Interaction of curcumin with glioblastoma cells via high and low linear energy transfer radiation therapy inducing radiosensitization effects

Jeong-Yub Kim¹,†, Chan-Woong Jung¹,²,†, Won Seok Lee³,†, Hee-Jin Kim¹, Hyeon-Jeong Jeong¹,⁴, Myung-Jin Park¹, Won Il Jang⁵,* and Eun Ho Kim³,*

¹Division of Radiation Biomedical Research, Korea Institute of Radiological and Medical Sciences, Seoul 01812, Republic of Korea
²Department of Life Sciences, Korea University, Seoul 02841, Republic of Korea
³Department of Biochemistry, School of Medicine, Daegu Catholic University, 33 17-gil, Duryugwongwon-ro, Nam-gu, Daegu 42471, Korea
⁴School of Biomedical Science, Korea University, Seoul 02841, Republic of Korea
⁵Department of Radiation Oncology, Korea Institute of Radiological and Medical Sciences, Seoul 01812, Republic of Korea
*Corresponding author. Eun Ho Kim, Department of Biochemistry, School of Medicine, Daegu Catholic University, 33 17-gil, Duryugwongwon-ro, Nam-gu, Daegu, Korea, Tel: 82536504480; E-mail address: eh140149@cu.ac.kr. Won Il Jang, Department of Radiation Oncology, Korea Institute of Radiological and Medical Sciences, Seoul 01812, Republic of Korea, Tel: 8229701262; Fax: 8229701262; E-mail address: zzang11@kirams.re.kr
†These authors contributed equally to this work.

ABSTRACT

Glioblastoma is a deadly cancer tumor in the brain and has a survival rate of about 15 months. Despite the high mortality rate, temozolomide has proven to increase the survival rate of patients when combined with radiotherapy. However, its effects may be limited because some patients develop therapeutic resistance. Curcumin has proven to be a cancer treatment due to its broad anticancer spectrum, high efficiency and low toxic level. Additionally, curcumin significantly enhanced radiation efficacy under high and low Linear Energy Transfer (LET) radiation conditions in vitro. In combination with radiation, curcumin increased the cell population in the sub-G1 phase and the reactive oxygen species (ROS) level, ultimately increasing GBM cellular apoptosis. The radiosensitizing effects of curcumin are much higher in neutron (high LET)-irradiated cell lines than in γ (low LET)-irradiated cell lines. Curcumin plus neutron combination significantly inhibited cell invasion compared with that of single treatment or curcumin combined γ-ray treatment. Curcumin enhances the radiosensitivity of Glioblastoma (GBM), suggesting it may have clinical utility in combination cancer treatment with neutron high-LET radiation.

Keywords: curcumin; neutron; glioblastoma; apoptosis; ROS

INTRODUCTION

Glioblastoma, with an average survival duration of 15 months, is among the most threatening of human malignancies [1]. Glioblastoma continues to be a very pernicious ailment despite substantial progress in cancer therapies in the previous few decades [2]. Modern classic treatments involve tripartite surgical resection surgery coupled with radiation therapy and simultaneous injection of the supplementary temozolomide, which is a DNA alkylating agent, which and is the most significant chemical therapeutic option for treating glioblastoma [3–5]. Modern chemotherapeutic methods have been found to enhance the longevity of glioma patients by engaging local or concomitant chemotherapy. In comparison with radiation therapy itself, temozolomide (TMZ) and radiology combined together have shown a substantial survival benefit [6]. The survivability advantages of TMZ were particularly found in high-intensity glioma therapies [7, 8]. However, these therapy methods yield little survival perks, mainly because therapeutic resistance develops frequently [6, 9]. Lately, the use of adjunctive and substitute medications for treating different forms of cancer has received greater attention [10].

A polyphenol named curcumin (diferuloylmethane; (1E,6E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is naturally found in the Zingiberaceae Curcuma Longa and is the primary
component of turmeric used in curries [11]. This research examines the possibility of curcumin’s usability as a radiosensitizer for GBM, in conjunction with traditional Low-linear energy transfer (LET) and high-LET radiation treatment, such as neutron radiations. During the last two decades, several research have examined curcumin’s therapeutic properties and specifically its anti-cancer effects are now validated and have altered several cell signaling processes [11–13]. Numerous research have shown the anti-hypertension and antihyperlipidemic benefits, oxidation, anti-infection, antioxidant roles, and anti-inflammatory activities of curcumin [14, 15]. Curcumin’s high effectiveness, minimal toxicity and wide range of anti-tumor properties render it as a third generation antiproliferative medicine [16]. In addition, curcumin was found to promote cancer cells’ radiosensitivity in vitro and in vivo [17–19] and change the expression of signaling pathways linked with radiosensitivity [20, 21]. The precise mechanism and cumulative effect of the radio-sensitizing consequences are poorly known, particularly high-LET radiation with combined curcumin on Glioblastoma (GBM). This study therefore investigated the ability of curcumin to enhance the toxicity to neutron radiation of GBM cell lines in vitro.

