

Modulation of P₂Y₁₂ Receptor Prevents Cognitive Decline and Oxidative Stress Induced by Chronic Cerebral Ischemia

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Article History

Received: 14.03.2022

Accepted: 26.04.2022

Published: 30.04.2022

Abstract: Stroke is a chronic disabling condition characterized by loss of motor skills and cognitive abilities. The unpredictability of stroke is one of the biggest challenges for its prevention. The time period after ischemic episode is so narrow that majority of the strokes end up in disabilities for life. Stroke - irrespective of hemorrhagic or ischemic origin leads to activation of platelet aggregation cascade and triggers the neuro and vascular inflammatory cascades. In stroke patients the levels of oxidative stress are measured very high and this increased ROS burden may be the reason behind the neuronal death. So, we have observed the role of P2Y12 receptors in reducing platelet aggregation and neuro-inflammatory pathways. The blockage of the P2Y12 receptors also leads to the reduced platelet aggregation and may lead to less instance of clot development and reduce the post stroke morbidities. Hence, we are hypothesizing that modulation of P2Y12 receptors may prove beneficial for the stroke subjects.

Keywords: Clopidogrel, P2Y12 receptor, Stroke, ROS, Platelet aggregation.

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1. INTRODUCTION

Cerebrovascular disease are associated with several pre-existing debilitating conditions like diabetes, hypertension, hypercholesterolemia, estrogen deficiency to name a few [1] Stroke is considered to be one of the important cause for the disability which results in hemiplegia, loss of consciousness and death [2].

Stroke is broadly distinguished into following two types, i.e. ischemia and hemorrhagic stroke. Ischemic stroke is caused by reduction in blood flow to the brain, generally due to artery blockage by an embolus, depriving the brain from oxygen and metabolic substrates. Immediate reperfusion may prevent the damage to the neurons but in cases with prolonged ischemic episodes, the reperfusion results in even more severe ischemia reperfusion injury [3] and infarctions in the various regions in the brain including brain edema,

hemorrhage and cell necrosis [4] Oxidative stress is one of the primary factors that play a major role in the development of tissue necrosis developed due to the occlusion of the artery followed by reperfusion resulting in the imbalance in production and utilization of reactive oxygen species (ROS) [5].

1.1 Risk factors

In our body, the arterial blood pressure, temp., arterial blood gases of body, blood glucose level, as well as free radical overproduction, Ca²⁺ overload, and excitotoxicity are the major ones [6]. Tobacco use, Total cholesterol levels that are too high, Brain hemorrhage and cerebral palsy are both increased by drugs like heroin, cocaine, and amphetamines. These medications can promote platelet adhesion, raise blood pressure (including blood pressure swings), produce artery spasms in the brain, and contribute to cardiovascular inflammation and thrombosis [7].

Citation: Rajat Bhardwaj, KM. Rajni Saini, Roopam Tomar (2022). Modulation of P2Y12 Receptor Prevents Cognitive Decline and Oxidative Stress Induced By Chronic Cerebral Ischemia, Glob Acad J Pharm Drug Res; Vol-4, Iss-2 pp- 41-50.

1.2 P2y12 receptor

It consists of seven transmembrane domains coupled with G proteins. They are triggered by endogenous nucleotides and extracellular agents released from by injured cells or secreted by non-lytic mechanisms during inflammation, ischemic, and hypoxia conditions [8].

The human P2Y12R has 342 amino acid residues at its extracellular amino terminus, as well as two potential N-linked glycosylation sites, which may influence its function [9].

Although ADP cannot cause platelet dense granules to be released on its own, it can enhance platelet secretion induced by powerful agonists. This action was not blocked by acetylsalicylic acid and

was independent of extracellular Ca^{2+} concentrations and the generation of large platelet aggregates. The stability of platelet aggregates generated by thrombin or TXA2 is dependent on P2Y12 [10].

P2Y12R is a key cofactor in platelet aggregation and secretion, which can be triggered by a variety of agonists such as collagen, TXA2, thrombin, and particular antibodies that crosslink Fc α R1a receptors, as well as serum from individuals as well as shear-induced platelet aggregation. Antagonism of P2Y1 receptor and P2Y12 receptor suppresses thrombogenesis more effectively than inhibition of either receptor alone under similar experimental settings [11].

