A Comparison of Ramipril and Bevacizumab to Mitigate Radiation-Induced Brain Necrosis: An Experimental Study

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■ BACKGROUND: **Bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, is a new treatment approach for radionecrosis. In our study, we compared the prophylactic and therapeutic usage of a promising agent, ramipril (an angiotensin-converting enzyme inhibitor), with that of bevacizumab for reducing radiation-induced brain injury after high-dose stereotactic radiosurgery (SRS).**

■ METHODS: **A total of 60 Wistar rats were used. The rats were irradiated with a single dose of 50 Gy using a Leksell Gamma Knife device. Bevacizumab and ramipril were administered in the prophylactic protocol (starting the first day of SRS) and in the therapeutic protocol (starting the fourth week of SRS). Their usage was continued until 12 weeks, and the right frontal lobes of the rats were examined histologically (hematoxylin and eosin stain) and immunohistochemically (hypoxia-inducible factor [HIF]-1α, VEGF, and CD31 antibody expression).**

■ RESULTS: **The expression of VEGF, HIF-1α, and CD31 had significantly increased at 12 weeks after SRS compared with the control group. The addition of bevacizumab or ramipril to SRS significantly mitigated the histological severity of radiation injury and the expression of VEGF, HIF-1α, and CD31. However, the prophylactic use of bevacizumab and ramipril seemed to be more effective than therapeutic administration. Our results also revealed that the greatest benefit was achieved with the use of prophylactic administration of bevacizumab compared with other treatment protocols.**

■ CONCLUSIONS: **Ramipril might be a promising agent for patients with radionecrosis. Clinical studies are required to investigate the effective and safe doses of ramipril, which is an inexpensive, well-tolerated drug that can cross the blood–brain barrier.**

INTRODUCTION

Stereotactic radiosurgery (SRS) is a key component in the treatment of intracranial tumors. Although SRS is an effective treatment option, the late-onset side effects, which include radionecrosis (RN) and brain edema, have limited its efficacy.[1] Radiation causes blood–brain barrier disruption and endothelial cell injury, followed by tissue edema and hypoxia. It triggers the increased expression of hypoxia-inducible factor (HIF)-1α, which stimulates vascular endothelial growth factor (VEGF) production.[2-4] VEGF has been identified as the most potent proangiogenic factor, and high expression levels result in neovascularization with abnormal and fragile vessels, which promote brain edema, neuronal demyelination, and, finally, necrosis.[3-7] The role of HIF-1α and VEGF in the pathogenesis of angiogenesis, edema formation, and cerebral necrosis after SRS has been shown in several preclinical and clinical studies.[5,8-10]

Key words
- Bevacizumab
- CD31
- HIF-1α
- Ramipril
- Radiation necrosis
- VEGF

Abbreviations and Acronyms
ACE: Angiotensin-converting enzyme
HIF: Hypoxia-inducible factor
MRI: Magnetic resonance imaging
P-B: Prophylactic bevacizumab
P-R: Prophylactic ramipril
RN: Radionecrosis
SRS: Stereotactic radiosurgery
T-B: Therapeutic bevacizumab
T-R: Therapeutic ramipril
VEGF: Vascular endothelial growth factor

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Citation: World Neurosurg. (2020) 144:e210-e220.
https://doi.org/10.1016/j.wneu.2020.08.081

*Journal homepage: **www.journals.elsevier.com/world-neurosurgery***

*Available online: **www.sciencedirect.com***

Although most cases of the RN will be asymptomatic and heal spontaneously, symptoms can develop and require treatment in some patients.[11] However, the treatment options are limited and include steroids, surgery, bevacizumab, and hyperbaric oxygen therapy.[2] Bevacizumab, a monoclonal anti-VEGF antibody, is one of these potential agents. It binds VEGF and prevents it from reaching the receptors on the endothelial cell surface, thereby pruning blood vessels and reducing vascular permeability and edema.[3] Treatment with bevacizumab has been shown to mitigate the development of RN in several animal studies.[12-14] Although bevacizumab appears to be a promising agent for the treatment of RN, few studies have evaluated its effectiveness in daily practice, and these have been restricted to retrospective clinical studies and one randomized double-blind study of 14 patients. In addition, the optimal scheduling, dosage, and duration of therapy have not yet been well established.[15] Finally, conflicting results have been reported regarding the efficacy of bevacizumab in terms of prophylactic or therapeutic usage.[12,14,16] New agents are urgently needed owing to the uncertainties in the protocol for bevacizumab and the potential side effects, including deep vein thrombosis and bleeding, which have limited its use.

One of the most promising approaches to pharmacologic blockade is the renin-angiotensin system pathway to reduce radiation-induced brain injury. The precursor angiotensin I is converted to angiotensin II by the angiotensin-converting enzyme (ACE). Angiotensin II induces VEGF production in the cells.[17] The use of ACE inhibitors or angiotensin blockade has resulted in the mitigation of radiation-induced injury in several organs such as the optic pathway, brain, and kidney in some studies.[18-21] Ramipril, one of the ACE inhibitors, was also shown to reduce VEGF expression, which also correlated with improved paralysis outcomes in irradiated rats.[22]

In our experimental study, we evaluated the effect of ramipril in the prevention and treatment of radiation-induced brain injury and compared its efficacy with that of bevacizumab in reducing RN in rat brain tissue.

METHODS

Animals

A total of 60 male Wistar albino rats, weighing ~250 g, were obtained from the Laboratory Animal Raising and Experimental Research Center. The rats were maintained at a standard temperature (22°–24°C) with a 12-hour light/dark cycle and standard rat chow in accordance with institutional and national guidelines. The local ethics committee for animal experiments approved the present experimental study. It was funded by Gazi University Institutional Projects of Scientific Investigation Unit (grand ID: 01-2018-01).

Experimental Schemes

The rats were divided into 10 groups of 6 animals each. The experimental scheme is summarized in Table 1. SRS was applied to the rats in groups 1–5 using the Leksell Gamma Knife Perfexion system (Elekta, Stockholm, Sweden). The rats were anesthetized with intramuscular injections of 50 mg/kg ketamine and 5 mg/kg xylazine and fixed in a specially designed stereotactic animal frame for the Gamma Knife device (Figure 1), as described previously.[14] Brain magnetic resonance imaging (MRI; 3 Tesla) was performed to determine the coordinates of the radiation target area in the right frontal lobe of the rats. Using the determined coordinates, SRS was applied using a 4-mm collimator to produce a 4-mm sphere at the 50% isodose line. A single-fraction, 50-Gy radiation dose (50% isodose) was applied using the Leksell GammaPlan, version 10.1, treatment planning system (Elekta). The Gamma Knife isodose distribution on the MRI scans is shown in Figure 2.

