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Journal Title: Magnetic Resonance Imaging
Volume: Volume 40
Publisher: Elsevier | 2017-07-01, Pages 24-30
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1016/j.mri.2017.03.011
Permanent URL: https://pid.emory.edu/ark:/25593/t0ndf

Final published version: http://dx.doi.org/10.1016/j.mri.2017.03.011

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Accessed January 31, 2020 6:25 AM EST
Longitudinal MRI Evaluation of Neuroprotective Effects of Pharmacologically Induced Hypothermia in Experimental Ischemic Stroke

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Abstract
Pharmacologically induced hypothermia (PIH) shows promising neuroprotective effects after stroke insult. However, the dynamic evolution of stroke infarct during the hypothermic therapy has not been understood very well. In the present study, MRI was utilized to longitudinally characterize the infarct evolution in a mouse model of ischemic stroke treated by PIH using the neurotensin agonist HPI201. Adult male C57BL/6 mice underwent permanent occlusion of the right middle cerebra artery (MCA). Each animal received a vehicle or HPI201 intraperitoneal injection. The temporal changes of stroke lesion were examined using T2-weighted imaging and diffusion-weighted imaging (DWI) in the acute phase (1–3 hours) and 24 hours post stroke. Significantly reduced infarct and edema volumes were observed in PIH treated stroke mice, in agreement with TTC staining findings. Also, the TUNEL staining results indicated apoptotic cells were widely distributed among the ischemic cortex in control group but limited in PIH treated mice. Dramatically reduced growth rate of infarction was seen in PIH treated stroke mice. These

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Conflict of interest statement
Dr. Dix is the Scientific Director of JT Pharmaceutical. All other authors claim no conflict of interest in this investigation.

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results demonstrate HPI201 has strong neuroprotection effects during acute stroke. In particular, MRI with the numerical modelling of temporal infarct evolution could provide a unique means to examine and predict the dynamic response of the PIH treatment on infarct evolution.

Keywords
HPI201; Drug-induced hypothermia; MRI; Ischemic stroke; logarithm pattern

Introduction

Ischemic stroke, which accounts for more than 80% of all strokes, occurs when the arterial blood supply to the brain is obstructed, leading to an early-onset neuronal death after stroke [1]. Although treatment with recombinant tissue plasminogen activator (rt-PA) can provide reperfusion of the ischemic territory and salvage the ischemic lesions, a very small portion of patients (less than 5%) are able to receive the reperfusion therapies due to highly restricted therapy windows (within 4.5 hours of insults) [2, 3]. Therefore, there is an imminent need for an efficacious and novel therapy for stroke disease.

Induced hypothermia is suggested to be a useful therapeutic strategy for stroke, traumatic brain injury, cardiac arrest, and kidney injury [4, 5]. It has been shown that mild to moderate hypothermia (2–5°C reduction) is generally safe, and could protect brain tissue from ischemic damage and improve functional outcomes after cerebral ischemia [6]. Hypothermia may attenuate free radical levels [7], inhibit cell apoptosis [8], and reduce energy demand of neuronal activity [9] as well as suppress inflammatory response in ischemic brain tissue [10]. In the clinic, therapeutic hypothermia is an approved therapy for cardiac arrest and children with hypoxic ischemic encephalopathy [11]. Phase II and III clinical trials for stroke treatment have been carried out using physical cooling methods to induce hypothermia in children [12]. However, physical cooling almost always triggers shivering and vasoconstriction responses, which makes it very challenging to reduce and accurately control the patient’s temperature [13]. Recently, novel neurotensin (NT) analogs have been introduced to produce pharmacologically induced hypothermia (PIH), which has been demonstrated to be effective in promoting functional recovery and protecting the brain from further damage from ischemic and hemorrhagic stroke [14, 15] and traumatic brain injury [16, 17]. However, there is a lack of translational imaging information regarding temporal and spatial evolution of infarction in the PIH-treated ischemic brain, which plays a critical role for monitoring the treatment efficacy and optimizing the treatment options in future clinic trials.

