

Sonodynamic Therapy with 5-Aminolevulinic Acid and Focused Ultrasound for Deep-seated Intracranial Glioma in Rat

TADAHIRO OHMURA¹, TAKEO FUKUSHIMA¹, HIROTOMO SHIBAGUCHI², SHIN YOSHIKAWA³, TOORU INOUE¹, MASAHIDE KUROKI², KAZUNARI SASAKI⁴ and SHIN-ICHIRO UMEMURA⁴

Departments of ¹Neurosurgery and ²Biochemistry, Fukuoka University Faculty of Medicine, Fukuoka, Japan;

³Department of Electrical and Communication Engineering, Tohoku University, Miyagi, Japan;

⁴Central Research Laboratory, Hitachi, Ltd., Saitama, Japan

Abstract. *Background:* 5-Aminolevulinic acid (5-ALA) has already been applied clinically as a photosensitizer. In this study, sonodynamically induced selective antitumour effect of 5-ALA for deep-seated lesions was evaluated. *Materials and Methods:* First, normal rat brains were sonicated via a transducer placed on the dural surface to confirm safe acoustic conditions for normal rat brains. One week after inoculation of brains with C6 rat glioma cells, brains with/without administration of 5-ALA (100 mg/kg body weight) were sonicated. *Results:* Sonodynamic therapy (SDT) with 5-ALA and focused ultrasound (10 W/cm², 1.04 MHz, 5 min) achieved selective antitumour effect against deep-seated experimental glioma. Mean tumour sizes in the largest coronal section in sham-operated rats and rats receiving ultrasound with/without 5-ALA were 29.94±10.39, 18.32±5.69 and 30.81±9.65 mm², respectively. Tumour size was significantly smaller in the SDT group than in other groups (*p*<0.05). *Conclusion:* This experimental rat model showed that SDT appears to be useful in the treatment of deep-seated malignant glioma.

Human malignant glial tumours, particularly glioblastomas, strongly invade neighbouring tissue and cannot be completely resected surgically, even with up-to-date technologies, such as the neuronavigator or photodynamic diagnosis (PDD). Fewer than half of such patients survive more than 1 year, and 5-year survival is only 5 to 8%, even when all treatment modalities, including radio-chemo-immunotherapy are applied (1). More aggressive therapy is required to eradicate unresectable nests of tumour cells invading adjacent normal brain tissue.

Correspondence to: Tadahiro Ohmura, MD, Department of Neurosurgery, Fukuoka University, Faculty of Medicine, 7-45-1 Nanakuma, Johnan-ku, Fukuoka 814-0180, Japan. Tel: +81 928011011, ext: 3445, Fax: +81 928659901, e-mail: tadaohm@kouhoukai.or.jp

Key Words: Sonodynamic therapy, focused ultrasound, 5-aminolevulinic acid, glioma, deep-seated, rat model.

5-Aminolevulinic acid (5-ALA) is a natural porphyrin precursor that has already been used in PDD and photodynamic therapy (PDT) for human glioma. Therefore, it is easy to apply to the treatment of human brain tumours, unlike other materials. PDT has been extensively investigated as a treatment for various brain tumours and has been applied in clinical trials (2). The selective antitumour effect of PDT is based on selective uptake of a photosensitizer by neoplastic tissue (3-5). However, it is quite difficult to focus PDT on deep-seated tumours and selectively kill the tumour cells without placing indwelling optical fibers directly into tumours (6). In contrast, the energy of focused ultrasound can be delivered into deep-seated lesions and can be focused into a small volume (7-9). Furthermore, Fry *et al.* reported that they were able to create focused ultrasound-induced lesions in brains of craniectomized cat through a formaldehyde-fixed human skull (10). Hynynen *et al.* also created focal lesions in rabbit brains through a piece of human skull (11). By adjusting the acoustic intensity of the ultrasound, hazardous effects to surrounding tissues can be minimized (12). In addition, the photosensitizing haematoporphyrin derivative and antitumour drugs (13, 14) have been found to localise selectively in some tumour cells and to be activated by ultrasound, resulting in a significant antitumour effect. Sonodynamic therapy (SDT) has the potential to be very a useful and noninvasive treatment in the future if it can destroy deep-seated brain tumour by sonication through the human skull while avoiding destruction of surrounding normal brain tissue.

