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The Neuroprotection Effect of GLP-1 Receptor in Murine Stroke Model

Rui Chen[#], Zetao Wu[#], Yi He, Xiaojia Liu, Hao Lyu*, Meng Zhang* and Jianming Wu

Department of Neurosurgery, Shenzhen Second People's Hospital, PR China

[#]Both authors contributed equally to this manuscript

***Corresponding author:** Hao Lyu, Department of Neurosurgery, Shenzhen Second People's Hospital, Shenzhen, Guangdong, 518035, P.R. China

Meng Zhang, Department of Neurosurgery, Shenzhen Second People's Hospital, Shenzhen, Guangdong, 518035, P.R. China

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ABSTRACT

Purposes: Stroke ranks as the world second most frequent cause of death. Eight-five percent of strokes are ischaemic, meaning a reduction of blood supply and permanent neurological disabilities with both mental and physical manifestations. Glucagon-like peptide 1(GLP-1) is a neuropeptide that has shown neuro-protective properties. In this study, we assessed the neuroprotective effect of GLP-1 receptor agonist in a murine model of global cerebral ischaemia stroke.

Methods: Adult male C57BL/6 mice (25 to 30g) were subjected to transient global ischaemia by means of occlusion of the bilateral internal carotid arteries for 20 minutes. The mice were separated into three groups of 10: normal control group (n=10); transient global ischaemia group (n=10); and transient global ischaemia with GLP-1 receptor agonist treated group (n=10). Behavioural tests were performed before the mice were sacrificed on day 5. Real-time polymerase chain reaction was used to identify the molecular markers of neuronal damage.

Results: Up-regulation of neuron nuclear, synapse and neuro-filament markers was found in the group treated with the GLP-1 receptor agonist.

Conclusions: GLP-1 receptor agonist has neuro-protective effect, as shown by real-time polymerase chain reaction and western-blot detection, and could improve loco-motor function on mice global ischaemic model.

Keywords: Glucagon-Like Peptide 1; Ex-4; Neuro-Protection

Abbreviations: GLP-1: Glucagon-Like Peptide 1; Ex-4: Exendin-4; GCI: Global Cerebral Ischemia; EEG: Electroencephalogram; PCR: Polymerase Chain Reaction; BCA: Bicinchoninic Acid; RIPA: Radioimmunoprecipitation Assay; RT-PCR: Reverse Transcription Polymerase Chain Reaction; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; Catwalk: CatWalk System; BCA: Bicinchoninic Acid

Introduction

Brain ischaemic, which is also called cerebral ischaemia or ischaemic stroke, occurs when the blood flow to the brain is insufficient [1-3], causing deprivation of both oxygen and glucose to the brain and upsetting the balance of metabolism in the nervous system [4,5]. These may include abrupt numbness or weakness in one side of the body, particularly in the arms or legs, as well as facial drooping and difficulty speaking or understanding speech. Other signs of a stroke can include confusion, trouble with coordination and balance, and loss of vision [6]. Based on the infarct region, ischaemia can be either focal or global, if an area of the brain is affected, it results in focal ischaemia, and if the whole brain's blood supply is blocked, it results in a global ischaemia. An ischaemic stroke may be transient or permanent depending on reperfusion. Global cerebral ischaemia (GCI) commonly occurrs after cardiac arrest, which causes a decrease in the whole brain blood circulation. If the blood supply is restored within a short time, the ischaemia may be transient, but, if a long period passes before reperfusion, the damage to the brain tissue can be permanent

[7,8]. Although restoration of the blood supply is significant to the brain tissue, it sometimes induces reperfusion complications that can also cause damage to the brain [9,10].

During the acute period of ischaemia, axons are lost quickly [11]. In the early stage,axon loss is triggered by pathophysiological issues and both intra-cortical and connected downward axons are involved from the cortex with deprivation of the oxygen, nutrients, and metabolic supply necessary to maintain a normal environment [12,13]. Exendin-4 (Ex-4), also called exenatide, is a glucagon-like peptide-1 (GLP-1) agonist drug that can decrease blood glucose levels. Its half of the amino-acid sequence is the same as that of the GLP-1 receptor, which allows Ex-4 to perform in a similar manner to GLP-1, and gives it a longer half-life, approximately 2 hours. After administration of Ex-4, the insulin level in plasma remains higher and is maintained for a longer time than GLP-1 [14,15]. Ex-4 was approved by the FDA in 2005 for the clinical treatment of type II diabetes mellitus and is regarded as a forward therapeutic approach for controlling blood glucose levels [16,17]. In addition to the treatment of type II diabetes, Ex-4 has shown neuroprotective functions after brain insult and can also enhance neurogenesis in patients with Parkinson's disease [18,19]. In this study, we aim to mimic brain tissue infarct condition after cardiac arrest by using mice global cerebral ischemia (GCI) model, and investigate the neuroprotection effect of GLP-1 receptor [20].