MATERIALS AND METHODS

Antibodies and chemicals

Anti-cleaved PARP1(#5625) and anti-caspase 3 (#9668) antibodies were purchased from Cell Signaling Technology (Danver, MA, USA). Anti- beta-actin (sc-4778) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Curcumin was purchased from Sigma, dissolved in DMSO to make a 10 mmol/l stock solution and stored at 4 °C.

Cell culture

Human glioma LN18 cancer cells were obtained from the Korean Cell Line Bank (Seoul, South Korea) and were cultured in Dulbecco’s minimal essential medium (DMEM; GIBCO, Gaithersburg, MD, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; GIBCO). 0.1 mM non-essential amino acids, glutamine, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid (HEPES), and antibiotics at 37 °C in a 5% CO2 humidified incubator. Human glioma LN428 cells were obtained from the KCLB and were grown in RPMI 1640 medium supplemented with 10% FBS, glutamine, HEPES and antibiotics at 37 °C in a 5% CO2 humidified incubator.

Irradiation

Cells were plated in 60-mm dishes and incubated at 37 °C under humidified conditions and 5% CO2 to 70–80% confluence. Cells were irradiated with a 137Cs γ-ray source (Atomic Energy of Canada, Ltd, Ontario, Canada) at a dose rate of 3.81 Gy/min. Fast neutrons (9.8 MeV, 30–40 keV/µm) were produced by the bombardment of beryllium by proton 4Be(p, n)7B, as a nuclear reaction in the cyclotron (MC-50; Scanditronix, Uppsala, Sweden). For measuring the absorbed dose and dose distribution of fast neutron beams or γ-rays, we used the dosimetry method of paired ionization chambers. Dosimetry data was measured before in vitro study and the neutron dose was calculated using RBE, 2.2, which has been used for neutron therapy in our institute and represents an equivalent cell-killing efficacy to γ-rays, as determined by clonogenic assay.

Cell viability assay

Cells were seeded at a density of 5000 cells/well in a 96-well plate and incubated for 24 h, in accordance with the indicated experimental conditions. To quantify cell viability, an equal volume of culture medium containing EZ-Cytox reagent (EZ3000, Daeilab Service, Chungcheongbuk-do, Republic of Korea) was added to the cells, and the mixture was incubated for 4 h. Cell viability was determined by measuring the absorbance at 450 nm using a Multiskan EX (Thermo Fisher Scientific; Waltham, MA, US).

 Colony-forming assay

Cells (500–1000) were seeded into 6-well plates in triplicate and maintained at the indicated doses of curcumin before 1R. After 7–10 days, colonies were stained with 0.4% crystal violet (Sigma, St Louis, MO, USA). The plating efficiency (PE) represents the percentage of seeded cells that grew into colonies under the specific culture conditions of a given cell line. The survival fraction, expressed as a function of irradiation, was calculated as follows: survival fraction = colonies counted/(cells seeded × PE/100). The PE of LN18 and LN428 were 0.88 ± 0.10 and 0.86 ± 0.12, respectively. To evaluate the radiosensitizing effects of curcumin, the ratio for radiation alone and radiation plus curcumin was calculated as the dose (Gy) for radiation alone divided by the dose for radiation plus curcumin at a surviving fraction of 10%.

Flow cytometry

Cells were cultured and treated with radiation or curcumin for 24 h. They were harvested at the indicated times, stained with propidium iodide (1 µg/mL, Sigma) according to the manufacturer’s protocol and then analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). A minimum of 10 000 cells was counted for each sample, and data analysis was performed with the use of CellQuest software (BD Biosciences).

Annexin V/PI staining assay

LN18 and LN428 cells were exposed to curcumin (10 µM) and/or 5 Gy γ-ray or neutron radiation for 48 h for Annexin V/PI staining. For measuring cell apoptosis induction, dissociated cells were washed with cold PBS, resuspended in binding buffer with FITC-Annexin V and PI using Annexin V-FITC Apoptosis Detection kit (BD Biosciences) in line with the manufacturer’s protocol and analyzed by flow cytometry using Flow Cytometer CytoFLEX (Beckman Coulter).

Cell death detection assay

LN18 and LN428 cells were treated with neutron and/or curcumin for 72 h. The treatment, harvesting and staining of cells were done with a Cell Death Detection ELISA kit (Roche Diagnostics GmbH) in line with the manufacturer’s instructions [22]. Multiskan EX (Thermo Fisher Scientific, Germany) was used to calibrate cell mortality at 450 nm.
**TUNEL assay**

Cells were seeded in 12 mm Ø cover glasses (marienfeld-superior, Germany). Cells were fixed with 4% paraformaldehyde for 10 min after 48 hours of irradiation and washed three times with PBS at room temperature. To measuring TUNEL assay, the fixed cells were performed using the DeadEnd™ Fluorometric TUNEL System (Promega, USA). The stained cells were analyzed with a Zeiss LSM 880 confocal microscope (Zeiss, Germany).

