Neuroprotective effect of paricalcitol in a rat model of transient global cerebral ischemia.

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Abstract

Background Paricalcitol has been known to attenuate ischemic-reperfusion injury of various organs. However, it is not known whether paricalcitol prevents neuronal injury after global cerebral ischemia. The purpose of this study is to investigate the neuroprotective effect of paricalcitol in a rat model of transient global cerebral ischemia.

Methods This is a prospective, randomized, experimental study. Male Sprague-Dawley rats survived from 10 min of four-vessel occlusion were randomized to the two treatment groups treated with paricalcitol 1 µg/kg IP and an equivalent volume of normal saline IP, respectively. Drugs were administered at 5 min, 1 day, 2 days, and 3 days after ischemia. Neurologic function score was assessed at 2 h, 1 day, 2 days, 3 days, and 4 days after ischemia. We tested the motor function 3 days after ischemia using the rotarod test. Also, we tested memory function 4 days after ischemia using the passive-avoidance test. We assessed neuronal degeneration in the hippocampus of surviving rats 4 days after ischemia.

Results 8 rats were allocated to each group. No significant differences were found in terms of survival rate, motor coordination, or memory function. The neurological function score 2 h post ischemia was significantly high in the paricalcitol group ($p = 0.04$). Neuronal degeneration was significantly less in the paricalcitol group compared with the control ($p = 0.01$).

Conclusions Paricalcitol significantly attenuated neuronal injury in the hippocampus. Although motor coordination, memory function, and survival rate were not significantly improved, paricalcitol remains a potential neuroprotective drug after global cerebral ischemia.

Background

Hypoxic-ischemic brain injuries (HIBI) such as ischemic stroke and post-cardiac arrest syndrome are a leading cause of mortality and disability. Many clinical trials of neuroprotective agents against HIBI have shown disappointing outcomes [1–5], and no effective drugs are currently available. Therefore, further studies are needed to identify potential agents for neuroprotection. Vitamin D exhibits various cellular effects in multiple organs. Vitamin D plays a key role in the regulation of calcium-phosphorus homeostasis. On the other hand, vitamin D is a well-known immunomodulator and has a protective effect on inflammatory processes [6–8]. Moreover, vitamin D has been reported to attenuate ischemic-reperfusion injuries of hepatic and cardiac ischemia. [9]. A recent study showed that vitamin D reduced HIBI in a rat model of transient middle cerebral artery occlusion via attenuation of oxidative stress and apoptosis [12]. Recently, a few investigators have shown that paricalcitol, a synthetic vitamin D2 analog, is protective against renal, cardiac and hepatic ischemic-reperfusion injury in experimental models [13–15].

Collectively, clinical and basic research studies suggest that vitamin D and its synthetic analog play a protective role in ischemic-reperfusion injuries of various organs. Nevertheless, whether paricalcitol prevents HIBI after global cerebral ischemia such as post-cardiac arrest syndrome is unknown. The

purpose of this study is to examine the neuroprotective effect of paricalcitol in a rat model of transient global cerebral ischemia.

Methods

Animal preparation

This is a prospective, randomized experimental study. Male Sprague-Dawley rats weighing between 250 and 280 g were provided with food and water ad libitum and held at a temperature of $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, under a 12-h light/dark cycle for 5 days prior to experiment. Transient global cerebral ischemia was induced via the four-vessel occlusion method described in our prior experiment [16, 17]. To inhibit secretion, atropine (0.01 mg/kg) was administered intraperitoneally before surgery, followed by a combination of tiletamine hydrochloride along with zolazepam hydrochloride (Zoletil; Virbac, Carros, France) (30 mg/kg) and xylazine (Rompun; Bayer, Monheim, Germany) (10 mg/kg) 10 min later, intra-abdominally to induce anesthesia. Thereafter, tracheal intubation was performed to maintain airway, and the rats were fixed in a prone position in the stereotactic frame. A thermometer probe was inserted into the rectum of the fixed rats, and an automatic temperature control blanket (Homeothermic blanket system, NP50-7053-r; Harvard Apparatus, Holliston, MA) was used to maintain normothermia ($37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) during surgery.