We investigated the antitumour effect of SDT in experimental rat glioma by using focused ultrasound in combination with 5-ALA.

Materials and Methods

Preparation of the sonodynamically active agent. The natural porphyrin precursor 5-aminolevulinic acid hydrochloride was purchased from Cosmo Biochemical Company (Tokyo, Japan). The material was supplied as a powder and was mixed in a sterile solution of distilled water (20 mg/ml).

Ultrasound source. The ultrasound transducer used was the same as that used in consecutive SDT experiments as described in detail in previous reports by Yumita *et al.* (15) and Nonaka *et al.* (16). The transducer was kept at 25°C by circulating water, and its resonant frequency was 1.04 MHz. The wave generator and RF amplifier used were also the same as those reported in the previous experiments (15, 16), and the signal of the transducer was monitored by an oscilloscope during ultrasound exposure. The output acoustic pressure was measured at a depth of 7 mm from the surface of the transducer as described previously by Nonaka *et al.* (16).

Determination of the effect of focused ultrasound in normal rat brain tissue. Female Wistar rats (250 to 300 g; Kyudo Co., Kumamoto, Japan) were used. The rats were anaesthetised intraperitoneally with sodium pentobarbital (40 to 50 mg/kg) and placed in a stereotactic frame. The scalps of the rats were prepared and exposed as described elsewhere (16), and a 10-mm-diameter craniotomy was performed over the left hemisphere. The transducer, previously coated with gelatine, was placed tightly on the dural surface, and the left caudoputamen was focused by the ultrasound. Ultrasound energy was applied at the peak focus intensities of 10, 15, 20 or 25 W/cm² at 1.04 MHz for 5 minutes. Each experiment used 6 to 8 rats. After irradiation, the wound was closed with 5-0 nylon suture. The animals were killed 7 days after irradiation by an overdose injection of sodium pentobarbital. After the brains were removed and fixed in 10% formaldehyde, they were sectioned through the area of irradiation and stained with hematoxylin and eosin. The area of the lesions was measured with image analysis software (Biozero BZ-8000; Keyence Co., Itasca, IL, USA).

To examine the long-term chronic effect of ultrasonic irradiation applied at the above-mentioned parameters, the experiment was repeated, and the animals were killed either immediately after or at 4 weeks after irradiation. The brains were removed, fixed in 10% formaldehyde, sectioned through the area of irradiation, and stained with hematoxylin and eosin. Each experiment used 2 to 8 rats.

Treatment of experimental rat brain tumour. C6 rat glioma cells (Human Science Research Resources Bank, Osaka, Japan) were used for implantation in brain and were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma-Aldrich). The cells were incubated at 37°C in humidified 5% CO₂/air, and the culture medium was changed twice a week. Cell suspension of C6 cells in PBS was injected into the brains of the female Wistar rats. Immediately thereafter, the animals were anaesthetised intraperitoneally with sodium pentobarbital (40 to 50 mg/kg of weight) and fixed in a stereotactic apparatus. The cells (5×10⁵ cells/5 µl) were inoculated into the caudoputamen in the left hemisphere by the same method described in the previous report (16). One week after C6 cell inoculation, the brain was irradiated through a 10-mm-diameter craniotomized area in the left hemisphere. The peak focus intensity was 10 W/cm² at 1.04 MHz for 5 minutes. The brains of 6 rats were irradiated in a standing-wave mode 3 hours after the oral application of 5-ALA (100 mg/kg body weight). Eight rats underwent a sham operation that consisted of an inoculation of C6 glioma cells, craniotomy and placement of the transducer on the dural surface for 5 minutes without irradiation. Another 8 rats also underwent a sham operation that consisted of an inoculation of C6

glioma cells, craniotomy and placement of the transducer on the dural surface without irradiation after oral application of 5-ALA (100 mg/kg body weight). Six rats inoculated with C6 glioma cells received focused ultrasound irradiation (10 W/cm² at 1.04 MHz for 5 minutes) without oral application of 5-ALA. The animals were killed 7 days after the operation by an overdose injection of sodium pentobarbital. The brains were removed, fixed in 10% formaldehyde, paraffin embedded, and sectioned through the area of irradiation. The 5-µm-thick sections were stained with haematoxylin and eosin, and the tumour was examined microscopically. For each rat, the largest lesion area was measured by microscope with image analysis software (Biozero BZ-8000; Keyence Co.). All protocols for animal experiments were approved by the Animal Subjects Committee Institutional Review Board of Fukuoka University Faculty of Medicine.