MRI is a powerful approach to evaluate ischemic stroke non-invasively [18–26]. In particular, diffusion-weighted imaging (DWI) could detect the infarct in very early stages of ischemic tissue damage, and the DWI-derived infarct volume correlates with clinical outcomes [27, 28]. HPI-201 is a novel neurotensin receptor 1 (NTR1) agonist. Recent studies have demonstrated it is effective in inducing therapeutic hypothermia, reducing inflammatory response, brain injury and promote long-term functional recovery after ischemic, hemorrhagic stroke and traumatic brain injury (TBI) [15, 16, 29, 30]. The main
aim of the present study is to longitudinally examine HPI201 induced therapeutic effects on acute ischemic stroke using MRI. We hypothesized that HPI201 could induce therapeutic hypothermia which would spatially and temporally inhibit the infarct progression during acute stroke, and MRI could provide promising diagnostic information to access the hypothermic treatment response non-invasively.

Methods

Animal model preparation

Focal cerebral ischemic stroke was induced by permanent occlusion of the right middle cerebral artery (MCA) plus 7-min ligation of both common carotid arteries (CCA) in adult male C57BL/6 mice (8–12 wk, 22–28 g.), as described previously [14]. Animals were anesthetized with 3% isoflurane for induction and were maintained at 1.5% isoflurane with 100 O\textsubscript{2}. Rectal temperature was monitored and maintained at 37±0.5°C during surgery, using a heating pad controlled by a homeothermic blanket control unit (Havard Apparatus, Holliston, MA, USA).

Pharmacologically induced hypothermia

Animals (n=10) were subjected to HPI201 injection to induce mild hypothermia. Briefly, HPI201 was dissolved in saline and injected intraperitoneally with the bolus injection (2 mg/kg) at 30 min after the ischemic insult and CCA reperfusion, followed by additional injections at half of the initial dose (1.0 mg/kg). The intervals between the following injections were around 1.5 hours, with adjustments made in order to keep a constant mild hypothermia (33–35°C) for 6 hrs. Animals in the control group (n=10) were injected with saline after stroke. Rectal temperature was monitored using a rectal probe (Harvard Apparatus) for 6 hrs after ischemia, with measurements performed every 15 min for the first hour and every 30 min thereafter. Supplemental heat was given if the body temperature dropped below 32°C. Awakened animals were returned to their home cages after surgery and examination in Day 0. The animals were sacrificed at 24 hrs post stroke (or immediately after their last MRI scans without recovery from anesthesia).

In vivo MRI data acquisition

Ten mice (n=5 in control group and n = 5 in treatment group) were randomly selected for MRI examination during acute stroke (1–4 hours post stroke) and rescanned 24 hrs post stroke. During MRI scanning, the animal body temperature, SpO\textsubscript{2} level, heart rate (HR) and respiratory rate (RR) of the mice were continuously monitored using a Mouse-Ox system (Starr life science, USA). For the mice in the control group, the body temperature was maintained at ~37 °C with a Homeothermic Blanket Control Unit (Harvard Apparatus). For PIH-treated mice, the same blanket control unit was used if the body temperature dropped below 32°C.

In vivo MRI was performed using a 7T animal MRI scanner (Bruker BioSpin, Billerica, MA) and a home-made surface coil (ID=2cm) for RF transmission and receiving. Mice were prostrated on a custom-made head holder with ear and teeth restraints to minimize head motion while under spontaneous respiration. Mice were anesthetized using isoflurane/O\textsubscript{2}
(3% for induction and 1.5% for maintenance) during surgery for about 15 min to 20 min. Heating pad was used during the surgery. Animals were imaged after surgery for about 3 and half hours (from 0.5 hours up to 4 hours post stroke) and rescanned 24 hours post-surgery. Coronal MRI sections were performed beginning from 2 mm anterior to the rostral end of corpus callosum and ending at the end of the cerebrum. T2-weighted imaging (T2WI) was acquired with the following parameters: field of view (FOV) = 3.0 × 3.0 cm², matrix size=256×256, repetition time (TR) =1000ms and echo time (TE)=50ms, slice thickness=1.0 mm. DWI images were acquired with a four-shot EPI sequence for diffusion tensor imaging (DTI). The DTI parameters were: TR=3000 ms, TE=32 ms, Δ=20 ms, δ=4 ms, field of view=3.0 × 3.0 cm², slice thickness=1.0 mm, matrix size=128×128, image resolution=250×250 μm², NEX=4, 30 gradient directions, b values= 1000 s/mm². T2 and DWI images were acquired every hour for 3 hours immediately after stroke and repeated 24 hours post stroke.

