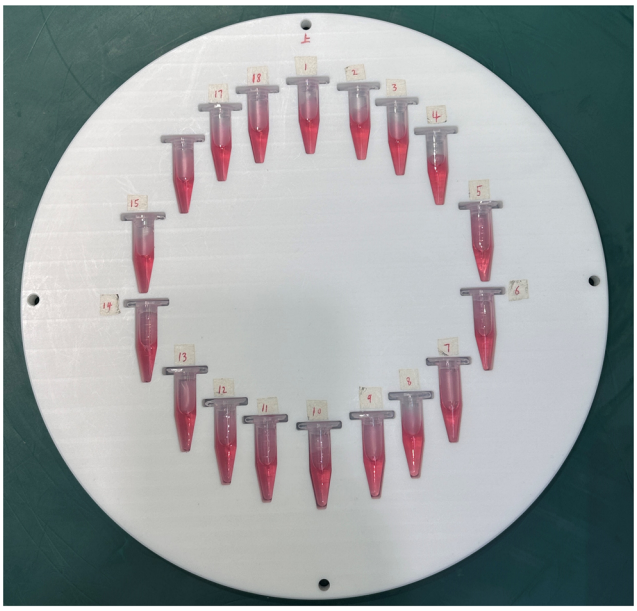

Figure 1. The fixing device for cells when they are irradiated with neutrons.

of 90% in U251 MG glioblastomas. The fixing device for the cell neutron irradiation is shown in Figure 1.

Trypan blue exclusion assay

Trypan blue solution (0.4%, Sbjbio, Nanjing, China) was added into U251 MG glioblastoma suspension at 24 and 72 h post-BNCT treatment, respectively. After a five-minute incubation, the cells were observed with a microscope (BZ-800X; KEYENCE Co., Osaka, Japan). The trypan blue exclusion rates were then calculated to analyze the integrity of the cell membrane. The formula of the trypan blue exclusion rate is the same as that used for the survival rate, i.e.

$$\text{Trypan blue exclusion rate}(\%) = \frac{\text{total number of cells} - \text{total number of blue cells}}{\text{total number of cells}} \times 100\% \quad (1)$$

Western blotting

The precipitates of U251 MG glioblastoma (48 h after BNCT treatment) were lysed on ice with RIPA buffer containing 1 mM PMSF. The cell lysates were centrifuged at 4 °C for 10 min (12,000 rpm/min). The protein supernatant was then transferred into a new cold tube. The protein concentration of the supernatants was detected using a Bicinchoninic Acid Kit (Pierce Biotechnology Inc., Rockford, IL). The sample with 20 μg of protein was resolved using 13% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked in Tris-buffered saline containing 5% nonfat milk and Tween 20 at room temperature for 4 h. Primary antibodies to detect microtubule-associated protein light chain 3 (LC3; 1:1000; ab51520, Abcam, Cambridge, UK) and Beclin-1 (1:1000; 3738, CST, Boston, MA) were incubated with the membranes overnight at 4 °C. The membranes continued to incubate with a horseradish peroxidase-conjugated secondary antibody (1:5000; ab6721, Abcam, Cambridge, UK) for 2 h. Protein detections were performed with an ECL kit (KeyGEN, Nanjing, China). GAPDH served as the internal loading control. The gray level of each band was quantified using ImageJ (National Institutes of Health, Bethesda, MD) (Mishra et al. 2017; Sule et al. 2023).

Cell counting kit-8 (CCK-8) detection

The cells were inoculated into a 96-well plate at a density of 1×10^4 cells per 100 μl per well, 48 and 72 h after BNCT treatment, respectively. The CCK-8 Kit (Solarbio, Beijing, China) was used to evaluate the survival rate of U251 MG glioblastoma according to the manufacturer's instructions.