Methods and Materials

Animal preparation and Global Cerebral Ischemia (GCI) Model

The protocols we use for animal were approved by the Animal Ethics Committee of Shenzhen Second People's Hospital. Forty eight-week-old C57BL/6 mice with body weights between 20 and 25g were used. All the animals were received standard welfare of 12h light/12 h dark housing with free access to food and water. The mice were anaesthetised with an intraperitoneal injection of ketamine100mg/kg and xylazine 10mg/kg body weight. A sterile cervical incision was made at the midline of the ventral surface of the neck. Both common carotid arteries were exposed. The tissue was carefully separated with fine forceps while ensuring that the vagus nerve was not damaged. A 5-0 silk suture was looped around the common carotid artery, a micro-clip was used to clip it by lifting the suture, and the blood flow was blocked for 20min. The animals were placed in a warming box (33~35°C). After 20 min, the clipswere removed, and the wound was closed with silk suture. Buprenorphine was administered by subcutaneous injection for 3 d at 0.02mg/kg body weight. The mice were returned to the warming box to maintain their body temperature to prevent hypothermia as a result of global ischaemia [21].

Electroencephalography (EEG)

Because cortical potential activity can be depressed during brain ischaemia infarct, electroencephalography can be used as a measurement method to evaluate ischaemic stroke [22]. Two recording probes were placed subdermally each side of the cranium,a reference electrode was placed subdermal on the nasal bone, and a ground electrode was connected to the tail.

Ex-4 Injection

After 1h of reperfusion, the anaesthetized mice were placed in a prone position. The tails were immersed in 40°C water for 3 to 5 min to enlarge the tail vein. A 1-ml syringe and 31-G needle were used to inject 330μg/kg body weight Ex-4 through the tail vein, followed by a 3-μg/kg low-dose supplement for 5 d.

Immunohistochemistry

The mice were given an overdose of phenobarbital and placed in a supine position. Saline solution (0.9%) was pumped into the body to replace the blood,Paraformaldehyde (4%) was pumped after the saline solution. After perfusion, the brain was dissected out and immersed in 4% paraformaldehyde and dehydrated by 15%, 20% and 30% sucrose subsequently. Six micrometer paraffin processed tissue sections were collected through the forebrain and stained with Neuron Nuclear (NeuN; 1:200; Abcam)antibody. Neuron cell lose was then observed by using Spot Imaging System.

Western-Blot Analysis

Western blot analysis was performed at day 5 after GCI. Brain cortex tissue and sufficient cold RIPA lysis buffer containing protease inhibitor cocktail were together mixed well. After the extraction, protein concentration was measured with a BCA kit. 10μg-20μg proteins were loaded into 8 to 10% SDS-polyacrylamide gel. Proteins were then transferred to nitroate cellulose membrane, following 5% non-fat milk blocking, incubated at 4°C overnight with first antibody. Washed membrane for three times with TBST for 10 min, and then incubated with secondary antibodies for another 2 h at room temperature, detected signals by using enhanced chemiluminescent (Thermo Fisher Scientific).

Real-Time PCR

After brain cortex RNA extraction by TRIzol (Ambion), 1μg of total RNAs were used in reverse transcription by using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). 100ng of total RNAs were then transcribed by using M-MuLV reverse Transcriptase (New England Biolabs) and a RT primer adding an anchored poly (T) tag to genes sequence (Alvarez and Nourbakhsh, 2014) for target genes detection. The following PCR procedure conducted with a universal primer and target genes specific primer. The products of PCR were detected with SYBR green dye, and SYBR assays were applied on an Applied Biosystems 7500 Real-Time PCR system. Related expression was calculated by delta threshold cycles (Ct), using normalization to GAPDH.

Behaviour- Catwalk Test

The Catwalk system consists of a glass walking plate, a green light

source, and two rectangular brace plates placed vertically along the glass plate; above the plate is a cover with red light source. A recording camera is placed below the glass plate and connected to a computer [23,24]. The Catwalk software is then used to analyse gait functions. Before the global ischaemia surgery, mice were trained for 3 days to get familiar with the system. On day 5 after ischaemia, catwalk gait analysis data were collected, and the mice were then sacrificed [25].