The rats in groups 2, 3, 6, and 7 received bevacizumab (Altuzan [Roche, Basel, Switzerland]) at a dose of 10 mg/kg intraperitoneally twice weekly. The rats in groups 4, 5, 8, and 9 received both bevacizumab and ramipril. Ramipril was given in the drinking water at a dose of 1.5 mg/kg/day. The freshly prepared solution was provided daily during the experiment. Bevacizumab or

Table 1. Experimental Scheme

Group	Gamma Knife	Bevacizumab	Ramipril	Treatment Protocol
SRS	Yes	No	No	50 Gy
SRS + P-B	Yes	Day 1	No	50 Gy plus bevacizumab from first day of SRS at 10 mg/kg intraperitoneally twice weekly
SRS + T-B	Yes	Week 4	No	50 Gy plus bevacizumab from fourth week of SRS at 10 mg/kg intraperitoneally twice weekly
SRS + P-R	Yes	No	Day 1	50 Gy plus ramipril from first day of SRS at 1.5 mg/kg/day in drinking water
SRS + T-R	Yes	No	Week 4	50 Gy plus ramipril from fourth week of SRS at 1.5 mg/kg/day in drinking water
P-B	No	Day 1	No	Bevacizumab from first day of experiment
T-B	No	Week 4	No	Bevacizumab from fourth week of experiment
P-R	No	No	Day 1	Ramipril from first day of experiment
T-R	No	No	Week 4	Ramipril from fourth week of experiment
Control	No	No	No	Only frame plus physiological serum at 0.1 mL intraperitoneally twice weekly

SRS, stereotactic radiosurgery; P-B, prophylactic bevacizumab; T-B, therapeutic bevacizumab; P-R, prophylactic ramipril; T-R, therapeutic ramipril.

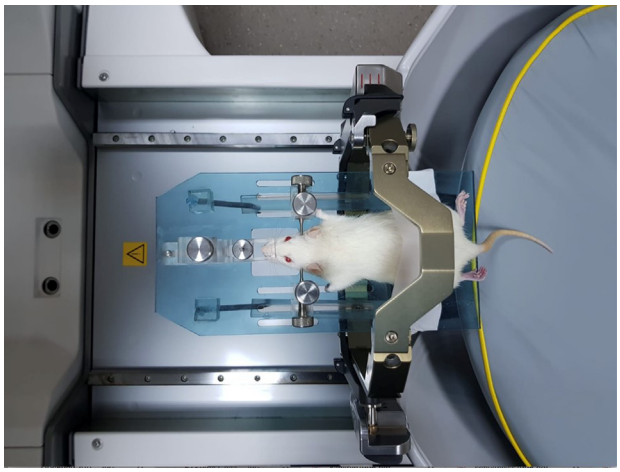

Figure 1. Placement of the rats using a specially designed stereotactic animal frame for the Gamma Knife device.

ramipril was provided using 2 different protocols: a prophylaxis protocol, which consisted of administration of bevacizumab or ramipril initiated on the first day of SRS, and a therapeutic protocol, which consisted of administration of bevacizumab or ramipril starting 4 weeks after SRS. In addition, sham control rats were anesthetized but did not receive SRS, bevacizumab, or ramipril. The control rats had had the stereotactic frame placed and had received physiological serum twice weekly at a dose of 0.1 mL intraperitoneally.

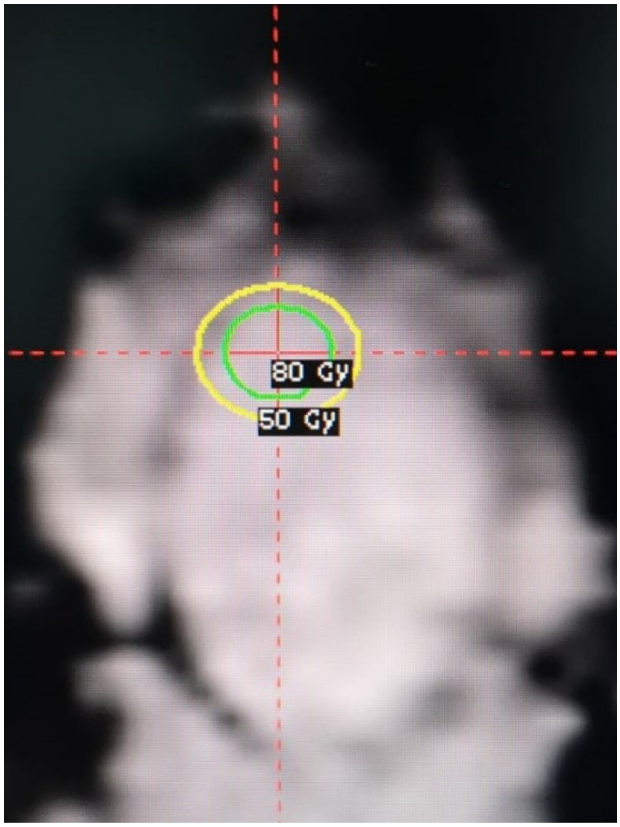

Figure 2. Gamma Knife isodose distribution on a coronal magnetic resonance image of a mouse (GammaPlan, version 10.1 [Elekta], was used as the treatment planning system).

The rats were sacrificed 12 weeks after the beginning of SRS. For euthanasia, the intracardiac blood was removed with the rat under general anesthesia. The brain was cautiously detached from the skull, and the right frontal lobe, which had been exposed to radiation, was separated from the rest of the brain and fixed in neutral formaldehyde for histological and immunohistochemical analyses.

Histological Analysis

The tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin after routine histological procedures had been completed. Sections 4-µm thick were then cut from the paraffin block and stained with hematoxylin and eosin. In each section, 6 random areas were evaluated at 200× magnification. Radiation injury was assessed using the following criteria, as identified in previous studies: neuronal loss, glial cell mitigation, congestion, edema, vascular lesions, and hemorrhage.[14,23-26] The total histopathological scores were determined using the histopathological criteria for each area (Table 2). The grade (grade 0–5) was defined according to the total scores (Table 3). The grade distribution for each group is presented as a percentage (Table 4). Photomicrographs were taken using a Leica DM 4000 light microscope (Leica Microsystems, Wetzlar, Germany).