Clonogenic formation assay

A clonogenic formation assay was used to evaluate reproductive death in BNCT-treated U251 MG glioblastoma (Franken et al. 2006). Six hundred cells were seeded into a six-well plate after BNCT treatment. Following an additional incubation period of eight days, the colony was observed with a phase contrast inverted microscope (BZ-800X; KEYENCE Co., Osaka, Japan). The formula for calculating the reproductive death rate is as follows:

$$\text{Reproductive death rate}(\%) = \left(1 - \frac{\text{total number of cell colonies}}{\text{total number of cell colonies in control group}}\right) \times 100\% \quad (2)$$

Cell cycle analysis

The cell cycle distribution was assessed on the fifth day post-BNCT. The cells were stained with propidium iodide and analyzed using a flow cytometer (BD, Franklin Lakes, NJ; Kim and Sederstrom 2015; Pozarowski and Darzynkiewicz 2004).

Statistical analysis

All data were presented as mean ± standard deviation (SD) and processed with GraphPad Prism 9.5 (GraphPad Software, San Diego, CA). Significant differences between groups were evaluated with a one-way analysis of variance, followed by a post hoc Student–Newman–Keuls test for multiple comparisons. A p value less than .05 was regarded as statistically significant.

Results

Sensitivity of the cellular membrane to BNCT

The trypan blue exclusion assay is a method used to assess the integrity of cellular membranes. Viable cells with intact membranes are impermeable to trypan blue, while cells with

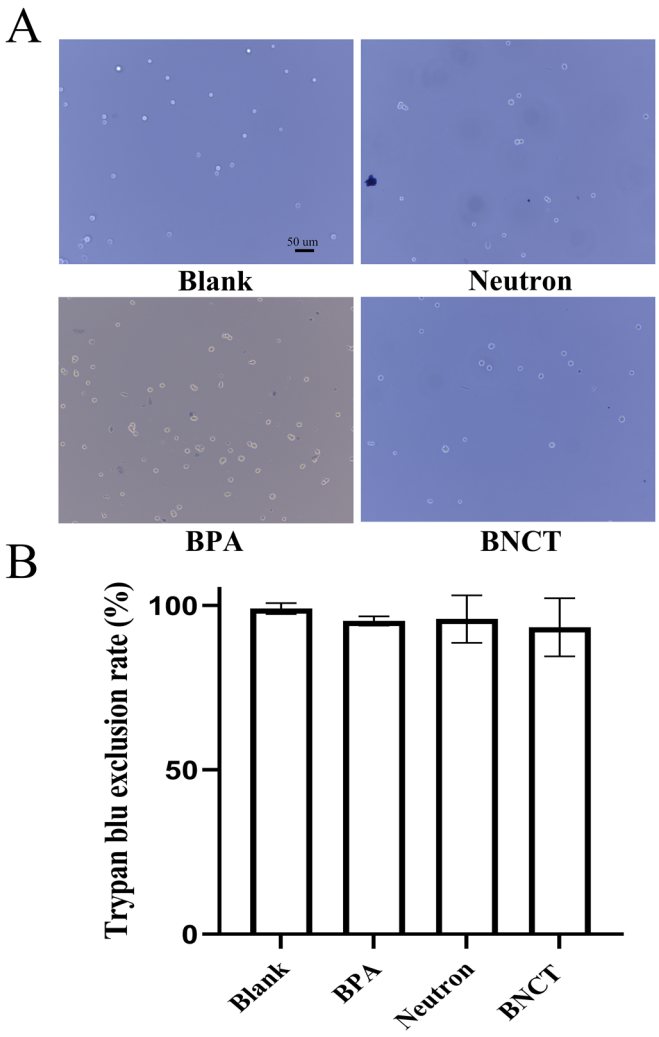

Figure 2. The trypan blue exclusion of U251 glioma cells 24 h after BNCT treatment (cells were irradiated with neutrons for eight minutes after they were incubated with 54 ppm BPA for 3 h, and the physical dose is 2.09 Gy). (A) The cell graph of trypan blue exclusion. (B) The trypan blue exclusion rate. The data were presented as the mean ± SD (n = 3).

compromised membranes become permeable and thus stained blue. We employed the trypan blue exclusion assay to evaluate the sensitivity of U251 MG glioblastoma cell membranes to BNCT. U251 MG glioblastomas were stained with trypan blue 24 and 72 h post-BNCT treatment. Figure 2 presents a trypan blue exclusion rate of 93.33% at 24 h after BNCT, while Supplementary Figure 1 presents an exclusion rate of 94.77% at 72 h post-BNCT. These results indicate that BNCT cannot significantly reduce the trypan blue exclusion rate. These data suggest that the cellular membranes of U251 MG glioblastomas are not sensitive to BNCT.

