Effect of Yokukansan on Nitric Oxide Production and Hydroxyl Radical Metabolism During Cerebral Ischemia and Reperfusion in Mice

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Background: The purpose of this study was to investigate the effects of yokukansan on forebrain ischemia. Because we can measure nitric oxide production and hydroxyl radical metabolism continuously, we investigated the effect of yokukansan on nitric oxide production and hydroxyl radical metabolism in cerebral ischemia and reperfusion. *Methods*: Yokukansan (300 mg per kg per day) was mixed into feed and given to 16 mice for 10 days. Sixteen additional mice received normal feed (control). Nitric oxide production and hydroxyl radical metabolism were continuously monitored using the salicylate trapping method. Forebrain ischemia was produced in all mice by occluding the common carotid artery bilaterally for 10 minutes. Levels of the nitric oxide metabolites nitrite and nitrate were determined using the Griess reaction. Survival rates of hippocampal CA1 neurons were calculated and 8-hydroxydeoxyguanosine-immunopositive cells were counted to evaluate the oxidative stress in hippocampal CA1 neurons 72 hours after the start of reperfusion. *Results*: Arterial blood pressure and regional cerebral blood flow were not significantly different between the 2 groups. The level of nitrate was significantly higher in the yokukansan group than in the control group during ischemia and reperfusion. Levels of 2,3- and 2,5-dihydroxybenzoic acid were significantly lower in the yokukansan group than in the control group during ischemia and reperfusion. Although survival rates in the CA1 did not differ significantly, there were fewer 8-hydroxydeoxyguanosine-immunopositive cells in animals that had received yokukansan than in control animals. *Conclusion*s: These data suggest that yokukansan exerts reducing hydroxyl radicals in cerebral ischemic injury.

Key Words: Nitric oxide (NO)—hydroxyl radical—yokukansan—microdialysis—forebrain ischemia—CA1 neuron

Abbreviations: NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; 8-OHG, 8-hydroxydeoxyguanosine; NMDA-R, N-methyl-D-aspartate receptor; nNOS, neuronal NOS; eNOS, endothelial NOS; PSD95, postsynaptic density protein 95; O_2^-, superoxide anion; DHBA, dihydroxybenzoic acid; MABP, mean arterial blood pressure; rCBF, regional cerebral blood flow; GSH, glutathione; BPSD, behavioral and psychological symptoms of dementia

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Received November 13, 2018; revision received December 22, 2018; accepted December 30, 2018.

Funding: The authors did not receive any specific grants for this research from funding agencies in the public, commercial or not-for-profit sectors.

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1052-3057/$ - see front matter

https://doi.org/10.1016/j.jstrokecerebrovasdis.2018.12.047

Introduction

Yokukansan is a traditional Japanese Kampo medicine which has been used since ancient times to reduce irritability in children. Yokukansan consists of 7 dried herbs. Recently, yokukansan has been used for treatment of the behavioral and psychological symptoms associated with many types of dementia.[1,2] It has been reported that yokukansan improves aggressiveness in patients with Alzheimer's disease and diffuse Lewy bodies.[3,4] In clinical research, it has also been reported that yokukansan is effective in the treatment of delirium following acute stroke.[5]

In basic research, it has been reported that yokukansan may affect glutamatergic neurons.[6,7] Yokukansan is useful for prevention of abnormal glutamate release and improvement of glutamate uptake via glutamate transporters.[6,7] It has been shown that yokukansan affects glutamate transporter activity[7] and attenuates abnormal glutamate release.[6] Glutamate is the major excitatory neurotransmitter in the brain.[8] However, excessive activation of glutamate receptors is harmful and extracellular glutamate is toxic in high concentrations.[9] In ischemia, a large amount of glutamate is released, causing excessive stimulation of N-methyl-D-aspartate receptors (NMDA-R) and increased interaction between neuronal nitric oxide synthase (nNOS) and postsynaptic density protein 95.[10,11] During cerebral ischemia, the NMDA-R forms a death-signaling complex with postsynaptic density protein 95 and nNOS, leading to calcium-dependent production of the superoxide anion (O^{2-}) and nitric oxide (NO).[11]

It has also been suggested that yokukansan may have neuroprotective effects with respect to oxidative stress, not only in vitro, but also in a gerbil model of global cerebral ischemia.[12] However, there have been no reports showing the effect of yokukansan on the chronological changes during and following cerebral ischemia. Furthermore, the effects of yokukansan on C57BL/6 mice (n=16) were used in *in vivo* microdialysis experiment. Yokukansan (300mg/kg) was given to 8 mice for 10 days, and the remaining 8 mice served as control. NO production and hydroxyl radical metabolism during cerebral ischemia and reperfusion in vivo have never been investigated. In this study, we investigated the effects of yokukansan on NO production and hydroxyl radical metabolism during cerebral ischemia and reperfusion in mice.