Immunohistochemical Analysis

The avidin-biotin-peroxidase method was used for immunohistochemical analysis. After deparaffinization, the cross-sections were subsequently incubated in citrate buffer (pH, 6.0) and 3% hydrogen peroxide (catalog no. TA-125-HP [Lab Vision, Thermo Scientific, Fremont, California, USA]). Ultra V block (catalog no. TP-125-HL [Lab Vision, Thermo Scientific]) was applied for blocking. After the blocking stage, the tissue sections were incubated with VEGF primer antibody (rabbit polyclonal; catalog no. PB9071 [Boster Bio, Pleasanton, California, USA]); HIF-1α primer antibody (rabbit polyclonal; catalog no. PB9253 [Boster Bio]), and CD31 primer antibody (rabbit polyclonal) at different dilutions (1 µg/mL, 0.5 µg/mL, and 1:300, respectively) overnight at 4°C.

Next, the tissue sections were incubated with secondary antibody (catalog no. TP-125-HL [Lab Vision, Thermo Scientific]) for 10 minutes. The reaction was revealed using streptavidin peroxidase complex (catalog no. TP-125-HL [Lab Vision, Thermo Scientific]) with aminoethyl carbazole (catalog no. TA-125-HA [Lab Vision, Thermo Scientific]). Mayer's hematoxylin was used for background staining. The slides were evaluated under a light microscope. In each preparation of VEGF, HIF-1α, and CD31 staining using the immunohistochemical method, 6 areas were determined randomly at 200× magnification. The immunostaining intensity was determined as a percentage using the ImageJ program (National Institutes of Health, Bethesda, Maryland, USA).

Statistical Analysis

The histopathological findings are presented as percentages, and the immunohistochemical and biochemical findings are presented

Table 2. Histopathological Assessment Parameters and Radiation Injury Score

	Score				
Parameter	**None**	**Mild**	**Prominent**	**Severe**	**Very Severe**
Neuronal loss	0	1	2	NA	NA
Glial cell loss	0	1	2	NA	NA
Congestion	0	1	2	3	4
Edema	0	1	2	3	4
Vascular lesion	0	1	2	3	4
Hemorrhage	0	1	2	3	4

NA, not applicable.

as the mean ± standard deviation. The Kruskal-Wallis H test was used to compare the differences between the groups. The parameters with statistically significantly differences between the 2 groups were assessed using the Bonferroni-corrected Mann-Whitney U test (statistical significance set at $P < 0.001$). SPSS for Windows, version 21 (IBM Corp., Armonk, New York, USA) was used for statistical analysis.

RESULTS

Histological Analyses

The results obtained by grading the radiation injuries are presented as percentages in Table 4. The findings were significantly different between the groups ($P < 0.001$). Although no grade 0, 1, or 2 radiation injury was observed in the SRS group, the proportion of grade 3, 4, and 5 radiation injury was 11%, 28%, and 61%, respectively. The frequency of grade 0 and 1 radiation injury in the control group was 92% and 8%, respectively. The differences between the SRS and control group were statistically significant ($P < 0.001$). The pure application of prophylactic bevacizumab (P-B), therapeutic bevacizumab (T-B), prophylactic ramipril (P-R), and therapeutic ramipril (T-R) without SRS did not significantly change the radiation injury grade.

After adding bevacizumab to the SRS group, no grade 4 and 5 injuries were observed in the P-B group. However, the proportion of grade 1, 2, and 3 injuries was 22%, 64%, and 14%, respectively.

Table 3. Histopathological Grade of Radiation Injury Determined by Radiation Injury Score

Grade	Total Score
0	<1
1	1–5
2	6–10
3	11–15
4	16–19
5	≥20

Although no grade 5 injury was observed in the T-B group, the proportion of grade 2, 3, and 4 injuries was 14%, 69%, and 17%, respectively. The addition of bevacizumab, either prophylactically or therapeutically, to SRS resulted in statistically significant reductions in radiation injury compared with the SRS-only group ($P < 0.001$). In addition, P-B plus SRS was found to be more effective than T-B plus SRS ($P < 0.001$).

The addition of ramipril treatment to SRS was also effective. The proportion of grade 2, 3, 4, and 5 radiation injuries in the P-R group was 9%, 61%, 22%, and 8%, respectively. The proportion of grade 2, 3, 4, and 5 radiation injuries in the T-R group was 3%, 58%, 28%, and 11%, respectively. The difference with the addition of ramipril either prophylactically or therapeutically to SRS was statistically significant in terms of reducing radiation injury compared with the SRS-only group ($P < 0.001$). However, no statistically significant differences were found between the P-R plus SRS and T-R plus SRS groups ($P = 0.333$).

The comparison of the efficacy of bevacizumab and ramipril revealed that P-B plus SRS was more effective than P-R plus SRS or T-R plus SRS ($P < 0.001$). However, no significant differences were found between the T-B plus SRS and T-R plus SRS groups ($P = 0.333$) or between the T-B plus SRS and P-R plus SRS groups ($P = 0.12$). The photomicrographs of the histopathological findings are shown in Figure 3.

Immunohistochemical Analyses

HIF-1α and VEGF expression was evaluated for angiogenesis, edema formation, and cerebral necrosis. CD31 expression was assessed for vascular density, which determined the changes in angiogenesis in the radiation target. The immunopositivity ratios of HIF-1α, VEGF, and CD31 were significantly different between the groups ($P < 0.001$). The ratios were significantly greater in the SRS group than in the control group ($P < 0.001$). Although P-B and T-B without SRS significantly reduced the VEGF, HIF-1α, and CD31 ratios ($P < 0.001$), the use of P-R and T-R did not significantly change the results. The immunopositivity ratios for VEGF, HIF-1α, and CD31 are summarized in Table 5.