Sensitivity of the endoplasmic reticulum to BNCT

Autophagic cell death refers to cell demise induced by autophagic vacuoles, which are formed by cytoplasmic portions enclosing by a double-level membrane exfoliated from the ribosome-free regions of the endoplasmic reticulum (Dunn 1990; Cui et al. 2016). The level of the LC3-II protein is a widely recognized indicator for quantifying autophagy processes (Warnes 2014; Ghosh et al. 2023). Beclin-1 protein is involved in the formation and maturation of these autophagic vacuoles (Wang et al. 2007; Wang 2008; Kaur and Changotra 2020). Therefore, the levels of LC3-II and Beclin-1 proteins were assessed to evaluate the sensitivity of the endoplasmic reticulum to BNCT. Figure 3 illustrates the expressions of LC3-II and Beclin-1 proteins in U251 MG glioblastomas after BNCT irradiation using western blotting. The expression level of LC3-II reduced significantly 48 h after BPA or neutrons treatment. However, it remained unchanged following the BNCT irradiation. No significant change in the level of the LC3-II protein indicates that BNCT does not induce autophagic death of U251 MG glioblastomas. Similarly, the level of Beclin-1 protein showed no change, suggesting that no autophagic vacuoles are formed or matured during this process. Given that the ribosome-free regions of the endoplasmic reticulum are the source of the membrane for the autophagic vacuoles (Militello and Colombo 2011; Osawa et al. 2022), the observation that BNCT fails to form the autophagic vacuoles and subsequently does not induce autophagic cell death in U251 MG glioblastomas implies that the endoplasmic reticulum is insensitive to BNCT.

Sensitivity of the mitochondria to BNCT

The CCK-8 is a method used to detect cell proliferation based on WST-8 ((2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt)). The principle behind this method is that WST-8 can be reduced by mitochondrial NADPH dehydrogenase to form a highly water-soluble formazan product. The intensity of the resulting yellow color is proportional to the number of living cells. We utilized the CCK-8 assay to measure the survival rate of U251 MG glioblastomas post-BNCT treatment, aiming to examine the impact of BNCT on the NADPH dehydrogenase function and to assess the sensitivity of mitochondria to BNCT. Figure 4 illustrates the survival rates of U251 MG glioblastomas at 48 and 72 h after BNCT treatment. The survival rate of U251 MG glioblastomas 48 h post-BNCT was about 77.78%, which suggests that BNCT decreased the function of the NADPH dehydrogenase. This decrease in the enzymatic function indicates that the mitochondria are sensitive to BNCT. However, this sensitivity appears to be relatively low, as the survival rate remained close to 80%. By 72 h post-BNCT, the survival rate increased to about 96.70%. This recovery from 77.78% at 48 hours to 96.70% at 72 hours suggests a restoration of NADPH dehydrogenase function, supporting the notion that mitochondria have a relatively low sensitivity to BNCT. Additionally, we utilized Annexin V and propidium iodide-based flow cytometry to assess apoptosis in U251 glioblastomas 48 h after BNCT treatment. Our result shows that BNCT induced approximately 33.03% apoptosis (the most are the early apoptosis, Supplementary Figure 2). The death rate measured by the CCK-8 assay (the survival rate is 77.78% at 48 h) was similar to the observed apoptosis rate. Since both external and internal signals that trigger apoptosis are mediated through the mitochondria, this apoptosis rate indicates