We shaved and sterilized the areas around the cervical regions, while the rat was fixed in the prone position and made a 3 cm median incision along the centerline from the border of the lower part of occipital bone to the back and cervical region. The muscles of the first cervical vertebra were dissected to expose the bilateral alar foramen. A thin needle-shaped electrocautery (SurgiStat™ II; Covidien, Boulder, CO) was inserted approximately 1 to 1.5 mm through the alar foramen, and the bilateral vertebral arteries were permanently occluded by cauterization. Next, the position was changed to supine, and the ventral cervical region was shaved and sterilized, a 3 cm median incision was made, and the bilateral common carotid arteries were dissected. Polyethylene tubes (PE-10; BD, Franklin Lakes, NJ) were loosely wrapped around the dissected common carotid arteries to allow both ends of the tubes to emerge approximately 3 cm from the skin, which was then sutured.

After the surgery, the rats were kept isolated in individual cages, and left to recover for 24 h under the same environment as before the surgery. On the following day, the rats were restrained without anesthesia, and the polyethylene tubes were pulled to expose the common artery outside the skin. The

common carotid arteries were occluded with microvascular clamps (RS-5422; Roboz, Chicago, IL) for 10 min, and global cerebral ischemia was confirmed by the loss of the righting reflex. A thermometer probe was inserted into the rectum to monitor the core temperature and maintain normothermia using a temperature control blanket throughout the ischemic period. The clamps and polyethylene tubes of the rats that survived global cerebral ischemia were removed 10 min later, and normothermia was maintained until the recovery of the righting reflex. We excluded rats maintaining righting reflex during the ischemic period, which meant incomplete global cerebral ischemia. Also, we rejected rats without any available outcome variables because of death during the ischemic period or early death within 2 h post-ischemia (Fig.1).

Study Protocol

Surviving rats were randomly assigned to one of the two treatment groups: paricalcitol group (n = 8), injected intraperitoneally with paricalcitol (Zemplar; Abbott Laboratories, Abbott Park, IL) (1 µg/kg); and normal saline group (n = 8), injected intraperitoneally with an equivalent volume of normal saline. We administered drugs 5 min after the end of the ischemic period. After recovery of righting reflex, rats were returned to the cages and observed until four days after cerebral ischemia. During the observational period, we intraperitoneally injected paricalcitol (1 µg/kg) or an equivalent volume of normal saline on days 1, 2 and 3 post-ischemia.

Neurological outcome

A researcher who was blinded to the treatment measured and recorded the neurological function score (NFS) at 2 h post-ischemia, and then on days 1, 2, 3, and 4 post-ischemia as previously described [18]. The test consists of five categories representing the level of consciousness, respiration, cranial nerves, motor and sensory function, and coordination. The score ranges from 0 (worst) to 500 (normal) (Table1).

Table1. Neurological function scoring in rats .

Parameter	Characteristic	Score Range
General		
Consciousness	Unresponsive, depressed, normal	0, 50, 100
Respiration	Abnormal, normal (60-120)	0, 100
Cranial Nerves		
Olfactory	Orient to smell	No = 0, Yes = 20
Vision	Visual stimulus startle response	No = 0, Yes = 20
Corneal reflex	Blink response to corneal stimulus	No = 0, Yes = 20
Whisker movement	Spontaneous	No = 0, Yes = 20
Hearing	Startle response to loud noise	No = 0, Yes = 20
Motor		
Left forepaw	Spontaneous or withdrawal from pain	No = 0, Yes = 10
Right forepaw	Spontaneous or withdrawal from pain	No = 0, Yes = 10
Left hindpaw	Spontaneous or withdrawal from pain	No = 0, Yes = 10
Right hindpaw	Spontaneous or withdrawal from pain	No = 0, Yes = 10
Tail	Spontaneous or withdrawal from pain	No = 0, Yes = 10
Sensory		
Left forepaw	Reaction to pain	No = 0, Yes = 10
Right forepaw	Reaction to pain	No = 0, Yes = 10
Left hindpaw	Reaction to pain	No = 0, Yes = 10
Right hindpaw	Reaction to pain	No = 0, Yes = 10
Tail	Reaction to pain	No = 0, Yes = 10
Coordination		
Ledge traverse		No = 0, Yes = 25
Righting reflex		No = 0, Yes = 25
Placing test		No = 0, Yes = 25
Stop to table edge		No = 0, Yes = 25
Total score		500

