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Stimulation of functional recovery via neurorepair mechanisms by the traditional Japanese Kampo medicine, Ninjin'yoeito, and physical exercise in a rat ischemic stroke model

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ABSTRACT

Ethnopharmacological relevance: Ninjin'yoeito (NYT), a traditional Japanese Kampo medicine consisting of 12 herbs, has been reported to improve cognitive dysfunction, depression, and neurological recovery in patients with neurovascular diseases such as Alzheimer's disease and stroke. Several studies have reported that the NYT components exert neurotrophic, neurogenic, and neuroprotective effects. In addition, exercise enhances neuroprotection and functional recovery after stroke. Rehabilitative exercises and pharmacological agents induce neurophysiological plasticity, leading to functional recovery in stroke patients. These reports indicate that NYT treatment and exercise may promote functional recovery following stroke through their beneficial effects. However, no study has determined the effects of NYT and the possible mechanisms of neurorepair and functional recovery after stroke.

Aim of the study: This study aimed to investigate the combined effects of NYT and exercise on neuroprotection and functional recovery and the underlying mechanisms in a rat ischemic stroke model.

Materials and methods: Stroke was induced with 60-min middle cerebral artery occlusion (MCAO) followed by reperfusion in adult male Sprague-Dawley rats. After stroke, the rats were assigned to four groups: ischemia reperfusion (IR), NYT, exercise (Ex), and NYT + Ex. NYT-treated rats were fed a diet containing 1% NYT one day after stroke. Exercise was performed using a motorized treadmill for 5 days a week (8–15 m/min, 20 min/day), starting 3 days after stroke. The NYT treatment and exercise were continued for 4 weeks after the stroke. Infarct volume, neurological deficits, sensorimotor functions, expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase A (TrkA) and B (TrkB), caspase-3 activity, and the p-Akt/Akt ratio were examined by immunohistochemistry and western blotting.

Results: Compared to the IR group, all treated groups indicated reduced infarct volumes. The NYT + Ex group showed significantly improved waking time and beam walking score compared with the IR group. The expression of NGF/TrkA/p-TrkA and BDNF/TrkB was significantly increased in the NYT + Ex group compared with those in the IR group, whereas the number of caspase-3 positive cells around the lesion was significantly lower in the NYT + Ex group than in the IR group. In addition, the ratio of p-Akt/Akt was significantly higher in the NYT + Ex group than in the IR group.

Conclusions: This study suggests that NYT in combination with exercise provides neuroprotective effects and improves sensorimotor function by stimulating NGF/TrkA and BDNF/TrkB, and by activating the Akt pathway in ischemic stroke of rats. NYT may be an effective adjunctive agent in post-stroke rehabilitation.

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1. Introduction

List of abbreviation

NYT	Ninjin'yoeito
IR	ischemia reperfusion
MCAO	middle cerebral artery occlusion
NGF	nerve growth factor
BDNF	brain derived growth factor
TrkA	tropomyosin receptor kinase A
TrkB	tropomyosin receptor kinase B
PI3K/Akt	phosphatidylinositol 3 kinase/v-akt murine thymoma viral oncogene homolog
BBB	blood-brain barrier

Stroke is a leading cause of disability and mortality worldwide. Although stroke mortality has decreased owing to advancements in acute stroke management, stroke survivors suffer from long-term disabilities due to motor and cognitive deficits. The mechanisms of action of stroke are complexly related to neuroprotection, oxidative stress, neuroinflammation, neural cell apoptosis, and neurogenesis. Consequently, several potential mechanisms underlying stroke therapy, such as exercise and drug to ameliorate brain injury, are complex. It involves multi-pathway and multi-target regulation, including the promotion of neuroprotection, regulation of oxidative stress and neuroinflammation, apoptosis inhibition, and neurogenesis promotion.

Physical exercise has been reported to have several potential mechanisms in stroke treatment, including increased levels of neurotrophic factors, particularly brain-derived neurotrophic factor (BDNF), reduction of oxidative stress, suppression of inflammation, improvement of cerebral blood perfusion, or combination (Alkadhi, 2018). In addition, rehabilitative exercises and pharmacological agents exert neurophysiological and neuroanatomical plasticity after stroke, leading to functional recovery. In animal studies, physical exercise has been shown to enhance neuroprotection and neurogenesis, stimulate neurorepair, and enhance functional recovery following ischemic stroke (Cheng et al., 2020).

Recently, herbal medicine has been shown to induce neurogenesis and neuroprotection in animal models of Alzheimer's disease (Zhu et al., 2020) and ischemic stroke (Liu et al., 2015a,b). For example, ligustilide, the main active component in *Angelica sinensis* (Zhu et al., 2020), and 1-deoxynojirimycin, the main alkaloid component (Chen et al., 2018), improve cognitive impairment by reducing neuroinflammation. Furthermore, *Rhizoma Dioscoreae* polysaccharides have a pharmacodynamic effect in cerebral ischemic reperfusion injury, which reduce the infarct volume, upregulate the anti-oxidant kinase, and downregulate inflammatory cytokines (Shi et al., 2022). Herbal medicine, with multifactorial effects including antioxidant, anti-inflammatory, neuroprotective, and vasculoprotective effects, may be a promising natural medicine for the treatment of stroke (Gaire, 2018). The advantage of herbal medicines is that they have fewer side effects than conventional medicines. Clinically, rehabilitative therapy with therapeutic agents may be a good strategy to induce great recovery in a patient with stroke (Gladstone et al., 2006). Therefore, the present study investigated whether herbal medicine in combination with rehabilitative exercise has a synergistic effect on stimulating the mechanisms of neurorepair or neuroplasticity, leading to significant functional recovery after stroke.

Ninjin'yoeito (NYT) is a traditional Japanese Kampo medicine consisting of 12 herbs, including *Atractylodes* rhizome, *Rehmannia* root, Japanese *angelica* root, ginseng, *Poria* sclerotium, cinnamon bark, *Polygada* root, peony root, *Astragalus* root, *Citrus unshiu* peel, *Glycyrrhiza*, and *Schisandra* fruit. A recent review article reported that NYT has multifunctional beneficial activities, such as improving fatigue, apathy, anorexia, night sweats, and anemia, and promoting recovery from several diseases (Miyano et al., 2018). NYT treatment ameliorates cancer cachexia-induced sarcopenia and anorexia (Ohsawa et al., 2018). In the central nervous system, NYT attenuates behavioral abnormalities via hippocampal neurogenesis in a mouse depression model (Murata et al., 2018). Each herbal ingredient of NYT has various effects, including anti-inflammatory, antioxidant, and neuroprotective effects that has been demonstrated in animal models of neurological diseases (Miyano et al., 2018). Ginsenoside Rd, the most active component of ginseng, promotes neurogenesis after brain ischemic reperfusion injury (Liu et al., 2015a,b). The extracts of *Polygada* root and ginseng facilitate hippocampal neurogenesis (Jiang et al., 2021). Several *in vitro* (Liu et al., 2015a,b; Pi et al., 2016) and *in vivo* (Liu et al., 2015a,b) studies have reported that NYT components have neurotrophic, neurogenesis, and neuroprotective effects. In clinical studies, oral administration of NYT improved cognitive dysfunction in patients with Alzheimer's disease (Kudoh et al., 2016). Kampo medicines, including NYT components, such as *Rehmannia* root, *Angelica* root, and *Citrus unshiu* peel, have been shown to improve thalamic pain and other symptoms in patients with stroke (Ueda et al., 2011). In addition, the use of Kampo medicine, including NYT components, could facilitate neurological recovery in older patients with acute ischemic stroke (Kimoto, 2003). Although few, these reports suggest that NYT treatment may promote functional recovery following stroke through its beneficial effects, such as neurotrophic and neuroprotective effects. However, no study has determined the effects of NYT or the possible mechanisms of neurorepair and functional recovery after stroke. Although some studies have been reported in the literature, further research is needed to ascertain the mechanisms of NYT function after stroke.