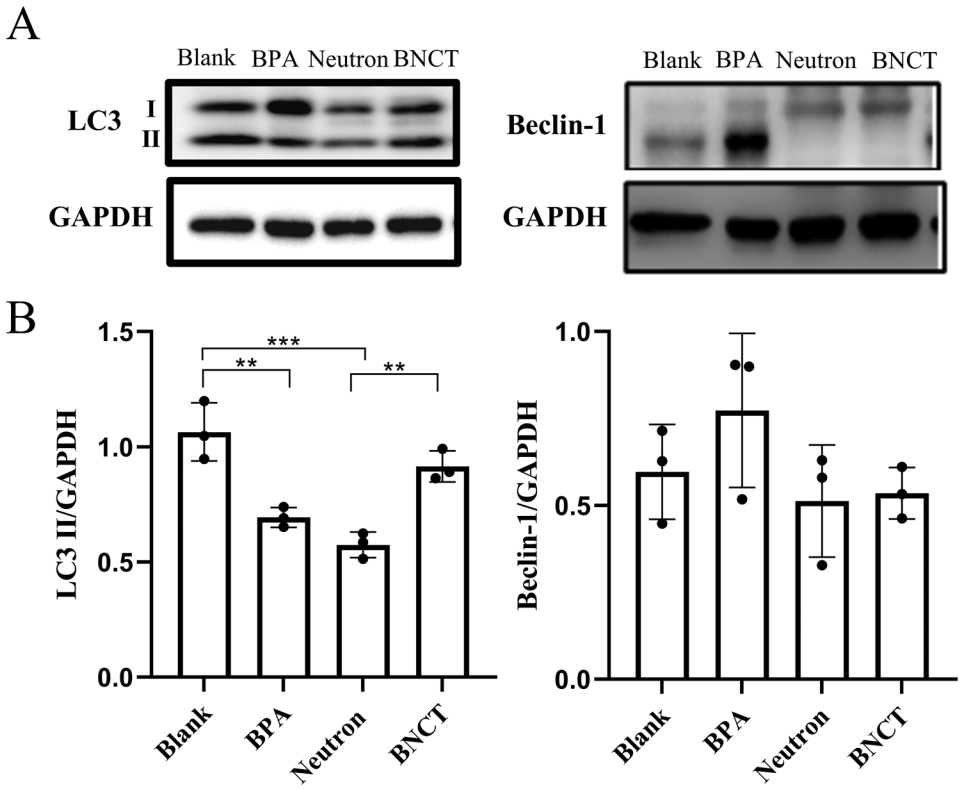

Figure 3. The level of autophagic proteins in U251 glioma cells 48 h after BNCT treatment (cells were irradiated with neutrons for eight minutes after they were incubated with 54 ppm BPA for 3 h, and the physical dose is 2.09 Gy). (A) The bands and the levels of LC3-II protein. (B) The bands and the levels of Beclin-1 protein. The data were presented as the mean ± SD (n = 3), $^{**}p < .01$, and $^{***}p < .001$ vs. Blank group.

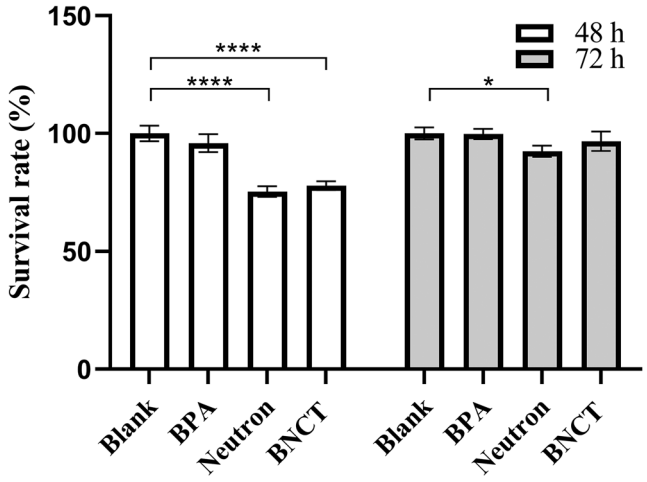

Figure 4. The survival rate of U251 glioma cells based on CCK-8 48 and 72 h after BNCT treatment (cells were irradiated with neutrons for eight minutes after they were incubated with 54 ppm BPA for 3 h, and the physical dose is 2.09 Gy). The data were presented as the mean ± SD (n = 3), $^{*}p < .05$, and $^{****}p < .0001$ vs. Blank group.

that mitochondria experienced only limited apoptosis-related changes. This further supports our claim that mitochondria have relatively low sensitivity to BNCT.

