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Obesity as a Limiting Factor for Remote Ischemic Postconditioning-Mediated Neuroprotection after Stroke

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Background: Remote ischemic postconditioning (RIPostC) may protect the brain from ischemia/reperfusion (I/R) injury. The association between RIPostC and obesity has not yet been extensively studied.

Methods: Twelve-week-old male Zucker diabetic fatty (ZDF; n=68) and Zucker diabetic lean (ZDL; n=51) rats were subjected to focal cerebral ischemia for 90 minutes, followed by 24 hours of reperfusion. RIPostC was performed with 5-minute I/R cycles using a tourniquet on the right hind limb.

Results: The results showed a negative association between obesity and neurological impairment in ischemic animals. We observed a 70% greater infarct size in ZDF rats compared with their lean counterparts, as evaluated by 2,3,5-triphenyltetrazolium chloride staining. To measure the total fragmented DNA in peripheral lymphocytes, comet assay was performed. Obese rats exhibited higher levels of DNA damage (by approximately 135%) in peripheral blood lymphocytes even before the induction of stroke. RIPostC did not attenuate oxidative stress in the blood in obese rats subjected to ischemia. Focal cerebral ischemia increased core and penumbra tissue glutamate release in the brain and decreased it in the blood of ischemic ZDL rats, and these changes improved following RIPostC treatment. However, changes in blood and tissue glutamate content were not detected in ischemic ZDF rats or after RIPostC intervention.

Conclusion: Our findings suggest that obese animals respond more severely to ischemia-reperfusion brain injury. However, obese animals did not achieve neuroprotective benefits of RIPostC treatment.

Key words: Obesity, Stroke, Neuroprotection, Ischemic postconditioning, Glutamates, Oxidative stress

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INTRODUCTION

Stroke is the leading cause of long-term neurological disability and the second leading cause of mortality worldwide.[1] Obesity is one of the most significant measurable risk factors for ischemic stroke, with obese individuals having a 2- to 3-fold increased risk of functional disability compared with the general population.[2] Obesity is defined by the U.S. World Health Organization (WHO) as a condition of having a body mass index (BMI) higher than 30 kg/m^2 and affects more than 650 million adults. It is important to note that for Asians, the WHO recognizes a lowed BMI cutoff of 25 kg/m^2 to identify individuals at risk for obesity-related health issues. The WHO predicts that approximately 1 billion adults will become less healthy as a result of being obese by 2025. Nearly 40% of obese people are at a higher risk of having a stroke, underscoring the importance of paying attention to this risk factor.[3]

Currently, the only approved therapies for ischemic stroke are recombinant tissue-plasminogen activator and endovascular thrombectomy.[4] Despite these treatments, a considerable number of stroke patients still end up with a lifelong disability. Therefore, more ef-

forts on developing strategies for ischemic stroke prevention and treatment are desperately needed. Much attention has been focused on developing novel neuroprotective strategies for stroke, such as ischemic postconditioning (IPostC). IPostC was first explored in myocardial ischemia and is defined as a series of brief mechanical occlusions and reperfusions.[5] The protective effect of IPostC on infarct size reduction in cerebral ischemia-reperfusion injury was also subsequently documented. Standard IPostC has been expanded to remote ischemic postconditioning (RIPostC), which can be applied to a distant organ, such as a limb.[6]

RIPostC has been shown to be a promising therapy in animal models of stroke, and several clinical studies in patients with acute ischemic stroke have subsequently been conducted.[7] However, only one small published clinical study confirmed its feasibility.[8] The lack of protection shown in other studies may be ascribed to several factors, including the use of animal models that do not sufficiently reflect clinical reality because of the absence of major comorbid conditions such as diabetes mellitus (DM), obesity, age, and sex. As obesity and DM are comorbidities for stroke, neuroprotective treatment should be investigated in the context of these comorbidities, as they may affect the efficacy of a therapeutic strategy.

In the present study, we investigated whether obesity influences RIPostC-induced neuroprotection against cerebral ischemia *in vivo*. We used a young 11- to 12-week-old Zucker diabetic fatty (ZDF) rat model with a *fa/fa* genotype.

METHODS

Ethics statement

All experimental procedures using animals followed the protocol for animal care approved by the European Communities Directive (2010/63/EU) and was approved by State Veterinary and Food Administration in Bratislava (decision No. 2978-5/2021-220) and the Ethical Council of the Institute of Neurobiology, Biomedical Research Center of the Slovak Academy of Sciences (BMC SAS). All efforts were made to minimize animal suffering.

Animals and experimental design

To minimize the influence of non-modifiable risk factors, such as age and sex, this study used 12-week-old male ZDF rats (n = 68) and the counterpart Zucker diabetic lean (ZDL) rats (n = 51), purchased from Dobrá Voda, Slovakia (Fig. 1A). Rats were housed in standard conditions (temperature of 20 ± 2 °C; humidity, 55% ± 5%; 12-hour light/dark cycle) and given food and water *ad libitum*. Prior to surgery, animals were assigned to one of the following three groups: (1) control group; (2) ischemia group (animals subjected to 90 minutes of right middle cerebral artery occlusion [MCAO]); and (3) ischemia group with ischemic tolerance (three cycles of 5-minute limb ischemia followed by 5 minutes of reperfusion; RIPostC). All animals were anaesthetized with chloral hydrate (300 mg/kg, intraperitoneal) and decapitated 24 hours after surgery, when the ischemic core expands to the maximum volume (Fig. 1B). Animals from groups (2) and (3) were used for histological analysis (to assess the range of neurodegeneration) and animals from all groups were used for infarct volume determination, fluorometric assessment of glutamate content in blood and brain tissue and oxidative stress determination (DNA damage, activity of antioxidant enzymes superoxide dismutase [SOD] and catalase [CAT]) (Fig. 1C).

Right middle cerebral artery occlusion model and neurological score

The intraluminal filament technique developed by Longa et al.[9] was used to induce MCAO. Neurological score was assessed after 1 hour and 1 day of reperfusion using the Bederson et al.[10] scoring system.

Remote ischemic postconditioning

RIPostC was achieved by three cycles of ischemia (5 minutes) and reperfusion (5 minutes) of the femoral artery up to 1 hour after MCAO.