Rotarod test

We assessed motor coordination using the accelerating rotarod test (Model 7750; Ugo Basile, Comerio, Varese, Italy). Four training sessions were performed 5, 4, 3 days and 1 h prior to the ischemic insult. We placed the rats on the stationary rod. After a while, the rod started to rotate at 2 rpm and accelerated to 40

rpm within 300 s. We recorded the latency to fall from the rotating rod. Rats not falling off within 300 s were scored a maximum of 300. We obtained the baseline score by averaging the two best scores out of the four training sessions. We performed the rotarod test three days after the ischemic insult and determined the post-ischemic latency to fall. We calculated the value relative to baseline for use in data analysis.

Passive-avoidance test

We assessed memory function using passive-avoidance test. The passive-avoidance apparatus (Model 7552; Ugo Basile, Comerio, Varese, Italy) consisted of two sections, the start and escape compartments. The start compartment was illuminated and surrounded by white walls, while the escape compartment was dark, with black walls. The two compartments were connected by an automatic sliding door. Three days after the ischemic insult, the rats were exposed to the passive-avoidance apparatus 60 min before the acquisition trial. During the pre-exposure, the rats were allowed to explore the start compartment for 1 min, without access to the escape compartment. Thereafter, the sliding door was opened and as the rat entered the escape compartment, the door was automatically closed. The rats were then allowed to explore the escape compartment for an additional 1 min. One hour after pre-exposure, the rat was again placed in the start compartment for the acquisition trial, and 10 s later, the door was opened. The latency to step through the door was recorded as baseline retention latency. After entering the escape compartment, the door was closed and an electrical current (0.8 mA, 2 s) was delivered through the grid floor. The next day, we performed the retention trial. The rat was placed in the start compartment, and 10 s later, the door was opened. The retention latency to enter the escape compartment was recorded. No shock was delivered during the retention trial. If the rat failed to enter the escape compartment within 300 s, it was removed from the apparatus and a maximum latency of 300 s was recorded. The data was expressed relative to baseline retention latency and used for data analysis.

Histopathological analysis

After the acquisition trial, tiletamine hydrochloride + zolazepam hydrochloride (1:1 solution, 30 mg/kg) was injected into the abdominal cavity for anesthesia, and the rats were euthanized with 4% paraformaldehyde via transcardiac perfusion fixation. Brains were then post-fixed in 4% paraformaldehyde for more than a day, followed by washing under running water for another day. Finally, they were fixed in paraffin and two 4- μ m-thick coronal sections of hippocampal cornu ammonis (CA) 1 region from each rat were obtained for hematoxylin-eosin staining. As each specimen section contained right and left CA1 regions, four histological images of a 1.13-mm-long stratum pyramidale were acquired using an optical microscope (IX71; Olympus, Tokyo, Japan). A blinded researcher calculated the percentage of degenerated pyramidal cells in each image using image analysis software (Image-Pro Premier; Media Cybernetics, Rockville, MD). The median value of 4 images was calculated for each rat.