Sensitivity of the cell nuclear to BNCT

Reproductive death refers to cell demise resulting from disruptions of the nuclear division process. Cells experiencing reproductive death do not die immediately; they can continue their metabolic activities and may undergo several rounds of mitosis before ultimately stopping the nuclear division. The characteristic of this type of cell death is the loss of the cell's ability to proliferate. We assessed the sensitivity of the cell nuclei to BNCT by detecting the reproductive death rate of U251 MG glioblastomas using the colony formation assay. Figure 5 depicts the reproductive death rate of U251 MG glioblastomas after BNCT treatment. The BNCT-induced reproductive death rate in U251 MG glioblastomas can reach as high as 90.25%. This suggests that the cell nuclei are the sensitive organelle to BNCT and also indicates that reproductive death is the primary form of cell death induced by BNCT.

Reduction in the duration of the S phase

The process of cell division is known as the cell cycle, which consists of the G1, S, G2, and M phases. Among these, the G1, S, and G2 phases are interphase, while the M phase represents mitosis. We examined the cell cycle distribution of U251 MG glioblastomas using flow cytometry to explore the mechanism behind reproductive death. Figure 6 illustrates the cell cycle distribution of U251 MG glioblastomas on the 5th day after BNCT treatment. A significant reduction in the S phase duration was observed following BNCT irradiation, accompanied by a slight increase in the G0–G1 and G2–M phase durations, although this increase was not statistically significant. The reduction in the S phase duration

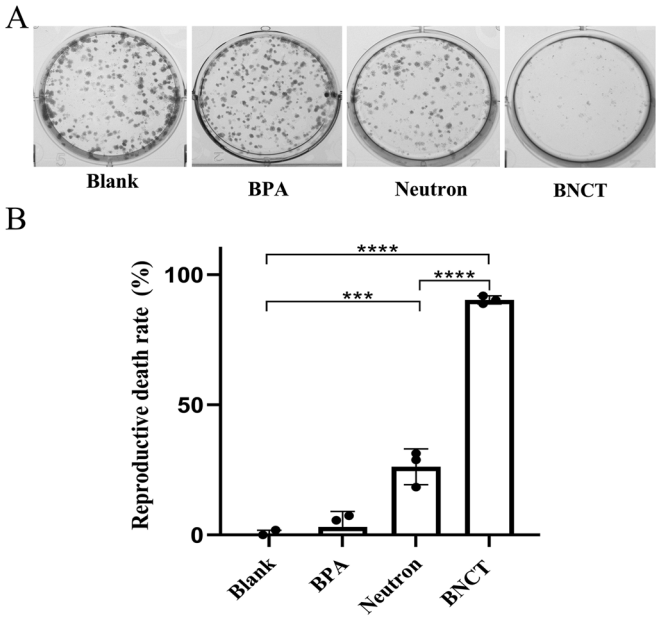

Figure 5. Reproductive death of U251 glioma cells after BNCT treatment (cells were irradiated with neutrons for eight minutes after they were incubated with 54 ppm BPA for 3 h, and the physical dose is 2.09 Gy). (A) The graph of the colony. (B) The reproductive death rate. The data were presented as the mean ± SD (n = 3), ***p < .001, and ****p < .0001 vs. Blank group.

suggests a decrease in DNA synthesis (Sluder and Hinchcliffe 1998; Wang 2021), which should contribute to the reproductive death observed in U251 MG glioblastomas.