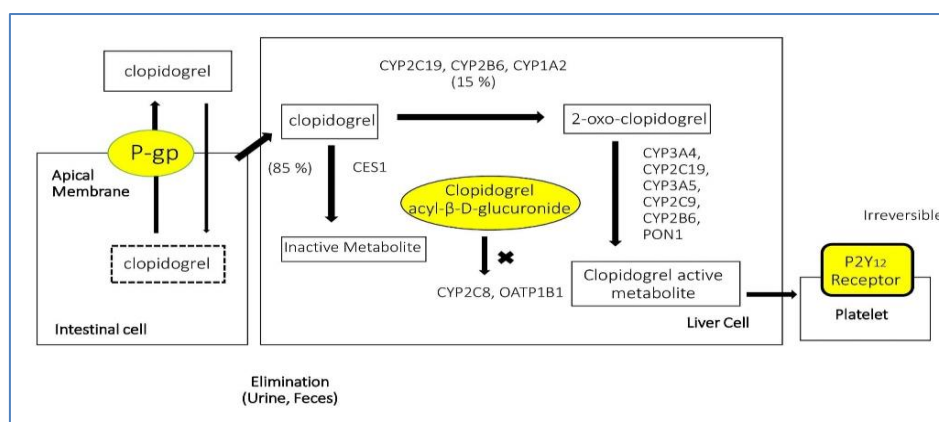


Fig-1: Clopidogrel metabolism via P2Y12 Receptor

Abbreviations

CES1, carboxylesterase 1; CES2, carboxylesterase 2; CYP, cytochrome P450; OATP1B1, organic anion transporter family 1B1; P-gp, P-glycoprotein; PON1, Paraoxonase-1.

1.3 Clopidogrel

Clopidogrel is an antiplatelet medication used to prevent myocardial infarction and to prevent blood clots in CAD and peripheral vascular disease. The medication operates by irreversibly blocking P2Y12 receptor regulated by ADP on thrombocytes membrane. Although there are other mechanisms that can contribute to thrombotic events, inhibiting P2Y12 is not the only strategy to reduce the risk of thrombosis [12]. Clopidogrel is a prodrug and is converted to its active metabolite by hepatic enzymes. P-Glycoprotein (P-gp), encoded by ABCB1, aids in clopidogrel absorption in the intestine. Only 15% of clopidogrel is accessible for hepatic metabolism after esterases hydrolyze it into an inert molecule. Clopidogrel is converted to its active form by two step oxidative. The first process produces 2-oxo-clopidogrel, which becomes the substrate for converting to the active form [13].

2. MATERIALS AND METHODS

2.1 Animals and approval of Ethics Committee

Adult male *Rattus norvegicus* (Wistar rat) , around 3-5 month of age and an average weight of about 200 - 250 grams required for our lab work. As per the guidelines the rats were under supervision providing proper conditions i.e. room temperature $22 \pm 3^{\circ}C$ and Relative Humidity : 30-70% and Natural day light cycle in the Animal House Facility, KIET School of Pharmacy, KIET Group of Institutions.

2.3 B CAO (Bilateral carotid artery occlusion)

Animals are anesthetized and kept on a heated surface maintained at $35-40^{\circ}C$ to prevent hypothermia. After anesthesia animal was allowed to move freely after observing loss of locomotor activity or movement it is tied to the platform, extra hair were removed from the neck and a clean midline incision was made in between sternum region and neck, to expose trachea as both the right and left carotid arteries. After that arteries were separated from tissue and Vagus nerve with extreme care. And B CAO was performed passing cotton thread below the carotid arteries. Global cerebral

ischemia was induced for 20 minutes of occlusion and followed by 24 hr. of reperfusion. The incision made was sutured back in layers and were cleaned with 70% ethanol. The rats were moved to their cages kept in a well-ventilated room at 25 ± 2°C until they gained full consciousness. Animals were housed individually till the healing of the surgical incision. Aseptic condition was maintained before, during and after the surgical procedure [14].