The VEGF (31% vs. 8%; $P < 0.001$), HIF-1α (15.5% vs. 5%; $P < 0.001$), and CD31 (9% vs. 4%; $P < 0.001$) ratios were significantly decreased with the addition of P-B to SRS compared with the ratios in the SRS-only group. Similar results were obtained

Table 4. Frequency of Histopathological Grade Stratified by Group

	Group (%)									
Grade	SRS	SRS + P-B	SRS + T-B	SRS + P-R	SRS + T-R	P-B	T-B	P-R	T-R	Control
0	0	0	0	0	0	81	75	72	64	92
1	0	22	0	0	0	19	25	28	36	8
2	0	64	14	9	3	0	0	0	0	0
3	11	14	69	61	58	0	0	0	0	0
4	28	0	17	22	28	0	0	0	0	0
5	61	0	0	8	11	0	0	0	0	0

SRS, stereotactic radiosurgery; P-B, prophylactic bevacizumab; T-B, therapeutic bevacizumab; P-R, prophylactic ramipril; T-R, therapeutic ramipril.

in the comparison of the SRS plus T-B and SRS-only groups (VEGF, 31% vs. 10%, $P < 0.001$; HIF-1α, 15.5% vs. 8%, $P < 0.001$; CD31, 9% vs. 4%, $P < 0.001$, respectively). The addition of P-B to SRS was more effective than T-B for decreasing the VEGF and HIF-1α ratios ($P < 0.001$). However, the differences were not statistically significant for the CD31 ratio ($P = 0.982$).

The VEGF (31% vs. 14%; $P < 0.001$), HIF-1α (15.5% vs. 6%; $P < 0.001$), and CD31 (9.2% vs. 6.7%; $P < 0.001$) ratios had decreased significantly with the addition of P-R to SRS. Similar results were also obtained with the addition of T-R to SRS (VEGF, 31% vs. 16%, $P < 0.001$; HIF-1α, 15.5% vs. 9%, $P < 0.001$; CD31, 9% vs. 7%, $P < 0.001$, respectively). The addition of P-R to SRS was more effective than the addition of T-R in decreasing the HIF-1α ratio ($P < 0.001$). However, the difference was not statistically significant for the VEGF ($P = 0.133$) and CD31 ($P = 0.982$) ratios.

When we compared the efficacy of the addition of prophylactic bevacizumab and ramipril to SRS, P-B was more effective in reducing the VEGF and CD31 ratios ($P < 0.001$). However, the difference was not statistically significant for the HIF-1α ratio ($P = 0.408$). Similar results were obtained in the comparison of the therapeutic usage of bevacizumab and ramipril (VEGF and CD31, $P < 0.001$; HIF-1α, $P = 0.640$). The comparison of P-B plus SRS to T-R plus SRS and T-B plus SRS to P-R plus SRS revealed that the prophylactic or therapeutic use of bevacizumab was more effective in reducing VEGF, HIF-1α, and CD31 rates ($P < 0.001$). The immunohistochemical results are summarized in Table 5. The photomicrographs of the histopathological findings are shown in Figures 4–6.

DISCUSSION

Improved survival with radiation therapy can come with a cost, including potential complications such as brain necrosis. RN will affect the quality of life of patients and could reduce the therapeutic ratio of radiation therapy.[2] The current clinical standard of care for RN has been the use of corticosteroids. However, because of the high rate of side effects and the limited activity of corticosteroids, alternative therapeutic options to these drugs are required.[27]

The use of ramipril was shown to reduce the incidence of radiation-induced optic neuropathy,[18] cognitive impairment,[20] and radiation-induced myelopathy.[22] Clausi et al.[22] reported that the use of angiotensin II might mediate radiation-induced vascular damage and that ramipril reduced VEGF expression in irradiated spinal cord tissue. From their results, we hypothesized that treatment with an ACE inhibitor would mitigate the incidence of RN by reducing the most potent proangiogenic factors, HIF-1α and VEGF, through a reduction in angiotensin II expression. We preferred the use of ramipril, as an ACE inhibitor, because it is rapidly absorbed, not affected by food after oral intake, and has superior stability in water and can cross the blood–brain barrier compared with other ACE inhibitors.[18,22,27,28]

In our study, bevacizumab and ramipril were administered using 2 distinct protocols: prophylactic and therapeutic. For the prophylactic protocol, they had been used from the first day of SRS. For therapeutic protocol, these agents had been used from the fourth week onward after 50 Gy of SRS, which has been reported to be the earliest time at which RN can be observed on MRI studies.[12,13] Bevacizumab and ramipril have been shown to significantly mitigated the histological severity of radiation injury in both prophylactic and therapeutic usage. In the present study, prophylactic usage of bevacizumab was more effective than T-B, P-R, or T-R. No difference was observed in the comparisons of the other treatment protocols. We also showed that the expression of VEGF, HIF-1α, and CD31 had significantly increased by the 12th week after SRS compared with the expression in the control group. The addition of bevacizumab or ramipril to SRS mitigated the expression of VEGF, HIF-1α, and CD31. However, the prophylactic use of these agents seemed to be more effective than therapeutic administration. Our results also revealed that the greatest reduction in the expression of VEGF, HIF-1α, and CD31 was achieved with prophylactic administration of bevacizumab compared with the T-B, P-R, and T-R groups.

Jiang et al.[13] reported on the efficacy of anti-VEGF antibodies on the incidence of RN after a 50-Gy dose of gamma knife radiosurgery. The irradiated mice had been treated starting from the initial appearance of RN on MRI (~4 weeks after irradiation) until the 13th week after SRS. The histological and MRI findings revealed that the therapeutic usage of anti-VEGF antibodies can mitigate RN in the brain.[13] Perez-Torres et al.[12] used a similar experimental model. In addition, they performed immunohistochemistry for VEGF. The