Experimental Procedures

Materials

Yokukansan was purchased from Tsumura & Co. (Tokyo, Japan).

Animal Preparation

C57BL/6 mice ($n = 32$; Charles River Laboratories, Atsugi, Kanagawa, Japan) were housed in the animal care facility at Saitama Medical University. Animals were initially anaesthetized with 2% halothane in air supplemented with O_2 and anaesthesia was maintained with 0.5%-1% halothane. Rectal temperature was maintained at 37.0°C-37.5°C with a disposable heat pack and small fan. A polyethylene catheter (PE-10; BD, Tokyo, Japan) was inserted into the right femoral artery to measure blood pressure.

Yokukansan (300 mg/kg) was mixed into feed and was given to 16 mice for 10 days. We checked carefully everyday feed containing yokukansan was completely eaten and disappeared. The remaining 16 mice served as controls and were given normal feed. All animal experiments were approved by the Institutional Animal Care and Use Committee of Saitama Medical University, Japan (approval numbers: 1659 in 2015, 1930 in 2016, 2199 in 2017, and 2466 in 2018).

In Vivo Microdialysis

NO production and hydroxyl radical metabolism were monitored continuously using in vivo microdialysis. Mice in both groups were initially anaesthetized with 2% halothane in air supplemented with O_2, then anaesthesia was maintained with 0.5%-1% halothane[10] (Fig 1). A microdialysis probe was inserted into the striatum in each hemisphere and perfused with Ringer's solution at a constant rate of 2 μL/min. The in vivo salicylate trapping method was used to monitor hydroxyl radical formation via 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) (Fig 1). A laser Doppler probe was placed on the surface of the right skull to measure regional cerebral blood flow (rCBF). After 2 hours of equilibration, fractions were collected every 10 minutes (Fig 1). Global forebrain cerebral ischemia was produced by occlusion of both common carotid arteries using MH-clips (Bear Medic, Tokyo, Japan) for 10 minutes.

All dialysis equipment was from Eicom (Kyoto, Japan), unless otherwise stated. Levels of nitrite (NO_2^-) and nitrate (NO_3^-) in the dialysates were determined using an ENO-20 high-performance liquid chromatography system with an M-510 automatic sample injector. Samples were analyzed based on the Griess reaction, with NO_2^- and NO_3^- separated on a packed column (NO-PAK) and NO_3^- reduced to NO_2^- in a cadmium reduction column (NO-RED).

Dialysates were collected into an autoinjector (EAS-20) every 20 minutes, and 2,3- and 2,5-DHBA levels were measured using an high-performance liquid chromatography system equipped with an electrochemical detector (HITEC-500) consisting of a graphite working electrode at +500 mV versus an Ag/AgCl reference electrode. Separation was done on an Eicompac SC-5ODS column (2.1 × 150 mm) at 25°C with a mobile phase consisting of 100 mM sodium phosphate buffer (pH 6.0) containing 134 μM EDTA and 2% methanol, at a flow rate of 230 μL/min. The locations of the dialysis probes were verified after each experiment.

Salicylic acid reacts with hydroxyl radical to form 2 main adducts, 2,3-DHBA and 2,5-DHBA.[13] Because