2.2 Drugs and Chemicals

Clopidogrel drug was given as a gift sample by Pure and cure Healthcare (p) ltd., Sidcul, Haridwar, India. Sodium Hydrogen Phosphate, 1,1,3,3-tetramethoxypropane, DTNB, Acetylcholine Iodide, BSA, Sodium Bicarbonate, Evans Blue, N-naphthylethylenediamine, Sulphanilamide, Sodium Nitrite, Reduced GSH, hydrogen peroxide, Hydroxyl Amine hydrochloride, Cupric Sulphate, n- butanol,

Nitroblue tetrazolium, Pyridine, Sodium Citrate, Sodium Dodecyl Sulphate,, Thiobarbituric acid, Trichloro Acetic Acid, Zinc Sulphate were purchased from CDH Laboratory Chemicals, India, HiMedia Laboratories, (p) ltd., India and Fisher Scientific, India.

All drug solution and aliquots were freshly prepared before use and 0.5% CMC was used as vehicle for our drug. Clopidogrel 10 and 30 mg/kg was given by oral administration. All animals were tested for Morris water maze test, Actophotometer, Rota rod and Inclined Beam Walking.

2.4 Experimental Protocol

The animals are randomly divided into six groups (as per the protocol). A total 30 consecutive days were given to all the treatments and 24 days to control groups.

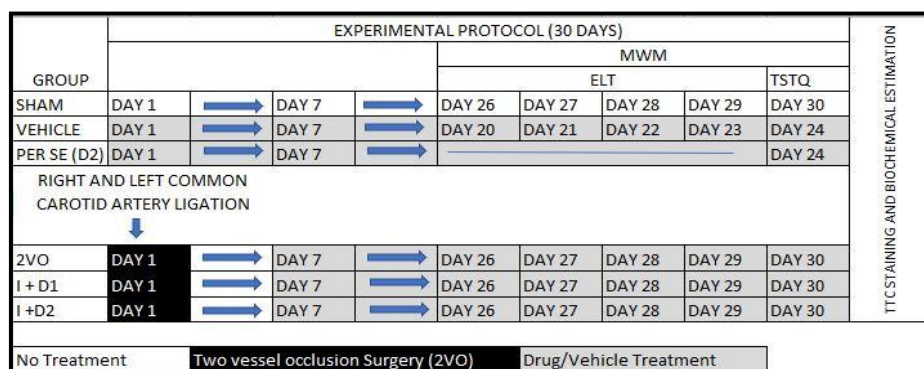


Fig-2: Schematic representation of experimental protocol

Abbreviations

2VO- Vessel Occlusion, CMC - Carboxymethyl Cellulose, D1 - Dose 1, D2 - Dose 2, ELT - Escape Latency Time (acquisition trials), MWS

- Morris Water Maze, TSTQ - Time spent in the target quadrant (retrieval trial), TTC - 2,3,5-triphenyltetrazolium chloride

Table-1: Experimental Groups

Group No.	Group Name	Dose Schedule
Group 1	Sham control	Surgical procedure to be performed without artery ligation.
Group 2	Solvent control	Vehicle to be given for 7 days.
Group 3	Target Per Se	Target to be given for 7 days.
Group 4	Induction	Carotid artery occlusion to be performed with artery ligation.
Group 5	Induction + target dose 1	Induction + clopidogrel (10 mg/kg)
Group 6	Induction + target dose 2	Induction + clopidogrel (30 mg/kg)

Group I - Sham Group

Surgical procedure to be performed without artery ligation and the animals were exposed to MWM from the 26th day onwards and retrieval trial will be on 30th day on MWM.

Group II - Solvent control

Vehicle to be given for 24 days followed by exposure to MWM and retrieval trials were performed on the 24th day.

Group III - Clopidogrel Per Se

Target to be given for 24 days followed by exposure to retrieval trial on 24th day

Group IV - Induction

Permanent bilateral common carotid artery occlusion (2VO) arteries then the animals were exposed to MWM from the 26th day onward. Acquisition trials were performed from the 26th to the 29th day, and retrieval trials were performed on the 30th day.