Statistics

According to our pilot study, we hypothesized that the percentage of injured neurons in the paricalcitol group would be $9 \pm 4.5\%$, while the percentage of injured neurons in the normal saline group would be $30 \pm 15\%$. Assuming a two-sided α of 0.05 at a power of 0.8, the number of rats per group was determined as 8 to reject the null hypothesis. Continuous data were expressed as medians with interquartile ranges (IQR). We conducted Mann-Whitney tests to compare the data between the groups. Log-rank test was used to compare the survival distribution between groups. Survival was presented by Kaplan-Meier curves. Values of p less than 0.05 were considered significant.

Results

Neurological function and survival

Out of a total of 29 rats subjected to four-vessel occlusion, 23 rats survived. Among those, four rats that maintained righting reflex during the ischemic period and three rats that died within 2 h after ischemia were excluded.

Paricalcitol treatment significantly improved the 2-h NFS compared with the control group: 295 (IQR 205 to 352.5) versus 105 (IQR 100 to 220) ($p = 0.04$). However, NFSs on days 1, 2, 3 and 4 post-ischemia did not show significant differences between the groups (Fig. 2).

The 96-h survival rate was 100% in the paricalcitol group and 62.5% in the control group. However, the survival rate was not significantly different between the groups according to the Log-rank test ($p = 0.06$) (Fig. 3).

Motor coordination

Three days after ischemia, the median latency to fall from a rotating rod relative to baseline in the control group was 0.97 (IQR 0.65 to 1.09) compared with 0.87 (IQR 0.69 to 1) in the paricalcitol group. Paricalcitol treatment did not significantly improve motor coordination compared with the control group ($p = 0.56$) (Fig. 4A).

Memory function

Four days after ischemia, median retention latency relative to baseline in the control group was 2.18 (IQR 0.51 to 4.76) compared with 4.75 (IQR 1.17 to 33.5) in the paricalcitol group. Although the retention latency was not statistically different between the groups ($p = 0.38$), five paricalcitol-treated rats never entered the escape compartment. By contrast, all the rats in the control group entered the escape compartment (Fig. 4B).

Neuronal degeneration

Four days after ischemia, the median percentage of injured neurons in the control group was 21.88% (IQR 7.74 to 51.26) compared with 2.04% (IQR 1.48 to 3.79) in the paricalcitol group. Paricalcitol treatment significantly attenuated neuronal degeneration in the CA1 region of hippocampus compared with the control group ($p = 0.01$) (Fig. 5).

Discussion

This study revealed for the first time that paricalcitol had neuroprotective properties. Our results demonstrate that paricalcitol accelerated the recovery time and attenuated neuronal degeneration. A non-significant trend toward improved survival and memory function was observed. We did not detect a significant improvement in motor coordination.

Experimental rat model of global cerebral ischemia does not result in long lasting focal neurological deficits [19]. Therefore, rats surviving longer than one day after four-vessel occlusion recovered well, showing equivalent NFSs between the groups. Global HIBI is caused by various clinical conditions such as asphyxia, profound shock, and cardiac arrest. Among those, cardiac arrest results the most devastating injury. In a rat model of cardiac arrest, neurologic deficits last longer than the four-vessel occlusion model [18]. Further studies are needed to identify the efficacy of paricalcitol in improving long-term neurological outcomes after severe HIBI such as cardiac arrest. Nevertheless, a significant improvement in NFSs 2-h post-ischemia in the paricalcitol group indicated that paricalcitol contributed to the short-term recovery of neurologic function.

Although there was no significant improvement in survival of paricalcitol-treated rats, all rats in the paricalcitol group survived for 4 days while three rats in the control group died. Similarly, in the passive-avoidance test, all rats in the control group entered the dark chamber while only 37.5% of the rats in the paricalcitol group entered the dark chamber, which suggested a trend toward preserved memory function in the paricalcitol group. Based on the calculated sample size using the hypothesis discriminating neuronal injury differences, the sample size in the present study may not have been adequate to evaluate the effect of paricalcitol on survival, cognitive and motor function.