Statistical analysis. Significance was assessed with one-way ANOVA followed by the Bonferroni multiple comparison test. A value of $p < 0.05$ was considered the level of significance for all tests. All data were analysed with a contemporary statistical software package (GraphPad Prism™; GraphPad Software, Inc., San Diego, CA, USA).

Results

Immediate effect of focused ultrasound in normal rat brain tissue. Even in the rats killed immediately after irradiation, there was sharply delineated necrosis in the rat brain at the peak focus intensities of 15, 20 and 25 W/cm² at 1.04 MHz for 5 minutes. A huge necrotic lesion was observed in the left hemisphere of each of the two rats when the peak focus intensity was 25 W/cm² (Figure 1A). The central part of the lesion contained only a few remnants of destroyed cells, and small lacerations and cavities were observed in the periphery. When the peak focus intensity was reduced to 20 W/cm², a lesion was observed in 2 out of 4 rats (Figure 1B), and when the peak focus intensity was further reduced to 15 W/cm², there was a small amount of necrosis in the caudoputamen in 1 out of 2 rats (Figure 1C). Intervening cerebral cortex in the immediate vicinity of the irradiated area did not show any significant damage. No lesions were found with the 10 W/cm² application (Figure 1D). These findings were observed consistently in each group of rats.

Chronic effect of focused ultrasound in normal rat brain tissue. We examined the long-term chronic effect of focused ultrasound at 10 and 15 W/cm², 1.04 MHz, and 5-minute exposure duration to confirm safety to the irradiated brain. At 15 W/cm², huge lesions with cavities were induced in 2 out of 2 rats (Figure 2A). At 4 weeks after irradiation, no lesions were present in the target areas of rat brains that received a peak focus intensity of 10 W/cm² (Figure 2B). Thus, the 10 W/cm² peak focus intensity was considered to be safe in normal rat brain, and this intensity was used for further experimentation with SDT in implanted intracerebral rat glioma.