Group V - Induction + target dose 1

Induction + clopidogrel (10 mg/kg) arteries with a 1-week interval between artery occlusions was performed then the animals were exposed to MWM from the 26th day onward. Acquisition trials were performed from the 26th to the 29th day, and retrieval trials were performed on the 30th day.

Group VI - Induction + target dose 2

Induction + clopidogrel (30 mg/kg) arteries with 1-week interval between artery occlusions was performed then the animals were exposed to MWM from the 26th day and Acquisition trials were performed from the 26th to the 29th day with the retrieval trials which were performed on the 30th day.

2.5 TTC Staining

It can easily differentiate viable tissue from infarction macroscopically. The colorless TTC is enzymatically reduced to a red formazan product by dehydrogenases, which are most abundant in mitochondria. After 24 hours of reperfusion, the rat was sacrificed and isolated brain was kept at -4°C temperature overnight. Then, frozen brain was cut into 1-2mm thick slices which were then incubated in 2% TTC solution containing TTC and 0.2M tris buffer (pH 7.4) for around 30 min at 37°C temperature. This turns the non-infarct region into red color due to the presence of NAD and Lactate dehydrogenase and at the infarcted region white patches can be seen [15].

2.6 Evans Blue Staining

The procedure comprises injecting Evans blue dye into the jugular vein to evaluate tissue plasma extravasation, as well as including exogenously given material to produce enhanced and readily quantified plasma extravasation results, in units of OD620/g dry weight [16].

2.7 Neurobehavioral activity**2.7.1 Assessment of Learning and Memory by MWM**

The rat's exceptional learning and memory abilities were assessed using this test. The animals

were taught to recognize the hidden platform in the acquisition experiment. During this time, animals were given four trials per day for four days in a row. Data was collected using a video camera that was linked to a computerized tracking device mounted above the pool's center [17].

2.7.2 Evaluation of locomotor activity using Actophotometer

A digital Actophotometer was used to measure the locomotor activity of the animals (Almicro, India). Locomotor activity can be used as a measure of mental activity wakefulness (alertness). The rat's movement cuts off the beam of light falling on the photo cell. The count was recorded digitally. The animals were first weighed and numbered. To acclimate the animal, it was placed in the Actophotometer for roughly 3 minutes. The baseline activity score was then monitored for the next 10 minutes. Between trials, the area was washed with dilute alcohol and dried. Reduced activity was used as an indicator of CNS depression [18].

2.7.3 Evaluation of motor coordination by Rotarod Test

According to a previous method, the Rotarod test (Almicro, India) was used to assess motor coordination and balance. Rats must maintain their balance while balancing on a revolving rod. Each rat was maintained in a separate chamber on a revolving rod with the velocity set to a constant 15 rpm. Magnetic trip plates were used to automatically record the latency (time) at which each mouse fell off the rotating rod. The time it takes for each mouse to fall off the rod is then recorded. Using 70% ethanol, clean the device [19].

2.7.4 Inclined Beam Walking

The inclined beam walking test was used to assess locomotor ability. Each rat was placed on a wooden beam that was inclined at a 60-degree angle and given 60 seconds to walk the beam. Before and 24 hours of BCAO, each rat's motor performance was scored (0-4), [20] as follows:

Table-2: Assessment Score

S.no.	Grade	Rat's Motor Performance
1.	0	Cannot to walk on the beam
2.	1	less than ¼ walking, of the beam length
3.	2	more than ¼ but less than ½ walking, of the beam length
4.	3	more than ½ but less than ¾ walking, of the beam length
5.	4	more than ¾ of the beam length or to walk the whole beam in about 60 s.

2.7.5 Lateral Push Test

The proportion of a rat's resistance to lateral pushes can be used to assess its motor coordination. The rat was held on a rough surface to

achieve a solid grip, and the retardation it showed against lateral pushes from any side of the shoulder was examined in this investigation. The test was conducted Ischemia and 24 hour reperfusion, and

variations in reading were documented and rated with positive or negative marks depending on whether lateral push resistance increased or decreased [21].