Quantitative analysis of infarct volume

Brain slices (2 mm) were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) in phosphate-buffered saline (PBS) for 30 minutes at 37 °C before being fixed in a 10% formaldehyde solution for 24 hours. Brain sections were scanned (Epson Perfection 4490 Photo) and processed using ImageJ 1.8.0 version software (National Institutes of Health). The size of infarct regions was calculated as described by Callaway et al.[11]

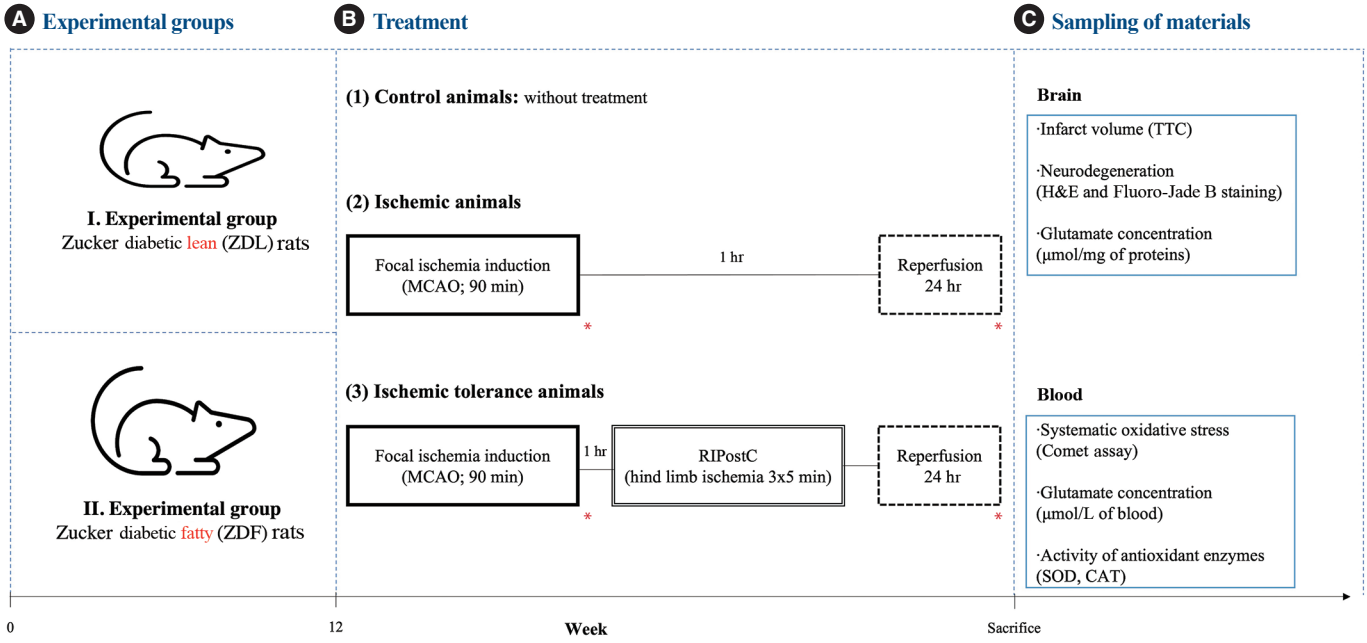

Figure 1. (A) Schematic representation of the *in vivo* study using Zucker diabetic lean (ZDL) and Zucker diabetic fatty (ZDF) rats. (B) ZDL and ZDF rats aged 12 weeks were randomized into three groups. The first control group received no surgery; the other two groups were subjected to 90 minutes of right middle cerebral artery occlusion (MCAO) without (ischemic animals) or with (ischemic tolerance animals) remote ischemic postconditioning (RIPostC) up to 1 hour after ischemia, followed by 24 hours of reperfusion. For ischemic tolerance animals, three 5-minute episodes of hind-limb ischemia, interspersed with 5 minutes of reperfusion, were provided, as described in the Methods. After the survival period (24 hours), the animals were sacrificed by decapitation and samples of brain tissue and blood were collected. (C) The infarct volume, neurodegeneration and glutamate concentration were determined in brain tissue. Blood samples were collected for biochemical analysis, such as measuring the activity of catalase (CAT) and superoxide dismutase (SOD), the amount of glutamate and oxidative stress. *Time of neurological score evaluation. TTC, 2,3,5-triphenyltetrazolium chloride.

Hematoxylin and eosin and Fluoro-Jade B staining

Coronal slices (2 mm) were stored in a 30% sucrose solution in PBS for 24 hours. Brain sections were cut into 25 μm thick sections (Leica CM1850) and mounted on gelatinized microscope slides. Hematoxylin and eosin (H&E) staining was performed using a H&E staining kit (Abcam) following the manufacturer's instructions.[12] Fluoro-Jade B (FJ B) staining was conducted (HistoChem Inc.) following the manufacturer's instructions.[13]

Ischemic damage was evaluated in H&E staining images and quantified as previously reported[14] and by counting pyknotic cells in the striatum and cortex of the penumbra region per mm^2 using ImageJ software. FJ B-positive neurons of the ischemic penumbra region were counted using ImageJ software in 10 random 1 mm^2 areas and results are expressed per 1 mm^2 of tissue.

Tissue and blood processing for glutamate detection

Coronal slices (2 mm) were dissected and separated as described previously,[15] with minor modifications. The tissue was weighed, homogenized (20 mM Tris-HCl pH 7.5 containing 1 mM dithiothreitol [DTT], 50 mM magnesium acetate, 140 mM potassium chloride [KCl], 1 mM ethylenediaminetetraacetic acid [EDTA], 2 mM aminopolycarboxylic acid [EGTA] with protease inhibitor cocktail tablets [Roche]) and centrifuged (12,000 rpm, 15 minutes, 4 °C). Total protein concentrations were determined by Bradford assay.[16] The core and penumbral post-mitochondrial supernatants were stored at –80 °C after precipitation with perchloric acid (PCA; 1:20; 10 minutes, 4 °C) and centrifugation (12,000 rpm, 10 minutes, 4 °C).

Whole blood samples, taken 24 hours after ischemia and RIPostC, were deproteinized by adding an equal volume of ice-cold 1M PCA, precipitated for 10 minutes and centrifuged at 12,000 rpm for 10 minutes at 4 °C.

Glutamate concentration

The glutamate concentration in blood and brain tissue was measured by a modified enzymatic fluorometric method based on an assay described by Graham and Aprison.[17] Glutamate concentration in the blood was expressed as μmol per liter of blood (μmol/L); brain tissue concentration was normalized to protein content (μmol/mg protein).