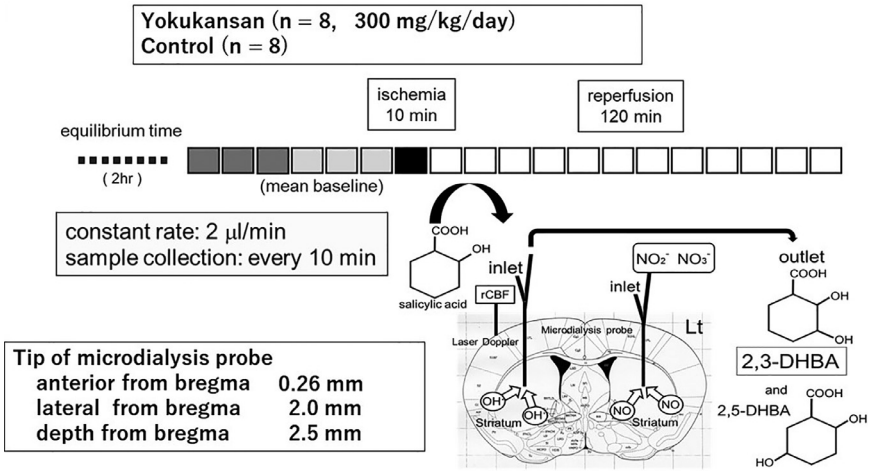

Figure 1. *Experimental procedures. NO production and hydroxyl radical metabolism were continuously monitored using in vivo microdialysis. A microdialysis probe was inserted into the bilateral striatum and perfused with Ringer's solution at a constant rate of 2 μL/min. A laser Doppler probe was placed on the surface of the skull over the right hemisphere. After 2 hours of equilibration, fractions were collected every 10 minutes. The in vivo salicylate trapping method was used to monitor hydroxyl radical formation via 2,3- and 2,5-DHBA. After 2 hours of equilibration, fractions were collected every 20 minutes. Abbreviations: 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid; Lt, left; NO, nitric oxide; rCBF; regional cerebral blood flow; Rt, right.*

2,5-DHBA is also endogenously formed by the cytochrome P450 system, only the change in 2,3-DHBA is a reliable hydroxyl marker in vivo.[14]

Morphological Classification of Neurons

Yokukansan (300mg/kg) was given to 8 mice for 10 days, and the remaining 8 mice served as control. Global forebrain cerebral ischemia was produced for 10 minutes by the same method as *in vivo* microdialysis experiment. At 72 hours after reperfusion, mice in the yokukansan and control groups were transcardially perfused with 0.9% saline followed by Bouin's fixative. Then the brains were removed and 5-μm thick paraffin sections were stained with hematoxylin and eosin. Viable neurons in the right CA1 were counted using a microscope (×40 magnification; BZ-X700, Keyence, Tokyo, Japan). Neurons were classified into 3 groups based on their appearance (severely ischemic, moderately ischemic, or no damage), and the ratio of no damage neurons to degenerated neurons was calculated as the ischemic damage in the yokukansan and control groups (Fig 2, A and B).

Only complete neuronal cells with a clearly defined cell body and nucleus were counted, as described in a previous study.[15] Neurons were counted in the hippocampal CA1 regions by an observer blinded to the study groups, as described previously.

Immunohistochemical Analysis of Neurons

Paraffin sections (5 μm thick) were immunostained with mouse anti-8-hydroxy deoxyguanosine (8-OHdG; Japan Institute for the Control of Aging, Tokyo, Japan) at 1:100 dilution, and visualized using the immunoperoxidase method (EnVision; Dako, Tokyo, Japan). To evaluate oxidative stress in the neurons, 8-OHdG-immunopositive neurons were counted 72 hours after the start of reperfusion (Fig 2, C and D).

Data are expressed as the mean ± standard deviation and were analyzed using a nonparametric analysis of variance, with $P < .05$ considered statistically significant (JMP version X; SAS, Cary, NC).

Results

Mean Arterial Blood Pressure

Preischemic mean arterial blood pressure (MABP) baselines shown are the average for the 60 minutes before ischemia. After reperfusion, MABP in control mice increased then returned to baseline. Changes in MABP were not significantly different between the yokukansan and control groups (Fig 3, A).

rCBF

During cerebral ischemia, rCBF decreased to less than 10% of baseline in the yokukansan and control groups, then increased during reperfusion. Changes in rCBF were not significantly different between the groups (Fig 3, B).

NO Metabolites

The levels of NO_2^- did not differ significantly between the yokukansan and control groups (Fig 4, A). The level of NO_3^- was significantly higher in the yokukansan group (5.7 ± 1.8%) than in the control group (3.3 ± 1.2%) from 20 to 120 minutes after reperfusion ($P < .05$; Fig 4, B). The level of total NO was significantly higher in the yokukansan group (5.4 ± 1.7 μmol/L) than in the control group (3.1 ± 0.9 μmol/L) at −10 minutes, from 20 to 60 minutes, from 80 to 90 minutes, and at 120 minutes after reperfusion ($P < .05$; Fig 4, C).