Neurotrophic factors, nerve growth factor (NGF), and BDNF are key mediators of neuronal plasticity, neuronal survival, and functional recovery through their binding to tropomyosin receptor kinase (Trk) receptors. They preferentially bind to NGF with TrkA receptors and BDNF with TrkB receptors (Gibon and Barker, 2017; Sims et al., 2022). Subsequently, downstream signaling activates several signal transduction pathways, such as PI3K/Akt (phosphatidylinositol 3 kinase/v-akt murine thymoma viral oncogene homolog) (Sims et al., 2022). NGF and BDNF are intrinsic neurotrophic factors that exert neuroprotective effects after insults to the mature brain. NYT increased NGF levels in cultured rat astrocytes, and *Polygala* root or *Panax ginseng* extracts strongly increased NGF levels compared with other NYT ingredients (Yabe et al., 2003). In addition, the mixed dried roots of *Polygala tenuifolia* and *Panax ginseng* promote hippocampal neurogenesis through the BDNF/TrkB signaling pathway (Jiang et al., 2021). Physical exercise, similar to NYT intake, increases NGF mRNA levels in the hippocampus and cortex of intact rat brains (Neeper et al., 1996). Furthermore, physical exercise reduces infarct volume and ameliorates motor function through BDNF/p-TrkB and by activating Akt (Sakakima et al., 2012). However, the potential of combination therapy with NYT and physical exercise and the underlying mechanisms have not been clarified in stroke treatments.

In this study, we investigated whether the application of NYT after stroke with or without physical exercise decreased brain infarct volume, increased NGF and BDNF expression levels, improved physical function via the NGF/TrkA and BDNF/TrkB pathways, and activated the Akt pathway. In addition, we examined whether NYT administration after stroke leads to increased neurotrophic factors and improved sensorimotor function, thus indicating the clinical relevance of NYT therapy and providing a rationale for combining NYT with physical exercise.

2. Materials and methods

2.1. Animals

Forty-five adult male Sprague-Dawley rats (278.5 ± 18.3 g, mean ± SD) were used. All animals were acclimatized for 7 days before the experiments began. Two rats were housed per cage with a 12-h light/dark cycles and kept at temperature-controlled conditions (23.0 ± 1.0 °C), with food and water available *ad libitum*. The experimental procedures were conducted in compliance with the guidelines established by the Institute of Laboratory Animal Sciences of Kagoshima University and were approved by the ethical committee of Kagoshima University (No. M19004).

2.2. MCAO

Rats were anesthetized intraperitoneally by a combination of 2.5 mg/kg butorphanol, 2.0 mg/kg midazolam, and 0.3 mg/kg medetomidine. Ischemic stroke was conducted through left middle cerebral artery occlusion (MCAO) using an intraluminal filament in accordance with our previous study (Sakakima et al., 2012). After 60 min of MCAO, reperfusion was performed by withdrawal of the filament. The rectal temperature was continuously monitored and maintained at 37 °C by a heating blanket (BWT-100A; Bioresearch Center Co., Ltd., Nagoya, Japan) during the surgical procedure.

2.3. NYT treatment and exercise training after stroke

After MCAO, the rats were randomly assigned to four groups: ischemic reperfusion (IR) injury without NYT treatment and exercise (IR group, n = 10), NYT treatment without exercise (NYT group, n = 10), exercise without NYT treatment (Ex group, n = 10), and NYT treatment with exercise (NYT + Ex group, n = 10). The inclusion criteria for MCAO model rats were neurological score 2 or 3 and weight loss less than 20% at 1 day after MCAO to minimize the variation in the bias of the effect of medication. Five intact animals were used as controls.

NYT (lot no. 39228490) was procured from Tsumura & Co. (Tokyo, Japan). To prepare 6 g of NYT extract powder, a mixture of 12 dried natural components was used (Table 1), which was manufactured as a spray dried powder from a hot water extract (yield 19%). Plant materials were verified by the identification of marker compounds (glycyrrhizic acid, paeoniflorin, and hesperidin) and external morphology of plant specimens according to the Japanese Pharmacopeia and company standards. NYT includes *Atractylodes* rhizome [4 g, *Atractylodes lancea* (Thunb) DC. or *Atractylodes macrocephala Koidz.*], *Rehmannia* root [4 g, *Rehmannia glutinosa* (Gaertn.) DC.], Japanese *angelica* root [4 g, *Angelica acutiloba* (Siebold & Zucc.) Kitag.], ginseng (3 g, *Panax ginseng* C.A. Mey.), *Poria sclerotium* (4 g, *Erythrococca* ulugurensis Radcl.-Sm.), cinnamon bark [2.5 g, *Cinnamomum verum* J. Presl or *Neolitsea cassia* (L.) Kosterm.], *Polygada* root (2 g, *Polygala tenuifolia* Willd.), peony root (2 g, *Paeonia lactiflora* Pall.), *Astragalus* root (1.5 g, *Astragalus mongholicus* Bunge), *Citrus unshiu* peel (2 g, *Citrus × aurantium* L.), *Glycyrrhiza* (1 g, *Glycyrrhiza uralensis* Fisch. ex DC. or *Glycyrrhiza glabra* L.), and *Schisandra* fruit [1 g, *Schisandra chinensis* (Turcz.) Baill. or *Schisandra sphenanthera* Rehder & E.H.Wilson]. The plant name has been checked with "World Flora Online" (www.worldfloraonline.org) or MPNS (http://mpns.kew.org). The extract quality was standardized based on good manufacturing practices, as defined by the Ministry of Health, Labor, and Welfare of Japan. The detailed plant source and medicinal part of each crude drug of NYT have been shown in a previous study (Matsumoto et al., 2021), and extensive profiling of NYT ingredients has also been performed via three-dimensional HPLC by Tsumura & Co (Supplementary Fig. 1). The NYT extract was mixed with the powder of a standard diet (Oriental Yeast Co., Tokyo, Japan) at a concentration of 1% (w/w) and then fed to the animals from 1 to 28 days after stroke. The animals in the IR and Ex groups were fed a standard diet without NYT from 1 to 28 days after stroke. Two animals were housed in each cage and fed 100 g of the diet per day. Feed intake was measured daily and the mean feed intake per week was calculated.

Three days after stroke, the rats in the Ex group were trained using a treadmill machine (MK-680; MUROMACHI KIKAI Co., Ltd., Japan) for 20 min/day, 5 days a week for a maximum of 28 days. Rats were trained at a speed of 8 m/min for the first 3 days, and the running speed was raised to 15 m/min for the remaining days after stroke, which was performed at room temperature during the day. Body weight was measured before injury, and at 1, 7, 14, 21, and 28 days. The experimental design is illustrated in Fig. 1.