All animal experimental procedures in this study were approved by the Emory University Institutional Animal Care and Use Committee and are in compliance with U.S. National Institutes of Health (NIH) guidelines.

**MRI data processing**

DTI images were processed using DTI Studio v2.4 [31](Johns Hopkins University, Baltimore, MD).

Final infarct volume was defined with ADC maps at 24 hrs using the threshold of the mean ADC value from the normal hemisphere plus two times the standard deviation. For each stroke mouse, the area of the stroke lesion and total brain area in every slice was derived from the ADC maps manually with the corresponding DWI and T2-weighted images as referenced by ImageJ 1.34 (National Institutes of Health, Bethesda, MD) at each time point. Then, the total stroke volume was calculated as the sum of the lesion areas across all slices, multiplied by the total slice thickness.

**Histopathology evaluation**

Mice were sacrificed immediately after their last MRI scan for histological evaluation at 24 hrs post stroke. Brain specimens collected after MRI scans were processed using standard histological protocols [32, 33]. Briefly, mice brains were perfusion-fixed through the left cardiac ventricle with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were quickly removed and cryoprotected in 30% sucrose. The brains were then frozen in OCT compound (Sakura Finetechnical) and stored at −80°C until sectioning. Coronal sections of 10 μm thickness were cryosectioned and mounted on slides which were then stored at −80°C until further processing.

Mice (n=5 for control and n=5 for PIH treatment) without MRI scans were used to examine the physiological parameter changes after HPI201 administration and sacrificed at 24 hours post stroke. The brains were removed and placed in a coronal brain matrix and then sliced into 1-mm sections. Slices were incubated in 2% TTC (Sigma) solution at 37°C for 5 min, then stored in 10% buffered formalin for 24 hrs. The digital images of the caudal aspect of each slice were obtained using a flatbed scanner. Infarct, ipsilateral hemisphere, and
contralateral hemisphere areas were measured using ImageJ software (NIH, Bethesda, MD, USA).

A TUNEL assay kit (Dead End Fluorometric TUNEL system; Promega, Madison, WI, USA) was used to assess cell death by detecting fragmented DNA in 10-μm-thick coronal fresh frozen sections. After fixation (10% buffered formalin for 10 min then ethanol:acetic acid (2:1) solution for 5 min) and permeabilization in 0.2% Triton-X 100 solution, brain sections were incubated in equilibration buffer for 10 min. Recombinant terminal deoxynucleotidyl transferase (rTdT) and nucleotide mixture were then added on the slide at 37°C for 60 min in the dark. Reactions were terminated by 2 × SSC solution for 15 min. Nuclei were counterstained with Hoechst 33342 (1:20,000; Molecular Probes, Eugene, OR, USA) for 5 min.

Infarct volume was calculated with TTC staining slices using the indirect method [34]. Cell count was performed on the TUNEL staining sections following the principles of design based stereology. Systematic random sampling was employed to ensure accurate and non-redundant cell counting. Brain sections (10 μm thick) of every animal were collected. The fields were chosen in each section in the penumbra region and viewed at x40 for cell counting. ImageJ was used to analyze each picture. All analysis was performed in a blinded fashion.

**Statistical Analysis**

All results were expressed as mean ± standard deviation (SD). The student’s t-test was applied to evaluate differences between the stroke volumes of the vehicle and hypothermia treatment groups. A one-way analysis of variance (ANOVA) test, followed by the Tukey test, was applied to analyze the longitudinal changes of ADC values. All statistical analysis procedures were performed using the SPSS for Windows statistical package (Version 18, SPSS, Chicago, IL). A p-value of <0.05 was considered statistically significant.

**Results**

**Longitudinal MRI evaluation of ischemia induced brain damage**

The progression of the focal ischemia induced brain injury was longitudinally evaluated using multiparametric MRI from 1 to 24 hrs post ischemia (Fig. 1 and 2). Compared to the control group mice, much smaller stroke lesion was demonstrated in HPI201 treated mice (Fig 1B). In both groups, ischemic regions were observed at cortex as early as 1 hour post occlusion. The stroke volume of control mice was significantly larger than that of the mice in the HPI201 treated group at 1 hour (57.4 ± 30.4 mm$^3$ vs. 4.6 ± 2.5 mm$^3$, p=0.01), 2 hours (65.9 ± 48.0 mm$^3$ vs. 7.3 ± 6.5 mm$^3$, p=0.05), 3 hours (79.8 ± 61.4 mm$^3$ vs. 8.5 ± 5.2 mm$^3$, p=0.06) and 24 hours (201.2 ± 109.7 mm$^3$ vs. 39.6 ± 27.9 mm$^3$, p=0.03) post ischemia (Figure 2). In other words, there were 80% reductions of infarct volume at 24 hrs in stroke mice with hypothermia treatment compared to that in the control group.