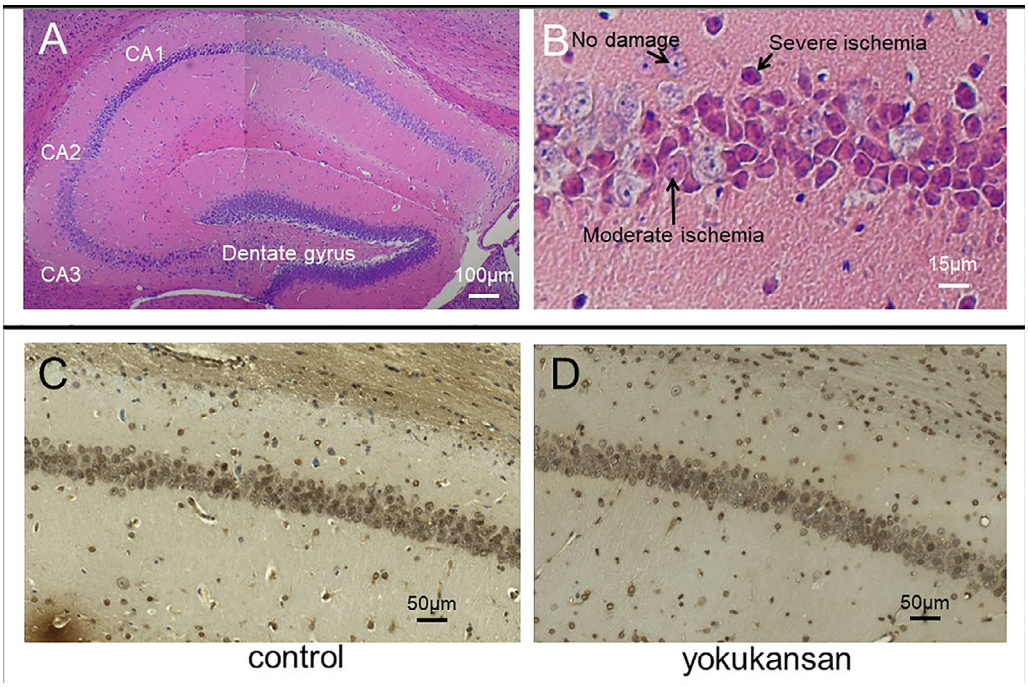

Figure 2. *Analysis of hippocampal CA1 neuron ischemic damage following cerebral ischemia and reperfusion in mice treated with yokukansan. (A and B) Pathological classification of neurons at 72 hours after reperfusion in control group. Hematoxylin and eosin-stained hippocampal CA1 neurons were classified into 3 conditions: severely ischemic, moderately ischemic, and no damage. The ratio of no damage neurons to degenerated neurons was calculated as the ischemic damage. (C and D) Immunohistochemistry. 8-Hydroxy deoxyguanosine-immunopositive cells were counted 72 hours after reperfusion to evaluate oxidative stress.*

Hydroxyl Radical Metabolites

The level of 2,3-DHBA was significantly lower in the yokukansan group (97.6 ± 24.3%) than in the control group (138.5 ± 21.2%) from during ischemia to 120 minutes after reperfusion ($P < .05$; Fig 4, D).

In addition, the 2,5-DHBA level was significantly lower in the yokukansan group (92.9 ± 25.9%) than in the control group (178.1 ± 30.5%) from 20 to 120 minutes after reperfusion ($P < .05$; Fig 4, E).

The level of total DHBA was significantly lower in the yokukansan group (64.7 ± 25.0%) than in the control group (107.0 ± 18.9 %) from 40 to 120 minutes after reperfusion ($P < .05$; Fig 4F).

Ischemic Damage in the Hippocampal CA1 Area

No significant difference between the yokukansan and control groups was observed in the neuronal ischemic damage in the CA1 area 72 hours after reperfusion (Fig 5, A).

Immunohistochemical Analysis of Hippocampal CA1 Neurons

The number of 8-OHdG-immunopositive cells in the hippocampal CA1 area 72 hours after the start of reperfusion was significantly lower in the yokukansan group (30.5 ± 7.6%) than in the control group (51.4 ± 15.9%; $P < .05$; Fig 5, B).

Discussion

The aim of our study was to investigate the effect of yokukansan on NO production, hydroxyl radical metabolism, and ischemic changes in hippocampal CA1 neurons during cerebral ischemia and reperfusion in mice. Recently, yokukansan has been used to treat the behavioral and psychological symptoms of dementia in patients with some types of senile dementia. Although previous studies have reported the effect of yokukansan in animal models of cerebrovascular dementia,[16] this is the first report of the effects of yokukansan on the chronological changes in NO production and hydroxyl radical metabolism during cerebral ischemia and reperfusion in vivo.

Yokukansan is usually given at a dose of 2.5-7.5 g/day. The 300 mg/kg yokukansan dose given to mice in this study equates to a 20 mg/kg dose in humans. In this study, mice received yokukansan for 10 days prior to the cerebral ischemia. The active ingredients of yokukansan are capable of penetrating the blood-brain barrier.[17] Therefore, there is a possibility that yokukansan produces direct effects on neurons in the brain.