Table 1
The galenical components of Ninjin' yoeito (NYT).

Latin name of crude drug	Original plant source and medical parts of crude drug	Amount (g)
Rehmanniae Radix	The roots of *Rehmannia glutinosa* Libosch. var. *purpurea* Makino or *Rehmannia glutinosa* Liboschitz	4.0
Angelicae Acutilobae Radix	The roots of *Angelica acutiloba* Kitagawa or *Angelica acutiloba* Kitagawa var. *sugiyama* Hikino	4.0
Atractylodis Rhizoma	The rhizome of *Atractylodes japonica* Koidzumi ex Kitamura or *Atractylodes macrocephala* Koizumi (*Astractylodes ovata* De Candolle)	4.0
Poria	The sclerotium of *Wolfiporia cocos* Ryvarden et Gilbertson (*Poria cocos* Wolf)	4.0
Ginseng Radix	The roots of *Panax ginseng* C. A. Meyer (*Panax schinseng* Nees)	3.0
Cinnamomi Cortex	The bark of *Cinnamomun cassia* Blume	2.5
Polygalae Radix	The roots of *Polygala tenuifolia* Willdenow	2.0
Paeoniae Radix	The roots of *Paeonia lactiflora* Pallas	2.0
Gitri Unshiu Pericarpium	The pericarp of *Citrus unshiu* Markowicz or *Citrus reticulata* Blanco	2.0
Astragali Radix	The roots of *Astragalus membranaceus* Bunge or *Astragalus mongholicus*	1.5
Glycyrrhizae Radix	The roots and stolons of *Glycyrrhiza uralensis* Fischer or *Glycyrrhiza glabra* Linne.	1.0
Schisandrae Fructus	The fruits of *Schisandra chinensis* Baillon	1.0

The weights indicate the amount of each herbal medicine used to produce 6 g of dry NYT extract.

2.4. Evaluation of ischemic infarction

The rats were anesthetized via intraperitoneal injection with 5% sodium pentobarbital and then transcardially perfused with 0.9% saline before being decapitated. The whole brain was removed and sliced into seven 2-mm-thick coronal sections using a brain slicer. The sliced tissues were immersed in a 1% solution of 2,3,5-triphenyltetrazorlium chloride (TTC) in phosphate buffered saline (PBS pH 7.4) at 37 °C for 15 min. After TTC staining, the sections were scanned to examine infarct volume. The ischemic infarct volume was measured using the ImageJ software 1.53 (NIH, USA). Infarct volume was calculated by the following formula to minimize the error of edema and liquefaction (n = 8 in each group): % of infarct volume = (volume of contralateral hemisphere − volume of ipsilateral non-infarction)/volume of contralateral hemisphere.

2.5. Assessment of neurological deficits, motor function, and sensorimotor dysfunction

Each group was evaluated for neurological deficits, motor function, and sensorimotor dysfunction using the neurological score, beam walking task, rotarod durability task, and sticky tape-removal task at 14 and 28 days after stroke (n = 10 in each group). All evaluations were conducted by 2 individuals. A neurological grading score on a 5-point scale (0–4) was used in accordance with our previous study (Otsuka et al., 2016). In the behavioral evaluation, the rats were subjected to a

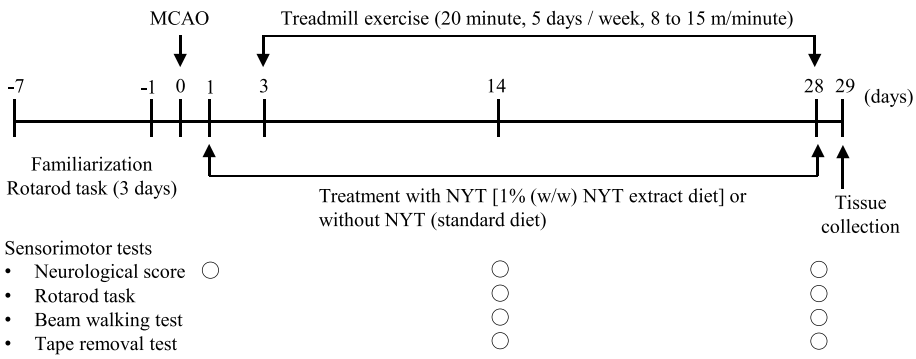

Fig. 1. Study procedures. The timeline of NYT administration, which began at 1 day after stroke and was continued for 28 days, and treadmill exercise, which began at 3 days after MCAO and continued until 28 days after stroke. Sensorimotor assessments were conducted at 1, 14, and 28 days after stroke. Neurological scores at 1 day after stroke were assessed as an inclusion criterion for MCAO model rats.

beam-walking task with an elevated beam (length × width, 100 cm × 2.5 cm), which was scored with a 6-point (0–5) scale, as described in our previous study (Terashi et al., 2019). For the balance and motor function tests, the rats were subjected to a rotarod durability task (MK-670; MUROMACHI KIKAI Co, Ltd). Each rat was placed on a rotarod cylinder, and the duration for which the animal remained on the cylinder was measured in accordance with our study (Otsuka et al., 2019). Sensorimotor dysfunction was evaluated using the adhesive sticky tape removal task in accordance with our study (Otsuka et al., 2021). In this study, the time to react to the square sticky label (1.3 cm × 1.3 cm) in the forelimbs was recorded in 2 trials for both forepaws, and the average time was analyzed.

2.6. Histological and immunohistochemical analyses

After TTC staining, brain sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight. Brain sections (# 3–5 out of seven consecutive TTC sections from cranial to the caudate region) were used for histological and immunohistochemical analyses. Paraffin-embedded coronal brain sections were stained with hematoxylin and eosin (HE) and the following antibodies: rabbit anti-NGF (Abcam plc, UK, ab52918), rabbit anti-BDNF (Bioss, Inc, bs-4989R), rabbit anti-TrkA (Bioss, Inc., bs-10210R), rabbit anti-TrkB (Bioss, Inc., bs-0175R), and rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Inc., P42574) in accordance with manufacturer's protocol. The sections were reacted with the following dilution ratio at 4 °C overnight: rabbit anti-NGF antibody (1:300), rabbit anti-BDNF antibody (1:500), rabbit anti-TrkA antibody (1:500), rabbit anti-TrkB antibody (1:500), and rabbit anti-caspase-3 (1:400). After washing with PBS, the sections were incubated with goat anti-rabbit IgG conjugated to a peroxidase-labeled dextran polymer (EnVision; Dako, CA, USA) for 60 min and immunoreactivity was observed by diaminobenzidine staining. Sections stained for caspase-3 were counterstained with hematoxylin for 1 min.

Two areas in the motor cortex surrounding the lesion of each section were imaged at 20 × magnification without visual field overlap and performed semi-quantitative analysis of immunostained areas (n = 10 in each group). The areas including cells positive for NGF, BDNF, TrkA, and TrkB were quantitatively examined using the ImageJ software 1.53. The craved caspase-3 positive cell numbers per unit area (0.37 mm^2) was counted in the motor cortex of the ischemic penumbra in each group. Quantitative analysis was conducted by two or three individuals who were blinded to each group. In addition, normal intact brains were subjected to immunohistochemical and semi-quantitative analyses to clarify the localization of expression in the intact brain.