Oxidative stress parameters in the blood

Comet assay

The comet assay was carried out under alkaline conditions as reported by Singh et al.,[18] with minor modifications to evaluate DNA strand breaks in peripheral lymphocytes. DNA was visualized using SYBR Green, and 100 cells per slide were scored under a fluorescence microscope (Olympus BX51). Images were analyzed using the Comet Score version 1.5 image analysis system (TriTek Corp.). DNA damage was assessed by the parameter "% DNA in tail" (100% of cell fluorescence intensity minus intensity of the head % DNA).

Enzymatic antioxidant defense

Catalase activity

The CAT activity in erythrocytes was estimated spectrophotometrically following the method of Góth,[19] which is based on the formation of a stable hydrogen peroxide–ammonium molybdate complex. One unit of CAT was defined as the amount of enzyme needed for the degradation of 1 μmol of hydrogen peroxide in 1 minute under these conditions. CAT activity was expressed as kU per liter of blood.

Superoxide dismutase activity

SOD activity was measured as described by Sun et al.[20] with modifications. One unit of SOD activity in blood cells was defined as the amount that reduced the absorbance change by 50%.

Statistical analysis

Data were analyzed and plotted using GraphPad Prism Software. Statistical analysis was performed using one-way and two-way analysis of variance, followed by the Dunnett *post hoc* test. The criterion for statistical significance was $P < 0.05$.

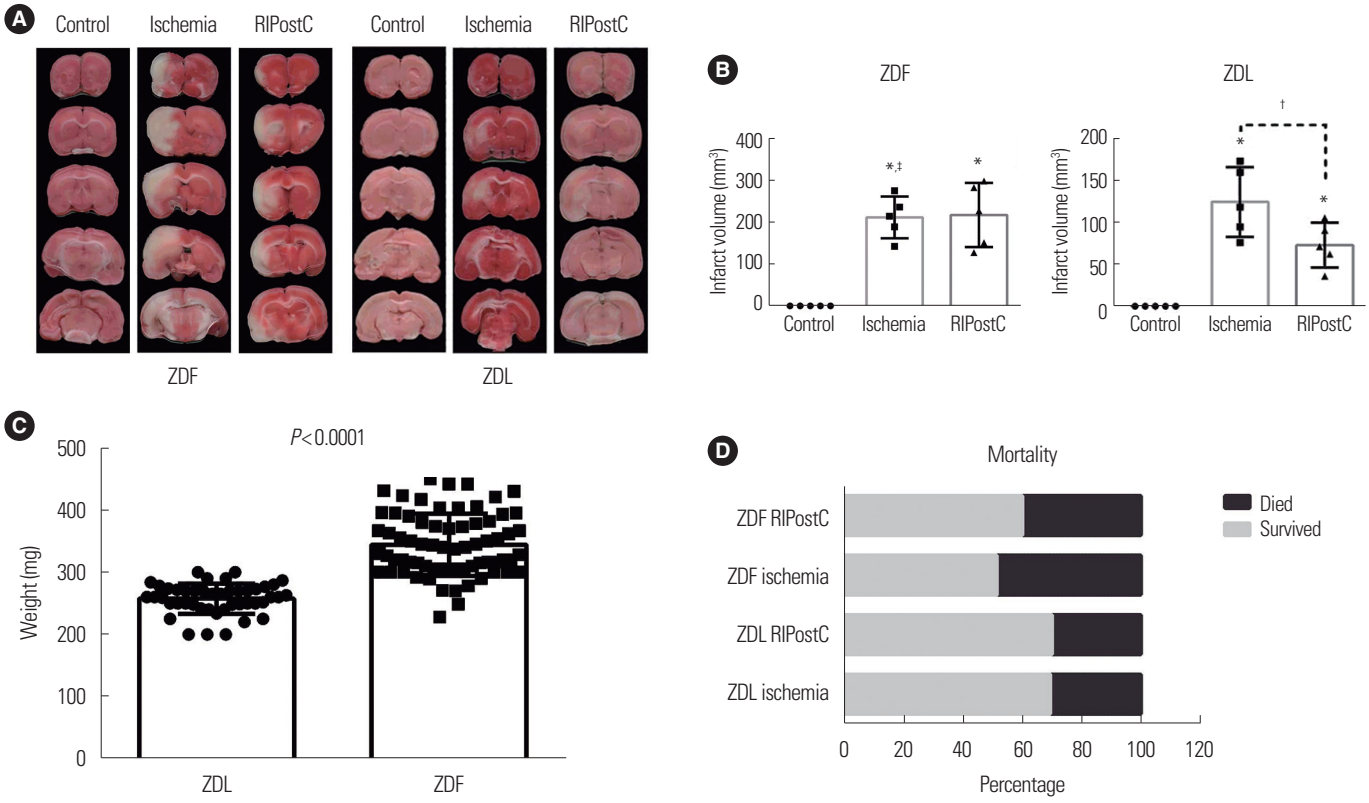

Figure 2. Effect of remote ischemic postconditioning (RIPostC) on post-stroke outcomes in obese Zucker diabetic fatty (ZDF) and Zucker diabetic lean (ZDL) rats. (A) Representative images of brain slices stained with 2,3,5-triphenyltetrazolium chloride (TTC) show cerebral infarction (white coloration) before and after RIPostC intervention. (B) Infarct volume analysis of control, ischemia and tolerance (RIPostC) groups in ZDF and ZDL rats. (C) Body weight of ZDL and ZDF rats. (D) Mortality of ZDL and ZDF rats after ischemia and RIPostC. Values are presented as mean ± standard deviation. *$P < 0.001$ vs. control; †$P < 0.05$ RIPostC vs. ischemia group difference; ‡$P < 0.05$ lean vs. obese group.

RESULTS

Obesity worsened stroke outcomes in obese rats and no improvement after RIPostC treatment was observed

The infarct volume of ZDF and ZDL rat brains in the ischemic and tolerance (RIPostC) groups was evaluated to assess outcome after stroke (Fig. 2A). Significant changes in the control pattern of ZDF rats (n = 5) compared with their lean counterparts ZDL animals (n = 5) were detected. At 12 weeks of age, i.e., adulthood, male ZDL rats weighed approximately 260 ± 3.24 g, while ZDF rats were 100 ± 6.00 g heavier (Fig. 2C).