**Autophagy Assay**

Cells were treated, harvested, stained with Cyto-ID® Green detection reagent (Cyto-ID® Autophagy Detection Kit 2.0, Enzo Life Science, Farmingdale, NY, US) and Hoechst 33342 in accordance with the manufacturer’s protocols, and observed under a confocal laser scanning microscope (LSM 880).

**Western blot analysis**

Total proteins from cells were extracted in RIPA buffer (50 mM Tris-Cl, pH 7.4; 1% NP-40; 150 mM NaCl and 1 mM EDTA) supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM Na3VO4) and quantified using the Bradford method. Protein samples (30 μg) were separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking non-specific antibody binding sites, the membrane was incubated overnight at 4°C with primary antibodies. After incubation with peroxidase-conjugated secondary antibodies at 37°C for 1 h, the protein bands were visualized using enhanced chemiluminescence reagent (GE Healthcare Biosciences, Pittsburgh, PA, USA) and detected using the Amersham Imager 680 (GE Healthcare Biosciences).

**Caspase3 activity assay**

Caspase-3 activity was analyzed in the 2 GBM cell lines 24 h after the treatment with radiation and curcumin using a Caspase-Glo 3/7 assay detection kit (cat. no. G8091; Promega Corporation). The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrates of DEVD-pNA (for caspase-3). The pNA light emission was quantified using a pNA light emission assay detection kit (cat.no.G8091; Promega Corporation). The assay was performed using LN18 and LN428 cells. The cell viability of two GBM cancer cell lines exhibited a significant largest reduction in neutron beams+ curcumin treated cells (Fig. 1b). Further, it was observed that the size of colonies in γ-ray + curcumin treated 3D cultures (LN18, LN428 average size: 13,11 μm) was larger than those formed by neutron beams+ curcumin -treated cells (LN18, LN428 average size: 9, 5 μm) (Fig. 1c). And it showed that the size of colonies in γ-ray treated 3D cultures (LN18, LN428 average size: 18,17 μm) was similar to that of neutron treated cells (LN18, LN428 average size: 16, 14 μm).

**ROS assay**

LN18 and LN428 cells were treated with neutron and/or curcumin for 24 h. ROS was detected with DCFDA/H2DCFDA - Cellular Reactive oxygen species (ROS) Assay Kit (ab113851) and quantified at 450 nm through a Multiskan EX (Thermo Fisher Scientific, Germany) [23]. The fluorescent ROS indicator C2′,7′- dichlorodihydrofluorescein diacetate (H2DCFDA; 5 μM, Molecular Probes) was used to observe the ROS.

**Transwell chamber assay**

The invasive ability of GBM cells was measured using Transwell chambers. Briefly, the cells were seeded onto the membrane of the upper chamber at a concentration of 4×10^4 cells/ml in 150 μl serum-free medium and were either left untreated or treated with radiation for 24 h. The medium in the lower chamber contained 10% (v/v) FBS as a source of chemoattractants. For the invasion assay, cells that passed through the Matrigel®-coated membrane (coating time, 30 min at 37°C) were stained with Cell Stain Solution containing Crystal violet (MilliporeSigma) for 30 min, and for the invasion assay, cells that passed through the gelatin-coated membrane were stained and examined after 24 h incubation. The wells were evaluated under a light microscope (Olympus CK40; Olympus Corporation).

**Statistical analysis**

Statistical significance was determined using Student’s t-test. Differences were considered significant if the P value was less than 0.05 or 0.001.

\[
* P < 0.05; ** P < 0.01; *** P < 0.001.
\]

**RESULTS**

**The dose dependency and radiosensitive effect of curcumin on GBM**

Initially, in this research, we performed the MTT analysis to determine the optimum concentration of curcumin (Fig. 1a). To find the curcumin dose for radio-combination treatment, we selected the 10 μM and 4 μM found among varied doses in LN18 and LN428 cells, which was 20% inhibitory concentration at 48 h exposure. And next, to evaluate the curcumin-induced cytotoxicity for radiation, a cell viability assay was performed using LN18 and LN428 cells. The cell viability of two GBM cancer cell lines exhibited a significant largest reduction in neutron beams+ curcumin treated cells (Fig. 1b).