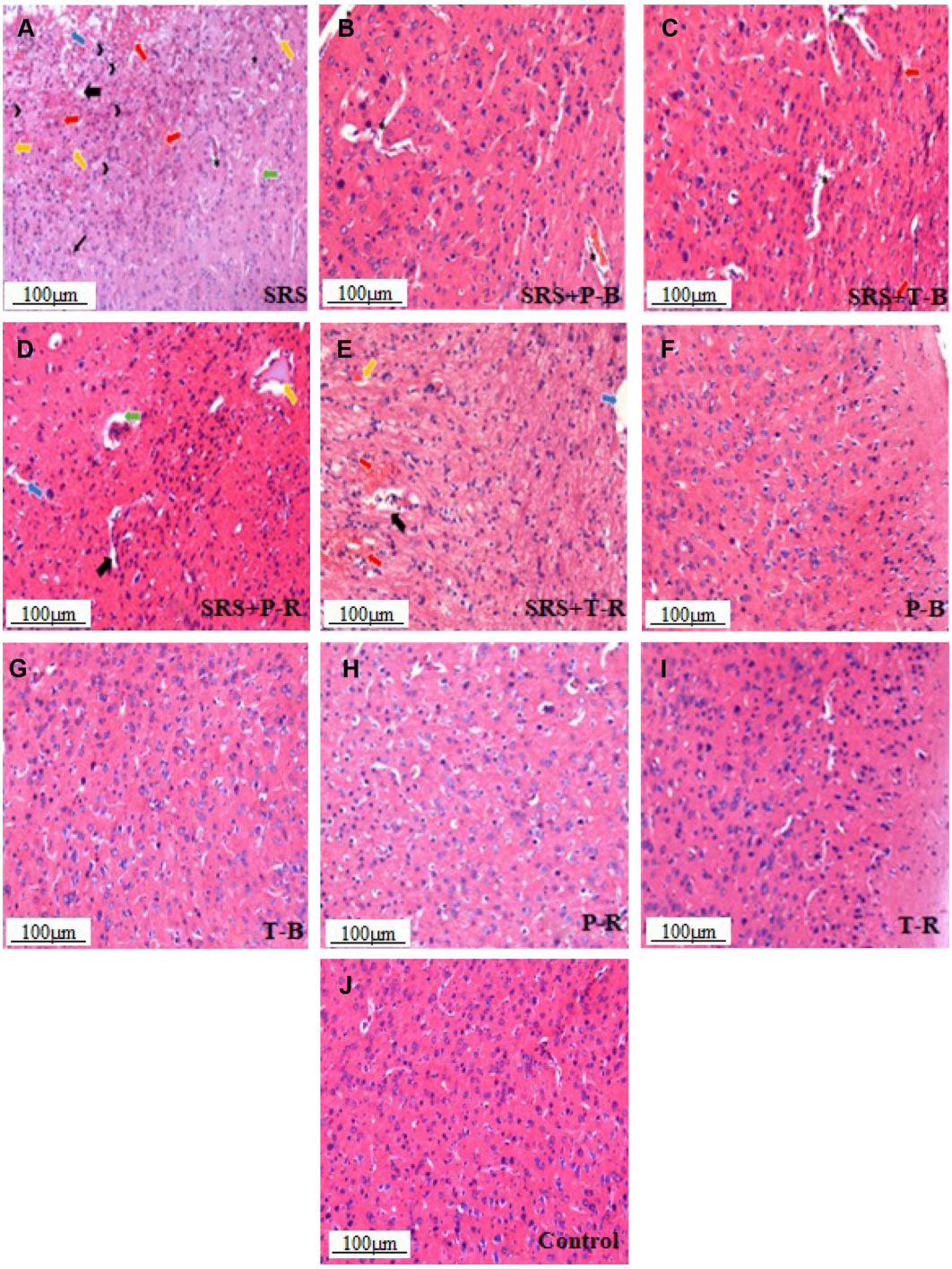

Figure 3. Representative photomicrographs of histopathological findings (hematoxylin and eosin stain, original magnification ×200). (**A**) Stereotactic radiosurgery (SRS) group: *yellow arrow*, necrotic hyalinized blood vessel; *red arrow*, hemorrhage; *black arrow*, neuronal loss; *green arrow*, vessel wall edema; *blue arrow*, demyelination; *thin black arrow*, protein denaturation; *arrowheads*, microglial infiltration; *asterisk*, vasodilatation; *star*, eosinophilic stroma. (**B**) SRS plus prophylactic bevacizumab (P-B) group: *asterisk*, vasodilatation. (**C**) SRS plus therapeutic bevacizumab (T-B) group: *asterisk*, vasodilatation; *red arrow*, hemorrhage. (**D**) SRS plus prophylactic ramipril (P-R) group: *yellow arrow*, necrotic hyalinized blood vessel; *black arrow*, neuronal loss; *green arrow*, vessel wall edema; *blue arrow*, demyelination. (**E**) SRS plus therapeutic ramipril (T-R) group: *yellow arrow*, necrotic hyalinized blood vessel; *red arrow*, hemorrhage; *black arrow*, neuronal loss; *blue arrow*, demyelination. (**F**) Prophylactic bevacizumab (P-B) group, (**G**) therapeutic bevacizumab (T-B) group, (**H**) prophylactic ramipril (P-R) group, (**I**) therapeutic ramipril (T-R) group, and (**J**) control group: normal appearance of cells and vascular structures in the brain cortex.

Table 5. Immunopositivity for VEGF, HIF-1α, and CD31 Ratios Stratified by Group

	Group									
Variable	**SRS**	**SRS + P-B**	**SRS + T-B**	**SRS + P-R**	**SRS + T-R**	**P-B**	**T-B**	**P-R**	**T-R**	**Control**
VEGF	31 ± 9	8 ± 1.8	10 ± 2.8	14 ± 4.7	16 ± 5.4	1 ± 0.98	2 ± 1.12	5.6 ± 2.5	6.2 ± 1.4	7.5 ± 3.7
HIF-1α	15.5 ± 5	5 ± 2	8 ± 2.7	6 ± 3	9 ± 4.4	2 ± 1	3 ± 2	2.7 ± 2	3.2 ± 1.9	1.4 ± 1
CD31	9 ± 2	4 ± 2	4 ± 2.3	6.7 ± 2.4	7 ± 2.5	1.94 ± 1.5	2.6 ± 1.8	2.8 ± 1.8	3 ± 2	4 ± 1.8

SRS, stereotactic radiosurgery; P-B, prophylactic bevacizumab; T-B, therapeutic bevacizumab; P-R, prophylactic ramipril; T-R, therapeutic ramipril; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α.

production of VEGF can be detected at 4 weeks after irradiation and will progressively increase. They also determined that VEGF overexpression and radiation-induced RN on MRI developed simultaneously, precluding preventative anti-VEGF treatment.[12] In our previous study,[14] the rats had received 2 different gamma knife doses (50 Gy to the 50% isodose line and 100 Gy to the 50%