We observed a 18.1% higher mortality rate for obese rats after MCAO induction, and RIPostC reduced mortality by 8.6% (Fig. 2D). The cerebral infarct volume was measured and expressed in mm^3 of the infarcted hemisphere. In the ischemia groups, the brain infarct volume was 70.2% higher in obese rats (210.50 ± 22.21 mm^3; n = 5) compared with lean rats (123.70 ± 18.47 mm^3; n = 5). RIPostC was neuroprotective in ZDL rats (n = 5); the infarct size was significantly smaller in the RIPostC group compared with the ischemia group (72.70 ± 11.91 mm^3 vs. 123.70 ± 18.47 mm^3, respectively). However, in obese rats (n = 5), RIPostC did not impact infarct size (Fig. 2B) or neurological impairments (data not shown). These results indicate a major impact of obesity on RIPostC-mediated treatment in animal rat stroke model where other potential limitations such as seniority or female sex were excluded.

RIPostC did not prevent neuronal damage in obese animals after stroke

Similar to TTC detection of the brain region containing metabolically inactive cells, as described above, TTC staining revealed

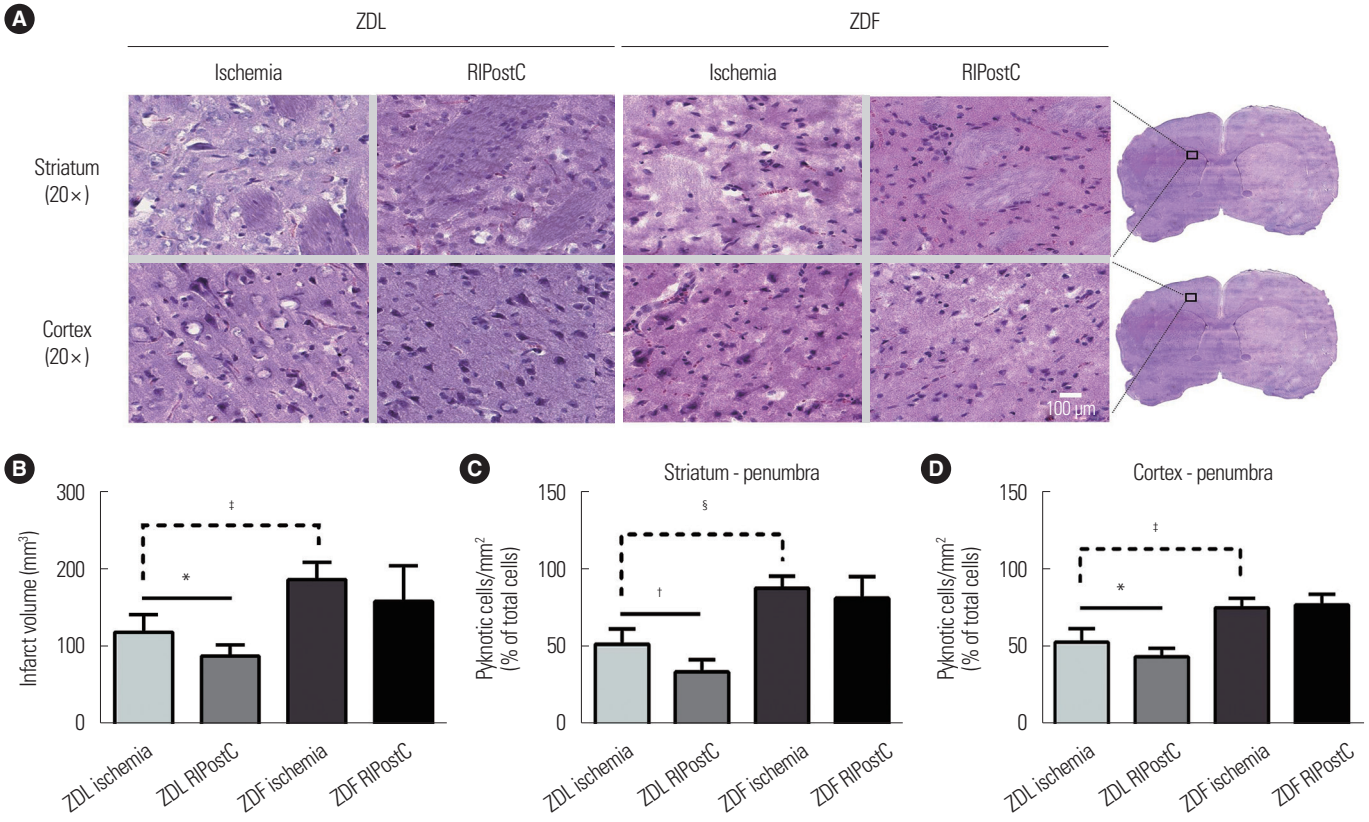

Figure 3. Histological assessment of brain damage in the Zucker diabetic fatty (ZDF) and Zucker diabetic lean (ZDL) rat model. (A) H&E staining of brain tissue sections of lean (ZDL) and obese (ZDF) rats shows cell morphologic changes in the penumbra after focal cerebral ischemia and remote ischemic postconditioning (RIPostC) treatment. The areas within the squares are shown at higher magnification (×20) to illustrate the pattern of cell destruction in the cortex and striatum of the penumbra of brain sections (scale bar = 100 μm). Quantification of the infarct volume (B) and quantitative analysis of the number of pyknotic cells per mm^2 in the striatum (C) and cortex (D) of the penumbra after ischemia and RIPostC. Values are presented as mean ± standard deviation. *P < 0.05, †P < 0.01 RIPostC vs. ischemia group; ‡P < 0.001, §P < 0.0001 lean vs. obese group.

differences between the ZDF (n = 7) and ZDL (n = 7) groups in the response to brain ischemia. The severity of ischemic injury after MCAO was significantly higher in obese animals (by 57.9%) compared with lean rats, as evidenced by a wider range of ischemic areas (186.50 ± 8.37 and 118.10 ± 9.33 mm^3, respectively), indicated by lighter H&E staining of brain tissue (Fig. 3A, B). In H&E staining, neurons in MCAO-induced rats had typically darkly stained pyknotic nuclei surrounded by pale cytoplasm. Obese rats had 71.4% more H&E pyknotic cells in the striatum of the penumbra than lean rats (87.43% ± 2.88% and 51.00% ± 3.73%, respectively) (Fig. 3C) and 42.8% more in the cortex of the penumbra (74.43% ± 2.34% and 52.14% ± 3.33%, respectively) (Fig. 3D). RIPostC intervention significantly reduced the infarct volume in ZDL rats (n = 6; to 87.23% ± 5.79%; $P < 0.05$). The percentage of pyknotic cells in the striatum of the penumbra was reduced by approximately 55% (to 32.90% ± 3.33%) and approximately 22.3% in the cortex of the penumbra (to 42.63% ± 2.29%) in the RIPostC-treated lean animals compared with ischemia lean group. In contrast, no significant differences in the infarct volume or the percentage of pyknotic cells were seen after RIPostC treatment in ZDF rats (n = 6). These results showed that this neuroprotective strategy failed to protect cells in the penumbra of obese rats against ischemic neuronal damage.