Discussion

Compound biological effect (CBE) is a relative magnitude of BNCT killing effect on cells, which arises from variances in the micro-distribution of boron compound and radiation types (Dagrosa et al. 2011; Fukuda 2021). Theoretically, enhancing CBE could be achieved by regulating the micro-distribution of boron compound to near the BNCT-sensitive organelles. However, a prerequisite for this is the identification of the sensitive organelles to BNCT. In this study, we found that the cell nucleus of U251 MG glioblastomas exhibited the highest sensitivity to BNCT, mitochondria displayed a low sensitivity, and the cellular membrane and endoplasmic reticulum were identified as insensitive organelles.

Reproductive death occurs due to the disturbance in the nuclear division. The reproductive death of 90.25% in U251 MG glioblastomas (Figure 5) indicates that the cell nucleus is very sensitive to BNCT. Wu et al. (2025) have investigated the micro-distribution of ^{10}B atoms (BPA) in the U251 cell through neutron autoradiography. Their findings indicated that the particle tracks produced by the ^{10}B atom capturing neutrons were predominantly concentrated in the projected area of the cell nucleus. This result strongly supports our claim that the cell nucleus is the most sensitive organelle to BNCT. In addition, as DNA molecules predominantly exist in the cell nucleus, the conclusion is consistent with the point of view of DNA as a sensitive molecule to BNCT (Rodriguez et al. 2018).

Based on the CCK-8 method, the survival rates of U251 MG glioblastomas were 77.78% and 96.70% at 48 h and 72 h post-BNCT treatment, respectively (Figure 4). Zhang et al. (2013) reported cytoplasmic irradiation with 10 alpha particles induced the reduction in the activity of cytochrome *c* oxidase and succinate dehydrogenase in human small airway epithelial cells, and the activities of these two enzymes were gradually restored over time following alpha particles irradiation. These findings support our results that BNCT induced the decline in the function of the mitochondrial dehydrogenase, with a corresponding gradual recovery. Shimura et al. (2023) found that the mitochondrial signaling (AMP-activated protein kinase) and mitochondrial metabolic activity of normal human diploid lung fibroblasts TIG-3 and MRC-5 were unaffected by X-irradiation, which suggests that the mitochondria of these cells are not sensitive to X-irradiation. This result is close to the sensitivity of the U251 mitochondria to BNCT. Although the types of radiation differ, these results may suggest that the mitochondria are not an organelle with a high radiosensitivity.

The trypan blue exclusion rate of 93.33% indicates that the cellular membrane of U251 MG glioblastomas is not the BNCT-sensitive organelle (Figure 2). Membrane lipids are the primary component of the cellular membrane. Hydroxy radicals generated following photon irradiation can initiate lipid peroxidation of polyunsaturated fatty acids within these membrane lipids. Consequently, the cellular membrane becomes a sensitive organelle to photon radiation. However, the alpha particles and ^{7}Li recoil nuclei produced from BNCT differ from photon radiation; these are charged particles that are unable to generate significant quantities of water radiolysis products required to initiate lipid peroxidation of the cell membrane, which results in the insensitivity of the cellular membrane to BNCT.

The exfoliated ribosome-free regions of the endoplasmic reticulum are the source of the membrane for autophagic vacuoles. The failure of BNCT to form the autophagic vacuoles and subsequently induce autophagic death of U251 MG glioblastomas (Figure 3) suggests that the endoplasmic reticulum is also insensitivity to BNCT. Since both the cell membrane and the endoplasmic reticulum are membranous organelles, their sensitivity to BNCT can be considered similar. Furthermore, the conclusion regarding the insensitivity of the cell membrane and endoplasmic reticulum to BNCT may also apply to other membranous organelles, such as the Golgi complex.