2.7. Western blot analysis

Western blot analysis was performed to evaluate protein levels in the ipsilateral brain (n = 6 in each group). TTC staining does not constrict quantitative gene and protein analyses (Kramer et al., 2010). Therefore, the ipsilateral brain, coronal sections of # 1, 2, and 6 out of 7 consecutive TTC sections from the cranial to the caudate region, was used for Western blot analysis in accordance with the manufacturer's protocol. Ipsilateral brain tissue was dissected on ice and homogenized in T-Per reagent (78,510; Pierce Protein Research Products). Approximately 12 μg of protein in each sample was loaded onto a 4–20% mini-protean precast gel and transferred to a nitrocellulose membrane. After blocking with 5% skim milk in washing buffer or PVDF Blocking Reagent (Can Get Signal; TOYOBO) for 1 h, the membrane was incubated with the following primary antibody: rabbit anti-NGF antibody (1:1000, ab52918; Abcam plc), rabbit anti-BDNF antibody (1:1000, bs-4989R; Bioss, Inc.), rabbit anti-TrkA antibody (1:500, bs-10210R; Bioss, Inc.), rabbit-anti phosphorylated-TrkA (Tyr680, Tyr681) antibody (p-TrkA, #PA5-104,740; 1:500, Invitrogen), rabbit anti-TrkB antibody (1:400, bs-0175R; Bioss, Inc.), rabbit anti-Akt antibody (1:1000, #9272S; Cell Signaling, Inc.), rabbit anti-p-Akt antibody (1:1000, #9271S; Cell Signaling, Inc.), and mouse anti-α-tubulin antibody (1:2000, 66031-1-Ig; Proteintech, USA) at 4 °C overnight and then with a secondary horseradish peroxidase-labeled antibody for 1 h at room temperature. Protein bands were detected by chemiluminescence (WSE-6100 LuminoGraph I; ATTO), visualized using an EzWestlumi plus detection system (ATTO), and semi-quantitatively measured using ImageJ software 1.53. α-tubulin was detected as the internal control. Semi-quantitative analysis was compared between the intervention groups without including the control group because the ratio to internal controls was calculated.

2.8. Statistics

All data were analyzed by GraphPad Prism software version 9.1.0 for Windows (San Diego, California, USA). Statistical analyses were conducted using parametric or nonparametric tests, following the Shapiro-Wilk test. The neurological score, beam walking score, rotarod endurance time, and tape removal time were analyzed by the Kruskal-Wallis test, and followed by Dunn's *post hoc* test. Infarct volume and NGF, BDNF, caspase-3, TrkA, p-TrkA, TrkB, Akt, and p-Akt expression were analyzed using one-way analysis of variance (ANOVA), and followed by Tukey's *post hoc* multiple comparisons. An independent Mann-Whitney *U* test or Student's *t*-test was performed for between -group analysis. The Cohen's effect size index (Cohen, 1992) was used to evaluate intergroup differences in the infarct volumes. Changes in feed intake, body weight, and neurological score were presented without including a normal control group to clarify the change in the time course between the

intervention groups. The infarct volume was also presented without including a normal control group because ischemic lesions were not identified in control animals. All data are presented as mean ± standard error of the mean (SEM). The measured values are plotted in graphs. The threshold for statistical significance was set at $P < 0.05$. Data were statistically processed after outlier removal using the ROUT method in GraphPad Prism software.

3. Results

3.1. Effect of NYT and/or exercise on feed intake and body weight after stroke

All ischemic animals were fed food with or without NYT 1 day after stroke. Feed intake was small for the first 2 days after MCAO but increased thereafter. Notably, the NYT + Ex group had a significantly increased mean feed intake per week compared with the other groups during 1 week after stroke (Fig. 2A). At 2 and 4 weeks after stroke, the NYT + Ex group indicated a significantly increased mean feed intake per week compared to the NYT group. However, there were no significant differences in the total feed intake during the experimental period, suggesting that there was no difference in the overall drug dose (Fig. 2B). Body weight decreased at 1 week but increased from 2 weeks after stroke (Fig. 2C). The NYT + Ex group showed increased body weight compared with the other groups at 2 and 3 weeks after stroke. However, there were no significant differences between the groups.

3.2. Effect of NYT and/or exercise on infarction, neurological deficits and sensorimotor functions after stroke

TTC and HE staining were used to examine the extent of brain injury and infarct volume (Fig. 3A, C). The infarct volume was reduced in all treatment groups compared to that in the IR group (Fig. 3A and B). However, there were no significant differences between the groups, as determined by one-way ANOVA. In a comparative test between the two groups, the NYT and NYT + Ex groups showed a significant decrease in the volume of infarction compared to the IR group (Student's *t*-test, $p < 0.05$), which had a high intergroup effect size ($r = 0.54$ and 0.52, respectively). Since it has been reported that exercise has decreased infarct volume via the inhibition of neuronal cell apoptosis (Matsuda et al., 2011), it was expected that 4-week exercise intervention would decrease infarct volume after stroke. However, no significant difference was detected between the IR and Ex groups, which had a median intergroup effect size ($r = 0.41$).

All groups showed improved neurological scores from 1 day (median score: 2) to 28 days (median score: 1) after stroke (Fig. 3D). The NYT + Ex group had the most improved neurological score 28 days after stroke compared with the other groups. However, there was no significant difference between the groups at 14 and 28 days after stroke, as examined by one-way ANOVA. In a comparative test between the two groups, the NYT + Ex group showed significantly improved neurological scores compared with the IR group at 14 and 28 days after stroke (Mann-Whitney *U* test, $p < 0.05$).

Sensorimotor function improved from 14 to 28 days after stroke. Therefore, we compared sensorimotor function in each group 28 days after stroke (Fig. 4). The IR group exhibited significantly worse sensorimotor function than did the control group (Fig. 4A–C, $p < 0.01$). All treatment groups showed improved sensorimotor function after stroke compared with the IR group. Notably, the NYT + Ex group showed significantly recovered waking time and beam walking score compared to the IR group, suggesting that NYT in combination with exercise enhanced walking ability (Fig. 4A, B, $p < 0.05$).

3.3. Effect of NYT and/or exercise on NGF, TrkA, and p-TrkA expressions

NGF is a neurotrophic factor that is involved in neurogenesis. NGF and its receptor levels were detected using immunohistochemical analysis or western blotting. The expression of NGF-positive cells surrounding the lesions was decreased in the IR group (Fig. 5A, C). The ratio of NGF-positive cells significantly increased in all treatment groups (Fig. 5C, $p < 0.05$). Similarly, the expression of TrkA positive-cells was remarkably increased in all treatment groups (Fig. 5A). Notably, the ratio of TrkA-positive cells in the NYT + Ex group was significantly higher than that in the IR group (Fig. 5D, $p < 0.05$). Furthermore, the protein levels of NGF and TrkA in the ipsilateral brain were significantly increased by NYT and/or exercise treatments compared with those in the IR group (Fig. 5B, E, F, $p < 0.05$). NGF protein levels were significantly higher in the NYT group than in the Ex group (Fig. 5E, $p < 0.05$). In addition, p-TrkA protein levels were significantly higher in the NYT + Ex group than in the other groups (Fig. 5G, $p < 0.01$).