Statistical Analysis

GraphPad Prism 8.3.0 software was used for statistical analysis and graphics production. A one-way ANOVA with Tukey's multiple comparison test was performed to assess the results of the behavioral tests and immunohistochemistry analysis. A p value of < 0.05 was considered significant to reject the null hypothesis. Normal distribution was checked by Kolmogorov-Smirnov test, a p value of $> 0.1$was considered pass the normality test.

Results

Mice Global Ischaemia Model Established

During the occlusion, mice EEG detection was set to investigate whether the blood flow to both left and right forebrain was decreased to a certain level. Figures 1 & 2 show the mice EEG wave change under normal, ischaemia and reperfusion situation, the upper line represents left brain EEG, while the lower line stands for right brain. Picture A in Figure 1 shows the initial mice EEG typical waveform. B, C, D, E show EEG changeat 1, 5, 10, 15 minutes after occluded both bilateral common carotid arteries, EEG change from wave shape to plate style. Picture A in Figure 2 illustrates the time removing the clips at 20 minutes after occlusion. B, C, D, E, F display EEG form gradually become normal from 1 to 30 min after removal of clips.

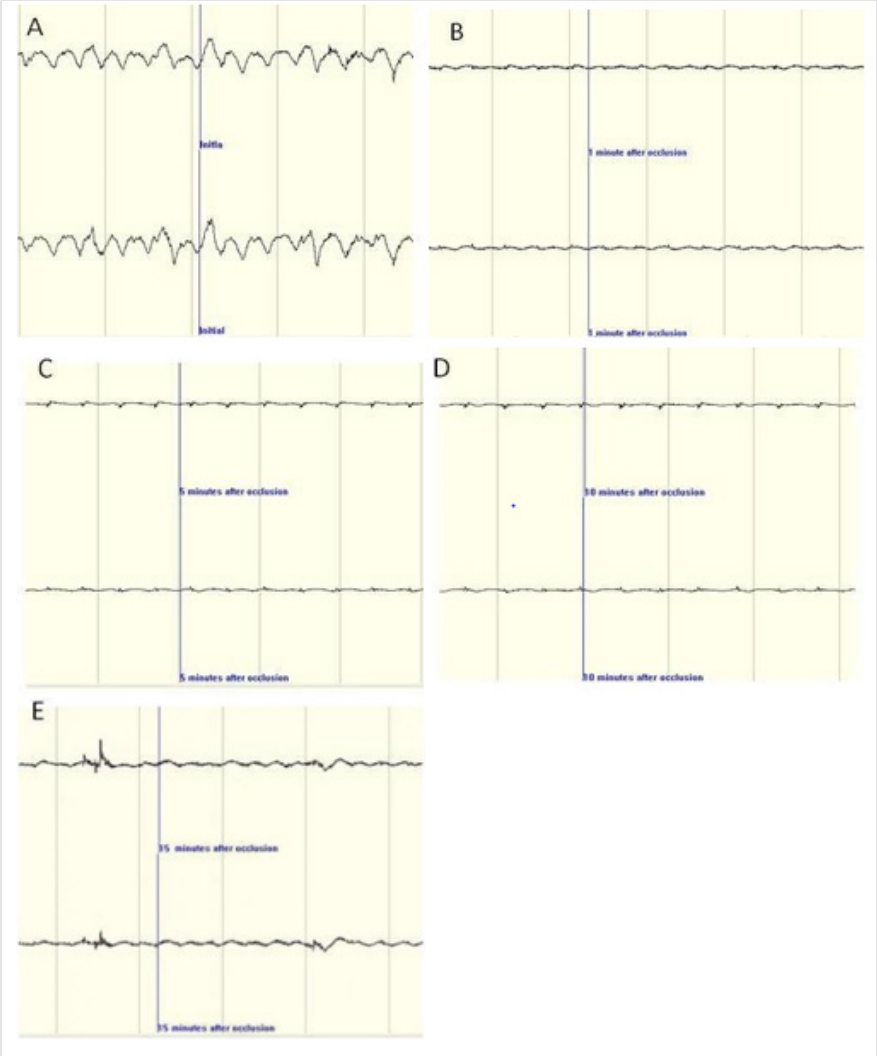

Figure 1: A demonstrates initial typical wave style on an electroencephalogram. B, C, D and E show the electroencephalogram after global ischaemia. The electroencephalogramstyles changed from wavesto plates.