The present in vivo data suggest that NO production was significantly higher in the yokukansan group than in the control group. Our previous data suggest that NO production in mice during cerebral ischemia and reperfusion is closely related to not only nNOS, but also endothelial NO synthase (eNOS).[18] NO derived from eNOS has been suggested to have a neuroprotective effect during cerebral ischemia. eNOS not only promotes vascular

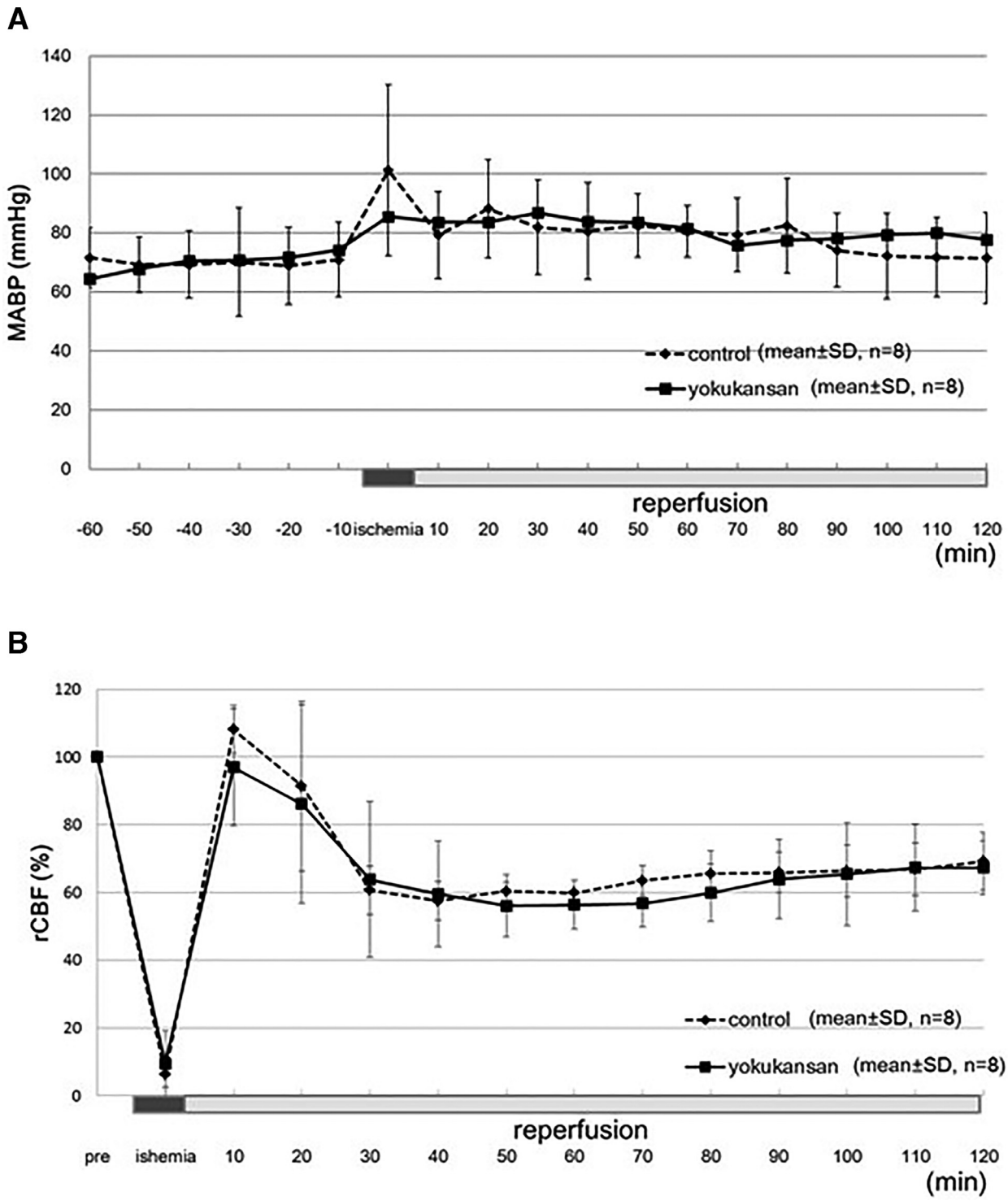

Figure 3. *Mean arterial blood pressure (MABP) and regional cerebral blood flow (rCBF) following cerebral ischemia and reperfusion in mice treated with yokukansan. A polyethylene catheter was inserted into the right femoral artery to measure blood pressure. A laser Doppler probe was placed on the surface of the right skull to measure regional cerebral blood flow. (A) The change in MABP was not significantly different between the yokukansan-treated and control groups. (B) The change in rCBF was not significantly different between the yokukansan-treated and control groups.*

dilation, but also increases vascular smooth muscle cell proliferation and migration, and thereby enhances arteriogenesis after stroke.[19] Yokukansan reduced the production of hydroxyl radicals in our study, which may protect the endothelial cells from hydroxyl radicals.