FJ B staining (Fig. 4A) showed significantly more morphologically degenerating neurons in the striatum ($P < 0.0001$) (Fig. 4B) and cortex ($P < 0.01$) (Fig. 4C) of the penumbra (evaluated regions marked in Fig. 3A) in the ischemia group of obese rats compared

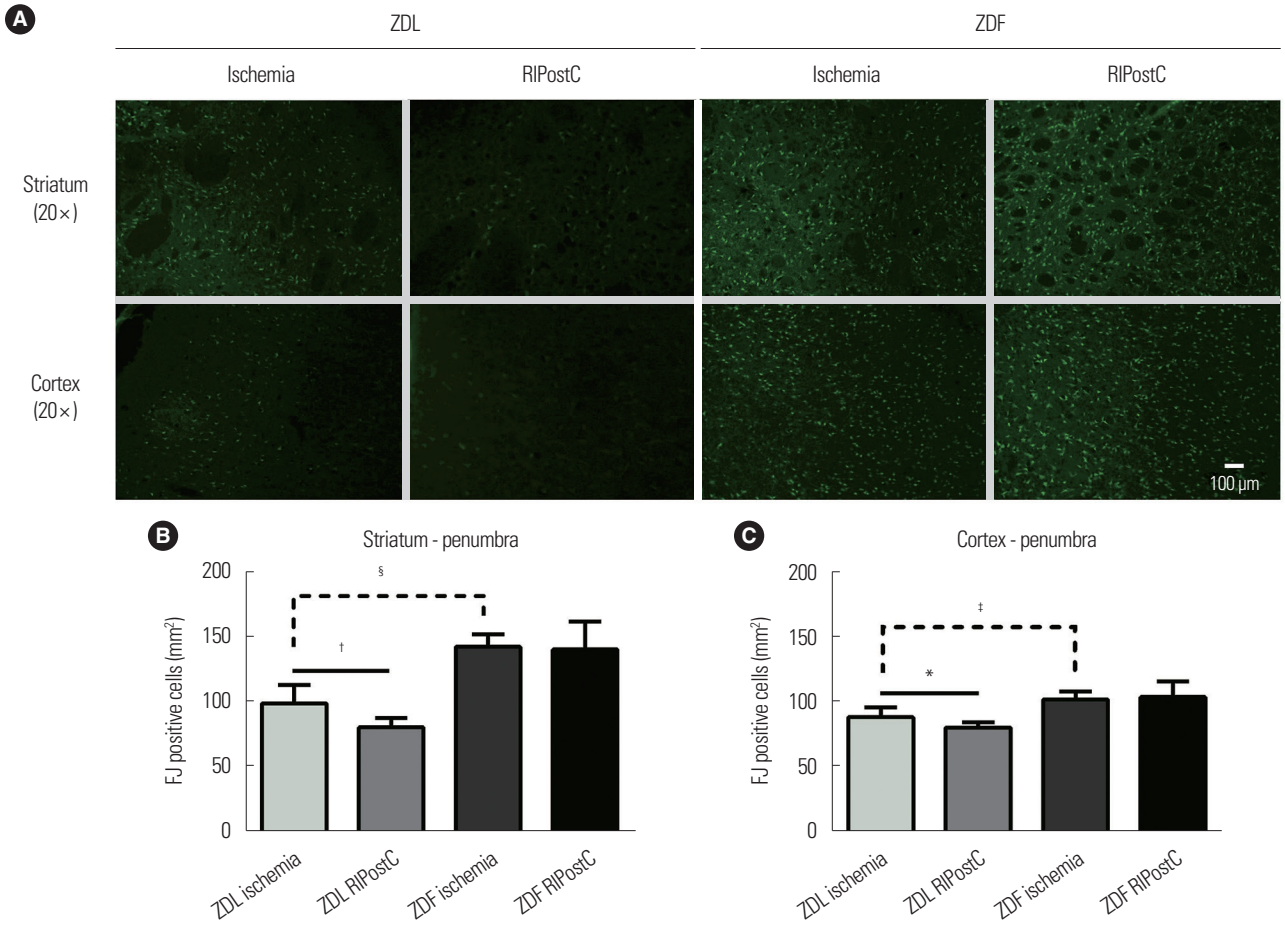

Figure 4. Fluoro-Jade B (FJ B) assessment of neuronal degeneration. (A) Representative fluorescence microphotographs of the investigated regions from the brain sections in ischemia and after remote ischemic postconditioning (RIPostC) condition of Zucker diabetic lean (ZDL) and Zucker diabetic fatty (ZDF) rats (magnification ×20, scale bar = 100 μm). Graphs showing the average number of FJ B-positive cells per mm^2 in the cerebral striatum (B) and cortex (C) of the penumbra for each treatment condition, respectively, 24 hours after middle cerebral artery occlusion and RIPostC. Values are presented as mean ± standard deviation. *$P < 0.05$, †$P < 0.01$ RIPostC vs. ischemia group; ‡$P < 0.01$, §$P < 0.0001$ lean vs. obese group.

with their lean counterparts. In ischemic lean animals, RIPostC significantly improved neuronal survival in the striatum (98.14 ± 5.32 FJ positive cells/mm^2 and 79.63 ± 2.52 FJ positive cells/mm^2, respectively) and cortex of the penumbra (87.71 ± 2.88 FJ positive cells/mm^2 and 79.71 ± 1.55 FJ positive cells/mm^2, respectively) after ischemia. However, RIPostC treatment did not prevent ischemic injury in both structures of the penumbra in the ZDF group.