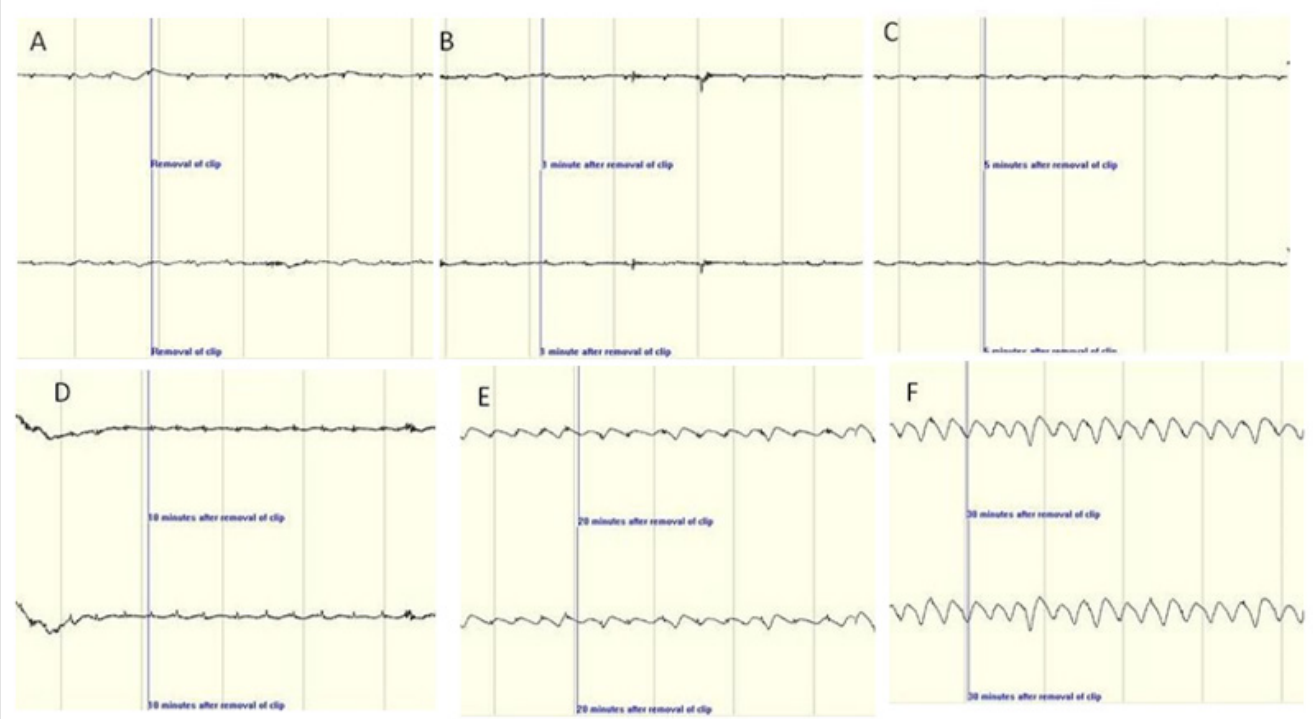

Figure 2: A demonstratesremoval of the artery clips. The electroencephalogramwave gradually normalised from 1 to 30 min after removal of clips, as shown in B, C, D, E and F.

Immuno-Staining, Real-time PCR and Western-Blot analysis

Picture A, B, C, D of Figure 3 show real-time PCR results of anterior cortex tissue, synapse (syn2), axon (Nefh) and neuron nuclear markers (NeuN) were down-regulated in ischaemia group, while in the Ex-4 treated group, remain the same level with normal control group. In the Western-Blot (Figure 3E), the expression of neuron markers we selectedwas also reduced in the ischaemic mice, and enhanced in Ex-4 treated group. F, G and H illustrate the immunostaining results of Neuron Nuclear (NeuN) marker on the section of anterior motor cortex. In the ischemia mice, a neuron cell body loss was observed, while after Ex-4 treated, such phenomenon was not found and remain the similar cell density compare with the normal mice.

GLP-1 Receptor Agonist Improves Loco-Motor Function on Ischaemic Mice Model

Ex-4 enhances ischaemic mice loco-motor function which was determined by Catwalk test. For behaviour test, we select single stance, stand, swing and swing speed as parameters. Figure 4 shows the Catwalk results and picture A, B, C represent parameters of single stance, stand and swing, both these three parameters of the ischaemia group are significantly shorter than the control group ($p<0.05$, t-test), while in the Ex-4 group stay the same level with the normal group. In picture D, the swing speed of ischaemic mice was faster than normal mice as well as ischaemic mice treated with Ex-4 ($p<0.05$, t-test).