The present in vivo data indicated that yokukansan reduced the production of hydroxyl radicals during ischemia and after reperfusion. Yokukansan is useful for prevention of abnormal glutamate release, and improvement of glutamate uptake by glutamate transporters.[6,7]

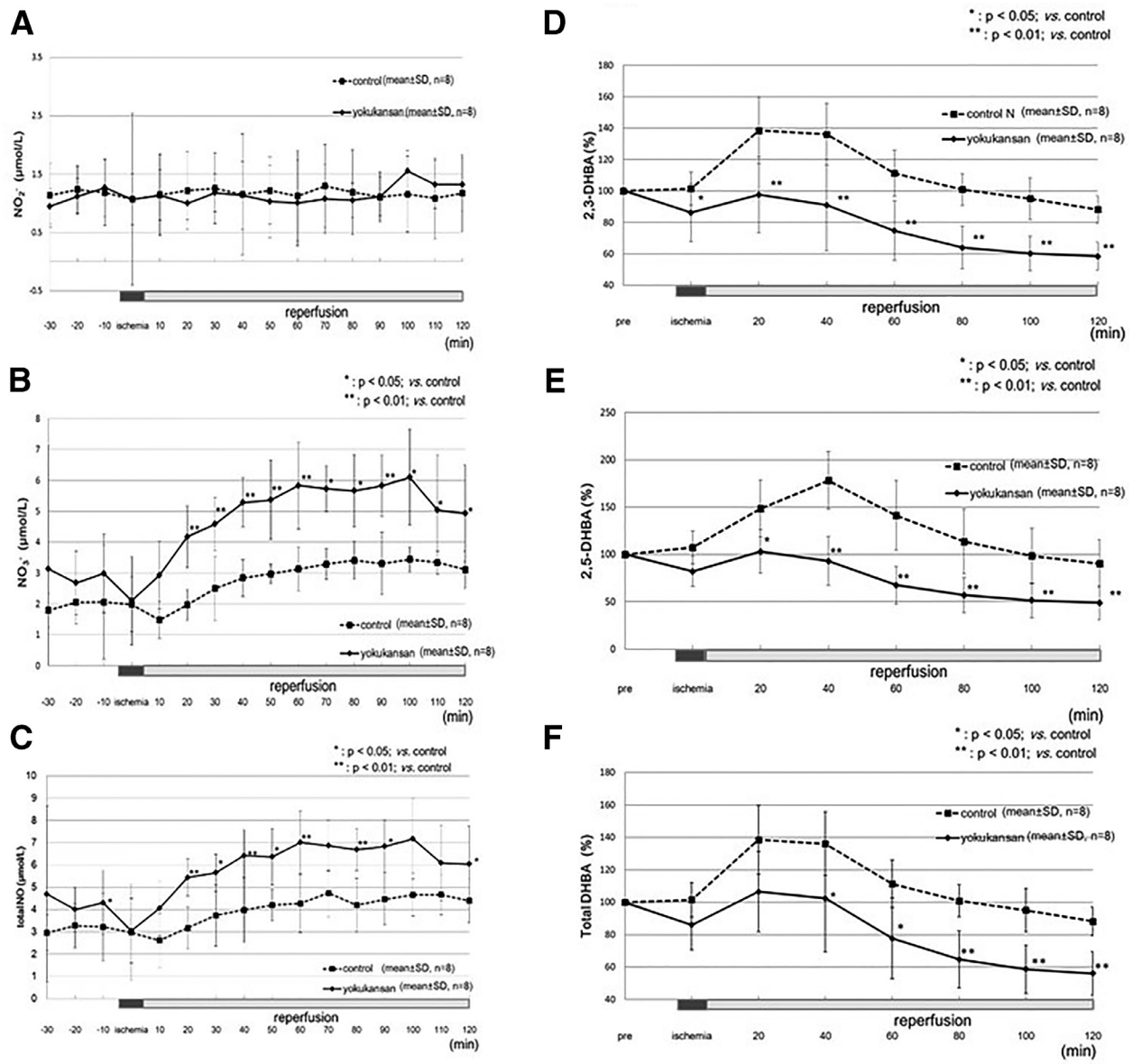

Figure 4. *NO and hydroxy radical metabolism following cerebral ischemia and reperfusion in mice treated with yokukansan. NO production and hydroxyl radical metabolism were monitored continuously using in vivo microdialysis. Levels of nitrite (NO_2^-) and nitrate (NO_3^-) in the dialysates were determined using an ENO-20 high-performance liquid chromatography (HPLC) system. The in vivo salicylate trapping method was used to monitor hydroxyl radical formation via 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA). (A) Levels of NO_2^- did not differ significantly between the yokukansan-treated and control groups. (B) The level of NO_3^- was significantly higher in the yokukansan group (5.7 ± 1.8 μmol/L) than in the control group (3.3 ± 1.2 μmol/L) from 20 to 120 minutes after the start of reperfusion ($P < .05$). (C) The level of total NO was significantly higher in the yokukansan group (5.4 ± 1.7 μmol/L) than in the control group (3.1 ± 0.9 μmol/L) at −10 minutes, from 20 to 60 minutes, from 80 to 90 minutes, and at 120 minutes after reperfusion ($P < .05$). (D) The level of 2,3-DHBA was significantly lower in the yokukansan group (97.6 ± 24.3%) than in the control group (138.5 ± 21.2%) from the time of ischemia to 120 minutes after the start of reperfusion ($P < .05$). (E) The level of 2,5-DHBA was significantly lower in the yokukansan group (92.9 ± 25.9%) than in the control group (178.1 ± 30.5%) from 20 to 120 minutes after the start of reperfusion ($P < .05$). (F) Level of total DHBA was significantly lower in the yokukansan group (64.7 ± 25.0%) than in the control group (107.0 ± 18.9%) from 40 to 120 minutes after reperfusion ($P < .05$). Abbreviations: 2,3-DHBA, 2,3-dihydroxybenzonic acid; 2,5-DHBA, 2,5- dihydroxybenzoic acid; NO_3^-, nitrate; NO, nitric oxide; NO_2^-, nitrite; SD, standard deviation.*

Consequently, yokukansan reduces intercellular glutamate, and inhibits glutamate-induced cell death, suggesting that yokukansan has a neuroprotective effect against glutamate toxicity.[20]

Kawakami et al demonstrated that yokukansan potently binds to NMDA-R,[21] a finding which suggests that yokukansan may have a neuroprotective effect as an NMDA-R antagonist. We have already reported the effect of the NMDA-R antagonist memantine on hydroxyl radical metabolism during cerebral ischemia and reperfusion in mice.[22] In our previous study, we reported that hydroxyl radicals were significantly lower in the