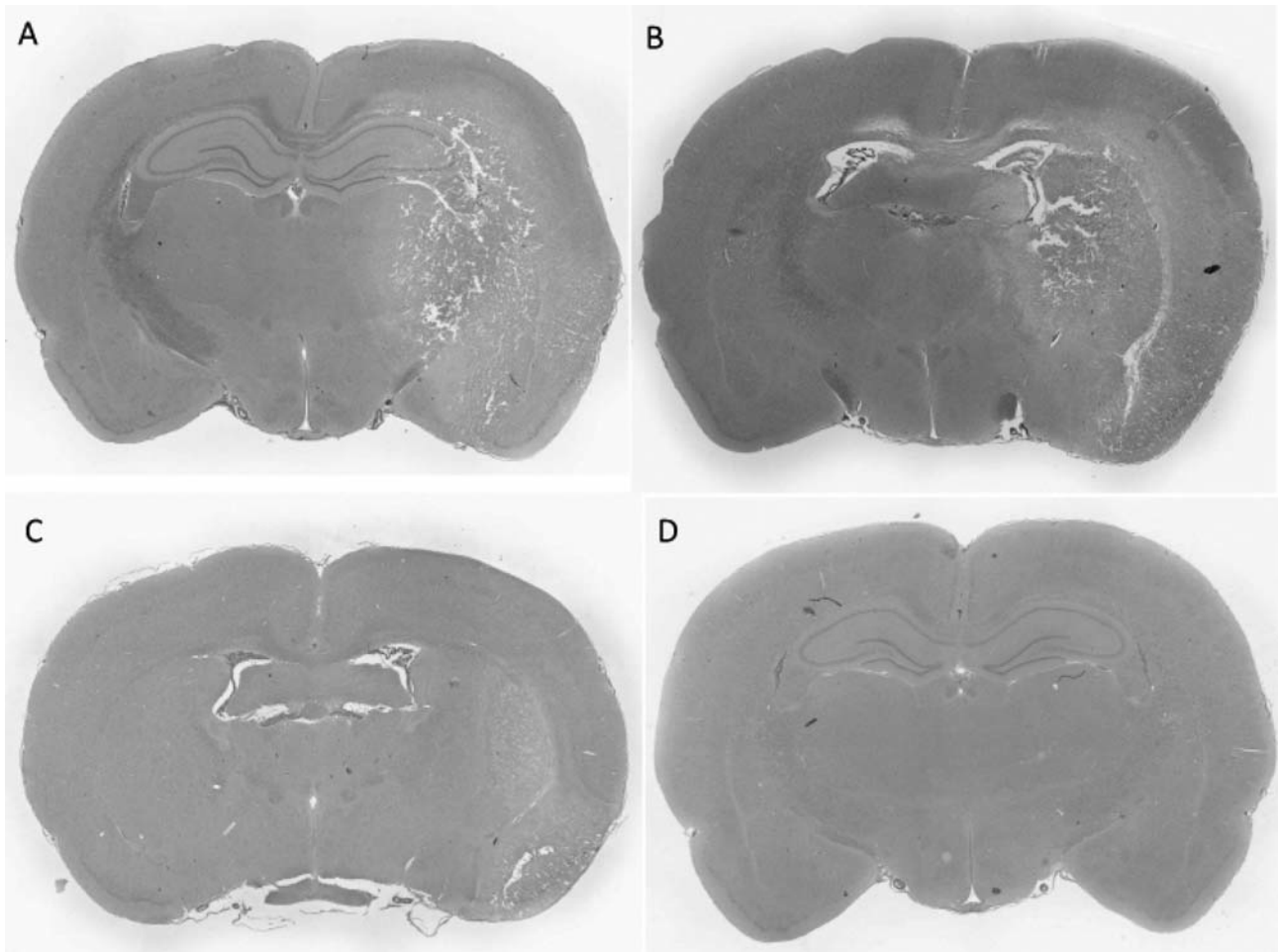


Figure 1. Immediate effect of focused ultrasound in normal rat brain tissue. The left caudoputamen was irradiated and rats were killed immediately. At the peak focus intensity of 25 W/cm² at 1.04 MHz for 5 minutes, a huge necrotic lesion was observed in the left hemisphere in each of the 2 rats (A). At peak focus intensities of 20 W/cm² (B) and 15 W/cm² (C), the necrotic lesions became successively smaller turn. No lesion was observed with 10 W/cm² at 1.04 MHz for 5 minutes of exposure (D).

Effect of SDT on rat C6 intracerebral tumours. A peak focus intensity of 10 W/cm² at 1.04 MHz and 5 minutes was used for ultrasound exposure in tumour treatment because safety in normal rat brain was confirmed under these conditions. C6 tumour cells (5×10^5 cells) were implanted at 5 mm below the dural surface, and at 2 weeks after inoculation, the tumour had grown to occupy the caudoputamen and surrounding structures (Figure 3A). Although the tumours were destroyed and tumour growth was inhibited, normal brain tissue was not affected in the group treated with SDT (Figure 3D). The largest tumour areas found were 29.94 ± 10.39 , 30.81 ± 9.65 , 32.98 ± 7.21 and 18.32 ± 5.69 mm² in the rats undergoing sham operation, ultrasound irradiation alone, 5-ALA alone and SDT, respectively. The tumours receiving SDT were significantly smaller than those of rats undergoing sham operation

($p < 0.05$), rats receiving ultrasound irradiation alone ($p < 0.05$) and rats receiving 5-ALA alone ($p < 0.01$) (Figure 4). No damage was observed to surrounding normal brain tissue in any rat of any group.