2.8 BIOCHEMICAL PARAMETERS

2.8.1 Catalase (CAT)

Before starting any biochemical parameter we need brain homogenate. So, the extracted brain was separated and cleaned with ice-cold (0.9 percent w/v) with isotonic saline. Then, the brain homogenization was started by adding it to a 4.5ml of ice-cold 0.1 M phosphate buffer (pH 7.4). Which is then centrifuged for 15 minutes at 10,000 rpm at 4°C, and aliquots of supernatant were separated and utilized for biochemical calculations.

Now, the Brain homogenate was added to 50 Mm phosphate buffer (pH 7.4). For our blank we have to make a mixture of hydrogen peroxide and phosphate buffer without brain homogenate. In UV Spectroscopy, the absorbance reading was taken at 240nm in contrast to blank [22].

2.8.2 Superoxide dismutase (SOD)

In a cuvette, 24 M NBT, 1Mm EDTA, 50 L carbonate buffer, and 5ml tissue homogenate were added. The reading was taken at 560nm every 60 seconds starting at zero time and ending with the creation of blue hue. The SOD activity in the tissue sample was extrapolated using a standard plot [23].

2.8.3 Lipid peroxidation (TBARS)

It's a method for calculating lipid peroxidation and MDA in the brain that's quantitative. In the test, 50 µl of brain homogenate was obtained and well mixed with 50 µl of SDS (8.1%). To this mixture, 350 µl of glacial acetic acid (20%) and thio-barbituric acid (0.8%) solution were added, and the volume was made up to 1.5 ml with distilled water. After that, the solution was heated for an hour at 95°C. The supernatant was collected by centrifugation at 10,000 rpm for 10 minutes after it had cooled to room temperature. The maximum absorbance of the supernatant was measured at 532nm. The MDA concentration was determined and expressed in micromoles of MDA per milligram of protein [24].

2.8.4 Glutathione (GSH)

To determine the reduced glutathione content of a brain homogenate sample. In short, a 1:1 mixture of supernatant and 10% (w/v) TCA was thoroughly mixed. The mixture was centrifuged at 1000g for 10 minutes (40C). After collecting the separated supernatant sample, it was mixed with 1000l of disodium hydrogen phosphate (0.3M) and 250µl of 5, 5-dithiobis(2-nitrobenzoic acid) (0.001M). The absorbance was recorded

spectrophotometrically at $\lambda_{max}=412nm$. Different concentration of glutathione (0-4µM) were used for standard curve preparation [25].

2.8.5 Serum Nitrite/Nitrate Level

Blood samples were taken utilising retro-orbital bleeding. The blood samples were stored at room temperature for 30 minutes before being centrifuged for 15 minutes at 4000 rpm to extract the serum, which was then used to calculate serum nitrite/nitrate levels. The amount of nitrite released from tissue homogenate was used to estimate nitric oxide generation. 100 µl of brain or standard sample and 400 µl sodium hydroxide (.35M) were combined with copper cadmium alloy to determine the nitrite concentration. After deproteination, serum samples are centrifuged at 4000 g for 10 minutes. Greiss reagent was added next, and the absorbance was calculated at 540 nm. The results were given in milligrammes per gramme of brain tissue [26].

2.8.6 Brain AChE Activity

In a total volume of 500 µl, a combination containing Acetylcholine Iodide (1 mM), DTNB (2 mM), potassium phosphate buffer (100 mM) at pH 7, and homogenate of brain was prepared. After 10 minutes of incubation at 37 oC, 500 µl of serine hemisulphate (0.5 mM) was added to stop the process. At $\lambda_{max} = 412nm$, the absorbance of the resulting yellow tinted solution was measured [27].