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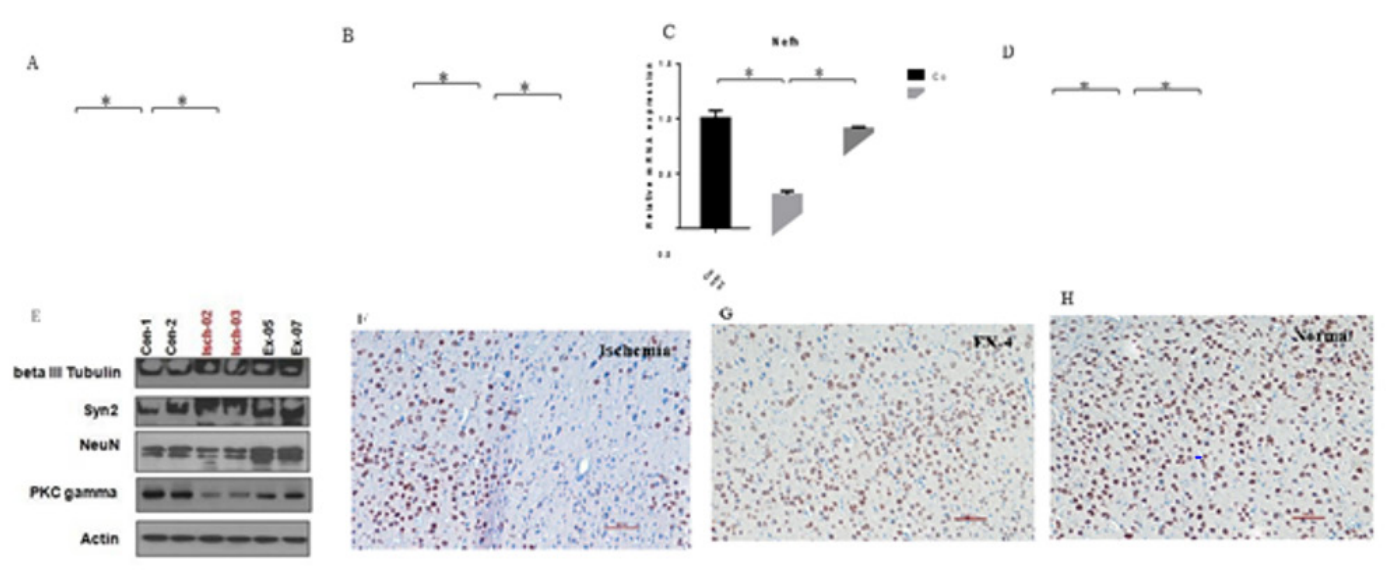

Figure 3: A, B, C, D and E show real-time PCR and Western-blot results of anterior cortex tissue, synapse, axon and neuron nuclear markers reduced in ischaemia group, while in the Ex-4 treated group, remain the same level as in the control group(*p<0.05, one-way ANOVA with Tukey post hoc multiple comparison, n=3 independent experiments. F, G and H illustrate the immunostaining results of Neuron Nuclear (NeuN) marker on the section of anterior motor cortex. In the ischemia mice, a neuron cell body loss was observed, while after Ex-4 treated, such phenomenon was not found and remain the similar cell density compare with the normal mice.