We found that thermal neutrons reduced the level of LC3-II (Figure 3). Yasui et al. (2011) reported that exposure to fast neutrons (2 Gy) increased the number of autophagosomes in U251 and U87 cells, as shown by transmission electron microscopy. This difference could be attributed to the varying energies of the neutrons, which result in different LET. We speculate that thermal neutrons may activate certain targets that inhibit autophagy. The return of LC3-II to baseline level in the BNCT group could be due to the insensitivity of endoplasmic reticulum membranes to BNCT. Additionally, a decrease in the expression of LC3-II related to BPA was also observed (Figure 3). However, there is limited research on BPA's effects on autophagy, making it

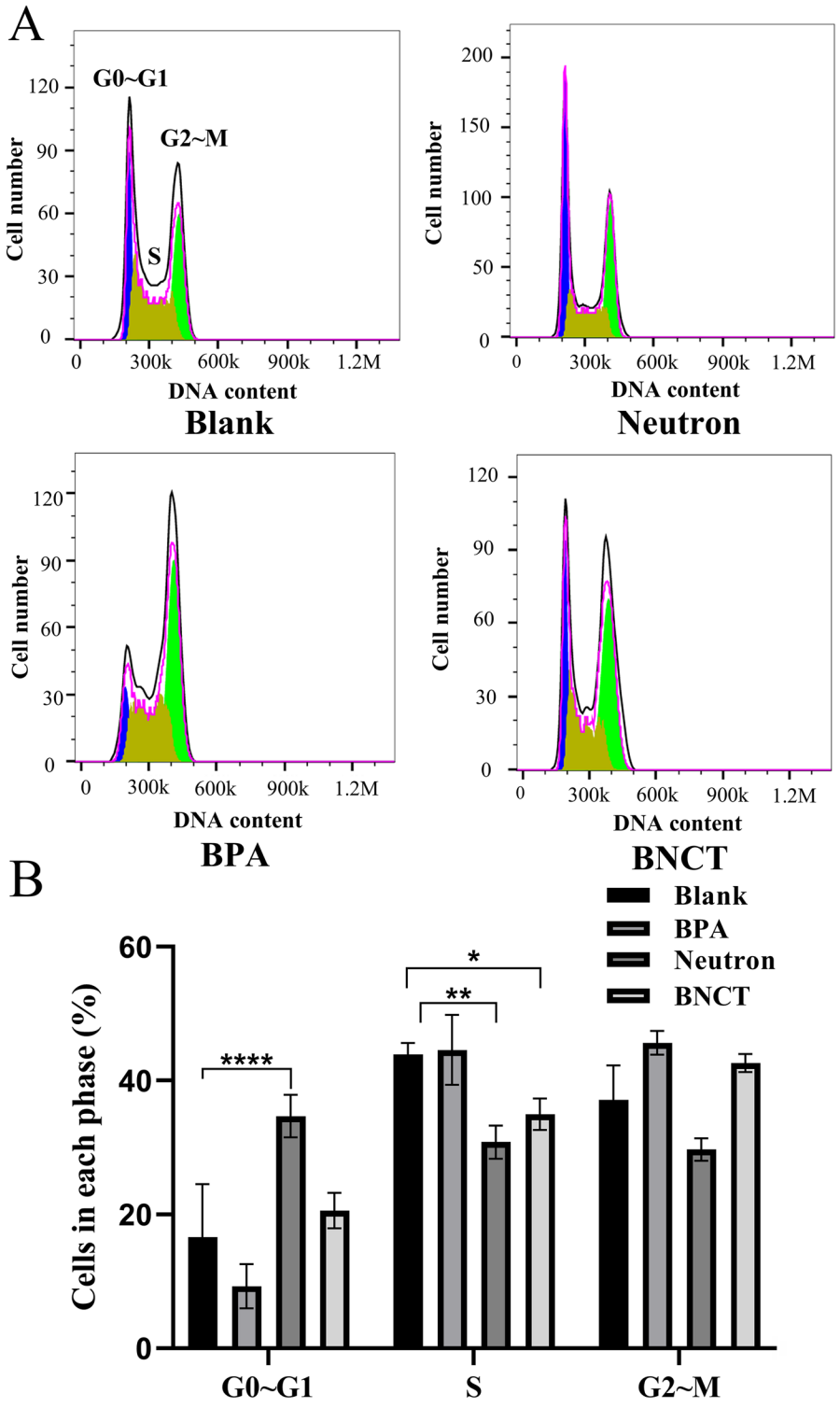

Figure 6. The cell cycle distribution of U251 glioma cells on the fifth day after BNCT treatment (cells were irradiated with neutrons for eight minutes after they were incubated with 54 ppm BPA for 3 h, and the physical dose is 2.09 Gy). (A) The graph of the cell cycle distribution. (B) The cell proportion in each phase. The data were presented as the mean ± SD (n = 3), *p < .05, **p < .01, and ****p < .0001 vs. Blank group.

challenging to explain this phenomenon, which requires further investigation.