2.8.7 Assessment of Total Protein

Using BSA as a standard, the total protein content of the brain was measured spectrophotometrically. The tissue homogenate supernatant (15 mL) was diluted to 1 and 5 mL of Lowry's reagent was added. The materials were gently combined, and the mixture was allowed to sit at room temperature for 15 minutes. The contents were vortexed vigorously and incubated for 30 minutes at room temperature with the addition of 5 mL Folin-Ciocalteu reagent (made by diluting 50 mL Folin-Ciocalteu reagent in 50 mL distilled water). The standard curve was plotted using BSA concentrations of 2-2.4 mg/mL. At 750 nm, the protein concentration was determined by spectrophotometry [28].

2.9 STATISTICAL ANALYSIS

The results are represented as means \pm S.E.M. and were analyzed by 1-way ANOVA, followed by Tukey's multiple comparisons post hoc test using a Graph Pad Prism version 5. A value of $P < 0.05$ was considered as statistically significant.

3. RESULTS

3.1 Behavioral Parameters

3.1.1 Assessment of learning and memory by Morris Water Maze

MWM was employed as a tool for assessment of learning and memory. The rats were subjected to 5 days of MWM trails, with day 1 to day 4 being the training phase where the animals

learned to locate and climb on the platform. On day 5 the platform was removed and the animal was left looking for the platform in the pool and the time spent by the rat in the target quadrant was noted. The elapsed latency was assessed by MWM and a significant decline was observed in day 1 vs day 4 ELT in Sham operated, solvent control and *per se* treated animals. ELT in day 4 of treatments were significant on comparison with the induction group.

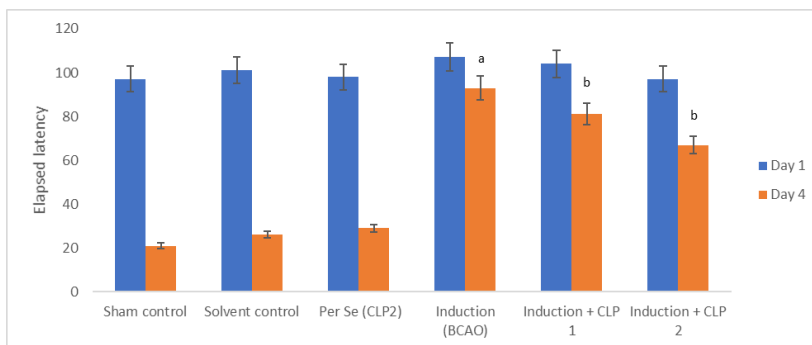


Fig-3: The results are shown as Mean ± SEM, analyzed by one way ANOVA followed by post hoc Tukey's test. ^ap<0.05 vs Sham control, ^bp<0.05 vs BCAO

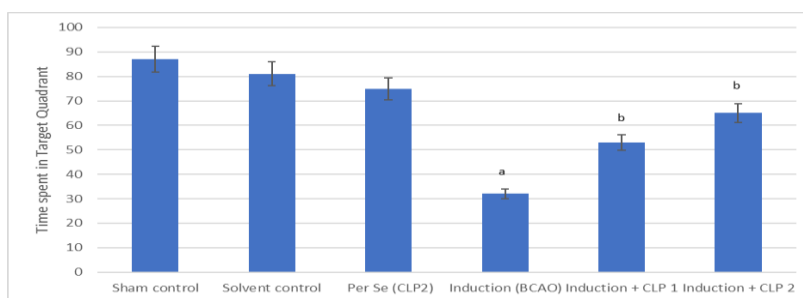


Fig-4: The results are shown as Mean ± SEM, analyzed by one way ANOVA followed by post hoc Tukey's test. ^ap<0.05 vs Sham control, ^bp<0.05 vs BCAO

The TSTQ was significant in with clopidogrel treatment in comparison to induction group animals. Clopidogrel treated animals showed an upward trend in day 5 TSTQ which indicated preservation of learning and memory.

3.1.2 Locomotion assessment by Actophotometer

Chronic hypoperfusion is known to reduce the locomotor activity of the animals and this was

assessed by Actophotometer. Animals were subjected to 5-minute exposure on the Actophotometer and the number of crossings by the animals in the photo emitting cells were recorded. Our result shows the sham control, solvent control, and *per se* group showing no significant difference but treatment group showing improvement in locomotion activity of animals.