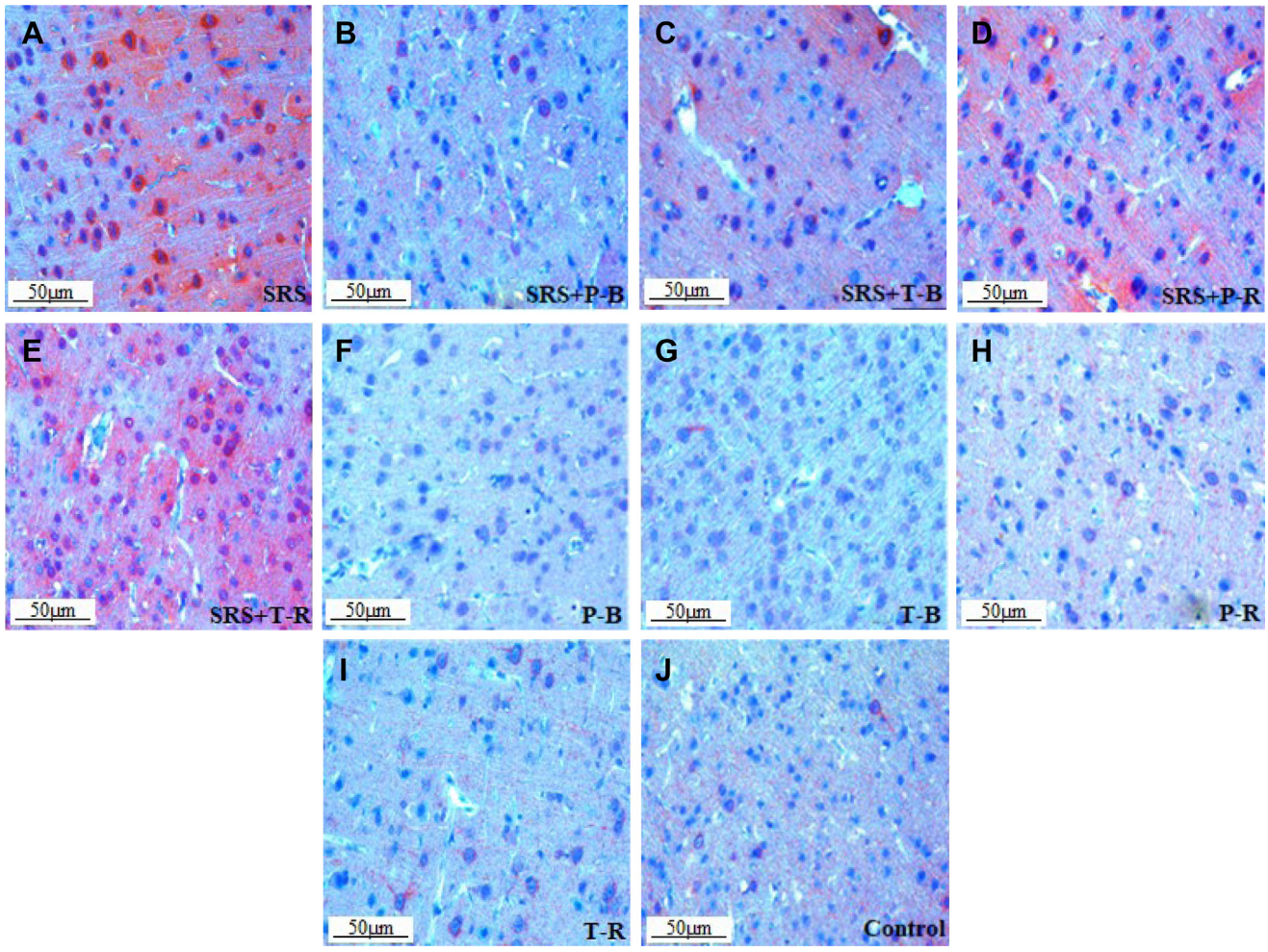

Figure 4. Representative photomicrographs of positive staining of vascular endothelial growth factor antibodies in all groups on immunohistochemical staining (aminoethyl carbazole, hematoxylin stain, original magnification ×400). (**A**) Stereotactic radiosurgery (SRS) group (**B**) SRS plus prophylactic bevacizumab (P-B) group (**C**) SRS plus therapeutic bevacizumab (T-B) group (**D**) SRS plus prophylactic ramipril (P-R) group (**E**) SRS plus therapeutic ramipril (T-R) group (**F**) Prophylactic bevacizumab (P-B) group (**G**) therapeutic bevacizumab (T-B) group, (**H**) prophylactic ramipril (P-R) group, (**I**) therapeutic ramipril (T-R) group, and (**J**) control group.