3.4. Effect of NYT and/or exercise on BNDF and TrkB expressions

BDNF is a neurotrophic factor associated with neurogenesis and neuroplasticity. We investigated the immunoreactivity of BDNF- and its receptor-positive cells surrounding lesions. The expression of BDNF and TrkB decreased in the IR group (Fig. 6A, C, D). NYT and/or exercise remarkably increased the ratio of BDNF and TrkB-positive cells compared to the IR group (Fig. 6C, D, $p < 0.05$). The protein levels of BDNF and TrkB were significantly higher in the NYT + Ex group than in the other groups (Fig. 6B, E, and F, $p < 0.05$).

3.5. Effect of NYT and/or exercise on the expressions of caspase-3 and p-Akt/Akt activity

Caspase-3 is an essential component of some apoptotic pathways (McIlwain et al., 2013). Therefore, we examined the craved-caspase-3

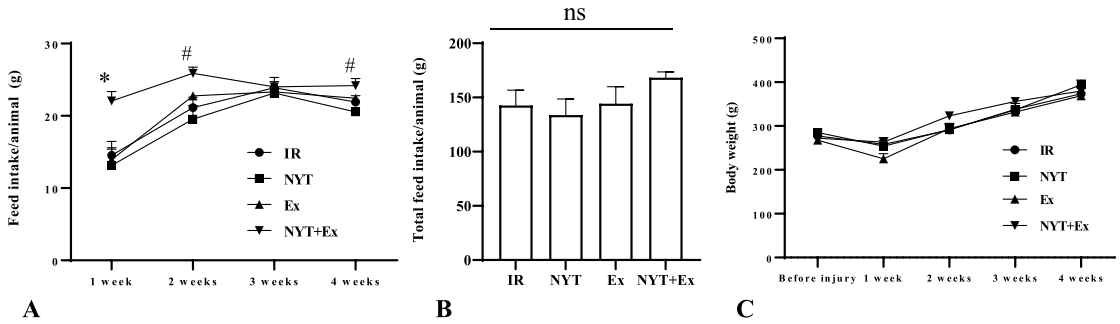

Fig. 2. Effect of NYT and/or exercise on feed intake (A, B) and body weight (C) after stroke. The NYT + Ex group exhibited remarkably increased mean feed intake per week compared to the other groups at 1, 2, and 4 weeks after stroke (A). The total feed intake was not significantly different during the experimental period (B). Body weight was decreased at 1 week, but increased from 2 weeks after stroke (C). Mean ± SE. *$p < 0.05$ (compared with the other groups), #$p < 0.05$ (compared with the NYT group). ns: no significant difference. (n = 6–10 in each group).

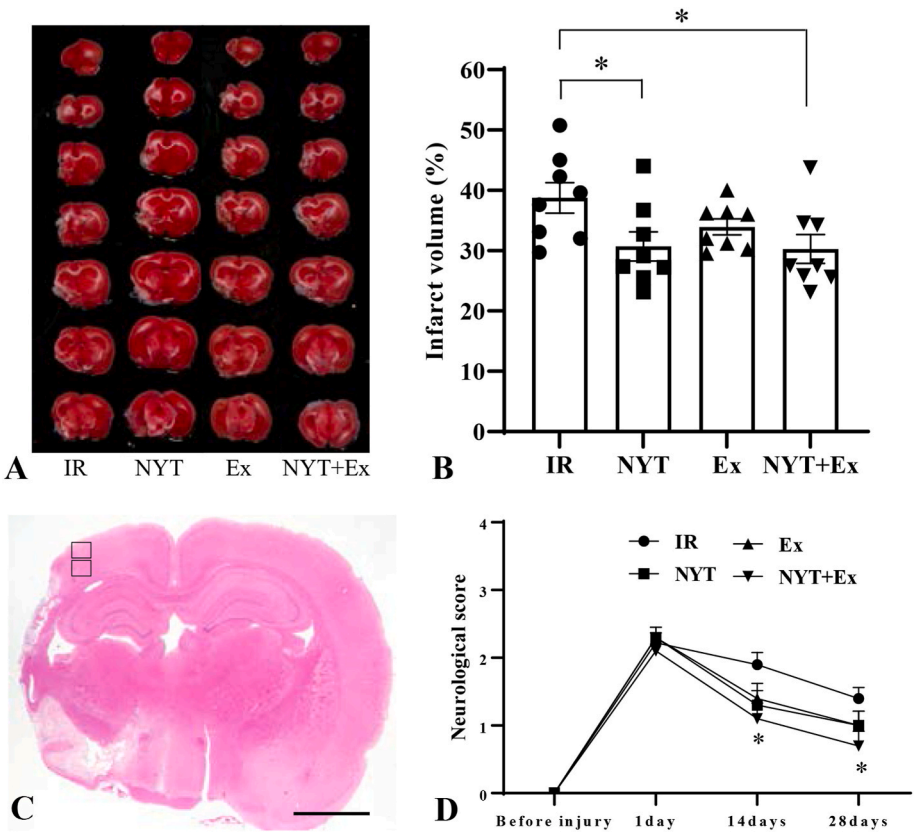

Fig. 3. Effect of NYT and/or exercise on infarct volume (A, B, C) and neurological deficits (D) after stroke. Representative TTC stained sections (A) and quantitative infarct volume (B). In a comparative test between the two groups, NYT and NYT + Ex groups indicated significantly reduced infarction compared to the IR group. H&E staining indicate the section of 5 out of 7 consecutive TTC in the IR group (C). Two rectangles were used for immunohistochemical analysis. All groups had improved neurological score after stroke (D). In a comparative test between two groups, the NYT + Ex group indicated remarkably improved neurological score compared to the IR group at 14 and 28 days after stroke. Mean ± SE. *p < 0.05 (compared with the IR group). Scale bar = 2 mm. (n = 8–10 in each group).

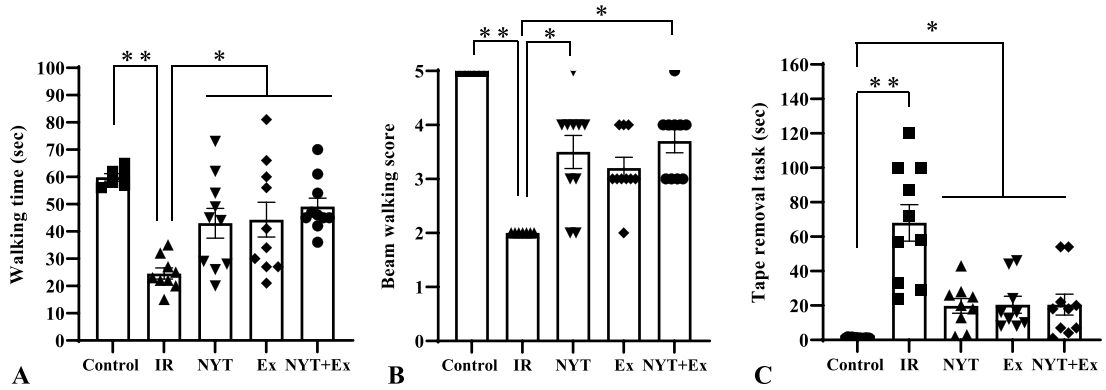

Fig. 4. Effect of NYT and/or exercise on walking time (A), beam walking score (B), and tape removal task (C) after stroke. The IR group exhibited significantly worse performance for all sensorimotor functions compared to the control group (B, C). All treatment groups had improved sensorimotor functions compared to the IR group after MCAO. The NYT + Ex group showed significantly improved waking time and beam walking score compared to the IR group (A, B). Mean ± SE. *p < 0.05, **p < 0.01. (n = 10 in each group).

immunoreactivity surrounding the lesions. The proportion of caspase-3 positive cells increased in the motor cortex surrounding the lesion in the IR group (Fig. 7A). The treated groups exhibited a reduced proportion of caspase-3 positive cells. Notably, the Ex and NYT + Ex groups showed a significantly decreased ratio of caspase-3 positive areas compared to the IR group, suggesting that this may be related to the reduced infarct volume (Fig. 7B, $p < 0.01$).