**Curcumin increased neutron-induced sensitivity in GBM cells**

Clonogenic survival ability was evaluated in LN18 and LN428 cells to examine the effect of curcumin on radiation-induced cytotoxicity. Cells exposed to radiations in curcumin’s presence showed a much lower survival fraction as compared to the ones irradiated in a dearth of curcumin. Table 1 provides linear quadratic model parameters for survival curves and the doses needed to decrease survival to 10%. Figure 2 depicts two GBM cell lines’ dose–response curves after being exposed to radiations of in γ-ray and neutron beams. Survival of curcuminized cells reduced in comparison to those irradiated without curcumin following γ-ray and neutron radiation. Identical cytotoxicity was reflected by equal doses of the γ-ray and neutron beams. The effectiveness of the neutron radiations outperformed in the presence of curcumin in comparison to that of γ-ray (Fig 2a, Table 1). The curcumin impact as the ratio of radiation sensitivity was determined at D50. The D50 of LN18 (LN428) for γ-rays combined with curcumin
Curcumin mediates neutron beam sensitization of GBM

Fig. 1. GBM cell viability in the dose-dependent manner: (a) Curcumin inhibited GBM cell viability in a dose-dependent manner. Cell viability was evaluated by MTT assay for LN18 and LN428 cells treated with the indicated doses of curcumin. Values are the means ± SD from three experiments; * P < 0.05, ** P < 0.01, *** P < 0.001; (b) MTT assays of LN18 and LN428 cells treated with 5 μM curcumin and irradiated with γ-rays and neutrons. Values are the means ± SD from three experiments; * P < 0.05, *** P < 0.001; (c) 3D spheroid growth assay of LN18 and LN428 cells treated with curcumin and radiation for four days. Phase-contrast images indicated that untreated cells formed polarized spheroids, but cells exposed to curcumin and radiation did not. Cells exposed to γ-rays and neutron radiation (5 Gy, 5 GyE).
Fig. 2. The radiosensitizing effects of curcumin on GBM cells: (a) Radiosensitivity of LN18 and LN428 cell lines with and without curcumin (5 μM) after various doses of γ-ray and neutron radiation was measured by colony-forming assay. The x-axis shows the equivalent dose, expressed as GyE (Gray equivalent). Values are the means ± SD from three experiments.

was 2.84 (4.06) Gy, whereas the neutron beam were 2.74 (3.70) Gy, respectively (Table 2). The radiosensitivity enhancement factor values for each IR treatment of curcumin-pretreated GBM cells are shown in Table 2.

Curcumin enhances neutron-induced apoptosis and autophagic cell death

We next examined the effects of curcumin alone or in combination with radiation on cell cycle progression using flow cytometry (Fig. 3a, Table 3). Sub-G1 cells, which represent apoptotic cells, were moderately increased by curcumin, but markedly increased by the combined treatment of curcumin and neutron beam irradiation compared with that of γ-ray irradiation. The combination of curcumin and neutron irradiation was more sensitive than curcumin and γ-ray irradiation, as depicted by the identical results produced by flow cytometry (Fig. 3b, Table 4). Maximum apoptotic cells were produced by neutron irradiation and curcumin. The cell death detection kit assisted in the examination of early apoptosis for ascertaining the introduction of apoptosis in GBM cells with curcumin and radiation collaboratively. The number of cells in apoptosis of GBM tumor cells increased substantially in 72 hours of neutron combined curcumin therapy (Fig. 3c, Table 5). Additionally, consistent with this observation, the rate of apoptosis was increased following curcumin and neutron beam therapy treatment, as determined by the TUNEL assay (Fig. 3d). To investigate the anticancer effects of curcumin and radiation further, we examined other further cellular responses associated with cell death upon neutron or curcumin treatment; in particular, we investigated the effects on autophagy, since both neutron and curcumin induce autophagy. As shown in Fig. 3e, increased accumulation of Cyto-ID Green, an autophagy indicator, was observed markedly around curcumin and neutron beam treated group.

Curcumin plus neutron enhance the apoptosis of GBM through increased ROS

In the presence of curcumin, the proportion of apoptotic cells increases substantially after being exposed to the radiation of two GBM cell lines (Fig. 4a). The combination therapy improved PARP1 fragmentation

Table 1. Fitting parameters α and β for survival curves in cell type

| Cell type | Radiation type | α (Gy⁻¹) | β (Gy⁻¹) |
|-----------|----------------|----------|----------|
| LN18      | γ-ray          | 0.066 ± 0.369 | 0.007 ± 0.040 |
|           | γ-ray + Cur 5 μM | 0.245 ± 0.363 | 0.245 ± 0.0003 |
|           | Neutron        | 0.230 ± 0.366 | 0.009 ± 0.040 |
|           | Neutron + Cur 5 μM | 0.207 ± 0.370 | 0.017 ± 0.040 |
| LN428     | γ-ray          | 0.120 ± 0.045 | 0.006 ± 0.007 |
|           | γ-ray + Cur 5 μM | 0.155 ± 0.055 | 0.004 ± 0.008 |
|           | Neutron        | 0.142 ± 0.070 | 0.004 ± 0.010 |
|           | Neutron + Cur 5 μM | 0.140 ± 0.041 | 0.013 ± 0.008 |