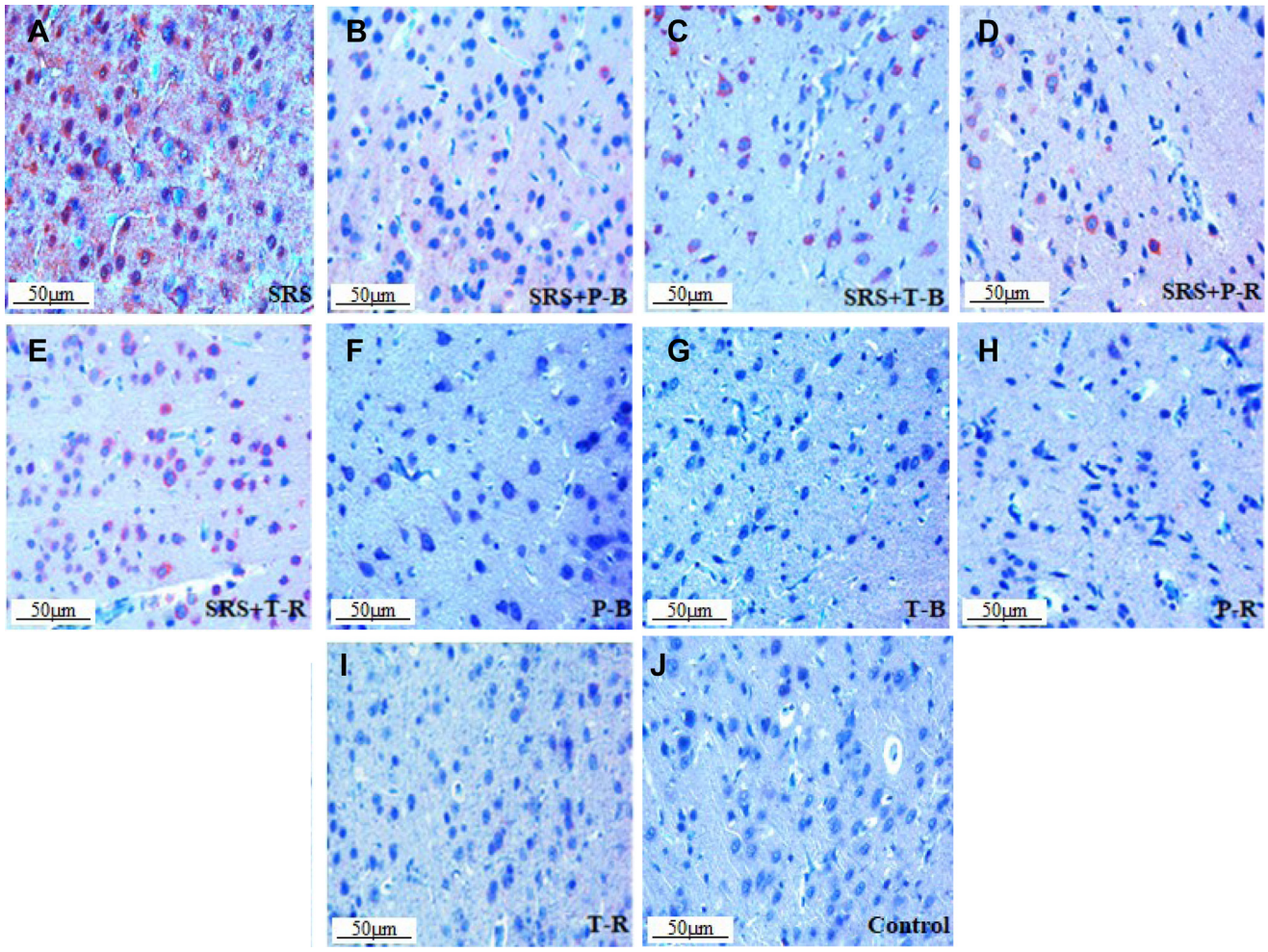

Figure 5. Representative photomicrographs of positive staining of hypoxia-inducible factor (HIF)-1α antibodies in all groups using immunohistochemical staining (aminoethyl carbazole, hematoxylin stain, original magnification ×400). (**A**) Stereotactic radiosurgery (SRS) group (**B**) SRS plus prophylactic bevacizumab (P-B) group (**C**) SRS plus therapeutic bevacizumab (T-B) group (**D**) SRS plus prophylactic ramipril (P-R) group (**E**) SRS plus therapeutic ramipril (T-R) group (**F**) Prophylactic bevacizumab (P-B) group (**G**) therapeutic bevacizumab (T-B) group, (**H**) prophylactic ramipril (P-R) group, (**I**) therapeutic ramipril (T-R) group, and (**J**) control group.

isodose line) and bevacizumab 10 mg/kg intraperitoneally twice weekly. Bevacizumab was applied prophylactically (administration initiated on the first day after SRS) and therapeutically (administration initiated on the day of the detection of necrosis on MRI). High-dose gamma knife radiosurgery resulted in the earlier development of RN with greater severity than low-dose radiosurgery (3 weeks, grade 2–3; 9 weeks, grade 1) on MRI. These findings differed from those reported by Jiang et al.[13] in terms of the timing of the onset of necrosis. This difference might have resulted from our less sensitive radiologic assessment using a non–animal-specific MRI scanner. The histological and radiological evaluations revealed that prophylactic bevacizumab successfully mitigated the effects of RN, although this effect was not prominent with the therapeutic bevacizumab protocol. The results of the present study have confirmed the superiority of the prophylactic usage of bevacizumab. Because we had previously shown radiological verification of histopathological brain necrosis after SRS at a dose of 50 Gy (50% isodose line),[14] MRI was not performed to reduce the amount of anesthesia for the rats in the present study.

Although our study evaluated 2 distinct treatment protocols, we did not address the important questions regarding optimal dose scheduling and treatment duration. We had decided to treat the rats for 12 weeks, in accordance with previous data,[5,9,12] which had demonstrated the existence of alterations in VEGF, HIF-1α, and CD31 expression. Cheng et al.[5] reported progressive increases in VEGF and CD31 expression in the irradiated cortex for ≤16 weeks after SRS. The maximal expression level was reached at 16 weeks, followed by a gradual decrease to the control level by