RIPostC is ineffective in reducing stroke-related oxidative stress in obese rats

The comet assay was performed to measure systemic oxidative stress in peripheral lymphocytes of ZDF and ZDL animals subjected to focal ischemia followed by RIPostC intervention (Fig. 5A). Notably, obese rats (n = 8) exhibited approximately 135% higher levels of DNA damage than their lean counterparts (n = 8), even before MCAO surgery (9.53% ± 0.70% and 4.05% ± 0.43%, respectively). Following the induction of stroke, the results showed an increased incidence of DNA single-strand breaks in cells exposed to ischemia. ZDL rats (n = 6) exhibited 13.20% ± 1.13% of DNA in tail after MCAO while ZDF rats (n = 6) exhibited 20.62% ± 1.46% of DNA in tail; the DNA breaks increased by about 56%. Our results showed that induction of RIPostC caused a significant reduction of oxidative stress in ZDL rats (n = 6) by approximately 36% compared with the ischemia group (9.66% ± 0.99% and 13.20% ± 1.13%, respectively). However, obese rats subjected to postischemic hind-limb tourniquets (n = 6) did not demonstrate any significant

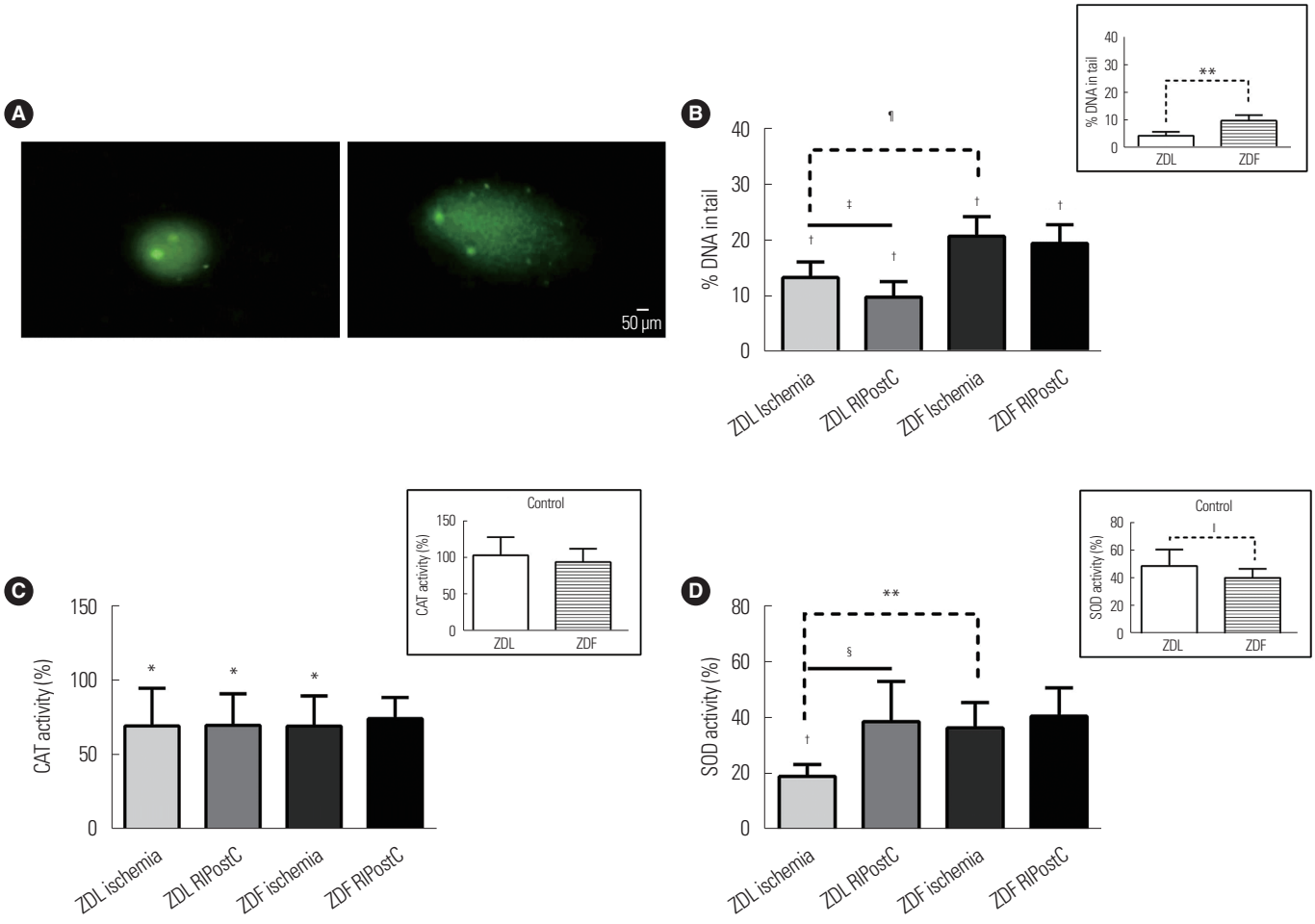

Figure 5. Antioxidant effect of remote ischemic postconditioning (RIPostC) on Zucker diabetic fatty (ZDF) and Zucker diabetic lean (ZDL) rats after stroke. (A) Representative immunofluorescence image of comet tail of fragmented DNA in lymphocytes in the control group and after focal ischemia at ×20 magnification (scale bar = 50 μm). (B) Statistical analysis of lymphocytic DNA damage in ZDL and ZDF rats of the control group, ischemia group and after RIPostC treatment. The activity of catalase (CAT) in plasma (C) and superoxide dismutase (SOD) in blood cells (D) is expressed as a percentage of the control group values. Values are presented as mean ± standard deviation. *P < 0.05, †P < 0.0001 vs. control; ‡P < 0.05, §P < 0.001 RIPostC vs. ischemia group; ǁP < 0.05, ¶P < 0.01, **P < 0.0001 lean vs. obese group.

differences. RIPostC treatment decreased stroke-induced oxidative stress of obese rats only by approximately 6.62% (Fig. 5B).

We next measured the antioxidant defense by evaluating the activity of SOD and CAT enzymes. CAT activity did not differ in obese rats compared with their lean counterparts (ZDL 102.80% ± 8.71%, ZDF 93.61% ± 6.41%). CAT activity significantly decreased by 48% in ZDL and 35% in ZDF rats after undergoing ischemia (ZDL 69.39% ± 8.99%, ZDF 69.25% ± 7.22%). However, CAT activity of tolerant rats stayed unchanged in both lean (to 69.76% ± 8.72%) and fat (to 74.26% ± 6.42%) animals when compared to ischemia group (Fig. 5C).