Table 2. Radiation dose required for 50% cell death and radiosensitivity enhancement factor (REF)

| Cell type | Radiation type | D50 (without Curc) | D50 (with Curc) | REF value |
|-----------|----------------|--------------------|-----------------|-----------|
| LN18      | γ-ray          | 6.27 Gy            | 2.84 Gy         | 2.21      |
|           | Neutron        | 3.51 Gy            | 2.74 Gy         | 1.28      |
| LN428     | γ-ray          | 4.63 Gy            | 4.06 Gy         | 1.14      |
|           | Neutron        | 4.31 Gy            | 3.70 Gy         | 1.16      |
Curcumin mediates neutron beam sensitization of GBM

Fig. 3. Effects of curcumin and radiation on apoptosis and autophagic cell death in GBM cells: (a) LN18 and LN428 cells were treated with neutron and/or curcumin for 24 h. Cell cycle distribution (subG1) was analyzed quantitatively by flow cytometry. Values are the means ± SD from three experiments; *P < 0.05, **P < 0.01, ***P < 0.001; (b) LN18 and LN428 cells were exposed to curcumin (10 μM) and/or 5 Gy γ-ray or neutron radiation for 48 h for Annexin V staining. Values represent means of three experiments ± SD; *P < 0.05, **P < 0.01, ***P < 0.001; (c) Analysis of cell death in two GBM cell lines 72 h after treatment with curcumin plus radiation using a cell death detection kit. Values are the means ± SD from three experiments; *P < 0.05, ***P < 0.001. (D, E) TUNEL staining and cyto-ID staining of LN18 and LN428 cells with and without neutron beam or with and without curcumin treatment.
and caspase-3 invigoration as compared to treatment with radiation solely, thus substantiating the role of curcumin in enhancing apoptosis. Additionally, curcumin’s benefits with neutrons were exceedingly evident in comparison to γ radiation. Curcumin combined with neutron beam treatment, in fact, produced increased caspase-3 activation as rate compared with the control group (Fig. 4b). Additionally, the production of ROS in GBM cancer cells was improved in both curcumin and neutron beam therapy (Fig. 4c, Table 6), indicating that curcumin and neutron beam treatment producing ROS augment the signalization of intracellular caspase, leading to apoptosis. The
succeeding step involved the investigation of whether the increased cell toxicity due to curcumin plus neutron beam is mediated by increased caspase activation, resulting in increased apoptotic cell deaths.

**Effect of curcumin and neutron on cell invasiveness**

We next evaluated the effect of neutron and curcumin on the invasive capacities of GBM cancer cells using transwell chamber assay. Result demonstrated that curcumin plus neutron treatment significantly inhibited cell invasion compared with the individual or \( \gamma \)-ray plus curcumin treated groups (Fig. 5a).

**DISCUSSION**

Since Glioblastoma is life-threatening, it requires urgent medical care. Although medics have tested various treatments, they have not succeeded in finding a cure for Glioblastoma. Curcumin has been suspected as a possible treatment due to its anti-cancerous property. By testing curcumin, the medics have been trying to determine whether or not it’s a better treatment than others. Curcumin is identified as a likely radiostimulator in last few years for peripheral blood lymphocytes, breast and prostate breast cancer cells excluding GBM cells [17, 23, 24]. A critical challenge is the development of anticancer medicines, like radiosensitizers to increase tumor radiosensitivity, so that curative fractions are finally improved and tumor radioresistance vanquished [25]. Anti-cancerous and cytotoxic impacts of curcumin on malignant cells in humans have been documented in few studies [26, 27]. The enhancement of high LET radiosensitivity has not been documented under any research until yet. The high LET radio sensitization ability

---

### Table 3. Detection of apoptotic cells by cell cycle detection on LN18 and LN428

| Cell type | Treatment | Sub G1 (% of Control) | Ratio |
|-----------|-----------|-----------------------|-------|
| LN18      | Control   | 1.00                  |       |
|           | Curcumin  | 1.52                  |       |
|           | \( \gamma \) -ray | 2.34             |       |
|           | \( \gamma \) -ray + Cur | 3.11          |       |
|           | Neutron   | 3.73                  |       |
|           | Neutron + Cur | 5.42            |       |
|           | \( \gamma \) -ray + Cur / Cur | 2.05   |       |
|           | \( \gamma \) -ray + Cur / \( \gamma \) -ray | 1.33 |       |
|           | Neutron + Cur / Cur | 3.57          |       |
|           | Neutron + Cur / Neutron | 1.45    |       |
| LN428     | Control   | 1.00                  |       |
|           | Curcumin  | 1.43                  |       |
|           | \( \gamma \) -ray | 1.34             |       |
|           | \( \gamma \) -ray + Cur | 2.14          |       |
|           | Neutron   | 2.14                  |       |
|           | Neutron + Cur | 3.85          |       |
|           | \( \gamma \) -ray + Cur / Cur | 1.50   |       |
|           | \( \gamma \) -ray + Cur / \( \gamma \) -ray | 1.60 |       |
|           | Neutron + Cur / Cur | 2.69          |       |
|           | Neutron + Cur / Neutron | 1.80    |       |