The PI3K/Akt pathway is involved in basic cellular processes, including protein synthesis, survival, and proliferation, and is activated by various signals containing growth factors and extracellular matrix components, such as the Akt signaling pathway (Sims et al., 2022). Therefore, we investigated p-Akt/Akt levels in the ipsilateral brain (Fig. 7C and D). The ratio of p-Akt/Akt increased in the treatment groups. Notably, the NYT + Ex group showed a significant increase in the p-Akt/Akt ratio compared with the IR group (Figure 7C, $p < 0.05$).

4. Discussion

Herbal medicines exert neuroprotective effects via multiple mechanisms (Gaire. 2018). Watanabe (1998) reported that Shimotsu-to, a traditional Chinese medicine containing *Rehmannia*, Japanese angelica, and peony roots, which are crude drugs contained in NYT, prevented the development of brain infarction and rarefaction induced by chronic brain ischemia in rats. However, the effects of NYT treatment and mechanisms underlying neurorepair and functional recovery after stroke remain unclear. The present study revealed that NYT and/or

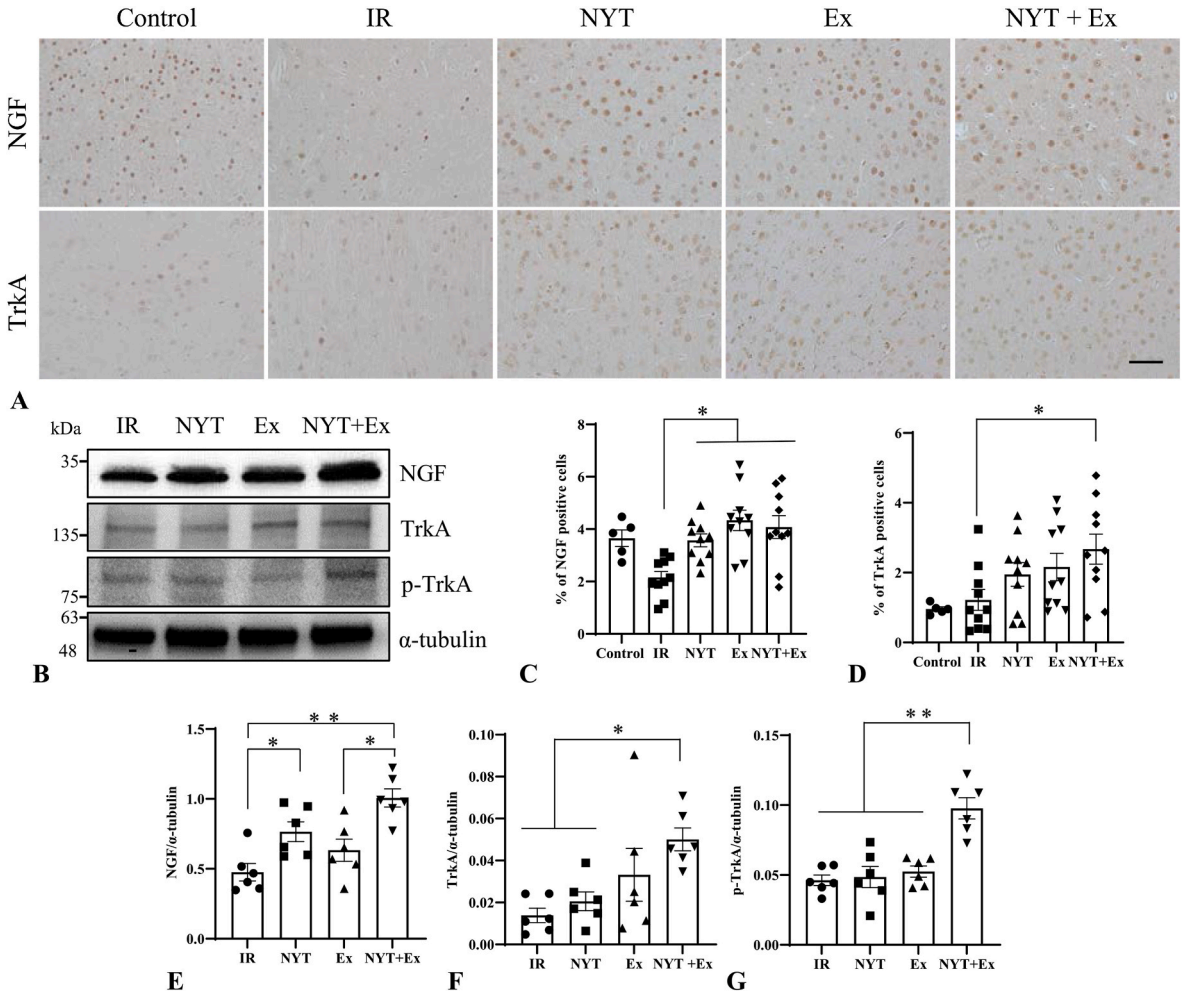

Fig. 5. Effect of NYT and/or exercise on the expressions of NGF (A, B, C, E), TrkA (A, B. D, F), and p-TrkA (B, G) after stroke. The expression of NGF positive cells surrounding the lesions was decreased in the IR group (A, C). The treatment groups exhibited significantly increased ratio of NGF positive cells. The NYT + Ex group showed significantly increased ratio of TrkA positive cells compared to the IR group (D). The protein levels of NGF and TrkA were significantly increased by the NYT and/or exercise treatments compared to those in the IR group (B, E, F). The protein levels of p-TrkA were significantly increased in the NYT + Ex group compared to those in the other groups (G). Mean ± SE. *$p < 0.05$, **$p < 0.01$. Scale bar = 50 μm in all panels. (n = 6–10 in each group).

physical exercise provided neuroprotection, enhanced neuronal plasticity, reduced apoptotic activity, and improved sensorimotor functions by stimulating the expression of neurotrophic factors NGF/TrkA and BDNF/TrkB and by activating Akt in ischemic stroke. Furthermore, our findings suggest that NYT combined with exercise may enhance the degree of sensorimotor function recovery following stroke. As stroke pathogenesis triggers multiple pathways resulting in neurodegeneration, NYT in combination with rehabilitation exerts neuroprotective or neuroplastic effects and enhances sensorimotor functions by stimulating the expression of neurotrophic factors and activation of the Akt pathway, which might be an appealing strategy for stroke management.