Previous studies have demonstrated neuroprotection by vitamin D in a rat model of transient focal cerebral ischemia. Furthermore, several studies have shown that vitamin D supplementation improved recovery from traumatic brain injury [20–24]. The proposed mechanism of vitamin D neuroprotection is mediated via anti-inflammatory and anti-apoptotic effects [6–8, 25]. Vitamin D also acts as an antioxidant and promotes axonal regeneration [26–28]. As paricalcitol is a vitamin D receptor agonist that diffuses through the blood-brain barrier and shows relatively fewer side effects such as hypercalcemia and hyperphosphatemia, it may be a more appropriate neuroprotective drug than vitamin D for clinical application [28, 29]. Until now, paricalcitol is mainly used for the treatment of hyperparathyroidism associated with chronic kidney disease. Despite its limited use in the management of neurological disorders, a study demonstrated the antiepileptic properties of paricalcitol mediated via antioxidant effects [30].

There are some limitations in this study. We did not validate optimal neuroprotective dose of paricalcitol. High dose of paricalcitol may result in complications such as hypercalcemia and hyperphosphatemia. However, we did not measure the calcium and phosphate levels. Paricalcitol dose showing high neuroprotective efficacy with minimal adverse effects remains an issue. In addition, this study did not investigate intracellular signaling pathways. Thus, the neuroprotective mechanism of paricalcitol was not evaluated. It can be assumed that the neuroprotective effects of vitamin D may be identical to those of paricalcitol. However, paricalcitol is a selective vitamin D receptor activator, with distinct and varying levels of non-selective vitamin D receptor activation [29]. Additional studies are required to elucidate the mechanisms underlying the neuroprotective effects of paricalcitol.

Conclusions

Paricalcitol administration attenuated neuronal injury after transient global cerebral ischemia. It remains to be determined if paricalcitol improves survival and neurological outcomes in a clinically relevant model such as cardiac arrest. Overall, paricalcitol should be considered as a potential neuroprotective drug prompting further study.

Abbreviations

HIBI

Hypoxic-ischemic brain injury

IACUC

Institutional animal care and use committee

NFS

Neurological function score

IP

Intraperitoneal.

CA

Cornu ammonis

IQR

Interquartile range

Declarations

Ethics approval and consent to participate

Our institutional animal care and use committee (IACUC) approved this study.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SWK participated in the experiment, study design, data collection, data analysis and writing. JSO participated in the experiment, study design, data analysis, data interpretation and writing, HHJ, JP participated in the experiment data collection, data analysis, YMO, SC participated in study design, critical revision, KHC participated in study design, literature search, critical revision. All authors read and approved the final manuscript.

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Figures

Figure 1

Timeline of experiment. NFS, Neurologic Function Score; IP, Intraperitoneal.

Figure 2

Impact of paricalcitol on neurologic function. Paricalcitol-treated rats showed significantly improved neurologic function at 2 h after cerebral ischemia. Neurologic function scores later than 1 day after ischemia did not demonstrate statistically significant differences between groups (* $p = 0.04$). The box-and-whisker plot represents medians and interquartile ranges.

Figure 3

Impact of paricalcitol on survival. Paricalcitol administration did not significantly improve 96-h survival ($p = 0.06$). However, all rats in the paricalcitol group survived 96 h after cerebral ischemia.

Figure 4

Impact of paricalcitol on motor function (A) and memory function (B). Paricalcitol administration did not improve motor coordination after cerebral ischemia ($p = 0.56$) (A). Although five rats in the paricalcitol group never entered the escape chamber, the median retention latency relative to baseline was not significantly different between the groups ($p = 0.38$) (B). Hematoxylin-eosin stain, original magnification $\times 200$. The box-and-whisker plot represents medians and interquartile ranges.

Figure 5

Neuronal degeneration of hippocampal CA1 regions four days after cerebral ischemia. Paricalcitol administration significantly attenuated neuronal injury ($p = 0.01$).