Also, we examined the temporal evolution of infarct volumes of the mice during acute stroke with and without hypothermia treatment using the formula reported previously [35]:

Magna Reson Imaging. Author manuscript; available in PMC 2018 July 01.
\[ V = C \times \ln(t) + V_0 \]

In which \( t \) is the time post occlusion, \( V \) is the lesion volume at time \( t \) (\( t > 0 \), in hour), \( C \) is the growth rate of lesion volume per logarithmic time scale, \( V_0 \) is the baseline value at the time \( t = 1 \), and \( \ln \) is the natural logarithm.

As shown in Figure 3, the evolution of the infarct volume also follows the logarithmic pattern in mice with/without treatment (\( R^2 = 0.995, 0.919 \), respectively), similar to those reported previously in macaque and rat brains with permanent MCAo. In particular, the infarct growth rate decreases remarkably in the treatment groups compared to that in control group (3.59 vs 19.51), indicating such hypothermia therapy significantly reduces both the infarct growth rate and volume during the acute phase. In addition, edema was seen slightly 1–3 hour post stroke. PIH treatment reduced brain edema 24 hours after stroke insult substantially (Fig 1).

**Physiological changes in PIH treated mice with stroke**

HIP201 induced pharmacological hypothermia were seen in adult C57BL/6 mice when administered intraperitoneally. The detailed physiological parameters at baseline and within 1 hour after injection of HIP201 are illustrated in Table 1. At the dosage of 2.0 mg/kg, HIP201 induced a core body temperature drop of 1°C within 10 min after injection, and the temperature continued to drop to ~30°C 1 hour post injection without detectable shivering. With 2 additional injections of HIP201 at 1.0 mg/kg, the low body temperature was maintained for 6 hrs before a gradual recovery to normal temperature (36.5°C). During this time, no obvious changes of the physiological parameters were observed (Table 1). These findings indicate that the neurotensin receptor agonist HIP201 is an effective hypothermic compound that does not show observable side effects in the experimental rodent model.

**Neuropathological evaluation of PIH’s neuroprotective effect in ischemic stroke**

Quantitative analysis of infarct volume using TTC staining showed that 24 hrs after stroke, the hypothermic treatment of HIP201 significantly reduced brain infarction formation (Fig. 5). In order to examine the therapeutic effect at cellular level, TUNEL and NeuN staining was performed in brain sections 24 hrs after stroke. We focused on the neuronal cell death in the ischemic core and penumbra (Fig. 6). TUNEL-positive cell levels were reduced in the HIP201 group compared with those in the control group. Neuronal cells were identified by NeuN staining, whereas the nuclei of all cells were visualized with Hoechst staining. Infarct volume and Neuronal cell death shown as TUNEL/NeuN double-positive cells are reduced significantly in the HIP201-treated group (Figure 3).

**Discussion**

The present investigation examined the neuroprotective effects of pharmacologically induced hypothermia by HIP201 in stroke brains longitudinally during acute attack using MRI. Our results show that drug induced hypothermia decreases infarct growth rate and infarct and edema volume substantially during acute stroke. These findings provide
complementary information about the temporal and spatial evolution of infarct on the neuroprotective action of HPI201-induced hypothermia by using a translational neuroimaging approach.

HPI-201 based PIH therapy has shown effective in protecting the brain from ischemic, hemorrhagic stroke, traumatic brain injury (TBI) in mice models [15, 16, 30]. Previous investigations evaluated the duration of hypothermia for achieving therapeutic effect after stroke. For the PIH treatment, we previously compared the neuroprotective effect of 6 hrs and 24 hrs hypothermia and did not observe additional benefits by the prolonged treatment [29]. There was no rebound over the basal body temperature after the PIH treatment. Also, the effect of PIH on infarct formation was evaluated up to 3 days and the neuroprotective benefits of functional improvement lasted for at least 21 day after stroke in mouse models, as reported in our previous investigations [14, 36]. It will be interesting and necessary to perform MRI examinations on the sub-acute and chronic effects of hypothermia in a long-term investigation.