### Table 4. Detection of apoptotic cells by annexin V/PI staining on LN18 and LN428

| Cell type | Treatment | Annexin V/PI (% of Control) | Ratio |
|-----------|-----------|-----------------------------|-------|
| LN18      | Control   | 1.00                        |       |
|           | Curcumin  | 1.49                        |       |
|           | \( \gamma \) -ray | 2.36             |       |
|           | \( \gamma \) -ray + Cur | 5.47          |       |
|           | Neutron   | 7.48                        |       |
|           | Neutron + Cur | 9.96          |       |
|           | \( \gamma \) -ray + Cur / Cur | 3.67   |       |
|           | \( \gamma \) -ray + Cur / \( \gamma \) -ray | 2.32 |       |
|           | Neutron + Cur / Cur | 6.68          |       |
|           | Neutron + Cur / Neutron | 1.33    |       |
| LN428     | Control   | 1.00                        |       |
|           | Curcumin  | 1.47                        |       |
|           | \( \gamma \) -ray | 3.00             |       |
|           | \( \gamma \) -ray + Cur | 5.66          |       |
|           | Neutron   | 5.95                        |       |
|           | Neutron + Cur | 9.56          |       |
|           | \( \gamma \) -ray + Cur / Cur | 3.85   |       |
|           | \( \gamma \) -ray + Cur / \( \gamma \) -ray | 1.89 |       |
|           | Neutron + Cur / Cur | 6.50          |       |
|           | Neutron + Cur / Neutron | 1.61    |       |

### Table 5. Detection of apoptotic cells by cell death kit on LN18 and LN428

| Cell type | Treatment | Cell death \((1X10^4)\) | Ratio |
|-----------|-----------|------------------------|-------|
| LN18      | Control   | 3.84                   |       |
|           | Curcumin  | 4.15                   |       |
|           | \( \gamma \) -ray | 4.30             |       |
|           | \( \gamma \) -ray + Cur | 6.02          |       |
|           | Neutron   | 6.11                   |       |
|           | Neutron + Cur | 8.95          |       |
|           | \( \gamma \) -ray + Cur / Cur | 1.45   |       |
|           | \( \gamma \) -ray + Cur / \( \gamma \) -ray | 1.40 |       |
|           | Neutron + Cur / Cur | 2.16          |       |
|           | Neutron + Cur / Neutron | 1.46    |       |
| LN428     | Control   | 5.44                   |       |
|           | Curcumin  | 5.66                   |       |
|           | \( \gamma \) -ray | 5.73             |       |
|           | \( \gamma \) -ray + Cur | 7.54          |       |
|           | Neutron   | 7.12                   |       |
|           | Neutron + Cur | 9.19          |       |
|           | \( \gamma \) -ray + Cur / Cur | 1.33   |       |
|           | \( \gamma \) -ray + Cur / \( \gamma \) -ray | 1.32 |       |
|           | Neutron + Cur / Cur | 1.62          |       |
|           | Neutron + Cur / Neutron | 1.29    |       |
of curcumin for GBM cells in vitro has been made evident in this study. However, it must be noted that a number of high-quality studies have been discussed concerning the presently controversial combination of curcumin and radiation therapy for the treatment of several cancers [28, 29]. While certain reviews confirm that there is no biological or clinical foundation for the use of curcumin as a radiosensitizer.
Curcumin mediates neutron beam sensitization of GBM

Fig. 5. Effect of combinatorial treatment with neutron beam and curcumin on the invasiveness of glioblastoma cell: (a) GBM cell invasion was assessed using a Transwell chamber assay 24 h after treatment with curcumin, neutron or combination. Values are the means ± SD from three experiments; * $P < 0.05$, ** $P < 0.01$.

in the therapy of GBM patients [29], the reports also mention the effectiveness of the interactive technique within the treatment of a variety of tumors including glioma, cervical cancer, prostate, colorectal, ovarian cancers and non-Hodgkin's lymphoma [17,30–33]. Having been cited in several similar reports, curcumin has garnered considerable attention in the medical industry due to its advantageous chemopreventive and chemotherapeutic activity. This is a positive consequence of its influence on multiple signaling pathways [33]. Although there may be particular arguments posed against the naturally occurring chemical compound, our results support that curcumin may prove to be a useful adjuvant in combination with other modalities of cancer therapy, which include high LET radiotherapy and chemotherapy [34]. This research manifests the significance of curcumin as an efficient technique for high LET radiosensitization of GBM cells. Two radiation modalities namely standard low LET photons and high LET neutrons have been examined in this study. As far as we are aware, no other work published evaluating curcumin as a high LET radiosensitizer has been published till yet. However, a handful of phase I and in-vitro investigations have studied curcumin in conjunction with low LET photon radiation.