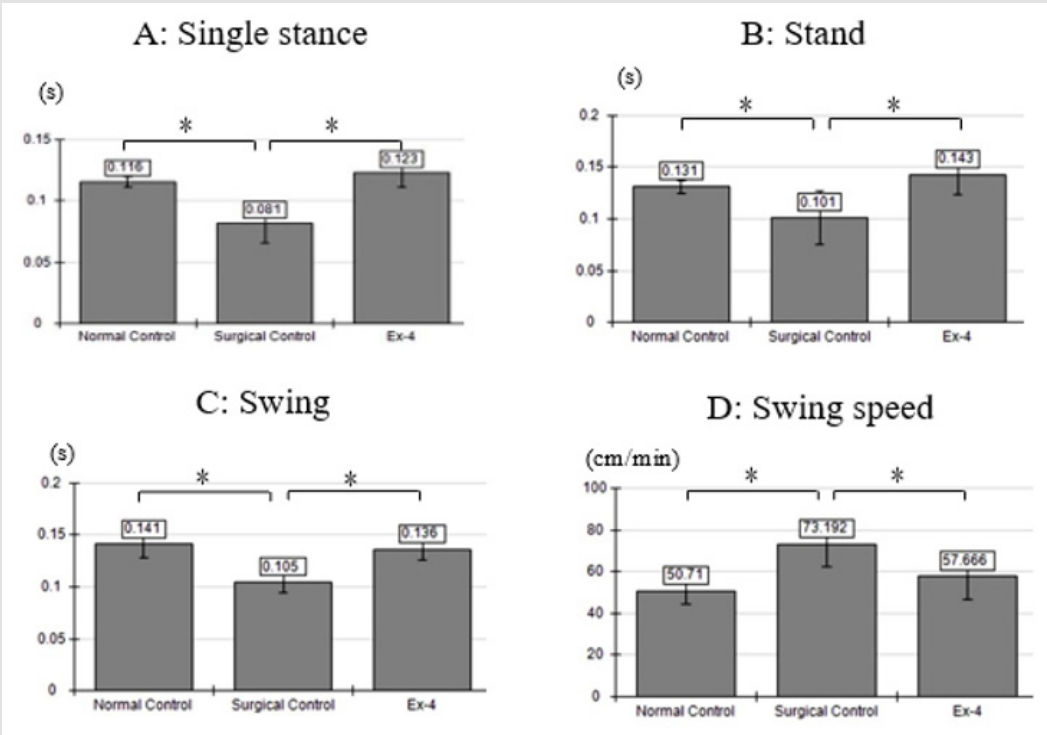

Figure 4: Single stance, stand and swing of the ischaemia group were both lower than those in the control group, while those in the Ex-4 group remained at the same level as in the normal group, which illustrates that motor function was affectedafter global ischaemia; as a result, the mice were not willing to put their paws on the plate. This effect was reduced by the administration of Ex-4. (* P<0.05, t-test).

Discussions

In this research, we investigated the effect of Ex-4, a GLP-1 receptor agonist, on axon outgrowth in animal model. For the cell culture part, we chose primary mice embryo cortex neuron culture as cell model. We selected C57 mice as research subjects which bilateral common carotid artery occlusion was established on it. In our study, we observed the phenomenon of EEG reduction in the global ischaemic model. Due to the usage of the EEG detection, we could know whether the model was established or not in a directly way and it is the first time EEG is administrated in global ischemia model. In previous research [26] observed there was a reduction in cortical spontaneous EEG after middle cerebral artery occlusion. Other research teams [27,28] also detected a reduction in EEG on unilateral common carotid artery occlusion. The neuroprotective effect of GLP-1 receptor was further proved by immunostaining, Real-time PCR and Western-Blot analysis. The immunostaining results showed a neuron cell body loss in the ischemia mice, after given Ex-4, remain the same cell intensity with normal mice. In the Real-time PCR detection, synapse (Syn2), neuron-specific nuclear protein (NeuN), neurofilament heavy chain (Nefh) and Glp1 receptor (Glp1r) markers were found down-regulated in global ischaemia mice, however, in Ex-4 treated mice, these markers' mRNA expression level were almost stay at the same level except Glp1R, which was up-regulated, cause the injection of the Glp1 Ragonist [29].

Moreover, in the Western-Blot analysis, another two markers were further detected, beta III Tubulin (Tubb3) and central nervous system specifically expressed PKC gamma, which was class III beta tubulin and Protein kinase C gamma protein markers respectively, which still showed an equal result in PCR detection. As a recommendation of future study [30], some cell proliferation and neurogenesis markers like Ki-67 [31], Mash-1 [32] and GFAP, an astrocytes and microglia cell markers [33] can be further detected to help making sure more effects of Ex-4 on rodent stroke model. Therefore, based on the results of animal model study, we draw the conclusion that, Ex-4 a Glucagon-like peptide 1 receptor agonist prevent neuron from death in the ischaemicmice model.

Contribution

Hao Lyu and Meng Zhang conceived and designed the study. Rui Chen and Zetao Wu contributed to the animal experiments, behavioral tests, histology, and statistical analysis. Xiaojia Liu, Yi He Raxida Umar contributed to the animal observation and care. Hao Lyu, Meng Zhang and Jianming Wu supervised the study. Rui Chen drafted the manuscript. Hao Lyu and Meng Zhang revised the manuscript.

Declarations of Interest

NO.

Ethics Declarations

The procedures involving animals and their care were conducted under the approval of the Animal Ethics Committee of the Second People's Hospital of Shenzhen.

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