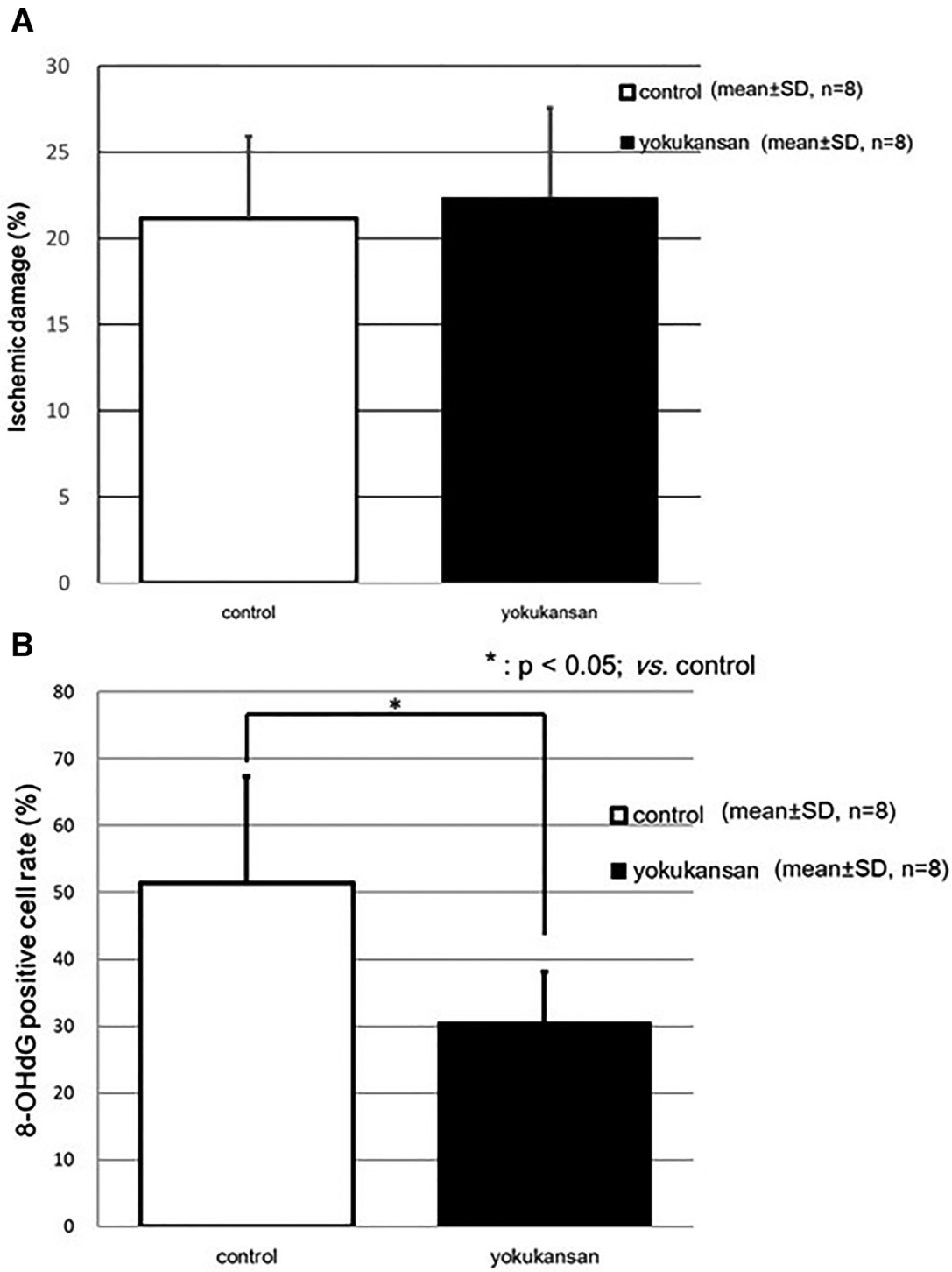

Figure 5. *Neuronal ischemic damage and oxidative stress following cerebral ischemia and reperfusion in mice treated with yokukansan. The neuronal ischemic damage and percentage of 8-OHdG-immunopositive cells were measured in the hippocampal CA1 area 72 hours after the start of reperfusion. (A) No significant differences in the ischemic damage of neurons were observed between the yokukansan-treated and control groups. (B) There were significantly fewer 8-OHdG-immunopositive cells in the yokukansan group (30.5 ± 7.6%) than in the control group (51.4 ± 15.9%; P < .05). Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; DHBA, dihydroxybenzoic acid; HPLC, high-performance liquid chromatography; MABP, mean arterial blood pressure; NO, nitric oxide; rCBF, regional cerebral blood flow; SD, standard deviation.*

memantine-treated group than in the control group. Similarly, it was also noted that yokukansan reduced hydroxyl radical metabolites in this study.

Edaravone is known as a free radical scavenger, which removes hydroxyl radicals and inhibits lipid peroxidation. It has been shown that edaravone normalizes the reduced

expression of eNOS mRNA that occurs following irradiation,[23] and reduces the induction of nNOS following transient ischemia in rabbits.[24] Yokukansan also reduced free radicals and increased the NO levels in this study.

Kawakami et al suggested that yokukansan ameliorates the ischemia-induced decrease in levels of glutathione (GSH), which is one of the antioxidant materials induced by glutamate.[20,25] Yokukansan also increases expression levels of components of a glutamate/cystine antiporter system, which takes in cystine as a precursor of GSH.[20,25] GSH has a neuroprotective effect against glutamate-induced oxidative cytotoxicity.[20,25]