Even a single beam of 2 Gy neutron was of low efficacy in reducing fractions of GBM than 4 Gy photon radiation for the two cell lines examined. Both techniques would yield comparably identical outcomes given that the assumption of an RBE is tentatively 2. The addition of curcumin to neutron radiations created a minimum of equivalent impact on radiosensitivity as commensurate photon-based therapies (cell line) or even exceeding than the same. Thus, even in the case of the usage of identical amounts of radiation, the radiostimulating action of curcumin becomes more evident with increased LET
Table 6. Detection of ROS on LN18 and LN428

| Cell type | Treatment          | ROS (1X10^4) | Ratio  |
|-----------|--------------------|--------------|--------|
| LN18      | Control            | 5.19         |        |
|           | Curcumin           | 5.66         |        |
|           | γ-ray              | 5.35         |        |
|           | γ-ray+Cur          | 7.29         |        |
|           | Neutron            | 5.79         |        |
|           | Neutron+Cur        | 8.69         |        |
|           | γ-ray+Cur / Cur    | 1.29         |        |
|           | γ-ray+Cur / γ-ray  | 1.36         |        |
|           | Neutron+Cur / Cur  | 1.54         |        |
|           | Neutron+Cur / Neutron | 1.50     |        |
| LN428     | Control            | 5.79         |        |
|           | Curcumin           | 5.41         |        |
|           | γ-ray              | 6.10         |        |
|           | γ-ray+Cur          | 7.26         |        |
|           | Neutron            | 5.96         |        |
|           | Neutron+Cur        | 8.57         |        |
|           | γ-ray+Cur / Cur    | 1.34         |        |
|           | γ-ray+Cur / γ-ray  | 1.19         |        |
|           | Neutron+Cur / Cur  | 1.58         |        |
|           | Neutron+Cur / Neutron | 1.44    |        |

ETHICAL STATEMENT

Our study did not require ethical board approval because it did not involve human or animal trials.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

This work was supported by a National Research Foundation of Korea (NRF) grant (No. 2019M2A2B4095150), funded by the Korean government (the Ministry of Sciences and ICT). It was a grant of the Korean Institute of Radiological and Medical Sciences (KIRAMS), funded by Ministry of Science and ICT (MSIT), Republic of Korea (No. 50571-2021).

REFERENCES

1. Gilbert MR, Dignam JJ, Armstrong TS et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. *N Engl J Med* 2014;370:699–708.
2. Chinot OL, Wick W, Mason W et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. *N Engl J Med* 2014;370:709–22.
3. Stupp, R, Mason, WP, van den Bent, MJ et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;352:987–96.
4. DeAngelis LM. Brain tumors. *N Engl J Med* 2001;344:114–23.
5. Laws ER, Parney IF, Huang W et al. Survival following surgery and prognostic factors for recently diagnosed malignant glioma: data from the Glioma Outcomes Project. *J Neurosurg* 2003;99:467–73.
6. Mirimanoff RO, Gorlia T, Mason W et al. Radiotherapy and temozolomide for newly diagnosed glioblastoma: recursive partitioning analysis of the EORTC 26981/22981-NCIC CE3 phase III randomized trial. *J Clin Oncol* 2006;24:2563–9.
7. Kapoor, R, Revesz, T, Powell, M. Solitary cervical lymphoma presenting as a neurofibroma. *Br J Neurosurg* 1992;6:583–6.
8. Perry, JR, Belanger, K, Mason, WP et al. Phase II trial of continuous dose-intense temozolomide in recurrent malignant glioma: RESCUE study. *J Clin Oncol* 2010;28:2051–7.
9. Mrugala, MM, Chamberlain, MC. Mechanisms of disease: temozolomide and glioblastoma—look to the future. *Nat Clin Pract Oncol* 2008;5:476–86.
10. Lev-Ari S, Lichtenberg D, Arber N. Compositions for treatment of cancer and inflammation. *Recent Pat Anticancer Drug Discov* 2008;3:55–62.
11. Bachmeier BE, Killian P, Pfeffer U et al. Novel aspects for the application of Curcumin in chemoprevention of various cancers. *Front Biosci (Schol Ed)* 2010;2:697–717.
12. Shishodia S, Amin HM, Lai R et al. Curcumin (diferuloylmethane) inhibits constitutive NF-kappaB activation, induces G1/S arrest, suppresses proliferation, and induces apoptosis in mantle cell lymphoma. *Biochem Pharmacol* 2005;70:700–13.
Curcumin mediates neutron beam sensitization of GBM

13. Wei, SC, Lin, YS, Tsao, PN et al. Comparison of the anti-proliferation and apoptosis-induction activities of sulindac, celecoxib, curcumin, and nifedipine in mismatch repair-deficient cell lines. J Formos Med Assoc 2004;103:599–606.