To date, the exactly mechanism of HPI201 induced therapeutic effects are still under evaluation. As an NRT1 agonist, HPI-201 acts on the receptors located in the hypothalamus and causes a downward shift of the temperature set point in the thermoregulatory center[37]. Therefore, NTR1 agonist by targeting the central thermoregulatory system could have synergistic effects of temperature reduction and associated neuroprotective benefits. We recently reported that the PIH therapy significantly suppressed inflammatory responses in stroke mice, such as reducing inflammatory factors and microglia activation [29]. The anti-inflammation effect and functional benefits lasted for 14–21 days after stroke. In addition, HPI-201 showed a significant effect of preventing tachycardia [38]. Although further studies are needed to reveal possible direct and indirect mechanisms related to the HPI-201 regulation of heart function, it seems that the prevention of tachycardia by HPI-201 is an additional benefit for clinical application of the combined hypothermia therapy.

As demonstrated in Fig 1 and 2, the infarct volume was reduced substantially at any given time point during the acute phase. In addition, the edema volume was decreased accordingly as well, suggesting the evident neuroprotective effects of PIH after stroke insult. The fundamental mechanism of hypothermia’s neuroprotection is the reduction of metabolic demand [39]. The cerebral metabolic rate for oxygen (CMRO$_2$) is used to express brain metabolism in terms of oxygen consumption [40]. Hypothermia reduces CMRO$_2$ by a rate of approximately 6% per 1°C change in temperature [39]. Such decrease in oxygen demand likely corresponds to a slower rate of ATP consumption, diminished acidosis, and improved glucose metabolism [41, 42]. Hypothermia may reduce cerebral blood flow (CBF) in the absence of ischemia [43]. However, therapeutic hypothermia may not decrease CBF either during or after cerebral ischemia [14, 44]. In particular, our results demonstrated that HPI201 did not significantly change the physiological parameters including blood pressure, blood glucose levels, or blood pH [14]. These observations are important for future clinical trials because it has been shown that severe hypothermia (body temperature <30°C) can result in altered blood glucose levels and increased blood pH values [45], which could exaggerate ischemic brain injury.
Ischemic infarct evolves quickly after stroke onset and approaches to its maximal volume at late acute phase or early subacute phase, then it decreases as seen in a prior patient study [46] and monkey study [47]. Zhang et al reported a logarithmic pattern of the infarct volume evolution during acute stroke in macaque brains with pMCAo [35]. In the present study, the formula was also applied to fit the infarct evolution in mice with pMCAo to examine the temporal-spatial changes of infarct with/without treatment. As seen in Fig 3B, the temporal changes of infarct volume in control and treatment groups exhibit a good fitting of logarithmic pattern, similar to those seen in macaque and rat brains with pMCAo [35, 48].

The logarithmic pattern was used to predict infarct volume successfully for macaque monkeys up to 48 hours [35] in which the maximal lesion volume was seen at ~32 hours in the same pMCAO model [47], suggesting the formula may be applicable to predict the infarct evolution up to early subacute phase. In the present study, the logarithmic pattern was used to predict the infarct volumes at 24 hr post stroke, and the calculated mean volumes were 118.0 ml for control and 16.0 ml for treatment, respectively. Obviously the predicted values are substantially smaller than the actual volumes at 24 hr (201.2 ml, 39.6ml respectively). Therefore, as demonstrated in prior and present studies, the logarithmic algorithm is not applicable for rats at 48 hours and mice at 24 hours, most likely due to the species difference of temporal stroke evolution after ischemic injury [49].

After PIH treatment, the growth rates of infarct volume dramatically decrease in PIH mice compared to control mice (3.59 vs 19.51 unit: mm$^3$ per hour (log scale)), indicating that treatment intervention altered the course of infarct evolution remarkably. The findings suggest that the treatment time window may be substantially extended after stroke insult due to the PIH intervention. In addition, the numerical fitting results indicate that the progressive changes of infarct volume during the hyperacute phase follow the logarithm pattern. Therefore the infarct evolution could be modelled and predicted in PIH treated stroke subjects, allowing for more accurate estimation of possible outcome of stroke during the temporal evolution of stroke infarct right after stroke insult. In particular, the reduced growth rate of infarct volume in PIH-treated mice suggested that the growth rate may be a complementary and sensitive indicator to assess the efficacy of pharmaceutical treatment in rodents, large animals, or even human subjects with stroke.