Several herbal medicines and their components can protect against ischemia/reperfusion injury, improve microcirculation in the ischemic brain, exert neuroprotective properties, and inhibit apoptosis (Gaire et al., 2014). NGF and BDNF play important roles in protecting the brain against ischemic injury and aging through various mechanisms, including ant-inflammatory, anti-oxidant, and anti-apoptosis mechanisms (Sims et al., 2022). Previous studies have reported that NYT and its crude drugs, such as *P ginseng* and *P tenuifolia* increase NGF and BDNF expression in the brain (Yabe et al., 2003; Jiang et al., 2021). Our findings showed that NYT and exercise increased NGF and BDNF expression in the motor cortex surrounding the lesions. Notably, NYT treatment in combination with exercise stimulated neurorepair mediators NGF/TrkA/p-TrkA and BDNF/TrkB. Post-stroke physical exercise increases NGF and BDNF expression surrounding the lesion, which is related to neuroprotection and functional recovery (Ang et al., 2003; Sakakima et al., 2012). Therefore, NYT combined with exercise may enhance the benefits of post-stroke exercise therapy by stimulating the expression of neurotrophic factors and their receptors. Our findings suggest that increased activation of the NGF/TrkA/p-TrkA and BDNF/TrkB pathways due to NYT and/or exercise may be related to neuroprotection, neuronal plasticity, and improved sensorimotor function after stroke.

Autophosphorylation and dimerization of Trk receptors activate major signaling pathways, such as PI3K/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), which suppress the apoptosis of its downstream mediators (Sims et al., 2022). NYT constituents, such as *P tenuifolia*, promote neurogenesis or neurite outgrowth via the MAPK/ERK and PI3K/AKT signaling pathways (Liu et al., 2015a,b; Pi et al., 2016). This study indicated that the p-Akt/Akt ratio was increased by NYT and exercise, whereas caspase-3 activity decreased in the treatment groups. Therefore, NYT and exercise are likely to have neurotrophic effects by stimulating NGF/TrkA and BDNF/TrkB and by activating the Akt pathway. Our findings suggest that NYT treatment in combination with exercise after stroke may enhance antiapoptotic activity by stimulating the NGF/TrkA and BDNF/TrkB pathways, which in turn activate Akt pathways. Stroke is a

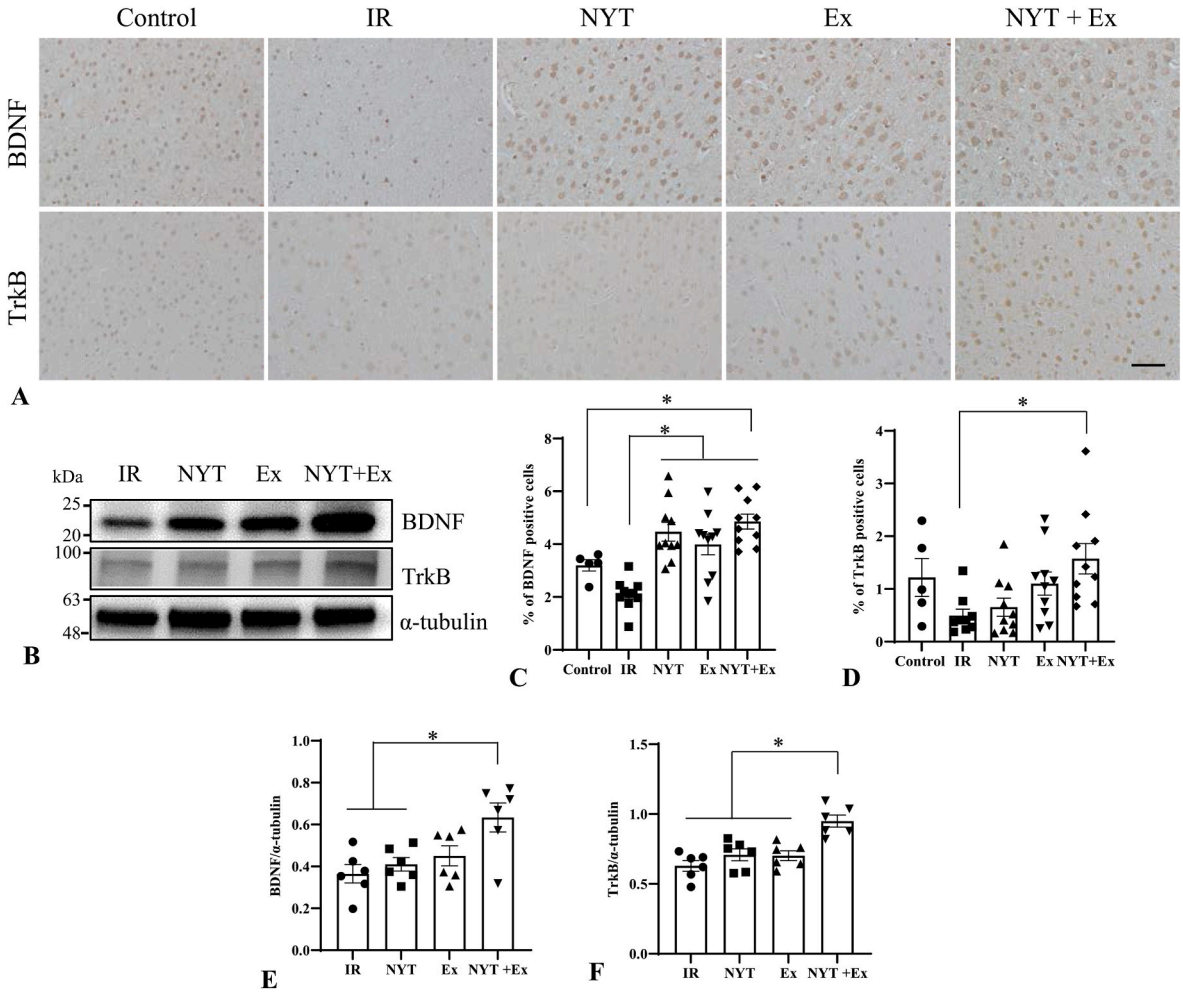

Fig. 6. Effect of NYT and/or exercise on BNDF expression (A, B, D, E) and TrkB expression (A, C, D, F) after stroke. The expression of BDNF and TrkB was decreased in the IR group (A, C, D). The ratio of BDNF and TrkB positive cells was significantly increased by NYT and/or exercise compared to that in the IR group (C, D). The protein levels of BDNF and TrkB were significantly increased in the NYT + Ex group compared to those in the other groups (B, E, F). Mean ± SE. *p < 0.05. Scale bar = 50 μm in all panels. (n = 6–10 in each group).