We observed a decrease in SOD levels of ZDF rats (by approximately 21.7%) compared with the control lean group. Stroke led to significant repression of antioxidant activity by approximately 154% in ZDL rats (from 48.23% ± 3.40% in control to 18.97% ± 1.29% after ischemia). Notably, SOD activity remained significantly reduced, even compared with obese rats with brain ischemia (by approximately 91.7%). This suggests that stroke did not affect blood antioxidant activity mediated by SOD. We observed an increase in SOD activity in RIPostC-treated ZDL rats by approximately 103.7% (to 38.65% ± 5.88%) in comparison with the ischemia group. However, RIPostC treatment only had a minimal influence on the SOD activity elevation in ZDF rats (Fig. 5D), supporting earlier findings.

Tissue and blood glutamate level remained unchanged in RIPostC-treated obese animals

We examined differences of the glutamate level in the post-mitochondrial supernatant of cells related to core and penumbra in both ZDF (n = 8) and ZDL (n = 8) control animals. Both groups did not differ significantly in the glutamate content of the core and penumbra region (ZDL 0.08 ± 0.00 μmol/mg, ZDF 0.09 ± 0.00 μmol/mg in the core; ZDL 0.07 ± 0.00 μmol/mg, ZDF 0.07 ± 0.00 μmol/mg in the penumbra). We focused on the intensively non-perfused region of the core, and the significant elevation of glutamate levels in lean rats after ischemia (n = 6; by approximately 77% to 0.15 ± 0.00 μmol/mg) was not reflected in the counterparts of obese rats (n = 6). Blood supply restriction in obese rats did not lead to any increase in glutamate excitotoxicity (and was even significantly lower compared with ZDL stroke rats). Compared with results in the ischemia group, RIPostC intervention of lean rats (n = 6) resulted in a significant drop in glutamate concentration in the ischemic core (0.15 ± 0.00 and 0.07 ± 0.01 μmol/mg, respectively). Postischemic

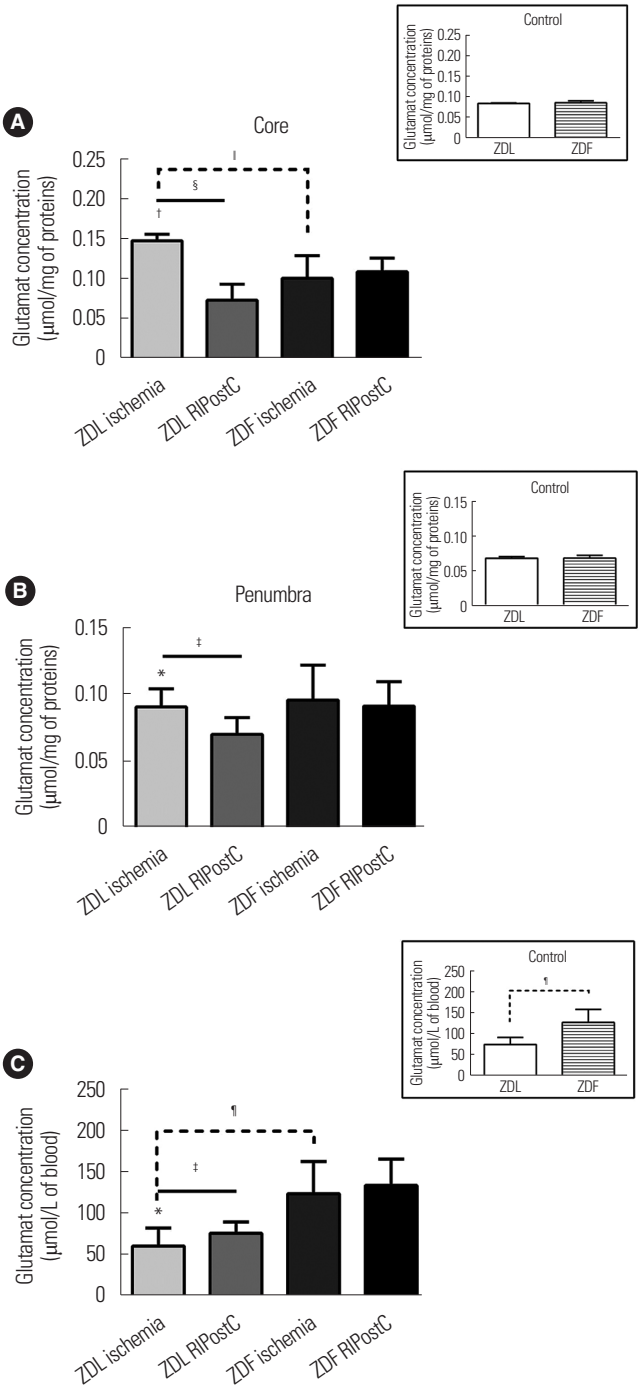

Figure 6. Effect of remote ischemic postconditioning (RIPostC) on glutamate concentration in the core (A) and penumbra (B) of brain tissue and (C) blood samples derived from the control, ischemic, and RIPostC groups of Zucker diabetic lean (ZDL) and Zucker diabetic fatty (ZDF) rats. Values are presented as mean ± standard deviation. *P < 0.05, †P < 0.01 vs. control; ‡P < 0.05, §P < 0.01 RIPostC vs. ischemia group; ǀP < 0.05, ¶P < 0.0001 lean vs. obese group.

hind-limb tourniquet treatment did not result in the lowering of core tissue glutamate in obese rats (n = 6) (Fig. 6A).

Additionally, our results showed that glutamate levels in the region of the penumbra of ZDL rats increased by approximately 34% following ischemia, whereas hind-limb postconditioning resulted in a significant reduction in glutamate content up to control values. In contrast, ZDF brain tissues were not significantly altered through the ischemia/reperfusion process. Likewise, RIPostC did not result in significant changes in excitotoxicity in the ZDF group (Fig. 6B).

We further observed a higher amount of glutamate levels in peripheral blood in ZDF rats by approximately 73% (126.23 ± 6.10 μmol/L) compared with ZDL rats (72.94 ± 3.15 μmol/L) before the MCAO surgery. Lean animals subjected to MCAO showed a decrease ($P = 0.0308$) of glutamate in blood circulation, with no change observed in ZDF ischemic rats. RIPostC treatment increased glutamate levels in ZDL rats by approximately 26% compared with the MCAO group. However, no difference was seen in obese rats following RIPostC intervention (Fig. 6C).

DISCUSSION

Obesity has been strongly associated with brain stroke. Therefore, we investigated the effect of this comorbidity on the effects of RIPostC treatment for stroke. We used male 12-week-old ZDF adult rats. This animal model allowed us to focus primarily on the impact of obesity on the efficacy of postischemic treatment by remote ischemic conditioning and minimized the influence of non-modifiable factors such as senior age or sex.