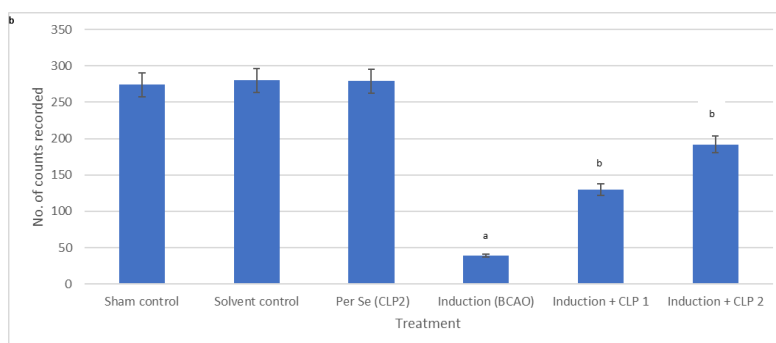


Fig-5: Result showing actophotometer activity with animals. The results are shown as Mean ± SEM, analyzed by one way ANOVA followed by post hoc Tukey's test. ^ap<0.05 vs Sham control, ^bp<0.05 vs BCAO

3.1.3 Motor coordination- Rota Rod

Hypoperfusion tends to reduce the motor coordination in the subjects, and this was assessed by rota-rod apparatus. The animals with BCAO had extreme difficulties in maintaining themselves over the beam and were falling in a short duration of

time. The animals treated with solvent, and drug *per se* had no significant effects on them either. The treated animals showed improvement in their motor coordination which was evident by their ability to stay on the rotating rod for a longer duration of time.

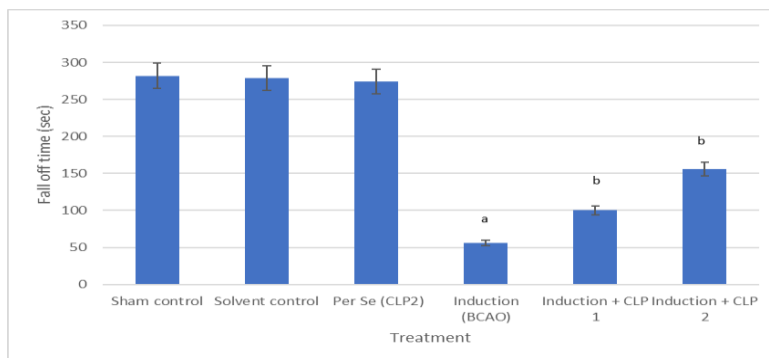


Fig-6: The results are shown as Mean ± SEM, analyzed by one way ANOVA followed by post hoc Tukey’s test. ^a p<0.05 vs Sham control, ^b p<0.05 vs BCAO

3.1.4 Motor coordination by Inclined Beam walking test

We have observed the Inclined Beam walking Test results, which shows that the group exposed to BCAO model show high amount of severity score for the impairment in motor

coordination. Treatment group, sham operated, *per se* group has less severity score and the treated animals showed improvement thus showing its positive effect in motor coordination induced sensory impairment.

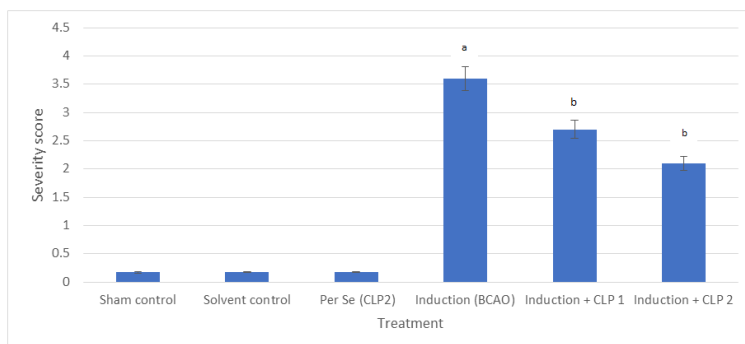


Fig-7: Result showing severity score of motor coordination assessed by inclined beam. The results are shown as Mean ± SEM, analyzed by one way ANOVA followed by post hoc Tukey’s test. ^a p<0.05 vs Sham control, ^b p<0.05 vs BCAO

3.1.5 Lateral Push

Animals subjected to lateral push test showed varied degree of resistance to the manual push. Sham operated, solvent control and *per se* animals showed high resistance to lateral push while the induction animals hardly showed any

resistance and they often turned to side when pushed. This observation was countered by clopidogrel treatment in a dose dependent manner. The results were scored and hence couldn’t be analysed statistically. The results are presented in percentage of animals showing resistance in a group.