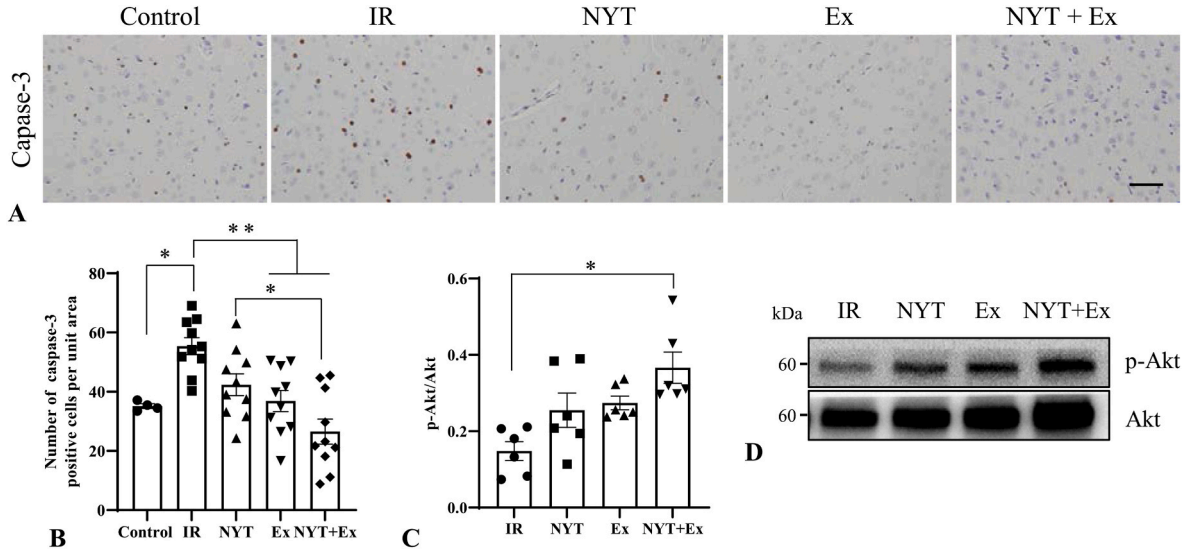

Fig. 7. Effect of NYT and/or exercise on the expressions of cleaved-caspase-3 (A, B) and p-Akt/Akt activity (C, D) after stroke. The Ex and NYT + Ex groups had significantly decreased ratio of caspase-3 positive cells compared to the IR group (B). The NYT + Ex group showed significantly increased p-Akt/Akt ratio compared to the IR group (C). Mean ± SE. *p < 0.05, **p < 0.01. Scale bar = 50 μm in all panels. (n = 6–10 in each group).

vascular-induced neurological disorder in which neuronal cell death is caused by serial pathophysiological events, and NYT administration, which showed neurotrophic and neuroprotective effects via these pathways, could be an appealing strategy for stroke management.

We hypothesized that some pharmacological ingredients enter the brain after oral administration of NYT, considering that the beneficial effects of NYT on cognitive function have been reported in clinical trials (Ohsawa et al., 2017). Previous studies have reported that NYT, paeoniflorin (Cao et al., 2006), and glycyrrhetinic acid (Tabuchi et al., 2012) enter the brain through the blood-brain barrier (BBB). Matsumoto et al. (2021) demonstrated, using pharmacokinetic analysis, that some NYT components could be absorbed into the circulating blood and transported into the brain, suggesting that several components of NYT may be transported across the BBB into the brain after oral administration. Notably, they reported that schizandrin, an ingredient of NYT, can be transported into the brain, which may be part of the beneficial effects of NYT on cognitive impairment and depression (Matsumoto et al., 2021). Taken together, some NYT ingredients, such as schizandrin, paeoniflorin, and glycyrrhetinic acid are transported into the brain following oral administration, which may contribute to the expression of neurotrophic factors and recovery of the sensorimotor functions. In addition, post-stroke physical exercise may enhance neuroplasticity and neuroprotection by directly stimulating the expression of NGF and BDNF in the penumbra lesions. Therefore, this study suggests that the beneficial effects of NYT administration may be enhanced in combination with exercise therapy after stroke. However, this study did not investigate which of the 12 NYT ingredients was effective. Further studies are required to elucidate the beneficial effects of NYT ingredients after stroke. However, herbal medicine is a drug whose pharmacological effects are enhanced by a combination of multiple crude drugs.

Among the non-physical aspects, post-stroke fatigue is associated with depression, motor impairment, physical deconditioning, and reduced health-related quality of life (Winward et al., 2009; Ponchel et al., 2015). Therefore, it may be important for stroke survivors to improve their fatigue, frailty, physical deconditioning, apathy, and motor deficits following stroke. Improvement in anorexia is a benefit of NYT treatment (Miyano et al., 2018). Our results showed that animals treated with a combination of NYT and exercise had increased feed intake and body weight in the early phase after stroke, suggesting that NYT treatment in combination with exercise may be associated with improved anorexia following stroke. However, feed intake in these animals increased in the early phase, but no significant body weight gain was observed after stroke, which might be affected by pathological conditions. In addition, NYT administration alone did not increase the feed intake after stroke. Therefore, further studies are required to elucidate the multifunctional beneficial effects of NYT, such as improving fatigue, apathy, and anorexia following stroke.

This study has some limitations. First, we did not examine the activation of the PI3K/Akt pathway using a PI3 kinase inhibitor because several studies have reported that the beneficial effects of NYT ingredients were blocked by LY294002, a PI3 kinase inhibitor (Liu et al., 2015a,b; Pi et al., 2016). Therefore, we examined the p-Akt/Akt ratio post-stroke. Second, we did not show the expression of phosphorylated TrkB (p-TrkB) by western blotting. We carried out Western blot analysis for p-TrkB, but it could not be detected. Third, immunohistochemical analysis was performed in the motor cortex; However, western blotting was performed in the ipsilateral brain, including both ischemic and non-ischemic tissues. Fourth, this study used an animal model of severe stroke, with a neurological score of 2 or 3. NYT treatment may be clinically relevant for mild patients after improving frailty, fatigue, apathy, depression, and anorexia following stroke. Fifth, we focused on the neuroprotective effects of NYT and exercise including increases in the levels of neurotrophic factors and apoptosis inhibition. However, the mechanism of action of stroke is not only related to neuroprotection, but also to oxidative stress, neuroinflammation, and neural cell activation. These processes are mediated by various cellular and molecular signaling pathways. Therefore, further studies are required to elucidate the potential mechanisms of NYT, such as the reduction of oxidative stress and suppression of neuroinflammation following stroke. Sixth, we did not examine the effects of long-term exercise interventions. In an animal model, early initiation of post-stroke aerobic exercise for 14 days or more produced beneficial effects including increases in the expression of neurotrophic factors, particularly BDNF (Ploughman et al., 2015). Therefore, this study investigated the neuroprotective effects of NYT and exercise therapy at 4 weeks. Despite these limitations, our findings suggest that NYT administration and exercise therapy after stroke enhance functional recovery and reduce apoptotic activity by promoting the expression of neurotrophic factors NGF/TrkA and BDNF/TrkB and by activating Akt. Notably, the combination of NYT and exercise may have synergistic effects in enhancing the expression of neurotrophic factors, exerting anti-apoptotic activity, and improving sensorimotor functions compared to NYT or exercise alone.

5. Conclusions

NYT and its components have multifunctional beneficial effects on several diseases. This study revealed that NYT administration improved sensorimotor dysfunction and enhanced the benefits of physical exercise after stroke by stimulating NGF/TrkA and BDNF/TrkB and by activating the Akt pathway. Stroke survivors may be treated with a combination of rehabilitative exercise and a mechanism-based therapeutic agent with proven efficacy. Our findings suggest that NYT treatment in combination with rehabilitation therapy such as exercise may have potential therapeutic effects in patients with stroke.

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CRediT authorship contribution statement

Akira Tani: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – review & editing. **Harutoshi Sakakima:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Validation, Visualization, Writing – review & editing. **Shotaro Otsuka:** Investigation. **Keita Mizuno:** Conceptualization. **Kazuki Nakanishi:** Investigation. **Kosuke Norimatsu:** Investigation. **Seiya Takada:** Investigation. **Teruki Matsuoka:** Investigation. **Ryoma Matsuzaki:** Investigation. **Ikuro Maruyama:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115927.

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