We first investigated the effect of RIPostC on post-stroke neurological outcomes in obese rats. Two independent methods of infarct volume assessment showed that obesity has a meaningful impact on the range of infarction in stroke model animals. The characteristic neurodegenerative cell phenotype was observed at a significantly higher amount in the penumbral region in obese rats compared with lean counterparts. While several studies reported a protective effect of obesity on stroke outcomes in patients (the so-called obesity paradox), there is a clear consensus in preclinical studies (primarily demonstrated in obese rodents) in line with our observations. Obese rats exhibit increased ischemic brain damage and have worse behavioral outcomes in comparison with control animals.[21,22] This unfavorable effect rises from a deficiency in the satiety hormone leptin or a defective leptin receptor (LEPR). Since leptin usually acts on the hypothalamus to limit feeding, Zucker rats rapidly gain weight because they are hyperphagic.

Neurons in the penumbra, which surrounds the ischemic core, are damaged but may be salvaged in the case of blood flow restoration.[23] This offers the possibility of rescuing brain tissue following RIPostC treatment.[6] However, in our study, the RIPostC intervention had no effect on the quantity of degeneration or dead neurons in the penumbra of obese rats. Therefore, the inefficacy of RIPostC after stroke may be related to the main characteristics of the ZDF rats, i.e., overweight and fat content (or BMI) and/or leptin deficiency.

Several studies have shown that LEPR deficiency in obese male rats increases the brain's vulnerability to ischemic injury, resulting in worse neurobehavioral outcomes.[21,22] Leptin, a common adipocytokine, has an anti-apoptotic effect on cerebral ischemia/reperfusion injury while also promoting neurogenesis and angiogenesis.[24] Its administration *in vivo* resulted in a decrease in cerebral infarction, morphological damage and neuronal apoptosis, demonstrating that leptin reduces the secondary events of brain injury after ischemia.[25] The fat content and BMI may be other factors that influence RIPostC inefficacy. To the best of our knowledge, the present study was the first to investigate RIPostC-induced neuroprotection in obese animals. One study on heart postconditioning was conducted in the context of obesity.[26] The authors demonstrated a lack of cardioprotection by IPostC in *ob/ob* leptin-deficient mice through a reduction in the phosphorylation of members of the reperfusion injury salvage kinase (RISK) pathway.

Postischemic neuronal damage is also characterized by the rapid production of single-strand breaks in DNA as a result of excessive free-radical species formation.[27,28] Our data confirmed that MCAO-induced focal cerebral ischemia is accompanied by increased oxidative DNA damage of circulating lymphocytes. Furthermore, approximately 135% more DNA damage was observed in obese animals compared with lean rats even before the surgery, suggesting that obesity status be associated with a naturally occurring predisposition to a worse oxidative state. Previous studies suggested that adipose tissue is the source of oxidative stress, which is then passed to other tissues and may cause obesity-related illnesses.[29]

Oxidative stress may also be induced by a lack of antioxidant

mechanisms. Therefore, we measured the activities of SOD and CAT. SOD activity was significantly lower in obese animals compared with lean rats, and ischemia did not affect SOD activity. CAT activity was comparable in both groups and decreased in both groups after ischemia. Several studies have shown that the activities of SOD, CAT and glutathione peroxidase are inversely related to BMI in obese children and adults.[30] As obesity progresses, the amount and activity of the antioxidant defense system components become depleted, increasing the susceptibility to oxidative tissue damage. In the study of Martinelli et al.,[31] SOD activity was decreased in the plasma samples of obese male ZDF animals compared with lean littermates. Additionally, the study showed an increase in oxidized protein concentrations and the expression of lipid-aldehyde 4-hydroxynonenal in the heart of obese male Zucker rats. Increased pro-oxidative elements and the decreased components of antioxidant defense indicate a condition of obesity-related oxidative stress in obese male rats. Our findings showed that RIPostC intervention did not prevent the increasing fragmentation of DNA in the lymphocytes of obese rats following MCAO/reperfusion injury and did not improve antioxidant defense, i.e., RIPostC did not positively affect the oxidative status of animals, including after stroke. In the context of myocardial injury, there is some evidence that obese Zucker rats were unable to respond to either ischemic or chemical preconditioning because of increased mitochondrial oxidative stress and the impaired activation of mitochondrial ATP-sensitive potassium (K_{ATP}).[32] Therefore, naturally elevated oxidative stress due to high amounts of adipose tissue and ischemia may mutually impact stroke outcomes and RIPostC treatment of ZDF rats.

Another very important pathological hallmark of brain ischemia is glutamate excitotoxicity, a mechanism triggered by excessive glutamate release from neurons. Therefore, reducing glutamate release or its rapid detoxication from the extracellular space of neurons is essential for preventing ischemia-reperfusion damage. Several studies have shown an increased efflux of extracellular glutamate from the ischemic brain to the peripheral blood circulation following the induction of ischemic tolerance.[28,33,34] Our results demonstrated comparable levels of glutamate in obese and lean rats in the control conditions, but only moderate postischemic glutamate increase in obese rats. RIPostC did not influence glutamate levels in the ischemic core or penumbra in ZDF rats. In addition, our findings revealed higher blood glutamate concentrations in non-operated obese animals by approximately 73%, suggesting that obesity is significantly correlated with higher levels of glutamate in the blood. Higher blood glutamate levels seem to be associated with an increased amount of abdominal fat and also represents a potential marker of visceral adipose tissue accumulation,[35] and this may be true for obesity. Nevertheless, no reports have explained the mechanism by which RIPostC is unable to mediate glutamate detoxication in obesity.

Liu and Zheng[36] reported the reduced expression of astrocyte-specific glutamate transporters and excitatory amino acid transporter 1 (EAAT1) and 2 (EAAT2) in obese animals. Therefore, we speculate that alterations in glutamate transport may be responsible for failures in the elimination of glutamate in brain tissue and in blood. However, this should be verified by future experiments.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Study concept and design: KK, JK, MG, MB, and PB; acquisition of data: KK, JK, MG, MB, and PB; analysis and interpretation of data: KK, JK, MG, MB, and PB; drafting of the manuscript: KK and PB; critical revision of the manuscript: KK, JK, MG, MB, and PB; statistical analysis: KK, JK, MG, MB, and PB; obtained funding: JK, MB, and PB; administrative, technical, or material support: KK, JK, MG, MB, and PB; and study supervision: PB.

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