Discussion

Focused ultrasound can penetrate deeply into tissue and can be focused on a deep-seated lesion. This property can be applied as an advantage in the treatment of deep-seated brain tumours, different from that of the laser light used in PDT. High-intensity focused ultrasound irradiation has been applied effectively in local cancer treatment by thermal effects and non-thermal cavitation (16-19). Ultrasound-induced cavitation causing sonoluminescence and sonochemical reactions were defined and investigated

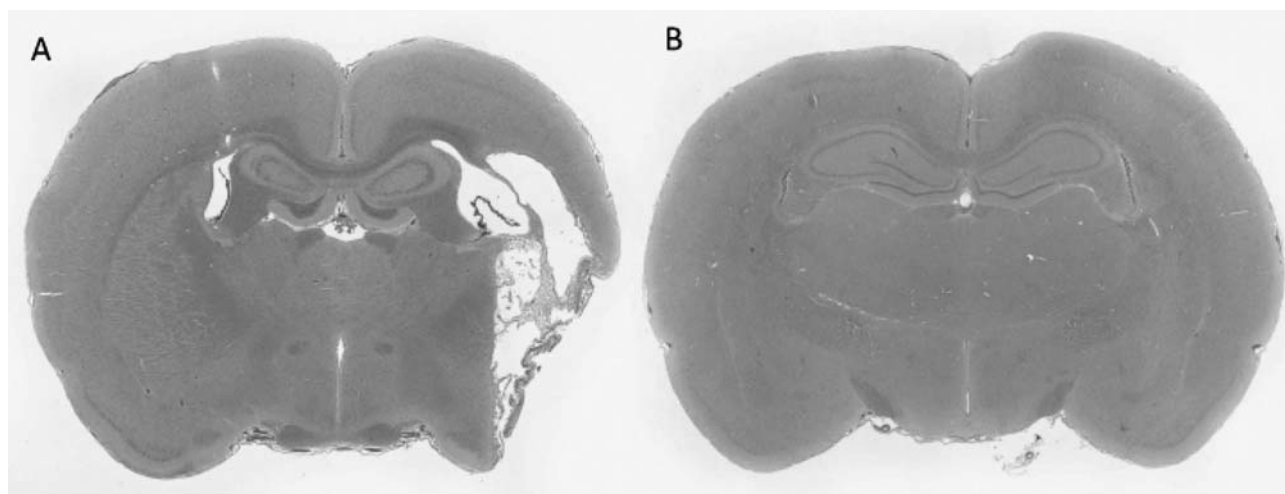


Figure 2. Chronic effect of focused ultrasound in normal rat brain tissue. The left caudoputamen was irradiated and the rats were killed 4 weeks after irradiation. A: At the peak focus intensity of 15 W/cm^2 at 1.04 MHz for 5 minutes, huge cavities were observed in 2 of 2 rats. B: There were no lesions in the target areas of 4 rat brains receiving 10 W/cm^2 at 1.04 MHz for 5 minutes.

macroscopically and ultrastructurally (19-21). Several morphological alterations, such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and apoptotic body formation, have been observed after ultrasound irradiation (18, 19, 22, 23). Umemura *et al.* (17) and Yumita and Umemura (24) reported that haematoporphyrin used as a photosensitizer can enhance the antitumour effect of ultrasound by singlet oxygen generated by ultrasonically activated haematoporphyrin. We have already reported the *in vitro* and *in vivo* efficacy of SDT using photofrin and rose bengal as sonosensitizers and found that the extent of destruction and the incidence of lesions increase with the intensity and duration of exposure (16, 19, 25).

5-ALA is specifically taken up by cancer cells and converted to photosensitizing protoporphyrin IX (PpIX) (26), and the 5-ALA-induced PpIX fluorescence signal correlates with histological malignancy grade, MIB-1 labelling indices, CD31 and VEGF expression (27).

Sasaki *et al.* reported that the peak wavelength of sonoluminescent light induced by ultrasound irradiation at 1 W/cm^2 at $361 \pm 3 \text{ kHz}$ in PBS was 415 nm (28). This result can be interpreted as indicating that the wavelength of sonoluminescent light is stable and does not occur randomly. Depending on the ultrasound source and the target, the wavelength of sonoluminescent light may vary. If the wavelength of sonoluminescent light induced by ultrasound corresponds to the absorption band of the photosensitizer, SDT is expected to have a selective antitumour effect similar to that of PDT.