The reproductive death rate of the BNCT group was significantly higher than that of the neutron group (Figure 5). However, the trypan blue exclusion rate (Figure 2) and the survival rate (Figure 4) of the BNCT group were similar with those of the neutron group, and the LC3-II level of the BNCT group was even higher than that in the neutron group (Figure 3). These results further support that the cell nucleus is the organelle most sensitive to BNCT.

The different endpoints selected can lead to different radiosensitivity in the same organ or tissue. Generally speaking, the functional endpoint is more sensitive than the morphological ones. We used reproductive death, the activity of

the mitochondrial dehydrogenase, the level of autophagic proteins, and the integrity of the cellular membrane to evaluate sensitivities of the cell nuclei, mitochondria, endoplasmic reticulum, and cellular membranes to BNCT. All these endpoints used are functional, which ensures a more accurate evaluation of the sensitive organelles to BNCT in U251 MG glioblastomas.

Photon radiation can trigger various types of cell death, including apoptosis (Alan Mitteer et al. 2015), autophagy (Zhai et al. 2022), necrosis (Alexiou et al. 2007), ferroptosis (Zhang et al. 2020, 2022), and reproductive death (Zhang et al. 2019). Some studies have reported that BNCT can initiate apoptosis. However, the reported apoptosis rate induced by BNCT is quite variable. Some researchers have found very low rates (Aromando et al. 2009), while others report very high rates (Wang et al. 2009; Faião-Flores et al. 2013). Our results indicate that BNCT induces approximately 33.03% apoptosis in U251 MG glioblastomas (Supplementary Figure 2). Different apoptosis rates reported may be attributed to various tumor cell lines used in these studies (Sun et al. 2013). In this investigation, BNCT cannot trigger autophagic cell death (Figure 3) but reproductive death (Figure 5) which is a functional endpoint relating to the disturbance of the nuclear division in U251 MG glioblastomas. The morphological characteristics of apoptosis, such as the condensation of chromatin, disassembly of nuclear scaffold proteins, and DNA degradation, are also nuclear-related (Prokhorova et al. 2015). Therefore, it is possible that 90.25% of the reproductive death observed may involve apoptosis in U251 MG glioblastomas. However, the discrepancy between the data on reproductive death and apoptosis suggests that BNCT may induce other types of nuclear-related cell death, such as mitotic catastrophe, which requires further investigation.

BNCT leads to a significant reduction in the duration of the S phase and a slight increase in the durations of the G0–G1 and G2–M phases in U251 MG glioblastomas (Figure 6). The mechanism behind reproductive cell death during interphase is primarily due to the shortened S phase, which results in insufficient DNA synthesis. Additionally, the slight prolongation of the G2 phase may not provide adequate time for repairing damaged DNA molecules, contributing further to reproductive cell death induced by BNCT.

To sum up, a hierarchical sensitivity of U251 MG glioblastoma organelles to BNCT is ranked as follows: the cell membrane (the endoplasmic reticulum) < the mitochondria < and the cell nucleus. The cell nucleus emerges as the most sensitive organelle to BNCT. These findings lay a theoretical groundwork for enhancing the boron-distribution-based killing efficiency of BNCT. Reproductive death was the primary death mode of U251 MG glioblastomas induced by BNCT. The shortening of the S phase duration during interphase was identified as a primary factor in this type of cell death. Further investigation into the M-phase mechanism for reproductive death is warranted.

Disclosure statement

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