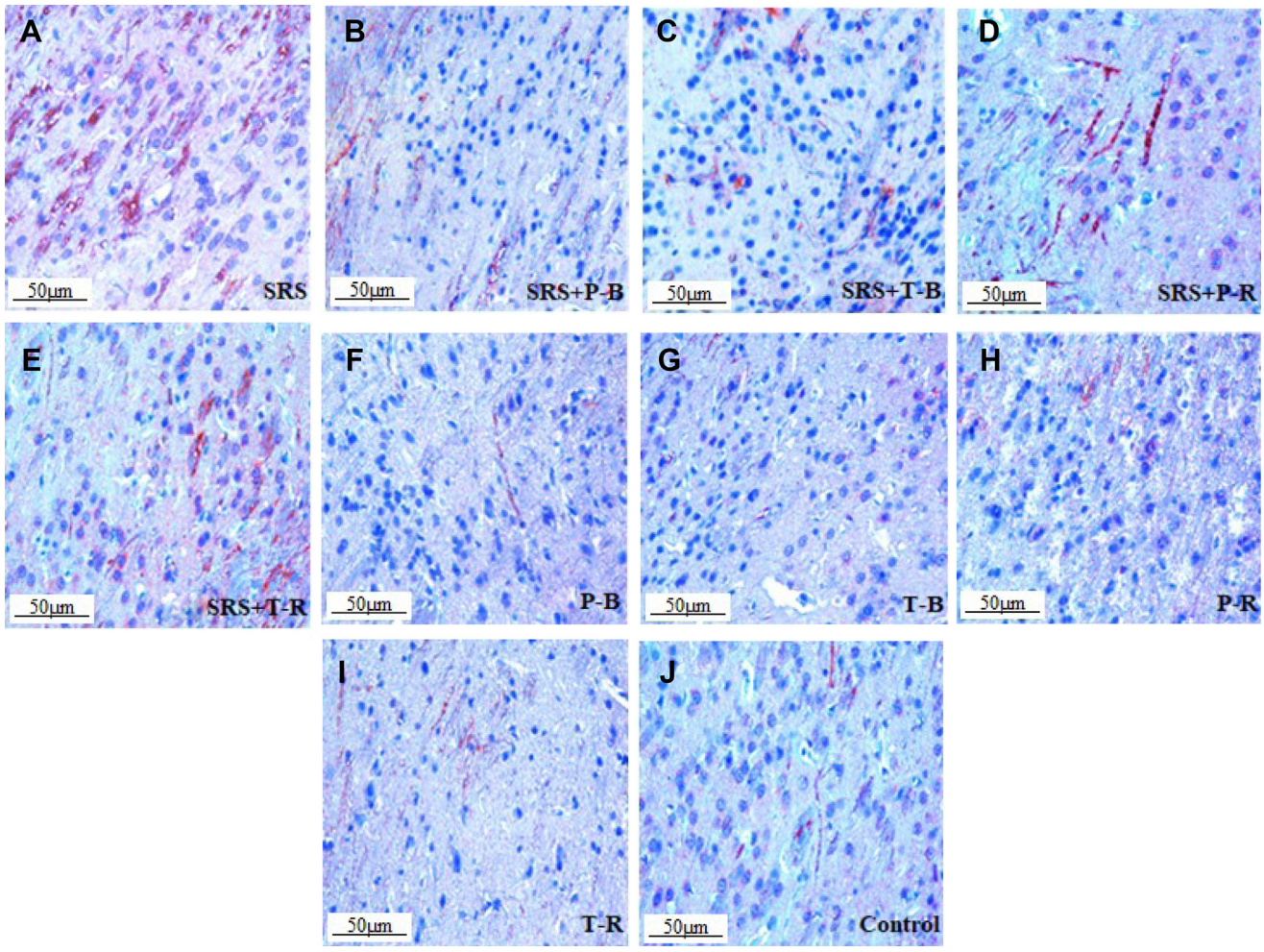

Figure 6. Representative photomicrographs of positive staining of CD31 antibodies in all groups by immunohistochemical staining (aminoethyl carbazole, hematoxylin stain, original magnification ×400). (**A**) Stereotactic radiosurgery (SRS) group (**B**) SRS plus prophylactic bevacizumab (P-B) group (**C**) SRS plus therapeutic bevacizumab (T-B) group (**D**) SRS plus prophylactic ramipril (P-R) group (**E**) SRS plus therapeutic ramipril (T-R) group (**F**) Prophylactic bevacizumab (P-B) group (**G**) therapeutic bevacizumab (T-B) group, (**H**) prophylactic ramipril (P-R) group, (**I**) therapeutic ramipril (T-R) group, and (**J**) control group.

24 weeks after SRS.[5] Additionally, Duan et al.[9] showed that VEGF and HIF-1α expression was continually upregulated in both the anti-VEGF and the control groups. Despite the improvements observed radiographically after anti-VEGF treatment, which was administered until week 12 after SRS, both VEGF and HIF-1α expression remained upregulated in the anti-VEGF group.[9]

Nevertheless, some clinical trials have demonstrated that the recurrence of RN can occur after bevacizumab treatment was discontinued.[15,29-31] These findings are consistent with the mechanism of treatment with an anti-VEGF antibody. Although it binds VEGF and prevents VEGF from reaching its receptors, bevacizumab should not directly affect its production. Therefore, using inhibitors of HIF-1α, which is an important regulator of angiogenesis through the activation of proangiogenic factors, might be a reasonable target for reducing VEGF expression.

In addition, no consensus has been reached regarding the optimal dosage schemes for bevacizumab. However, investigators have used different doses, ranging from 5 to 10 mg/kg in human and animal studies.[13,15,32] Patients have typically received bevacizumab every 2–4 weeks, because it has a half-life of ~20 days.[3] In animal studies, once or twice weekly doses of 10 mg/kg in mice have been reported.[13,14] Because we had sought to reduce the incidence of radiation-induced injury, we had preferred to use the higher dosing scheme of 10 mg/kg twice weekly.

The optimal dose scheduling and treatment duration of ramipril were evaluated in the study by Ryu et al.[19] They assessed the optimal dose and administration of ramipril to mitigate the incidence of radiation-induced optic neuropathy. To determine the optimal dose of ramipril for the preservation of the optic nerve and chiasm, 3 doses of ramipril were evaluated: 1.5, 1.0, and 0.5 mg/kg/day. To

assess the optimal time for starting ramipril treatment, ramipril was started at 2 or 4 weeks after SRS.[19] The higher dose of ramipril at 1.5 mg/kg/day, starting within 2 weeks of the initiation of SRS was found to be more effective for preserving optic nerve function. The use of ramipril, starting at 4 weeks after SRS, did not show differences compared with the radiation-alone group.[19] Because ramipril had been shown to be effective and feasible when given in drinking water at a concentration of 1.5 mg/kg/day, the same protocol was used in our study.[10] However, we chose to start ramipril from the first day of SRS as a prophylactic protocol.

CONCLUSIONS

The results from our study have demonstrated that treatment with bevacizumab and ramipril can significantly reduce the incidence of radiation-induced brain necrosis in the rat brain. The most prominent effect among these treatment protocols was achieved with the prophylactic addition of bevacizumab to SRS. Nonetheless, ramipril is an inexpensive, available, and well-tolerated drug that can also cross the blood–brain barrier. Thus, it might be an alternative therapeutic agent for patients with RN. Another treatment option would be to use the combination of ramipril and bevacizumab, which we plan to evaluate in our next experimental study. Although the findings from the present study are important because they show the efficacy of ACE inhibitors for reducing the incidence and severity of RN, additional studies are needed to determine the effective and safe doses for use in humans.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Ozge Petek Erpolat: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Niyazi Volkan Demircan:** Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Project administration, Funding acquisition. **Gulistan Sanem Sarıbas:** Investigation, Formal analysis, Methodology. **Pelin Kuzucu:** Formal analysis, Investigation, Methodology. **Ertugrul Senturk:** Investigation, Formal analysis, Methodology. **Cigdem Elmas:** Investigation, Formal analysis, Methodology. **Alp Borcek:** Conceptualization, Methodology, Writing - review & editing, Visualization, Supervision. **Gokhan Kurt:** Methodology, Visualization, Supervision.

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Conflict of interest statement: The present study was supported by the Gazi University Projects of Scientific Investigation Unit, Turkey (grant ID: 01-2018-01).

Received 27 May 2020; accepted 12 August 2020

Citation: World Neurosurg. (2020) 144:e210-e220. https://doi.org/10.1016/j.wneu.2020.08.081

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