From our experiments, we considered focused ultrasound irradiation at 10 W/cm^2 at 1.04 MHz for 5 minutes to be safe for normal rat brain. In previous studies on PDT and PDD, 5 to 200 mg/kg of 5-ALA (mainly 100 to 200 mg/kg) was administered orally, intravenously or intraperitoneally (29-36). Maximum fluorescence intensity of PpIX in tumours was detected 2 to 6 hours after administration of 5-ALA (30, 31, 33, 36). Because fluorescence intensity was not significantly different by any route of administration, we considered oral intake of 5-ALA to be preferable to intravenous or intraperitoneal injection because the latter require buffering to avoid adverse side-effects. Therefore, the rat brains were sonicated 3 hours after oral administration of 5-ALA (100 mg/kg body weight).

Extracellular PpIX showed an enhanced cell-killing effect through microbubble-enhanced ultrasound. However, intracellular PpIX enhanced the cell-killing effect by hyperthermia, which can be produced by ultrasound exposure in a moderately acidic environment (pH 6.6) (37). We previously reported that no significant temperature change was detected during focused ultrasound irradiation at 25 W/cm^2 at 1.04 MHz for 5 minutes, but target brain temperature rose to close to 50°C by exposure to 110 W/cm^2 at 1.04 MHz for 2 minutes. These results suggested that peak focus intensity correlates with target brain temperature, and when peak focus intensity is below 25 W/cm^2 at 1.04 MHz for 5 minutes, there is no significant temperature change in target brain (16). In the present study, selective tumour destruction and tumour growth inhibition were obtained by non-thermal effect with weak focused ultrasound and 5-ALA.