Our present data also showed that 8-OHdG-immunopositive cells in the hippocampal CA1 area were significantly fewer in the yokukansan group than in the control group. These data suggest the production of hydroxyl radicals in the yokukansan group is reduced.

However, the ischemic damage of CA1 neurons in the yokukansan group was almost the same as the control group. Although Liu et al showed that yokukansan increased the number of viable neurons in the hippocampal CA1 region following cerebral ischemia in gerbils,[12] we did not find the same effect in our model. There are several possible reasons for the difference between the data of Liu et al and ours. The first reason may be the different species of animals. The second reason may be the different length of the yokukansan administration period; we gave yokukansan for 10 days, but Liu et al gave it for 30 days.[12]

In our experiments, we demonstrated that yokukansan accelerates the production of NO in the reperfusion phase and suppresses the production of hydroxyl radicals in vivo. Investigation of pathology using immunohistochemistry for 8-OHdG also showed a decrease of free radicals in yokukansan-treated animals. The underlying mechanisms of yokukansan action will be further investigated in the future.

Conclusions

The present in vivo data suggest that yokukansan may enhance NO production after reperfusion, reduce hydroxyl radical metabolites during ischemia and reperfusion, and may reduce oxidative stress in hippocampal CA1 neurons 72 hours after reperfusion.

Acknowledgment: We thank Melony Black, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jstrokecerebrovasdis.2018.12.047.

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