There are some limitations in this study. Firstly, only acute period (6 hours and 24 hours after stroke) was evaluated to access the treatment response of HPI201. Subacute and chronic morphological, behavior and MR imaging results are needed to fully assess the HPI201 hypothermia treatment effect in future studies. Secondly, we just collected three time points to illustrate the temporal evolution of infarct within the first 4 hours post stroke. Longer and multiple time points of MRI acquisitions in the mice stroke model will allow us to better demonstrate the actual evolution pattern of ischemic infarction with/without treatment and define the applicable time window of logarithmic algorithm after stroke insult. In addition, the anesthetic coma could potentiate the effect of metabolic depression of hypothermia [50]. As a limitation, the anesthesia depth was not evaluated in the present study. EEG is a valid means to monitor the depth of anesthesia [51]. It will be very interesting to evaluate such effect by monitoring the EEG pattern and burst suppression with/without hypothermia induction. Meanwhile there are still challenges in translation of
preclinical results to clinical applications in regarding of toxicity, clinical evaluations and monitoring [52].

Conclusion

Pharmacologically induced hypothermia has evident neuroprotection effects in ischemic brains and the infarct development is inhibited dramatically during acute stroke. The temporal evolution of infarct volume might follow the logarithm pattern during the PIH treatment in acute stroke. In particular, the hypothermia treatment intervention reduces the growth rate of infarct evolution remarkably, allowing for extended time windows for reperfusion therapy. Also, our present results suggest that MRI with the logarithm model could provide unique information to examine and predict the dynamic response of the PIH treatment on infarct evolution in preclinical study or clinic trials of stroke disease non-invasively.

Acknowledgments

The authors thank Ruth Connelly, Paul Chen, and Doty Kempf (DVM) for animal handling. This project was supported in part by NCRR (P51RR000165) and currently by the Office of Research Infrastructure Programs of NIH (OD P51OD011132). It is also supported by NIH grants NS085568, R44NS090629, and a VA Merit grant RX00666.

References


Figure 1.
Longitudinal stroke lesion changes (red arrow) in vehicle (A) and PIH treated mice (B) at 1, 2, 3 hr and 24 hr post occlusion. In comparison to the vehicle group, PIH mouse has significant smaller stroke volume in ADC (black arrow, hypointense lesion) and DWI (red arrows, hyperintense lesion) among all time points and reduced edema at 24 hours post stroke (T2WI, white arrow).
Figure 2.
Temporal evolution of ischemic infarct in stroke mice with/without PIH treatment during acute phase evaluated using MRI. Significantly larger infarct volume was found in vehicle mice at any time point, compared to hypothermia treated mice. *: p<0.05.
The temporal evolution of infarct volume after stroke insult was fitted with a logarithmic pattern. The growth rate and infarct volume were reduced dramatically in PIH treated stroke mice.
Figure 4.
A) Infarct volume was reduced significantly as shown with TTC staining. * P<0.05. n=5 per group.

B) Total cell death (TUNEL/Hoechst positive) and neuronal cell death (TUNEL/NeuN positive) in the PIH group were fewer than those of the control group at 24 hours post occlusion. TUNEL-positive cells were significantly reduced in the PIH group compared with those in the control group. ** P<0.01. n=5 per group.
Figure 5.
TTC staining showed stroke lesion 24 hrs after stroke, and the hypothermic treatment of HPI201 significantly reduced brain infarction formation compared to control mice.
Figure 6.
The immunohistological results of control and treated mice at 24 hours post-surgery. Nuclei of all cells were visualized with Hoechst staining (blue); Neuronal nuclear were identified by NeuN staining (red) and apoptotic cells were identified by TUNEL staining (green). TUNEL-positive cells (green) were fewer in HPI201-treated group compared with the vehicle group. TUNEL (green) and NeuN (red) double-positive cells represent neuronal cell death in the penumbra. Scale bar =10μm.
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Values are expressed as mean ± SD, n=5 mice at each time point. HR=heart rate, RR=respiratory rate.