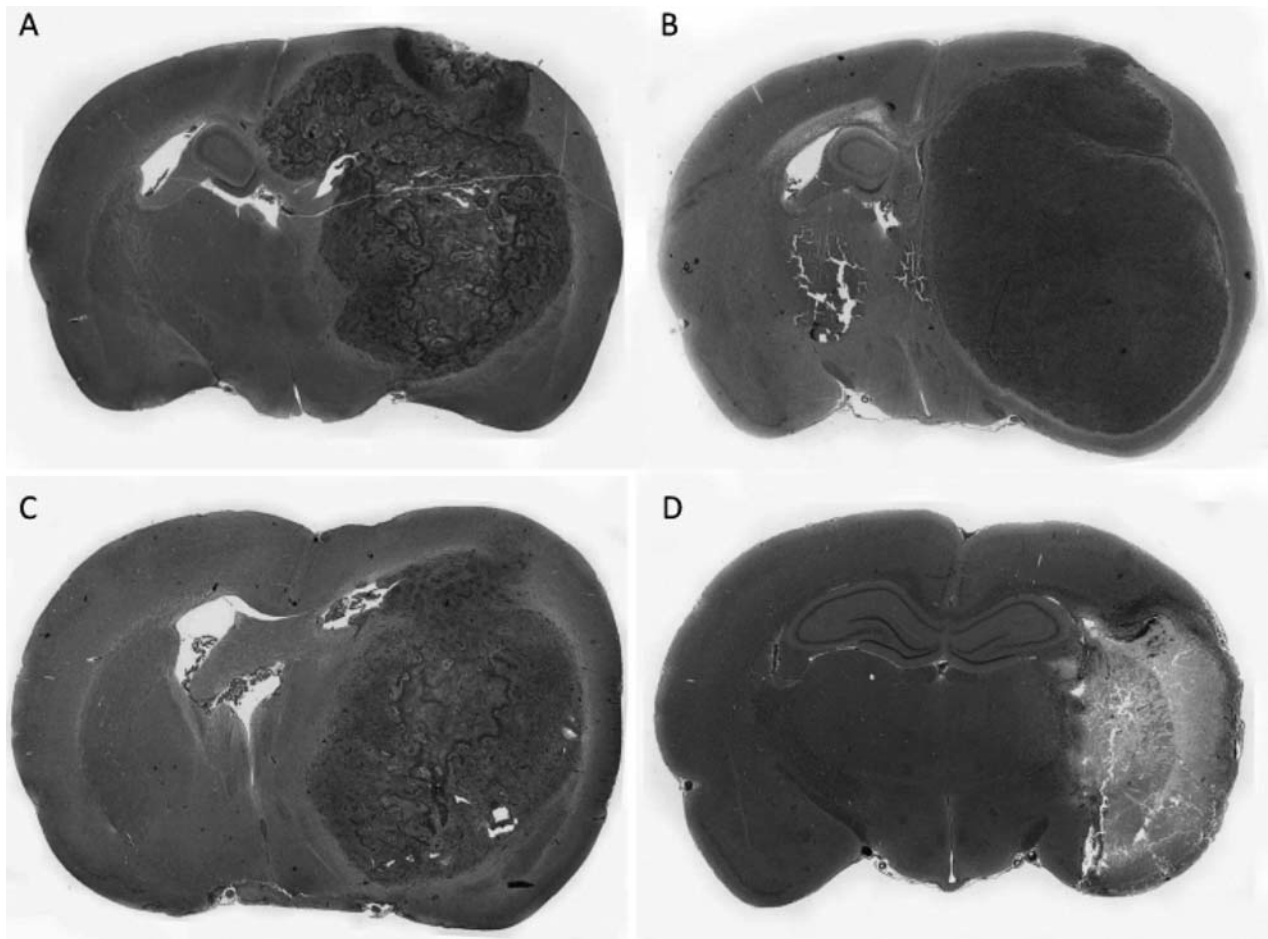


Figure 3. Effect of sonodynamic therapy (SDT) on rat C6 intracerebral glioma. Ultrasound exposure at 10 W/cm² at 1.04 MHz for 5 minutes was selected for tumour treatment. Rat brains were inoculated with 5×10^5 C6 glioma cells, and tumours grew and occupied the caudoputamen and surrounding structures within 2 weeks (A). There was no destruction of tumours in the rat group receiving 5-ALA administration alone (B), or in the group receiving focused ultrasound irradiation alone (C). However, selective destruction of the tumour and inhibition of tumour growth were observed in the group treated with SDT (D).

Conclusion

SDT with 5-ALA and focused ultrasound is considered to be effective for the *in vivo* treatment of deep-seated intracranial glioma in rat. Selective tumour destruction and tumour growth inhibition were obtained by non-thermal effect of weak focused ultrasound which was enhanced by 5-ALA, without causing damage to surrounding normal brain tissue. Because 5-ALA has already been applied clinically as a photosensitizer, SDT with this sensitizer is expected to be applied clinically to treat deep-seated tumours in humans in the future.

Acknowledgements

This work was supported in part by a Grant-in Aid for Scientific Research® to T.F. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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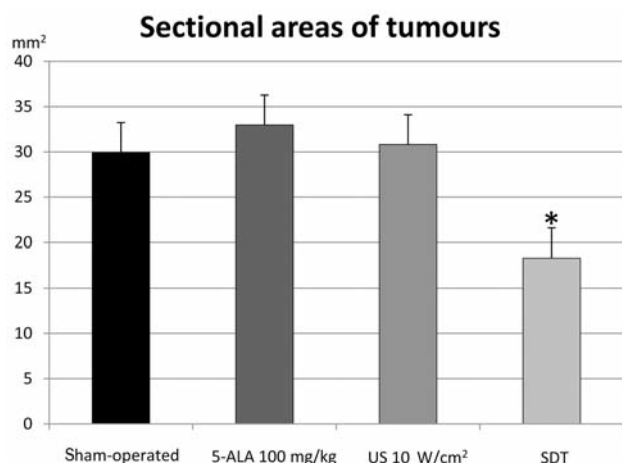


Figure 4. Tumour size in coronal sections for each treatment group. The largest tumour size was $29.94 \pm 10.39 \text{ mm}^2$ in the sham-operated rats, $30.81 \pm 9.65 \text{ mm}^2$ in the rats receiving ultrasound alone, $32.98 \pm 7.21 \text{ mm}^2$ in the rats receiving 5-aminolevulinic acid (5-ALA) alone and $18.32 \pm 5.69 \text{ mm}^2$ in the rats receiving sonodynamic therapy (SDT). *Tumours treated with SDT were significantly smaller than those in rats undergoing sham operation ($p < 0.05$), receiving ultrasound alone ($p < 0.05$) and receiving 5-ALA alone ($p < 0.01$). Data represent the mean \pm S.D.

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Received April 27, 2011

Revised June 16, 2011

Accepted June 17, 2011