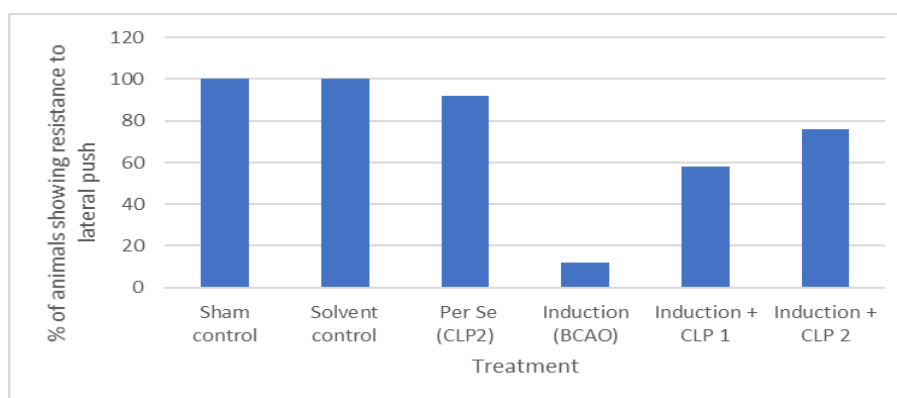


Fig-8: Result showing percentage of animals showing resistance to lateral push

4. DISCUSSION

Our study was designed for evaluating the role of P2Y₁₂ receptor modulation in BCAA induced chronic cerebral hypo perfusion. The brain needs continuous oxygen and blood supply and if there is any type of interference, can cause decrease in locomotor activity and neurodegeneration [29]. With the available literature we made a hypothesis and worked around it that the modulation of ADP receptors may play a protective role in hypo-perfusion induced cognitive declines in the animals.

The neuroprotective effects were assessed using a battery of behavioral tests and biochemical analysis. Hypoperfusion affects the brain hippocampus causing cognitive declines that's why we used Morris water maze. It is a widely used to assess the spatial memory and learning. Our results show that the animals treated with clopidogrel showed significant improvement in the challenges offered to the animals. The animals with sham operations and vehicle and *per se* treatments did not show any significant decline in their cognition [30].

Among several behavioral tests that measure motor performance, the rotarod is a suitable test for evaluation of cerebellar deficits in rodents [31], we tried to extract the acquisition of motor skill from the original procedure. In Rotarod test animals must maintain their balance while balancing on a revolving rod shows marked decrease in cerebral infarct size. There is a strong correlation between cerebral infarct size and motor coordination [32].

BCAO resulted in impairment of motor coordination and biochemical alterations so, The inclined beam walking test and Lateral push test shows improvement as we have seen in sodium dependent glucose co-transporters (SGLT) in neuroprotective mechanism of ischemic postconditioning (iPoCo) [33].

The involvement of microglial P2Y₁₂ receptors in the activity-dependent neuronal plasticity and our findings shows the clopidogrel as a potent drug for ischemia [34].

Administration of clopidogrel in animals significantly curbed the neuronal death and prevented the sensorimotor decline in animals. It also reduced the oxidative stress in the animals and proves that Clopidogrel involved in chronic cerebral hypoperfusion induced cognitive declines may be seen as a potential target therapy in stroke patients.

In our study we also planned to assess biochemical parameters that for the oxidative load. Assessment of TBARS, GSH, AChE, Serum Nitrite/Nitrate Level, total proteins, SOD and CAT activity and shows significant results.

Declaration of Interest

The authors declare that there is no *conflict of interest*.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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