14. Qin, S, Huang, L, Gong, J et al. Efficacy and safety of turmeric and curcumin in lowering blood lipid levels in patients with cardiovascular risk factors: a meta-analysis of randomized controlled trials. Nutr J 2017;16:68–77.

15. Oshimo M, Nakashima F, Kai K et al. Sodium sulfite causes gastric mucosal cell death by inducing oxidative stress. Free Radic Res 2021;73:1–43.

16. Dai, XZ, Yin, HT, Sun, LF et al. Potential therapeutic efficacy of curcumin in liver cancer. Asian Pac J Cancer Prev 2013;14:3855–9.

17. Chendil, D, Ranga, RS, Meigooni, D et al. Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3. Oncogene 2010;62:919–30.

18. Goel A, Aggarwal BB. Curcumin, the golden spice from Indian saffron, is a chemosensitizer and radiosensitizer for tumors and chemoprotector and radioprotector for normal organs. Nutr Cancer 2010;62:919–30.

19. Shehzad, A, Park, JW, Lee, J et al. Curcumin induces radiosensitivity of in vitro and in vivo cancer models by modulating pre-mRNA processing factor 4 (Prp4). Chem Biol Interact 2013;206:394–402.

20. Orr, WS, Denbo, JW, Saab, KR et al. Curcumin potentiates rhabdomyosarcoma radiosensitivity by suppressing NF-kappaB activity. PLoS One 2013;8:e51309.

21. Pan Y, Wang M, Bu X et al. Curcumin analogue T83 exhibits potent antitumor activity and induces radiosensitivity through inactivation of JAB1 in nasopharyngeal carcinoma. BMC Cancer 2013;13:323–31.

22. Liu, C, Zhu, Y, Lou, W et al. Inhibition of constitutively active Stat3 reverses enzalutamide resistance in LNCaP derivative prostate cancer cells. Prostate 2014;74:201–9.

23. Ji, WO, Lee, MH, Kim, GH et al. Quantitation of the ROS production in plasma and radiation treatments of biotargets. Sci Rep 2019;9:19837.

24. Sebastia N, Monotoro A, Hervas D et al. Curcumin and transresveratrol exert cell cycle-dependent radioprotective or radiosensitizing effects as elucidated by the PCC and G2-assay. Mutat Res 2014;766-767:49–55.

25. Malik A, Sultana M, Qazi A et al. Role of Natural Radiosensitizers and Cancer Cell Radioreistance: an update. Anal Cell Pathol (Amst) 2016;2016:6146595–603.

26. Vallianou NG, Evangelopoulos A, Schizas N et al. Potential anticancer properties and mechanisms of action of curcumin. Anticancer Res 2015;35:645–51.

27. Teymouri M, Pirro M, Johnston TP et al. Curcumin as a multifaceted compound against human papilloma virus infection and cervical cancers: a review of chemistry, cellular, molecular, and preclinical features. Biofactors 2017;43:331–46.

28. Verma V. Relationship and interactions of curcumin with radiation therapy. World J Clin Oncol 2016;7:275–83.

29. Sminia, P, van den Berg, J, van Kootwijk, A et al. Experimental and clinical studies on radiation and curcumin in human glioma. J Cancer Res Clin Oncol 2021;147:403–9.

30. Zhang L, Ding X, Huang J et al. In vivo Radiosensitization of human glioma U87 cells induced by upregulated expression of DUSP-2 after treatment with curcumin. Current Signal Transduction Therapy 2015;10:119–25.

31. Javvadi, P, Segan, AT, Tuttle, SW et al. The chemopreventive agent curcumin is a potent radiosensitizer of human cervical tumor cells via increased reactive oxygen species production and over-activation of the mitogen-activated protein kinase pathway. Mol Pharmacol 2008;73:1491–501.

32. Qiao, Q, Jiang, Y, Li, G. Curcumin enhances the response of non-Hodgkin’s lymphoma cells to ionizing radiation through further induction of cell cycle arrest at the G2/M phase and inhibition of mTOR phosphorylation. Oncol Rep 2013;29:380–6.

33. Yallapu MM, Maher DM, Sundram V et al. Curcumin induces chemo/radio-sensitization in ovarian cancer cells and curcumin nanoparticles inhibit ovarian cancer cell growth. J Ovarian Res 2010;3:11.

34. Mortezae, K, Salehi, E, Mirtavoos-Mahyari, H et al. Mechanisms of apoptosis modulation by curcumin: Implications for cancer therapy. J Cell Physiol 2019;234:12537–50.

35. Caretti, V, Zondervan, I, Meijer, DH et al. Monitoring of tumor growth and post-irradiation recurrence in a diffuse intrinsic pontine glioma mouse model. Brain Pathol 2011;21:441–51.